ORIGINAL ARTICLE

Hepatitis Virus-related microRNA-122 gene and Biochemical Parameters in Iraqi Patients with Hepatocellular Carcinoma

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ABSTRACT

Background: *Chronic HBV and HCV infections may modify hepatocyte function via comparable pathways that impact the development of hepatocellular carcinoma. Our understanding of the molecular biology of HBV and HCV, as well as the cellular signal transduction pathways affected by these illnesses, has advanced significantly. Several cancer types have aberrant expression and deregulation of microRNA-122, which raises the possibility that these molecules may function as prognostic and diagnostic biomarkers. In a global context, noninvasive biomarkers have limited application for the detection of HCC. Early identification is vital to enhance the survival percentage of individuals with HCC.* ***Methodology****: Clinical diagnosis of HCC was made, and laboratory work was done. Patients were divided into two groups: those getting therapy and those not. After that, a blood sample was taken from 24 individuals of various ages and genders. A handful of them had just been diagnosed with HCC.* ***Results****: This study was significance of HCV and HB V and liver cancer, as well as the decline in research and studies in Iraq. The microRNA-122 expression levels were marginally increased in individuals who received therapy and decreased in those who were diagnosed early and did not receive any therapy. In the first phase of hepatitis samples, the microRNA-122 expression levels were considerably lower than in standard samples, with (P < 0.0001), suggesting the possible use of microRNA-122 as a biomarker to track treatment success. It is suggested that further study should be done in this field to aid diagnosis.* ***Conclusion:*** *MicroRNAs have a high degree of specificity and dependability, making them an invaluable tool for tracking illness development and recurrence as well as for early diagnosis. Additionally, these indicators may function as a predictor of HCC advancement in individuals with a history of hepatitis B or C. Our findings show that, in comparison to healthy controls, HCC patients with HBV and HCV had lower expression levels of miR-122. Untreated HCC patients had considerably decreased expression of miR-122 compared to treated HCC patients and healthy controls.*

INTRODUCTION

Hepatitis B and C viruses can both cause chronic infection, which can modify hepatocyte function and potentially employ comparable pathways to encourage the development of hepatocellular carcinoma (HCC)[1]. For the majority of patients with advanced stage HCC, systemic therapy is required, and sorafenib is now the most often recommended medication.[2,3,4].

This virus has been divided into seven primary patterns with nucleotide variations ranging from 30% to 35% and 67 subtypes with less than 15% variance[5,6].

Though the main cause is hepatitis viruses, infections can also be brought on by alcohol, certain drugs, and other autoimmune disorders. Liver cancer and cirrhosis are 15%–40% more common in hepatitis C patients, among other chronic liver illnesses. 21–23 nucleotides combine to form microRNA [7]. One of the several diseases that have been connected to abnormal miRNA expression is cancer. MicroRNA is essential for several biological processes, including as apoptosis, proliferation, differentiation, and metastasis [8].

Numerous inflammatory illnesses and malignancies are brought on by dysregulation of the synthesis of microRNA-223, which is crucial for innate immunity[9,10,11].

One of the many cancer types connected to circulating imbalances in microRNA (miRNA) is HCC. The plasma microRNA (miR-122) has a good diagnostic accuracy when it comes to recognizing early-stage HCC. Most common miRNA in the adult liver is miR-122, which is necessary for both disease and

normal liver function. Furthermore, miR-122 is an essential host in the HCV transmission process. Antivirals can target miR-122 and can be utilized in addition to already existing therapies such as interferons and direct-acting antivirals. Since microRNA-223 is a crucial component of innate immunity, its expression is deregulated in a number of inflammatory illnesses and malignancies[11,12].

On the other hand, miR-223 expression is frequently downregulated in leukemia and HCC and elevated in colorectal and recurrent ovarian malignancies. MiR-223 downregulation has occasionally been linked to a large tumor burden, an aggressive illness, and a dismal prognosis for the patient. Therefore, it is essential to comprehend the complex role that miR-223 plays in cancer diagnosis and therapy. Numerous studies have examined the function of miRNAs, including miR-122, miR-223, and others, in various cancer types[13]. Aim of this study investigating the expression levels of two miRNA genes (miRNA-122) in serum samples from Hepatocellular carcinoma patients and Hepatitis B and C patients by RT-PCR, analyzing the blood and biochemical test results of patients.

METHDOLOGY

Specific primers of microRNAs

The primers varied in length from 18 to 23 nucleotides, whereas the PCR amplicons ranged in length from 75 to 150 base pairs.

Table 1: Design primer utilized in the molecular investigation

Primer	Sequence	Origin
miR-122 RT	5′GTCGTATCCAGTGCAGGGTCCGAGGTG CACTGGATACGACCAAACAC 3′	Applied Biological Materials Macrogen – South Korea
miR-122F	5′ TGCGGTTGGAGTGTGACAATGG 3′	
miR-122R	5′ CAGTGCAGGGTCCGAGGT 3′	
U6-F	5′ GAGAAGATTAGCATGGCCCCT 3′	
U6-R	5′ ATATGGAACGCTTCACGAATTTGC 3′	

Sample collection

Samples from around 75Iraqi patients in hospitals in Baghdad were meticulously collected. Three groups were created from the patient samples: thirty samples from patients with HCC and five samples from people with liver cancer who were not treated. A total of 10 patient samples infected with HBV, 10 patient samples infected with HCV, and 20 samples from healthy individuals between February 2023 and July 2023 were collected. HCC was diagnosed clinically, and laboratory testing was conducted. Two groups of patients—those receiving treatment and those who were not—were established. Next, a sample of blood was drawn from 24 patients of both genders with varying age. A few of them had recently received a diagnosis of HCC, but they had not yet started treatment. In addition to chemotherapy medications, such Taxotere, ADM, Zometa, carboplatin, and Herceptin SC 600 mg, Using Gemzar, Endoxan, Taxol, and Herceptin IV 440 mg, some patients with advanced cancer received therapy with external beam radiation. Patients with hepatitis C virus were isolated from patients infected with hepatitis B virus (10), hepatitis C virus (10), and hepatitis C virus (six males and four women). Yarmouk Teaching Hospital, University of Baghdad's College of Science for Girls, and Medical City's Oncology Teaching Hospital. These people had no prior medical history of hepatitis B or C virus infection or liver cancer. Additionally, they did not exhibit any signs of viral infections such hepatitis B or C or hepatocellular cancer.

Molecular study

Quantification of microRNAs

According to the manufacturer's protocol. Single- and double-stranded RNAs, siRNAs, and microRNAs were easily identified with the use of this miRNA quantification kit. Salts, solvents, and detergents are among the impurities it can withstand. It is very selective for short RNA over big mRNA or rRNA

Specific primers of MiRNAs

Primers (miRNA-122) with melting temperatures ranging from 60°C to 95°C were developed and manufactured by Applied Biological Materials (Macrogen)/South Korea for use in laboratory experiments. The PCR amplicons ranged in length from 75 base pairs to 150 base pairs, and the primers had a length of 18 to 23 nucleotides.

Amplification of Housekeeping Gene

Little nuclear housekeeping genes, such as RNAU6, were utilized as an internal control to determine the Ct value [14]. In order to amplify RNAU6, the Real Time-PCR experiment was run using the temperature profile given in table (2).

Table 2: The program for Real-time PCR was setup with indicatedthermocycling protocol

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95 ° C	8 mins	1
Denaturation **Extension**	95 ° C 60 ° C	15 seconds 30 seconds (+plate read)	50
Melt Curve	60°C-90°C	40 minutes	1

*The Livak formula was used to collect and analyze the result.

Calculate the Gene Expression

By applying the relative cycle threshold (2^-ΔΔCt) method, which was initially presented in fold fluctuations of the mature RNAs' measured expression[15].

This ratio was determined by comparing the relative gene expression levels of the test and control groups. No change is indicated by a 1-fold change, up-regulated or increased gene expression is shown by numbers larger than 1, down-regulated or reduced gene expression is indicated by numbers between 0 and 1. By establishing the proper thresholds, the target genes' expressions were standardized in order to acquire exact Ct values from the RT-qPCR apparatus.

ΔCt

This "normalized raw data," which is the required normalization factor, was computed from the Ct value of each gene of interest. ΔCt = Ct (Interesting Gene) - Ct (Maintenance/Reference) Gene The ratio of expression was **ΔΔCt**

Transcript levels were compared between many samples using the 2-ΔΔCt technique[15]. The ΔΔCt value was computed by subtracting each test group's ΔCt values from the control group. ΔΔCt is equal to ΔCt (control) - ΔCt (patient).

The fold change value was then calculated using the following equation: The target gene was up-regulated in the case of positive fold change findings and down-regulated in the case of negative fold change results. Because of this, the data were shown as fold changes from the control sample, which was thought to reflect the typical value [16].

Biostatistical analysis

The following tools and tests were used in statistical analysis to compare the relative fold changes in gene expression for each illness between patient samples and healthy control samples:

1. Graph Pad Prism (version 8.0.0 for Windows, Graph Pad Software, San Diego, California, USA; www.graphpad.com) was used to conduct unpaired t tests.
2. Tukey multiple comparisons test was conducted after two-way ANOVA using Graph Pad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com).

RESULTS

Expression of microRNA-122 in HCC

According to the RT-qPCR data, miR-122 levels were lower in HCC patients than in healthy controls. As can be shown in Figure (1), the relative fold change average of miR-122 in the HCC patients was 0.15 [Table (3)] in comparison to the control (1). It was determined that differences between the HCC and healthy control samples were statistically significant when P <0.00001. Dysregulation and abnormal expression of miR-122 have been associated with a variety of cancer types, suggesting the potential use of miR-122 as a biomarker for diagnosis or prognosis.

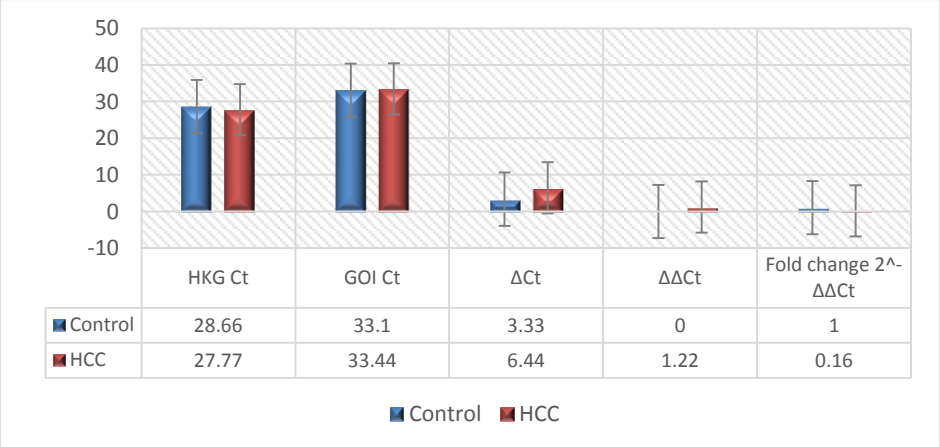

Fig. 1: Relative fold change utilizing RT-qPCR between samples from healthy controls and those from patients with HCC.

Table 3: Value of ΔCt, ΔΔCt, and fold change 2^-ΔΔCt for housekeeping gene and gene of interest microRNA122 in control samples and patients with HCC.

Sample 122	HKG Ct	GOI Ct	ΔCt	ΔΔCt	Fold change $2^{\wedge-\Delta\Delta Ct}$	Pvalue
Control	28.66	33.1	3.33	0	1	0.0001****
HCC	27.77	33.44	6.44	1.22	0.16	

MicroRNA-122 expression in HBV

Both Figure (2) and Table (4) show the relative fold change (RT-qPCR) of the miR-122 gene expression data comparing samples from HBV patients and healthy control samples.

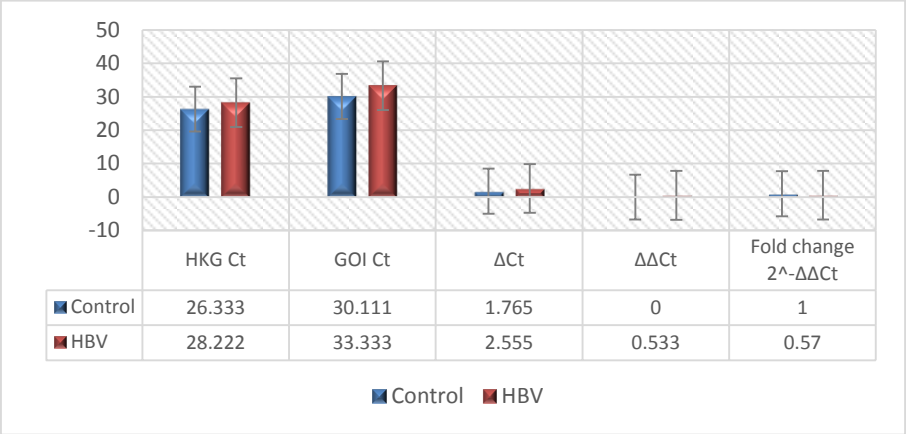

Fig. 2: Relative fold change between samples from healthy controls and those from HBV patients as determined by RT-qPCR

Table 4: Value of ΔCt, ΔΔCt, and fold change 2^-ΔΔCt for housekeeping gene and gene of interest microRNA122 in control samples and patient with HBV.et al., 2012).

Sample122	HKG Ct	GOI Ct	ΔCt	ΔΔCt	Fold change $2^{\wedge-\Delta\Delta Ct}$	P value
Control	26.333	30.111	1.765	0	1	0.0001****
HBV	28.222	33.333	2.555	0.533	0.57	

MicroRNA-122 expression in HCV

Both Figure (3) and Table (5) show the relative fold change (RT-qPCR) of the miR-122 gene expression data comparing samples from HCV patients and healthy control samples. .

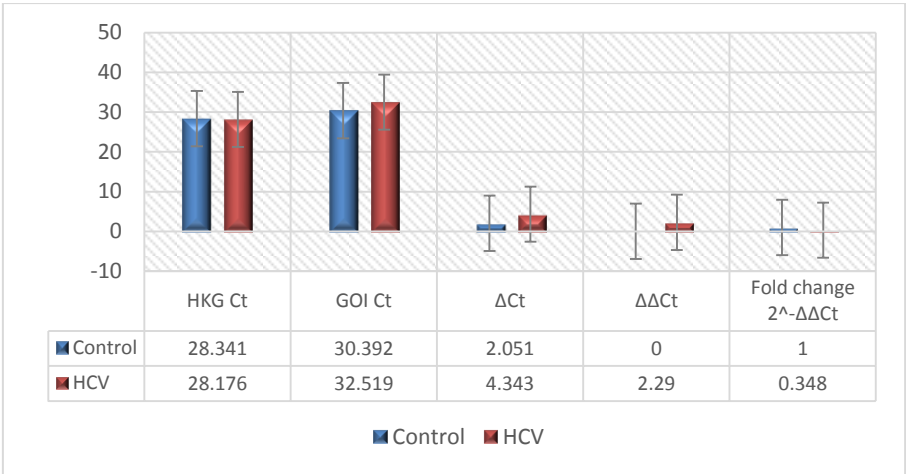

Fig. 3: Relative fold change, as determined by RT-qPCR, between samples from HCV patients and samples from healthy controls.

Table 5: Value of ΔCt, ΔΔCt, and fold change 2^-ΔΔCt for housekeeping gene and gene of interest microRNA122 in control samples and patients with HCV

Sample122	HKG Ct	GOI Ct	ΔCt	ΔΔCt	Fold change $2^{\wedge-\Delta\Delta Ct}$	P value
Control	28.341	30.392	2.051	0	1	0.0001****
HCV	28.176	32.519	4.343	2.29	0.348	

In order to present the complete picture of every HCC sample on a single graph, the data were shown as a heat map. Hierarchical grouping was utilized to construct the heat maps using GraphPad Prism, a freeware application. The total number of differently expressed miR-122 and miR-223 for all patient samples with HCC compared to healthy controls is shown graphically in Figure (3). Different colors are used to display values. A heat map is a two-dimensional data representation where values are represented colors. Apply the previously described relative fold change approach.

Biochemistry Parameters in Patients with HCC

In HCC patients, Table (6) displays the distributions of normal (8. %) and abnormal (92%) indirect bilirubin. These differences are statistically significant (P=0.002). Furthermore, a substantial connection was seen between normal (52.0%) and abnormal (48%) levels of creatinine and GOT (AST)

Table 6: Statistical correlation between biochemistry parameters of HCC

AnalysisName	Normal	Percentage %	Abnormal	Percentage %	Total	P value
Blood Sugar	18	72%	7	28%	25	0.8111
Urea	20	80%	5	205	25	0.499
Creatinine	13	52%	12	48%	25	0.0344*
GPT (ALT)	22	88%	3	12%	25	0.877
GOT(AST)	16	64%	9	36%	25	0.0399*
Alkaline Phosphatase	19	76%	6	24%	25	0.876
Total Bilirubin	18	72%	7	28%	25	0.765
IndirectBilirubin	2	8%	23	92%	25	0.002***
Chi Square	$P = 1.45x10^{-9}$					

Biochemistry parameters in patients with HBV

Table (7) demonstrates that, in comparison to normal persons, HBV patients have higher levels of GPT (ALT), GOT (AST), Alkaline Phosphatase, and Indirect Bilirubin, all of which have extremely significant p values ($p \leq 0.001$). While GOT (AST) is normal (20.00%) and abnormal (90.00%), GPT (ALT), Alkaline Phosphatase is normal (10%) and abnormal (90%) , and Indirect Bilirubin are abnormal (80%) and normal (20.00%). The other factors, however, did not significantly correlate with one another. Each of these tests serves as a gauge for liver function.

Table 7: Correlation between biochemistry parameters and HBV infection

AnalysisName	Normal	Percentage %	Abnormal	Percentage %	Total	P value
Blood Sugar	9(10)	90%	1(10)	10%	10	0.888
Urea	8(10)	80%	2(10)	20%	10	0.077
Creatinine	4(10)	40%	6(10)	60%	10	0.0511*
GPT (ALT)	1(10)	10%	9(10)	90%	10	0.002***
GOT(AST)	2(10)	20%	8(10)	80%	10	0.003**
Alkaline Phosphatase	1(10)	10%	9(10)	90%	10	0.002***
Total Bilirubin	7(10)	70%	3(10)	30%	10	0.666
Indirect Bilirubin	2(10)	20%	8(10)	80%	10	0.002***
Chi Square	$P = 3.806\times10^{-9}$					

Analysis of the Biochemistry of Patients with HCV

Table (8) indicates that GOT (AST), Total Bilirubin, and Indirect Bilirubin are higher in HCV patients. a linked significant P value of (P=0.002) and an abnormal (90%) to normal ratio of (10.00%). In GPT (ALT), the normal percentage is30.00%, whereas the abnormal proportion is 70.00%. Similarly, in Alkaline Phosphatase, the normal percentage is 30.00%, while the abnormal percentage is 70.00%. The two metrics did not, however, significantly correlate with one another.

Table 8: The correlation coefficient between biochemistry parameters relatedto HCV.

Analysis Name	Normal	Percentage %	Abnormal	Percentage %	Total	P value
Blood Sugar	9(10)	90%	1(10)	10%	10	0.888
Urea	8(10)	80. %	2(10)	20%	10	0.866
Creatinine	4(10)	40. %	6(10)	60. %	10	0.0255*
GPT (ALT)	3(10)	30. %	7(10)	70. %	10	0.008884**
GOT(AST)	1(10)	10%	9(10)	90%	10	0.001***
Alkaline Phosphatase	3(10)	30%	8(10)	70.00%	10	0.003**
Total Bilirubin	1(10)	10 %	90(10)	90%	10	0.002***
Indirect Bilirubin	0(10)	0%	10(10)	100%	10	0.001***
Chi Square	$P = 2.805\times10^{-9}$					

DISCUSSION

Using in-situ hybridization in zebrafish, it was discovered that additional species shared the distinct liver-specific expression pattern of miR-122. The liver has a conserved target area for hepatocyte nuclear factor 4, a liver-enriched transcription factor that stimulates the production of miR-122. On the other hand, miR-122, which is expressed at a frequency of around 66,000 copies per adult liver cell, is one of the most common miRNAs in the body. Since every species in which miR-122 has been discovered has a conserved sequence, every aspect of its function is essential to the living thing[12,17]. Downregulation of the tumor suppressor miRNA-122 in HCC promotes tumor growth, metastasis, treatment resistance, and apoptosis. According to a few ideas, miR-122's deregulation and aberrant expression in carcinogenesis and tumor growth may make it useful as a diagnostic and/or prognostic marker for human cancer18. Additionally, MiR-122 has been proposed as a biological marker for prognosis and diagnosis, as well as a possible novel target for cancer treatments. Tumor cells that are susceptible to treatment could respond to MiR-122 12 more readily.

MiR-122 has been linked to hepatic illness and physiological processes according to Ma et al.[19]. Stated that miR-122 inhibits hepatocyte growth, malignant transformation, and HBV expression and replication. Compared with healthy controls, patients with HBV infection have a substantial decrease in the expression of miR-122, and a negative correlation was found between the levels of miR-122 and the intrahepatic viral load and hepatic inflammation [19]. When natural miR-122 was decreased using its antisense inhibitor, HBV replication increased; however, when miR-122 was overexpressed using an expression vector or a mimic, viral replication was controlled. The effect of miR-122 on liver disease and hepatic function led researchers to investigate its potential influence on HBV replication and expression. HBV expression is inhibited by miR-122 transfection, but HBV production is increased by miR-122 antisense suppression[20]. In preclinical settings, it has been demonstrated that the most sensitive and accurate way of differentiating between intrahepatic and extrahepatic damage is by the levels of the more prevalent miRNA in the liver, liver-specific miR-122, in circulation. It is now possible to detect newborns infected with HBV using early miRNA testing. MiRNAs can be used to track the course of the disease and evaluate how well antiviral therapy is working. They could also function as undiscovered biomarkers for HBV-induced hepatocellular cancer [9].

On the other hand, no significant link was seen with other factor. This study supports the findings of Mehinovi and colleagues[16]. Who discovered that men with HCC have greater blood bilirubin levels (total, direct, and indirect and amyloid particle presence than women. The findings of this investigation align with those of Lala and associates [21,22,23]. They observed in their study that these tests might help identify the location of liver injury and could help develop a differential diagnosis based on the pattern. Elevations of ALT and AST that are not proportionate to increases in bilirubin and alkaline phosphatase are indicative of hepatocellular illness. Elevated bilirubin and alkaline phosphatase values in relation to ALT and AST are indicative of a cholestatic pattern[24]. Serum AST and ALT were statistically substantially higher in the HCV +ve group ($P<0.001$) when comparing the control group with the HCV +ve group. On the other hand, the HC +ve group had a considerably reduced prothrombin concentration ($P= 0.001$). Clotting factors by the liver is a key indicator of its health.

CONCLUSIONS

MicroRNAs are therefore a valuable tool for early diagnosis as well as for tracking the progression and recurrence of illnesses due to their high levels of accuracy and reliability. Furthermore, in those with a history of HBV or HCV, these biomarkers may serve as a predictor of the development of HCC. Our findings indicate that miR-122 expression is decreased in HCC, HBV, and HCV patients compared to healthy controls. Compared to treated HCC patients and healthy controls, the expression of HCC patients who had not received therapy was much lower.

Recommendation

Making use of these microRNAs as biomarkers for disease development, early identification, and post-treatment recurrence. Furthermore, these biomarkers showed excellent sensitivity and accuracy, and it was advised that patients have follow-up testing both during and after therapy.

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None

Conflict Of Interest

The authors have declared that no conflict of interest exists

FUNDING

None

Ethics Statement

The Ministry of Higher Education and Scientific Research, University of Anbar, Scientific Study Ethics Committee, Number 144, Dated 1-5-2022. The Institutional Review Board approved experimental investigations involving humans or animals before the research began and samples were collected. In order to protect patient confidentiality and identity, no pictures of patients, healthy people, or parts of them were used in**.**

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