Relation Between Serum MicroRNA-122 Expression Level and Both Laboratory and Clinical Profile in Chronic HBV Patients

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Abstract

Background

MicroRNA-122 (miRNA-122) is involved in the pathogenesis of chronic liver diseases and it is assessed for ability to predict the liver injury caused by chronic hepatitis B (CHB) infection.

Methods

Serum miRNA-122 expression levels were assessed in 15 patients with CHB infection and 15 healthy controls by qRT-PCR. MiRNA-122 level between CHB patients and controls was compared and the relationship between the miRNA-122 and clinical parameters was performed. The receiver operating characteristic (ROC) curve was used to test the sensitivity and specificity of miRNA-122 in differentiating patients with CHB from controls.

Results

Compared with controls, the serum miRNA-122 relative expression was increased in CHB patients ($P < 0.001$). The level of miRNA-122 was 4.2-fold higher in the serum of CHB patients. There was a significant positive correlation between the serum expression level of miRNA-122 and ALT activity ($r=0.93$, $p<0.001$). PTT ($r=0.61$, $p=0.02$), AFP ($r=0.65$, $p=0.009$) in CHB patients was observed. Furthermore, at a cutoff value of 2.83, the sensitivity and specificity of miR-122 to predict liver injury resulted from CHB were73% and 100%, respectively.

Conclusion

Serum miRNA-122 expression is increased in adult CHB patients and could be used as a biomarker to diagnose liver injury resulted from CHB.

Keywords: chronic hepatitis B; microRNA-122; biomarker

Tob Regul Sci.™ 2022; 8(1): 324-334
DOI: doi.org/10.18001/TRS.8.1.30

1. Introduction

Hepatitis B virus (HBV) is a small, partially double-stranded DNA affecting many people worldwide. Global reports showed that HBV infects about 2 billion persons worldwide,15-25% of whom were reported as chronic hepatitis B (CHB). It is also estimated to cause approximately 887 thousand deaths every year due to hepatocellular carcinoma (HCC) or advanced cirrhosis. [1-3]

Although HBV is a non-cytopathic hepatotropic virus, the human immune response for HBV infection usually causes hepatic destruction through CD8 cells mediated destructive immunological mechanism against Hepatitis B antigen. The oncogenic disruption of cellular genes caused by HBV DNA incorporation into the hepatocyte genome causes death and/or genomic instability. [4, 5]

Different markers such as HBsAg, HBcAg, HBeAg, HBsAb, anti-HBc, anti-HBc IgM, and anti-HBe are involved in the initial assessment of HBV infection. Other serological diagnostic assays, such as enzyme and chemiluminescenceimmunoassays, are used for HBV assessments.[6]

Currently, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), enzymes released from the liver in response to damage and disease, are used to determine the severity of liver fibrosis. More recently, the molecular diagnostic methods as UltraQualandCOBAS AmpliScreen HBV tests are FDA approved and mainly used for diagnosis of HBVthroughHBV DNA quantification, genotyping, monitoring antiviral therapy, and mutation analysis. [7, 8]

MicroRNAs (miRNAs) are single-stranded non-coding RNAs that play a key role in gene regulation by binding to the 3′-untranslational site on the target mRNA and causing either protein synthesis inhibition or RNA destruction. [9] However, miRNAs have a role in the pathogenesis of chronic inflammation like hepatitis and malignancy. [10] MiRNA may also govern a variety of cell changes, indicating that miRNA gene expression has the potential to improve our understanding of HBV pathophysiology and pathogenesis, as well as create new HBV diagnostic and therapeutic approaches. [11]

It was confirmed previously that dysregulation of miRNA function commonly occurs in all the stages of liver cell damage. Additionally, different miRNAs detected in the serum and tissue may have an important role in predicting HBV infection and its related complications. [12, 13] MicroRNA-122 (miR-122) represents 70% of the total miRNAs in the liver.[14]

Recent studies tested some valid miRNA markers on a variety of serums and plasmas from both HBV-infected patients and healthy people. A combination of these findings showed that variations in miRNA-122 plasma concentrations are related to disease severity and able to differentiate between HBV-infected patients and healthy peoples. [15-17] This study investigated whether the circulating miRNA-122 can be used as molecular biomarkers to predict liver injury by chronic hepatitis B (CHB).

2. Material and methods

Blood samples were collected from 30 subjects (15 patients with CHB infection and 15 healthy control subjects) attending the Tropical Medicine Department at Zagazig University Hospitals during the period from December 2019 to December 2020.

The Institutional Review Board at Zagazig University approved the study (IRB#4620). This study was conducted in accordance with the Declaration of Helsinki, and written informed consent was taken from each participant after explaining the nature of the study.

2.1. Case definition

Chronic hepatitis B patients defined as patients who were seropositive for HBVs-Ag for > 6 months and confirmed by quantitative PCR. Exclusion criteria included: (1) age <18 years, (2) patients with HBV/HCV or HBV/HIV coinfections, (3) patients who were previously or currently treated for HBV, HIV, HCV, or HCC, (4) patients with any malignancy other than HCC, (5) liver transplantation patients, and (6) osteoporotic bone fractures patients. Healthy controls were chosen with clinical laboratory investigations confirming no signs of HBV infection or co infections.

2.2. Laboratory and clinical data

10 ml of blood was withdrawn from each participant under complete aseptic conditions and centrifuged at 4000 rpm for 10 minutes for separation of serum. The serum samples were processed at the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University. For each participant, we performed the following tests: serum ALT(SGPT) and AST(SGOT) , total serum albumin and , total bilirubin using spectrophotometry on cobas c701 analyser (Roche diagnostics, Germany) using dedicated kits according to manufacturer instructions, alpha-fetoprotein(AFP) by electrochemiluminescence method using Roche Cobas 602 (Roche diagnostics, Germany) with dedicated kits according to manufacturer instructions, PT, PTT and INR using Sysmex CA1500 coagulometer (Sysmex corporation, Japan) using Siemens Thromborel and Siemens Pathromtin kits (Siemens healthineers, Germany) according to manufacturer instructions. HBV, HCV, and HIV serum viral load by quantitative PCR using Roche cobasTaqman 48 real time PCR analyser (Roche diagnostics, Germany) using dedicated reagents according to manufacturer instructions. Abdominal ultrasound was performed as well. Child-Pugh score was calculated as presented in **table (1).** The score values range from 5 to 15 points and are classified into 3 classes, A, B and C (Score A, 5 to 6 points, B,7 to 9 and C, 10 to 15).

Table 1: Child-pugh score calculation.

Factor	1 Point	2 Points	3 Points
Total serum bilirubin (mg/ml)	<2	2-3	>3
Serum albumin (mg/ml)	>3.5	2.8-3.5	<2.8
Prothrombin time (sec.)	<4	4-6	>6
Presence of ascitis	no	mild	Moderate to severe
Presence of encephalopathy	no	Grade 1 and 2	Grade 3 and4

2.3. Real-time PCR (RT-PCR)

Total RNA extraction from each serum sample was performed for isolation of miRNA using miRNeasy Mini Kit (Qiagen, Germany). To perform a reverse transcription (RT) reaction, the miScript II RT Kit was used. The master mix was prepared by adding the following ingredients: HiSpec Buffer (4µ, 5x), miScript Nucleics Mix (2µ, 10x),miScript Reverse Transcriptase Mix (2µ), template RNA (2µ), and RNase-free water (10µ).
RT-PCR semi-quantification of mature miRNA-122, using miScript SYBR Green PCR Kit (Qiagen, Germany) was applied using target-specific miScript Primer Assays. This step was conducted using two primers, one for miRNA-122 and the other for the human RNU6 as a reference gene for measurement of miRNA-122 relative expression according to kit manufacturer instructions. The qRT-PCR System (Applied Biosystems, USA) was used according to the manufacturer's protocols using the following cycling conditions; initial activation step at 95°c for 10 min followed by denaturation step at 95 °C for 15 seconds, then annealing at 55°C for 30 seconds, and finally the extension step at 70°C for 40 seconds.

2.4. Interpretation of PCR data

The cycle threshold (CT value) is defined as the number of cycles required for the fluorescent signals to cross the threshold in semi-quantitative PCR. Fold changes of miRNA-122 expression were estimated using Livak method.[18] At first normalization of microRNA-122 gene to the RNU6 housekeeping gene (relative expression ΔCT = CT of miRNA-122 gene – CT of RNU6 housekeeping. Then calculation of the ΔΔCT = ΔCT in HBV patients – ΔCT in the healthy control. The fold change ($2^{-\Delta\Delta CT}$) is the relative gene expression in which the positive value represents the number of fold increases in gene expression more than the control group, and the negative value represents the number of fold decrease in gene expression less than the control group.

2.5. Statistical Analysis

SPSS version 25.0 and MedCalc 10 Software were used to contact the statistical analysis of this study. Continuous variables were presented as mean, standard deviation (SD), and range. While the frequency and percentages were used to describe the categorical variables. Comparisons between continuous variables were made using Mann Whitney test. But, chi-square or Fisher's Exact tests were used to compare categorical data as applicable. Correlations between the expression levels of miR-122 and the laboratory data in HBV cases were done using Spearman's correlation coefficient. We used the receiver operating characteristic (ROC) curve to determine the best cutoff in the miRNA-122 fold change for HBV diagnosis. P-value <0.05 was considered statistically significant.

3. Results:

This study included 15 CHB patients and 15 healthy controls. The demographic and laboratory findings of the two groups are presented in **Table (2)**. The CHB group included 46.7% females and 53.3% males, while the control group included 40% females and 60% males. Serum levels of Albumin, AST, and ALT were significantly higher in CHB group compared to the control group (p <0.05). For the CHB patients, normal US findings were observed in most of the included patients (73%), while coarse cirrhosis was observed in

20% only. In addition, Child-Pugh score A was reported in 93% of the CHB patients **Table (3**).

The performed miRNA-122 analysis revealed a significant difference in serum miRNA-122 relative expression between CHB patients and healthy control (mean ΔΔCT (±SD) = -1.93 (±1.09) and 0.55 ± (1.5), respectively, P <0.001).The level of miRNA -122 was 4.2-fold higher in the serum of CHB patients(**Figure 1)**.

The relative miRNA-122 expression and ALT activity of each study participant in both CHB and control groups are shown in (**Figure 2)**. For the control samples, ALT activity, a biomarker frequently used for liver injury , was under the reference range of ALT with lower miRNA-122 gene expression, while, in the CHB groups, ALT activity was above the reference range of ALT with higher miRNA-122 expression.

The results showed a significant positive correlation between the miRNA-122 fold change and the ALT activity (r=0.93, p<0.001). PTT (r=0.61, p=0.02), AFP (r=0.65, p=0.009), and HBV viral load (r=0.54, p=0.04) as seen in **Table (4)**.

A ROC curve analysis was performed to assess the values of miR-122 as biomarkers for CHB (**Figure 3**). The best cutoff of miRNA-122 in the diagnosis of CHB is >2.83 with an area under curve (AUC) of 0.92 (95% CI [0.76 to 0.98]), sensitivity=73%, and specificity=100%. On the other hand, the best cutoff of ALT in the CHB diagnosis is >40 IU/L with an area under curve (AUC) of 0.86 (95% CI [0.69 to 0.96]), sensitivity=87%, and specificity=80%.

Table 2: Demographic and laboratory data of the included HBV cases and healthy controls.

	CHB Cases (n=15)	Control (n=15)	P-value
Age (year) Mean (±SD), [Range]	34.07 (±6.9), [21-45]	34.47 (±14.74), [18 - 60]	0.62
Gender, n (%) Female Male	7 (46.7%) 8 (53.3%)	6 (40%) 9 (60%)	0.71
T.bilirubin (mg/dl) Mean (±SD), [Range]	0.83 (±0.19) [1.97 – 1.21]	0.65 (±0.38) [0.27 – 1.7]	0.02
S.Albumin (mg/dl) Mean (±SD), [Range]	3.74 (± 0.7) [1.97 – 4.7]	4.31 (±0.42) [3.4 – 5]	**0.01**
AST (IU/L) Mean (±SD), [Range]	32.43 (±9.04) [21 – 56.1]	17.39 (± 9.9) [10 – 49.4]	**<0.001**
ALT (IU/L) Mean (±SD), [Range]	26.45 (±13.62) [12 – 53]	17.38 (±8.06) [8 – 35]	**0.04**
PT (sec.)	14.09 (±2.37) [12 – 21]	11.55 (±2.96) [1.4 – 13.6]	**0.01**

Mean (±SD), [Range]			
PTT (sec.) Mean (±SD), [Range]	31.85 (±12.03) [10 – 62.7]	27.93 (±4.22) [22.8 – 39]	0.26
INR Mean (±SD), [Range]	1.07 (±0.27) [0.9 – 1.89]	0.99 (±0.07) [0.86 – 1.1]	0.29
AFP (ng/ml) Mean (±SD), [Range]	3.35 (±1.97) [0.9 – 7.8]	--	--
HBV Viral load Mean (±SD), [Range]	882.87 (±1032.93) [240– 2600]	--	--

AFP: alpha-Fetoprotein, ALT: alanine aminotransferase, AST: aspartate aminotransferase HBV: hepatitis C virus,INR: international normalized ratio, SD: Standard deviation, PT: prothrombin time, PTT: partial thromboplastin time.
Mann Whitney test, independent t test, and Chai square test were used as appropriate**.**

Table 3: Clinical characteristics of the included HBV cases and healthy controls.

		HBV Cases (n=15)	Control (n=15)	P-value
US findings	Normal	11 (73.3.%)	15 (100%)	0.10
	Fine cirrhosis	1 (6.7%)	0	
	Coarse cirrhosis	3 (20%)	0	
Child Pugh score	A (5-6)	14 (93%)	15 (100%)	1.0
	B (7-9)	1 (7%)	0	
	C (10-15)	0	0	

Table 4: Correlation between MicroRNA-122and laboratory findings among the studied CHB cases.

Variable	r	P value
T.bilirubin (mg/dl)	0.45	0.09
S. albumin (mg/dl)	-0.48	0.07
PT (sec)	0.39	0.15
PTT (sec)	0.61	0.02
INR	0.19	0.49
AST (IU/L)	0.61	0.06
ALT (IU/L)	0.93	<0.001
AFP	0.65	0.009
HBV Viral load	0.54	0.04

AFP: alpha-Fetoprotein, ALT: alanine aminotransferase, AST: aspartate aminotransferase HBV: hepatitis C virus, INR: international normalized ratio, PT:

prothrombin time, PTT: partial thromboplastin time, and r: Spearman's correlation coefficient.

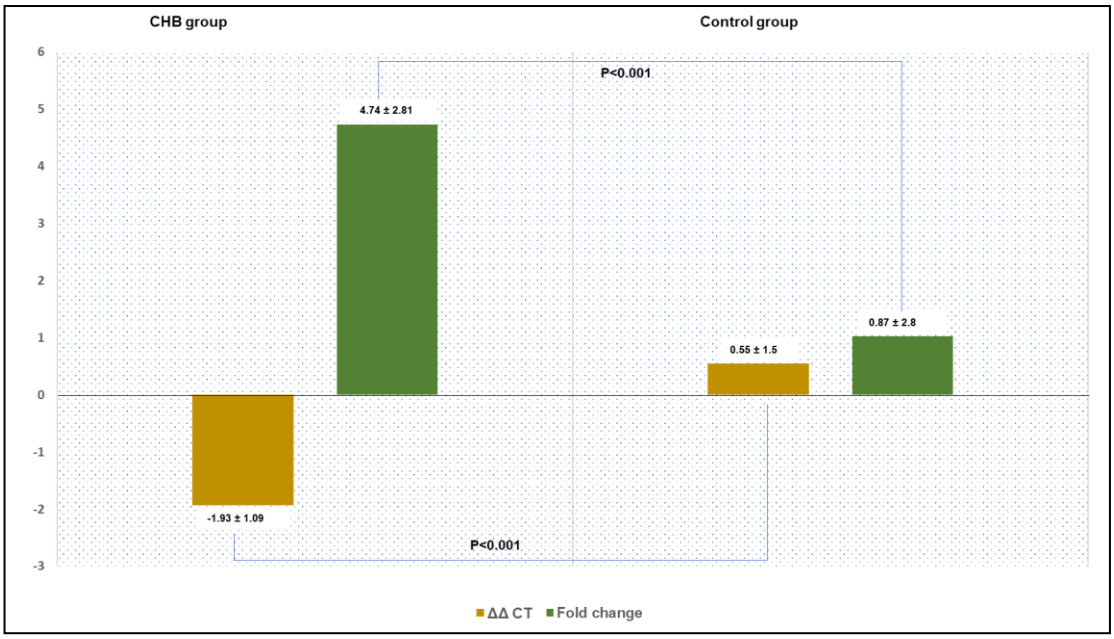

Figure 1: MicroRNA-122 relative expression and fold change among the studied groups.

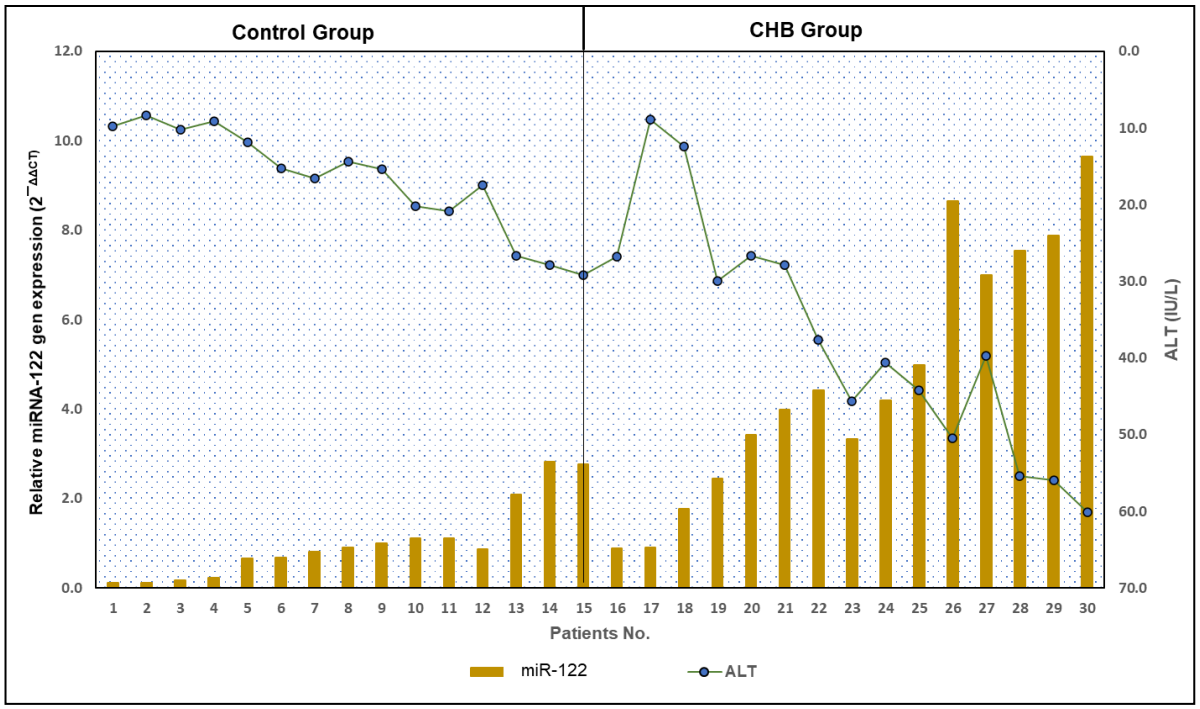

Figure 2: Analysis of Relative miRNA-122 gene expression ($2^{-\Delta\Delta CT}$) and ALT activities in plasma from patients with HBV infections compared to control group

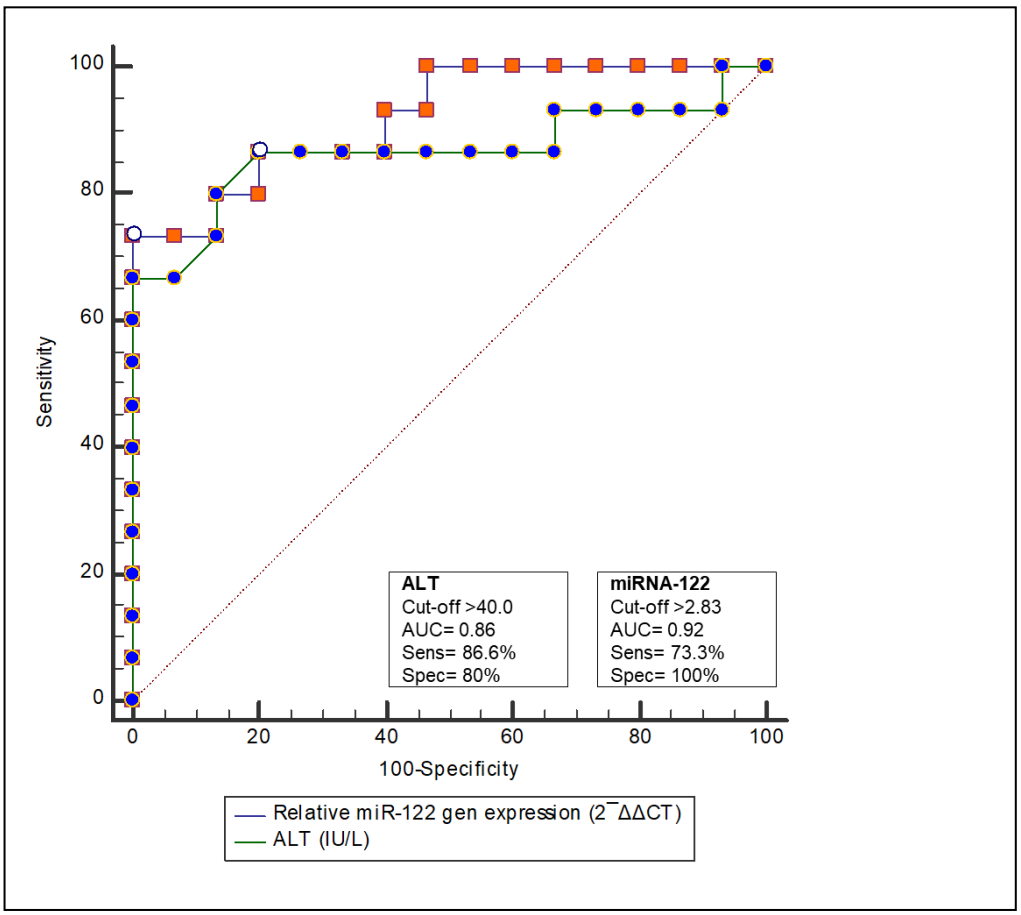

Figure 3: ROC curve showing the performance of miRNA-122 fold change in diagnosing liver injury caused by CHB.

4. Discussion

Damaged hepatocytes can release miRNAs into circulation. Circulating miRNAs could present in lipid/lipoprotein, apoptotic cells, microvesicles, or exosomes. Recent research has found that the number of circulating miRNAs can change dramatically depending on physiological phases and pathological situations. The levels of miR-122, miR-133a, and miR-124 are all raised in the blood of individuals with liver, muscle, and brain damage, respectively.[19-22]Recent studies have proven that miRNAs, mainly miRNA-122 (counting up to 70% of the total miRNAs in the liver), are involved in the pathogenesis of chronic liver diseases due to their important biological roles .[15, 23]Despite considerable advancements in diagnostic methods over the years, diagnosing HBV-associated chronic viral hepatitis conveniently remains a clinical issue. As a result, the use of miRNAs has gotten a lot of interest.[24, 25]In the present study; we investigated whether the circulating miRN-122 can be used as molecular biomarkers for CHB infection. Our molecular Analysis showed a significant difference in serum miRNA-122 relative expression between CHB patients and healthy controls. The level of miRNA-122 was 4.2-fold higher in the serum of CHB patients compared to the control group. Zhang et al., 2012 reported similar results that the level of miRNA-122 was 3.4-fold higher in CHB patients than that in controls.[13] Furthermore, Zhang and Jia et al. showed that, in 20 CHB patients compared to 12 controls, the level of expression of miRNA-122 and miRNA-192 were increased

significantly in HBV patient compared to the controls, While, no significant differences was observed between the two groups in the level of expression of miR-194, miR-21, miR-22, and miR-15a.[15]On the other hand, Wang et al., reported decreasing in the miR-122 levels and increasing in the miR-130a levels in training occult hepatitis B virus infection samples.[26]

Comparing the relative miRNA-122 gene expression and ALT activity in both CHB and control groups revealed that the abnormal ALT was associated with the change in the miRNA-122 gene expression level. For example, in the CHB groups, ALT activity was above the reference range of ALT with higher miRNA-122 gene expression. Various parameters are used to evaluate the necro-inflammatory process in the liver, including serum ALT and AST enzymes. There was a statistically significant difference between serum ALT and AST in HBV and healthy controls. In addition, a statistically significant correlation was reported between miRNA-122 fold change and ALT, AFB, and PTT in CHB patients. These results were consistent with the data by Waidmannet al. 2012 who reported highly significant correlations between serum miRNA-122 levels and ALT, AST, and γ-glutamyl transferase.[27] Furthermore, Zhang and colleagues highlighted the significant correlation between the miRNA-122 levels and ALT ($r=0.69$, $p<0.001$) and AST ($r=0.56$, $p<0.001$).[13] The higher level of miRNA-122 was not only linked to liver pathological characteristics (ALT, AFB, and PTT), but it was also a more accurate predictor for CHB than frequently used markers like ALT as presented in Figure 3.With a cutoff >2.83, miRNA-122 could be used to predict the liver injury caused by CHB (sensitivity=73%, and specificity=100%). That necrosis of hepatocytes could explain this will release cellular miRNA into the blood directly. This may be why the diagnostic performance of serum miRNAs was superior to ALT in diagnosing CHB.[13, 20] Zhang et al. performed ROC curve analysis to differentiate serum miRNA-122 expression levels in the CHB and control groups. ROC curve area of miRNA-122 was found to be 0.98 (95% CI: 0.88-1.00), with sensitivity and specificity of 87.5% and 100%, respectively.[13]

Conclusions

MiRNA-122expression level is increased in the serum of adult CHB patients compared to healthy controls. Therefore, analyzing the changes of serum miRNA-122 level may represent a future strategic non-invasive biomarker to diagnose liver injury resulted from CHB.

Data availability

The data of this study are available from the corresponding author upon reasonable request.

Acknowledgments

None

Funding

The authors have no funding to report.

Conflicts of interest

The authors have nothing to declare.

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