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Molecular and biochemical analysis of MicroRNA-125b and MicroRNA489-3p in chronic hepatitis B virus

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ABSTRACT

Hepatitis B virus (HBV) is a blood-borne virus that poses chief public health threats, and it is the frequent reason for cirrhosis all over the world. MicroRNA could be used as a disease biomarker. This investigation was carried out to investigate mRNA -125b, mRNA 489-3p, and biochemical analysis as markers for chronic HBV. This work included 20 patients who had chronic liver disease admitted to the Gastroenterology Hospital and Oncology Institute in Mansoura. After investigation, serum samples were obtained from five patients that were positive for PCR chronic HBV. For control, negative serum samples were collected at the same age range and sex. Liver function and alpha fetoprotein (AFP) parameters were estimated. RNA was extracted from serum for gene expression of mRNA125b and mRNA 489-3p. The main results showed that the HBV group had a significant increase in ALT, AST, TBIL, GGT, ALP, and AFP, while there was a significant decrease in albumin levels compared to the control group. HBV group showed a significant increase (6.70 ± 0.18) in mRNA125b, but a significant decrease in mRNA489-3 (0.51±0.03). In conclusion, mRNA125b and mRNA489-3p expressions along with biochemical analysis could be used as accurate markers for HBV diagnosis.

1. INTRODUCTION

Hepatitis B (HBV) is a blood-borne viral infection that has a high global prevalence, WHO estimates that 296 million People (Razavi-Shearer et al., 2018), and can cause both acute and chronic liver damage and cirrhosis. After a persistent infection has been established, HBV-infected patients are at risk of developing complications. About 30% of patients will develop liver cirrhosis, and about a quarter of cirrhotic patients will acquire decompensated liver disease within 5 years. Cirrhosis also raises the risk of liver failure and hepatocellular carcenoma (HCC) significantly, which are the most prevalent causes of HBV-related death (Ismail et al., 2017; Lim et al., 2020) .

In Egypt, viral hepatitis is a major public health issue, with an estimated 8 to 10 million persons, or 10% of the population, living with the illness and millions more at risk for infection. Statistics of the Egyptian Ministry of Health estimates that In Egypt, 150, 000 new cases of viral hepatitis are diagnosed each year (Elbahrawy et al., 2021). Patients can go years without showing symptoms, but they can unknowingly spread the virus to others through perinatal, percutaneous, and sexual contact, as well as close interpersonal interaction (e.g., open cuts and sores) (Terrault et al., 2018). Depending on viral features and host-related variables, between 8% and 20% of untreated HBV-associated fibrosis patients can advance to liver cirrhosis in five years (age, gender, other comorbidities, or coinfections).

The HBV genome is extremely compact, with the majority of nucleotides used in many overlapping open reading frames (ORFs). Protein-primed reverse transcription, which is linked to but mechanistically separated from retroviral replication, is a characteristic of HBV replication. Interestingly, all viral transcripts have an identical polyadenylation signal, and any smaller transcript's sequence is found within any bigger transcript (Madihi et al., 2020).

The clinical course of hepatitis B is dynamic and differs greatly between patients, ranging from self-limited infection following acute hepatitis to chronic infection with clinical manifestations ranging from asymptomatic carrier state to severe disease manifestations such as active chronic hepatitis, liver cirrhosis, and HCC (Seeger et al., 2000).

MiRNA expression levels in HCC patients with diverse etiological variables may differ (Ladeiro et al., 2008). Because HBV is the most common cause of HCC, this study on miRNAs implicated in HBV-related HCC (HBV-HCC). Cellular miRNAs influence both viral replication and the immune antiviral response. Viruses can produce their miRNAs and control the cellular miRNome to favor viral multiplication or latency. Because of their varied actions, miRNAs have been studied as biomarkers for diagnosis, prognosis, and treatment of viral infections, as well as a range of disorders, including liver fibrosis. (Iacob et al., 2020).

DNA methylation and histone modification are crucial for chromatin remodeling and the control of both coding genes and miRNAs. Several miRNAs have abnormal DNA methylation patterns in HCC. Among these miRNAs, only

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miR-125b has a consistent dysregulation pattern of expression and was up-regulated in both tissue (Burchard et al.,2010) and serum (Liu et al.,2017) of HCC patients. The methylation inhibitor 5-aza-2'-deoxycytidine dramatically boosted miR-125b expression in viral hepatitis and HCC cells, indicating that miR-125b expression is epigenetically modulated (Alpini et al., 2011). Because SIRT7 is targeted by miR-125b, it is possible that miRNA-based therapies might be employed to treat HCC. Kim and colleagues (2015). Several miRNAs were found to be expressed at various levels in the blood or liver tissue, and these levels were linked to patient survival and disease severity in HBV-HCC patients. MiR-125b has been connected to longer survival. In individuals with chronic HBV, miRNA-125b has been shown to play a significant role in controlling the hepatic pro-inflammatory response and to correlate with both viral replication and liver necroinflammation (Giray et al., 2014).

MiRNA489-3p is confirmed for its tumor-suppressive effect in several types of cancers including hepatic carcinoma (Chen et al., 2015). In *vitro* chronic HBV infection, MiR 489-3p was significantly reduced, but JAG1 was significantly upregulated.

The purpose of this research was to examine the value of Micro RNA-125b and Micro RNA 489-3p, together with other biochemical analyses, as trustworthy diagnostic markers for the identification of HBV.

2. MATERIAL AND METHODS

The current investigation follows a cross-sectional design which received approval from the Gastroenterology Hospital in Mansoura and Oncology Institute in Mansoura. Aconcent was obtained from all patients,and the study was carried out according to the institutional committee for human studies,The Mansoura Gastroenterology Hospital admitted 5 patients from the 20 patients with chronic liver disorders, were positive PCR chronic HBV. Serum samples were obtained from those patients who ranged in age from 30 to 50 and weight from 60 to 90 kg. From the control healthy group at the same age range and sex., five samples of negative serum were taken.

2.1. Biochemical Analysis:

All five HBV patients and the control group provided serum samples for the following investigations: Liver function parameters included serum alanine aminotransferases (ALT) (Ookoian and Pirola ,2015), aspartate aminotransferase (AST) (Ookoian and Pirola ,2015),Total bilirubin (T. Bil) (Pagana et al., 2019),Dirct bilirubin (D. Bil) (Pagana et al., 2019). γ-Glutamyltransferase (γ-GGT) (Beleta and Gella, 1990) alkaline phosphatase (ALP) (Harris, 1989) and Albumin (ALB) (Young, 2001). In addition to the analyses of tumor marker indicators utilizing alpha-fetoprotein (AFP) (Zhang et al., 2020).

2.2. Molecular analysis:

- RNA extraction:

The RNeasy Mini kit Catalogue no.74104 To extract RNA from enzymatic procedures and crude RNA preparations (for example, DNase digestion, proteinase digestion, RNA ligation, and labelling reaction). Applichem ethanol, 96 percent (thinned down to 70% using DDW).

Material used for mastermix preparation for SYBR Green real time PCR.

- Quantitect SYBR green PCR kit (Cat. No. 204141)
- RevertAid Reverse Transcriptase (Thermo Fisher) (200 U/µl).
- Oligonucleotide primers and probes utilized in SYBR Green real time PCR Molecular analysis (Table 1).
- Real time PCR machine (Stratagene MX3005P)

Table 1 Oligonucleotide primers and probes.

Gene		Primer sequence (5'-3')	Ref.
U6	F	GCTTCGGCAGCACATATACTAAAAT	Chen et al.
(housekeeping)	R	CGCTTCACGAATTTGCGTGTCAT	2003
miR-25b	F	CCCCCGCTAGCTCTTGTTTTGCTTTGC TTTGTC	Chen et al., 2019
	R	CCCGAATTCACCAAATTTCCAGGATGCAA	
miR-89-3p	F	CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA	Zheng and Chen, 2020
	R	GTT GAG AGC TGC CGT	

SYBR green rt-PCR were analyzed as:

The Stratagene MX3005P software was used to compute ct values and amplification curves. The ct of each sample was compared to the ct of the control group to measure the difference in gene expression on RNA of diverse samples using the "ΔΔCt" technique proposed by Yuan et al. (2006) using the following ratio: ($2^{-\Delta\Delta ct}$).

Meanwhile, ΔΔCt = ΔCtreference – Δcttarget.

Δ*Ct target = Ct control – Ct treatment* and Δ*Ct reference = Ct control- Ct treatment.*

2.3. Statistical analysis:

Biochemical results were expressed as mean ± standard error of mean. using the Statistical Package for Social science Software (Version 18, SPSS Inc., USA). A significant difference was used at the $P < 0.05$ probability level.

3. RESULTS

3.1. Biochemical analysis:

Table (2) displays the findings of the biochemical investigation of liver function. In contrast to the control group, the HBV group displayed a significant increase in ALT, AST, GGT, and ALP activity, a significant increase in TBil, DBil, with a significant decrease in albumin (ALB), but HBV group showed a significant increase (15.90 ± 0.35ng/ml) in AFP amount contrasted with control (3.28 ± 0.21 ng/ml). HBV group showed a significant elevation (73.08 ± 6.76 U/L) ($P < 0.001$) in ALT activity compared to control group (23.79 ± 1.60 U/L)

3.2. Molecular analysis:

The relative expression level of "miRNA-125b" and "miRNA-489-3P" genes, in liver of control and HBV groups of patients (Table 3). Concerning miR-125b gene expression of HCV group displayed a substantial rise (6.70 ± 0.18) ($P< 0.05$) in mRNA125b as opposed to control (1.00 ± 0.05). However, miR-489-3p gene expression displayed that HBV group had a significant decrease in mRNA489-3p (0.51 ± 0.03) ($P< 0.05$) in comparison with control group.

Table 2 The biochemical analysis of the chronic hepatitis B virus (HBV) group and comparing to control group.

Parameter	Unit	Patients groups	
		Control healthy group	HBs group
ALT	(U/L)	23.79 ± 1.60	73.08 ± 6.76***
AST	(U/L)	1.30 ± 31.12	55.52 ± 6.40*
ALP	(U/L)	52.20 ± 4.45	*4.35 ± 84.14
γ-GGT	(U/L)	27.59 ± 2.85	56.52± 4.25***
Albumin	(g/dl)	4.23 ± 0.21	3.07 ± 0.18*
T. Bili	(mg/dl)	0.90 ± 0.04	1.48 ± 0.11*
Direct Bili	(mg/dl)	0.23 ± 0.05	0.45 ± 0.07
AFP	(ng/ml)	3.28 ± 0.21	15.90 ± 0.35*

Data are presented as (Mean ± S.E), S.E = Standard error. Represents statistically Significant at $P < 0.05$)

Table 3 The relative expression level of "miRNA-125b" and "miRNA-489-3P" genes, in liver tissue of control and HBs groups of patients.

Parameter	Unit	Patients groups	
		Control healthy group	HBs group
MiRNA-125b	Fold change (Mean ± SE)	1.00 ± 0.05	6.70 ± 0.18***
	Average ct	23.04	19.47
MiRNA-489-3P	Fold change (Mean ± SE)	1.00 ± 0.06	0.51±0.03***
	Average ct	25.94	30.85

Data were statistically analyzed as (Mean ± S.E), S.E = Standard error. *** Represents statistically Significant at ($P < 0.05$)

4. DISCUSSION

HBV has a restricted host range and only normally affects humans. Chronic HBV is one of the leading etiology of hepatic fibrosis (Iacob et al., 2020). The detection of miRNAs as mediators of RNA-induced silencing has generated a motivating novel investigation into miRNAs' role in cell physiology (Lamontagne et al., 2015). miRNAs may play a role in the complicated relationship between the HBV and the host, according to evidence (Winther et al., 2014) .

The results in table (2) showed that the hepatic enzymes were employed as indications of liver damage (Parikh et al., 2017). This study showed a significant increase in ALT, AST, ALB, TBIL, DBIL, GGT, AFP, and ALP activity, with a significant decrease in albumin concentration in the HBV group compared to the control group. These results were in accordance with Liu et al. (2019) in terms of ALT, AST, and TBIL. Also, Al-Madany and Sarhat (2018) showed a statistically significant increase in the concentration of T. BIL with ALT, AST, GGT, and ALP activity among viral hepatitis patients. Coman et al. (2015) also revealed a significant increase in ALT, AST and ALP activities. These increases indicate a significant risk for the progression of the disease and the development of complications like cirrhosis and HCC among HBV-infected patients (Khan et al., 2021). ALT is the most accurate biochemical assay for diagnosing liver damage in individuals with acute and chronic viral hepatitis because it has a cytoplasmic distribution and a longer half-life in the blood than AST (Kalu et al., 2014) .

Furthermore, former studies (Sehrawat et al., 2006; Mahmoud, 2011) recorded a considerable rise in serum AST, ALT, and ALP that may be caused by liver cell death, which leads to the leakage into the bloodstream. Serum AST, ALT, and GGT are sensitive indicators used in the detection of liver illnesses. However, when the plasma membrane of the liver cell is destroyed, several enzymes typically found in the cytosol are released into the blood. Prati et al. (2002) stated that elevations of serum alanine aminotransferases (ALT) and aspartate aminotransferase (AST) activities are important markers for liver injury.

The ALP and GGT levels increase in chronic liver diseases. This rise is due to greater synthesis and release of the enzyme into serum rather than reduced biliary secretion since enhanced cholestasis stimulates the bile ductules' cell to create ALP, which gives more ALP that eventually enters the circulation. The amphiphilic character of bile salts facilitates the release of ALP from its membrane-bound location and entry into the bloodstream (Lowe et al., 2017). Also, levels of serum bilirubin increase significantly and remain elevated in patients with liver failure (López-Velázquez, 2014). Because bilirubin is processed in the liver and secreted through the biliary ducts, any failure in hepatocytes causes an increase in TBIL, but it is only modestly raised in chronic HBV infection (Hassan and Monem, 2013). However, a low serum albumin level is a sign of a liver that is not functioning properly. The most prevalent cause of reduced albumin is cirrhosis-related chronic liver failure caused by chronic liver disorders such as HBV. Because it takes many weeks for blood albumin levels to fall following reduced albumin synthesis, decreasing serum albumin levels are observed in acute liver failure (Al-Madany and Sarhat, 2018) .

Although it has been clinically stressed that AFP levels are susceptible to hepatic inflammation, inconsistent with the present results, Jasirwan et al. (2020) revealed that the cause was among the statistically significant influences ($P = 0.011$) in the possibility of making amounts of AFP in patients with HCC to elevate above 10 ng/ml. rising AFP levels in HBV-infected patients could be used as a diagnostic biomarker for the identification of hepatocellular carcinoma.

The results are in table (3) in line with earlier research, adult chronic HBV patients' serum miRNA-125b was linked with viral loads. The *in vitro* outcomes confirmed these clinical results. Furthermore, miRNA-125b level in immune-tolerant individuals was less than it was in immune-reactive patients, indicating that Hepatitis B (HBV) and serum HBV DNA positively related with miRNA125b. Previous research has suggested that an increase miR-125b is connected to HBV replication and liver necroinflammation (Li et al., 2016) and the reason for chronic HBV infection (via regulating BsAg expression) (Zhang et al.,2014). Because miR-125b affects the production of HBV DNA intermediates as well as the release of HBsAg and HBeAg (Ninomiya et al., 2016). MiR-125b can also control multiple oncogenes, which are linked to HBV hepatocarcinogenesis, implying that miR-125b plays a role in HBV hepatocarcinogenesis. MiR-1, miR-21, and miR-125b expressions in peripheral blood immune cells were considerably higher in chronic HBV infection patients compared to controls, and this expression was responsible for decreased immune responses in chronic HBV carriers. By targeting the sodium channel epithelial 1 subunit, miR-125b suppresses the production of HBV DNA intermediates as well as the release of HBsAg and HBeAg (Ninomiya et al., 2016). The varied roles of miR-125b in HBV-related liver disorders imply that miR-125b levels may have clinical significance. However, the relevance of these miRNAs in chronic HBV infection is still unknown and has to be investigated further (Momeni et al., 2014). miRNA-125b and miRNA-124 both correlated with liver necroinflammation, only miRNA-125b also correlated with viral replication. Wang et al. (2015) found that miRNA-125b was superior to miRNA-124 and ALT in differentiating grades of liver necroinflammation in patients with CHB.

Le et al. (2009) reported that overexpression of miRNA-125b may repress the endogenous level of p53 protein and then suppress apoptosis in human neuroblastoma cells and lung fibroblast cells. Therefore, miRNA-125b may repress the level of p53 protein and then correspondingly enhance HBV replication. The counteraction of miRNA-122 and miRNA-125b on HBV replication may contribute to viral persistence and liver histopathological lesions. Chen et al. (2011) found that persistent HBV infection can lead to cirrhosis and hepatocellular carcinoma (HCC) with a high mortality rate. Long-term changes in both serum HBV DNA and ALT levels independently predict the risk for hepatocellular carcinoma. (Wong et al.,2004) reported that intrahepatic DNA levels correlate strongly with serum HBV DNA levels and with the degree of fibrosis, Advanced fibrosis, and severemiRNA-125b enhanced hepatitis B virus (HBV) replication.

In line with the findings of this study, in peripheral blood immune cells, miRNA 489-3p expression was significantly lower in patients with chronic HBV infection compared to controls, and the expression of these miRNAs was responsible for impaired immune responses in chronic HBV

carriers (Jin et al., 2007). By interacting with the 3'-UTR of ligand 1 (JAG1) in LX-2 cells, researchers discovered that miR489-3p was significantly reduced while JAG1 was upregulated in chronic HBV infection both in vivo and in vitro. Tsochatzis et al. (2014) found that miR 489-3p inhibits chronic HBV and HSC activation by blocking the JAG1/Notch3 signaling pathway. In a calcium clorid (CCL4) -induced fibrosis model, miR-489-3p expression was significantly reduced, while the expression of jagged canonical NOTCH ligand 1 (JAG1) was increased. Overexpression of miR-489-3p reduced the expression of pro-fibrosis markers and inhibited the activation of HSCs by inhibiting the JAG1/NOTCH3 signaling pathway (Dewidar et al., 2019). In the present study miR489-3p gene expression was significantly decreased among HBV patients.

5. CONCLUSIONS

From the outcomes of the current research, concluding that mRNA125b as well as mRNA489-3p's expression; along with various biochemical analyses can act as perfect diagnostic biomarkers for diagnosis of chronic HBV.

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