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Expression of miRNA-122-5p as Diagnostic and Prognostic Biomarkers for Hepatocellular Carcinoma Susceptibility in Hepatitis B-Infected Iraqi Patients

[1] Khalid R Majeed, [2] Wiaam Ahmed AL-Amili, [3] Safaa A. A. Al-Waysi

[1]Department of Medical Laboratory Technique, Al-Nasiriyah Technical Institute, Southern Technical University, Thi-Qar, Iraq.
[2] Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / University of Baghdad / Baghdad / Iraq.
[3] Medical City, Hepatology and Gastroenterology Teaching Hospital.

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Abstract

Background. Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death. For this reason, researchers must have a solid grasp of molecular biology. Abnormal miRNA expression has been associated with metastasis, differentiation, proliferation, and apoptosis in several illnesses, including cancer. Research on miR-122 and other microRNAs has factualized on their potential roles in cancer. The leading cause of hepatocellular carcinoma (HCC) worldwide is the hepatitis B virus**. Aim.** This study aimed to evaluate expression of Circulating miR-122-5p in relation to the pathogenesis of HCC in hepatitis B Iraqi patients and as a promising diagnostic, prognostic and predictive biomarkers**. Methods.** A total of 120 samples were collected, 40 newly diagnosed patients with hepatocellular carcinoma representing group I, 40 patients chronic HBV representing group II who were admitted to hospital from the period among January 2023 to August 2023 and 40 apparently healthy individual's volunteers representing group III. In order to measure the miR-122 method gene expression, 5 ml of blood were drawn from each subject using disposable syringes during venipuncture. By using RT-qPCR, the miRNA expression in eighty serum samples was examined**. Results.** Serum *miR-122* levels were much higher in HCC and chronic hepatitis B virus (CHB) patients compared to healthy controls (HCs). The mean ΔCt values were −8.27733 for the HCC group, −8.0715 for the CHB group, and −6.517 for the control group. Accordingly, the mean $2^{-\Delta Ct}$ values were 310.260 in HCC patients, 269.007 in CHB patients, and 91.582 in the control group. When the control group was used as a calibrator (fold change = 1.00), the relative fold expression of miR-122-5p increased to 3.4-fold in the HCC group and 2.9-fold in the CHB group. *miR-122* expression was upregulated in individuals with HCC and when compared with healthy controls. **Conclusions.** The relative expression analysis revealed a increase in HCC patients and CHB patients, indicating a strong association between elevated *miR-122* levels and HBV-related liver pathology.

Keywords: Micro RNA-122, HBV, Hepatocellular carcinoma, RT-qPCR, Gene expression.
Corresponding author: (Khalid.gen12@stu.edu.iq).

Introduction

Liver cancer remains a global health challenge, with an increasing incidence worldwide. Statistics from the World Health Organization show that in 2030, more than 1 million patients worldwide will die of liver cancer, which reflects its serious disease burden (1). Hepatocellular carcinoma (HCC), one of the most common and lethal tumors

worldwide and considered as a malignant tumor of hepatic parenchymal cells, constitutes 90% of primary liver malignancies, followed by cholangiocarcinoma. In 2022, HCC was the 6th most common malignancy in adults and the 3rd to lung and colorectal cancer as a leading cause of cancer-related death. Liver cirrhosis and fibrosis are the common precursors of HCC, which may be brought on by things like toxins, chronic viral infection, lipid buildup, heavy alcohol use, and chronic viral infection (2). The molecular and other factors that contribute to the development of HCC are not well understood. Therefore, there is an immediate need for further research into these pathways (3).

Hepatitis B virus (HBV) is a member of the family *hepadnaviridae* and a virus with partially double-stranded DNA. More than 250 million individuals throughout the world are dealing with a long-term infection of HBV. Cirrhosis, acute hepatitis, and HCC account for the majority of HBV-related deaths, which exceed 800,000 annually. The hepatitis B virus is a major contributor to the development of hepatocellular carcinoma. Few details are known about the mechanisms that lead to hepatocellular carcinoma; a disease cautilized by hepatitis B (4). Iraq is home to a high frequency of hepatitis B, with estimates placing the disease's incidence anywhere from 1% in the north to 3.5% in the south (5). According to certain studies conducted in Iraq, the prevalence of HBV infections ranged from 0.77 to 1.37 percent (6).

A microRNA molecule has 21–23 nucleotides and is tiny, single-stranded, and non-coding RNA. It is thought that microRNAs (miRNAs) function to regulate gene expression after transcription and to silence RNA; they are present in mammals, plants, and even some viruses. A short non-coding RNA that interacts with genes that code for messenger RNA and may control the expression of target genes after transcription has taken place. In cancer and many other disorders, abnormal miRNA expression has been linked to several biological processes, such as cell death, proliferation, differentiation, and metastasis. Recent research has linked microRNAs (miRNAs) to metabolic pathways, liver maturation, and repair (7). As dependable biomarkers, miRNAs are utilized to ascertain the aetiology and progression of liver illnesses (8).

Among microRNAs, *miR-122* has received the greatest amount of attention from researchers. Among tissue miRNAs, it stands out due to its high concentration (over 70% of the entire liver miRNA population) and high copy number (more than 130,000 copies per cell) in human hepatic cells (9). Since *miR-122*-5p is highly concentrated in the liver, it is reasonable to assume that these miRNAs have a role in the development and advancement of HCC (10).

This study aimed assess the role of circulating *miR-122*-5p in hepatitis B patients' development of hepatocellular carcinoma (HCC) and its potential as a biomarker for diagnosis, prognosis, and prediction in Iraqi patients.

Materials and Methods

The collection of samples:

A case-control study was conducted on the following study groups during the period from the January 2023 to August 2023 in the Hepatology and Gastroenterology

Teaching Hospital in Medical City - Baghdad - Iraq. The study was approved by the Institute for Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad and the study protocol was approved by the Ethics Committee of the Iraqi Ministry of Health and Environment. A total of 120 samples were collected, 40 newly diagnosed patients with hepatocellular carcinoma representing group I, 40 patients chronic HBV representing group II and 40 apparently healthy individual's volunteers, from the staff members of hospital where the study was performed representing group III. The following were the inclusion criteria:(1) a healthy control group; (2) a chronic hepatitis B (CHB) group consisting of individuals who fulfilled all diagnoses of chronic hepatitis, Group 3: Hepatocellular carcinomas (HCCs) linked to herpes simplex virus (HBV), HBV history + clinical imaging + laboratory markers.

The following were the exclusion criteria: Liver cirrhosis and cancer due to other chronic liver diseases, individuals undergoing dialysis or organ transplantation, and a mix of other malignant tumours; liver damage cautilized by other causes, such as HCV, HIV infection, drug consumption, kidney, lung, severe heart, and/or systemic diseases; and liver damage cautilized by other substances. After an oncologist diagnosed hepatocellular cancer and Hepatitis B infection in Iraqi patients, a large number of samples were taken from their blood.

Quantitative Reverse Transcription Real-Time PCR

Table 1 shows the sequence of primers used for *miRNA-122-5P* and miRU6 (reference gene) the quality control run using primer 3plus, V4, and verification by the University Code of Student Conduct (UCSC) programs and the National Centre for Biotechnology Information (NCBI) database of the *miRNA-122-5P* reference sequences utilized in this study's Real Time PCR primers:

Table (1): The qPCR Primers with their product sizes and nucleotide sequences

qPCR primer	Sequences (5'-3')
miRNA-122-5P F.P.	TGGAGTGTGACAATGGTGTTTG
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

Preparing the Primers

The manufacturer sent the primers in lyophilised form. The nuclease-free water was utilized to dissolve the lyophilised material, as directed by the manufacturer. A stock solution was then made at a concentration of 100µM and kept at -20°C. A workable solution with a concentration of 10µL was produced by diluting the primer stock solutions with 90µL of nuclease-free water. From then on, it was kept at -20°C until it was required.

Total RNA extraction

Each subject had five millilitres of blood drawn by venipuncture using disposable syringes. After letting the blood coagulate in disposable gel tubes at temperature of room for five minutes, it will be centrifuged for five minutes and then pipetted out. After thoroughly mixing 250 ml of serum with 500 ml of Trizol in an Eppendorf tube, the mixture was refrigerated at -20 °C until analysis. After blood samples were separated into serum, total RNA was isolated. We

utilized TRIzolTM Reagent (ER501-01) to extract total RNA in accordance with the directions provided by the manufacturer.

Assessment of RNA quantity and purity

The One Nanodrop spectro-photometer (Thermo Fisher Scientific, USA) was utilized in accordance with the manufacturer's instructions to ascertain the amount and purity of the RNA that had been extracted. For samples with low RNA abundance, this is a very accurate and selective way to measure the sample. All samples have miRNA concentrations ranging from 73 to 147 ng/µl, suggesting that miRNA is quite selective for miRNA relative to other types of RNA. While two different wavelengths (260 and 280 nm) were utilized to assess the samples' absorbance in order to ascertain the RNA purity. A260/A280 ratios among 1.95 and 2.0 were indicative of a pure RNA sample.

Complementary DNA (cDNA) Synthesis, with Specific Primer

To synthesise first-strand miRNA from total RNA templates, EasyScript® offers a One-Step gDNA Removal and cDNA Synthesis SuperMix Kit Table (2). Total RNA was reverse-transcribed to obtain complementary DNA (cDNA) in line with the manufacturer's instructions.

Quantification of microRNAs

The method utilized to measure short RNA (~20 nucleotides or base pairs) is specified by the manufacturer in their procedure. Rapid identification of microRNAs and other short RNAs, including single-stranded and double-stranded RNAs, is possible with this miRNA quantification kit. It can withstand impurities like salts, solvents, or detergents, and it is very selective for tiny RNA over long mRNA. Table (3) shows the optimised cycles that were programmed into the cycling routine based on the thermal profile

Table (2): Strand cDNA synthesis reaction component.

Components	Volume
MiRNA	6
Anchored Oligo(dT)18 Primer(0.5µg/µl)	1 µl
Random Primer(0.1µg/µl)	1 µl
GSP	2 pmol
2×EX Reaction Mix	10 µl
Easy Script®RT/RI Enzyme Mix	1µl
gDNA Remover	1µl
RNase-free Water	To 23µl

Step	Time (sec.)	Temperature (ºC)	Cycles
Enzyme activation	30	94	1
Denaturation	5	94	40
Annealing	15	58	
Extension	20	72	
Dissociation	55 ºC-95 ºC		1

Table (3): The *miRNA 122-5P* genes expressions thermal profile.

Calculation of the gene expression:

The amount of miR-122 expression in patient samples was determined using quantitative real-time PCR (qRT-PCR). The expression of miR-122 was assessed using a relative cycle threshold ($2^{-\Delta\Delta Ct}$) technique. U6 served as a housekeeping gene for the healthy control samples.

Statistical Analysis

The data was described, analysed, and displayed using SPSS version 26, which is statistical software for the social sciences. Standard deviations (SD) and means (MA) were utilized for quantitative variables. Qualitative factors were quantified using percentages and frequencies. When comparing more than two means, a one-way analysis of variance (ANOVA) was used, however when comparing the means of the two groups, an independent T-test was utilised. Two quantitative variables were shown to be related using the Pearson correlation method. P≤0.05 was utilized to determine the significance value.

Results

The results of the age distribution for all studied groups are shown in table (4). The mean age of HCC was 52.12± 12.470 years old, 45.58± 13.177 years old for CHB, and that of HCs was 49.23± 13.465 years old and there was non-significant variation among patients and HCs in mean age (P = 0.08).

Table (4) Comparing among patients and control groups in Age.

Groups	Mean ±	SD	p-value
HCC	52.12	12.470	0.08
CHB	45.58	13.177	†
Control	49.23	13.465	NS

SD: standard deviation; †: one way ANOVA; NS: not significant at $P > 0.05$

Table 5 shows that there were 65 males and 55 females, or 54.2% and 45.8% of the total, respectively; of the instances of HCC, 22.5% were of the male variety. 19 cases (47.5%) were female, 26 cases (65.0%) were male, and 14 cases (35.0%) were female in the HBV group; in the control group, 18 cases (45.0%) were male, and 22 cases (55.0%) were female; and there was no significant difference in the frequency distribution of patients and HCs according to sex (P = 0.1).

Table (5): Comparing among patients and control groups in sex.

		SEX		Total	p-value
		Male	Female		
Groups	**HCC**	21 (52.5%)	19 (47.5%)	40	0.1 ¥ NS
	CHB	26 (65.0%)	14 (35.0%)	40	
	Control	18 (45.0%)	22 (55.0%)	40	
Total		65 (54.2%)	55 (45.8%)	120	

NS: not significant at P > 0.05; SD: standard deviation; ¥: Chi-square test.

Viral load

The comparing of patients with Hepatocellular carcinoma and chronic Hepatitis B virus according to Viral load was shown in table (6). The mean of Viral load was significantly higher in patients with HCC in comparison of patients with CHB, 6574698.30 versus 583319.62 copies /ml, respectively, (P= 0.008)

Table (6): Comparing among patients' groups in Viral load.

Groups	Mean ±	SD	p-value
HCC	6574698.30	13573598.443	0.008 † S
HBV	583319.62	1306264.310	

SD: standard deviation; S: significant at P < 0.05; ¥: Chi-square test

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Expression of *miR-122* in HCC, HBV and Control

This research compared the expression of miR122-5P in the control group, the HCC group, and the CHB group using a quantitative RT-PCR analysis. A relative quantitative measurement was utilized to determine the change in gene expression (30). This demonstrates the dispersion among the mean Ct values of the miR122-5P cDNA amplification replica for every case and U6 case, and it is dependent on normalizing the Ct data in order to compute ΔCt. The $2^{-\Delta Ct}$ values were utilized in table (7) to calculate the relative expression of the miR122-5P gene across all research groups. The control sample with the highest miR122-5P expression served as a calibrator. The control group had an average of 91.582 $2^{-\Delta Ct}$ values and the HCC group had an average of 310.260, whereas the normalization Ct values for the HCC group were (-8.27733), (-8.0715), and (-6.517) correspondingly. A mean of 269.007 was recorded for the CHB group. In terms of gene expression, the HCC group outperformed the control group when all factors were included. The number of folds in the HCC group was 3.4. The CHB group has a fold number of 2.9. As represented in the Figure 1. An examination of RT-qPCR data showed that, in comparing to HCs, patients with HCC and HBV had significantly upregulated *miR-122* levels (P < 0.05). In comparison to the HCs, which had a relative fold change of 1.0189 ± 0.57017, the average relative fold change of *miR-122* in patients with HCC was 3.6793± 1.48251, and in patients with HBV it was 3.2537± 1.39529.

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Table (7): Comparison of (Ct, $2^{-\Delta ct}$ and folding) among patients and healthy controls.

Groups	*Means Ct of miRNA-122-5P*	*Means Ct of U6*	*ΔCt (Means Ct of miR122-5P)*	$2^{-\Delta Ct}$	*experimental group/ Control group*	*Fold of gene expression*
HCC	13.20233	21.47966	-8.27733	310.260	310.260/91.582	3.4
CHB	13.45875	21.53025	-8.0715	269.007	269.007/91.582	2.9
Control	15.04866	21.56566	-6.517	91.582	91.582/91.582	1.00

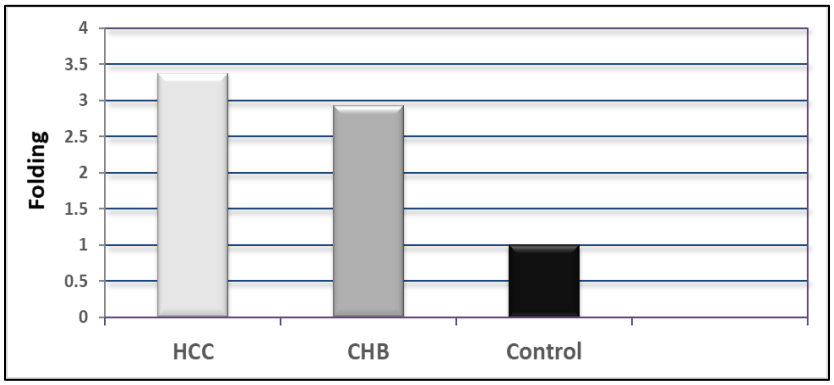

Figure (1): The variations among the HCC, CHB patient and apparently healthy control according to *miRNA-122-5P* gene expression.

Discussion

With an annual incidence of 906,000 cases and a mortality rate of 830,000, hepatocellular carcinoma (HCC) ranks third among malignancies in terms of mortality (11). Today, the most common risk factors for liver neoplasms are hepatitis B virus, alcohol misuse, and obesity (12). Factors affecting the clinical course of chronic HBV infection and HCC are hence of great public health significance. An important objective of HBV research is to shed light on this constellation of risk variables and comprehend the hepatocarcinogenesis associated with HBV (13). Although HCC can affect individuals at wide range of age, and that also confirms the need for screening and early detection of liver cancer. The present study shows the high rate of patients was in more than 50 years. Where show the mean age of HCC patients was 52.12± 12.470 years old, 45.58± 13.177 years old for HBV, and that of HCs was 49.23± 13.465 years old and there was non-significant variation among patients and HCs in mean age (P= 0.08), these results agree with results of Majeed and Qadurie (14), who found that age ranged from (21-86) year, with mean age of (57.5) year the commonest age group affected was (50-59) years old. Also, this result was similar to study by Gairing and Schattenberg (15), who highlights that age is a crucial risk factor for hepatocellular carcinoma development, especially in older

individuals. Most of the participants were between the ages of 51 and 60, which is consistent with the results published by Qasim et al., (16). One possible explanation for the positive correlation between age and HCC incidence is that CHB has an increasingly substantial impact on HCC development as people become older. The probable higher incidence of HCC in Korean elderly individuals may account, at least in part, for this finding. Two independent risk variables for HCC were being male and being older than 50 years old, according to earlier research (17). The incidence of HCC is minimal before age 40 but then climbs exponentially after that, suggesting a clear association between age and HCC risk (18). The patients aged older than (60) year have the least percentages (8.8%) since most Iraqis get the HBV infection at younger age and this age is active and more insecure to HBV infections The infected patients are likely to take antiviral drug and the probability for mutations occurrence will be high in these patients at any age if the treatment continue (19).

Patients with chronic viral hepatitis are at increased risk for developing hepatocellular carcinoma (HCC), and sex is a major factor in these risk scores (20). Generally, HCC is more prevalent among men (21). The present study revealed higher prevalence of HCC and HBV in male 21 (52.5%) and 26 (65.0%) than female 19 (47.5%) and 14 (35.0%) respectively, and there was no significant variation in the frequency distribution of patients and HCs according to sex (P= 0.1), table (5). Where the present results indicating preponderance of liver disease in male. This similar to the finding of McGlynn et al., (22) who found there was an estimated 2:1 to 4:1 male-to-female ratio for HCC. According to another research, the occurrence of HCC in male patients is much more than in female ones (20). Similarly, Japanese research found that males, especially those over the age of 70, had a higher risk of developing HCC than women (23). Among the many known causes of HCC, including HBV, it is well-established that males have a greater incidence than females (24).

Biological differences between the sexes and differences in exposure to risk factors may play a role in illness progression, and our finding lends credence to this idea. The result of present study was in agreement with the study by AL-Hawaz et al., (25) in Basra, Iraq, that showed there is a high prevalence of hepatitis viruses in males (59.7%) than females (40%). Tobacco use, excessive calorie eating, and alcohol use are more common among males than women, which may explain this phenomenon (26). Furthermore, the fact that men and women are at distinct risk of developing HCC may be due to the fact that sex hormones have different characteristics. Whereas oestrogen protects against hepatocarcinogenesis, androgens and androgen receptor signaling promote cancer (27). Approximately 5-7:1 is the male-to-female ratio for the prevalence of HBV-related HCC. This data points to men being at a higher risk of developing liver cancer due to HBV. Furthermore, men had a chronic HBV carrier rate of 10.7% and females of 4.4% when vaccinated at birth and followed up for more than 18 years. Various research has put up the idea that the sex influence on HBV-induced HCC starts in the early stages of chronic hepatitis B. It is believed to affect HBV titers and antigens via direct regulation of viral gene expression or indirect regulation of host immune responses (28).

An interesting but still unexplored subject is the physiological processes that explain why men have a worse prognosis

and are more likely to develop HCC following an HBV infection (13). Complex and multi-factorial sex differences in behavioral risk factors, hormones, metabolism, and tumor biology are believed to be the root cause of this gender gap (24). Additional research is needed to fully understand the underlying pathways; however, it has been suggested that genetic and epigenetic changes contribute to the sex discrepancy in HBV-related HCC (29).

The HBV in HCC patients was evaluated using the real-time PCR method. Research on the risk factors for HCC, including the host, the environment, and the virus itself, is necessary to understand how the disease progresses. On a global scale, the prevalence of chronic HBV infection is still the greatest risk factor for HCC, with viremia level ranking highest (31). The current findings corroborate research that found that greater levels of HBV viral load are the greatest predictor of future HCC risk (32), as the mean viral load was higher in patients with HCC compared to CHB. Research conducted by Marugán and Garzón (32) shown that patients with elevated DNA levels should be closely monitored. This is due to the fact that DNA levels serve as a predictor of prognosis, help identify the stage of CHB infection, and provide evaluation of the effectiveness of antiviral treatment.

Hepatocarcinogenesis may be aided by overexpression of HBxAg, the gene for the herpes simplex virus. This overexpression has the potential to change the signal transduction pathways of hepatocytes and bind to and deactivate genes that regulate negative growth. A high HBV-DNA viral load has been suggested by many studies to be a possible indicator of individuals at higher risk of developing liver cirrhosis (33). Hepatitis B virus infection may cause a variety of symptoms and complications, including asymptomatic carrier condition, acute or fulminant hepatitis that resolves on its own, chronic hepatitis that develops into cirrhosis and hepatocellular cancer, and many more (34). The immune response and the age range of patients may have a significant influence on the viral load's variability. greater viral loads are linked to greater death rates in chronic HBV-infected individuals, as shown by subsequent investigations (35).

Renal cirrhosis is characterised by inflammation and fibrosis, two hallmarks of the disease, and microRNAs, tiny non-coding RNA molecules, regulate genes involved in both processes (36). Controlling the expression of certain genes is the overarching role of microRNAs (37). Abnormal miRNA expression has been associated with cancer and other diseases. miRNA is essential for several biological processes, such as cell death, proliferation, differentiation, and metastasis (38). Emerging evidence suggests that microRNAs (miRNAs) are critical for liver growth, regeneration, and metabolic processes (39). Liver disorders like cirrhosis, hepatitis, steatosis, and hepatocellular carcinoma may be exacerbated by alterations in intrahepatic miRNA networks.

The adult liver is home to the most abundant miRNA, *miR-122*, which is involved in both healthy and diseased liver function. Along with existing therapies like interferons and direct-acting antivirals, *miR-122* has been shown to be a target for antivirals and a critical host component in hepatitis virus transmission. New research suggests that *miR-122* differently regulates the host gene, an essential step in HBV replication (40). Research has shown that miRNAs in the blood may serve as

diagnostic biomarkers for a variety of disorders (41). They make a big difference in the way a patient responds to therapy as well (42).

Both the HCC and HBV patient groups had a significantly higher expression of miRNA-122 compared to the healthy control group, with a variation that was highly significant ($P < 0.001$). When utilized to detect early-stage HCC, *miR-122* expression offers a good diagnostic accuracy for the disease (43). *MiR-122* suppresses hepatocyte growth, cancerous transformation, and HBV replication and expression (44). Tumour samples from patients provided more evidence that dysregulation of *miR-122* is critical for cancer progression. It seems that the physiological role of *miR-122* in carcinoma differs according to the type of cancer, according to examination of *miR-122* expression levels in different organs (39).

The current findings go counter to those of Al-Juboori and Al-Juboori, (45), who discovered a statistically significant downregulation of *miR-122* (with a fold change average of 0.15 and 0.59) in HCC and HBV patients compared to healthy controls. According to Wischhusen et al., (46), reduced *miR-122* expression is linked to worse prognosis, bigger tumour growth, less differentiation, invasion, and metastasis in HCC. Another finding is that HCC is associated with down-regulated levels of *miR-122*, a tumour suppressor miRNA, which leads to tumour development, treatment resistance, apoptosis evasion, and metastasis. Inflammation causes greater cell necrosis and death when hepatocytes are exposed to HBV, and enormous quantities of *miR-122* are released into the blood. Serum *miR-122* was significantly higher in CHB patients compared to normal persons. In mature hepatocytes, *miR-122* expression is high, but in HCC, it is dramatically downregulated. There is a strong correlation between this down regulation and liver carcinogenesis, poor prognosis, and metastasis in HCC (47). Based on our findings, *miR-122* has the potential to be a more sensitive and reliable biomarker of liver infection than liver enzyme testing. The results of this investigation are in agreement with those of an earlier study showing that measuring *miR-122* blood levels is a handy and suitable way to diagnose HBV infection (48).

Conclusions:

In contrast to HCs, patients with HCC and CHB had higher levels of *miR-122* expression, according to our results. Therefore, we suggest using these microRNAs as biomarkers to track the development of the illness, its course, and any signs of recurrence after therapy has ended. Furthermore, patients were advised to have follow-up testing using these biomarkers due to their high sensitivity and accuracy. Additionally, it may serve as an indicator for the progression of HCC in patients infected with HBV. Because of this, microRNAs are a gold mine of information for illness recurrence and progression monitoring, as well as for early detection.

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