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COMPARING MIRNA-224 EXPRESSION AS A BIOMARKER TO DISCRIMINATE DISEASES IN SERUM CHRONIC HEPATITIS B AND HBV- RELATED CIRRHOSIS

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ABSTRACT

Hepatitis B virus (HBV) is a partially double-stranded circular DNA genome with a high tendency to liver tissue. Chronic hepatitis B varies from an asymptomatic infection to symptomatic chronic hepatitis, cirrhosis and hepatocellular carcinoma. In this study, serum miR-224 expression in healthy people, chronic hepatitis B and cirrhosis caused by hepatitis B was compared. The indices achieved were 35.13, 35.51 and 36.29, respectively. Difference in miR-224 expression level of patients with cirrhotic hepatitis B was significant, but it was not significant in people infected with hepatitis B. However, the significant difference between people with cirrhosis and hepatitis B infection is a measure to determine the developing from chronic to cirrhotic state which can be a valuable criterion for the diagnosis of disease in people infected with hepatitis B.

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Introduction

Hepatitis B is one of the infectious diseases caused by the hepatitis B virus (HBV) that affects the liver. It can cause both acute and chronic infections. Many people have no symptoms during the initial infection. Some develop a rapid onset of sickness with vomiting, yellowish skin, tiredness, dark urine and abdominal pain. Hepatitis B virus impairs cellular signaling pathways through targeting various protein factors by its HBx protein which among these are the effect on P53 signaling pathways, Ras, MAPK which leads to cell proliferation by affecting MAPK signaling pathway (1). It also leads to cell proliferation and Jak-STAT signaling pathways by affecting JACKs and STAT3. In addition, it affects NFκB signaling pathway and leads to apoptosis. CDK2 and CycE also induce cell proliferation by disorders in the functions of these factors as well as lead to inefficiency in the mitochondrial function by affecting on VDACs. Hepatitis B can also cause phosphorylation of beta-catenin, increased expression of Frizzled-7, reduced expression of pRb and P16, each of these factors play important roles in cellular signal transduction pathways (2). Chronic liver diseases include hepatitis B, cirrhosis, and hepatocellular carcinoma and are one of the major medical challenges in many parts of the world in terms of public health. Biochemical, serological markers such as aminotransferases, including: ALT (alanine aminotransferase), AST (aspartate aminotransferase), AFP (alpha

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fetoprotein) have been clinically used for liver disease for decades; however, with the development of liver cancer, they are limited in sensitivity and specificity (4). Non-coding genes comprise 98 to 98.5 percent of the human genome, which are known as ncRNA. These RNAs are divided into several groups according to the number of nucleotides. RNA (ncRNA) molecules with a length of 18 to 24 nucleotide**s** belong to the miRNA and siRNA family. Non-coding RNAs are involved in different pathways such as gene silencing, gene transcription, DNA methylation, demethylation and RNA interfering routes (5). miRNAs have an internal source that are involved in regulating gene expression after translation. Recent studies suggest that miRNAs play a role in different human diseases from cancer to cardiovascular disease. MiRNA expression profiles can significantly vary from naturally occurring to pathogenesis and are considered as a new biomarker in diagnosis and prognosis of potential human diseases. Emerging data on the role of miRNA in the pathogenesis of various human diseases, has provided a way to test their ability to act as a new diagnostic and therapeutic tools (6). In infectious diseases, miR-224 impacts on CD40, CDC42, SMAD4, CXCR4 and in liver metastases on SMAD4, CXCR4, in cancer on CD40, CDC42, SMAD4, CXCR4, API5, in hepatitis on CXCR4, in disease hepatic on CD40, in bile ducts cancer on SMAD4 and in the liver cancer, CD40, CDC42, FOSB, SMAD4, PEBP1, KLK1 and CXCR4 (7-8). All of these factors play certain roles in the cellular pathway and show that there is a close relationship between infection with hepatitis B and course of the disease from acute to chronic fibrosis and cirrhosis and eventually liver cancer and non-coding factors in the cell. According to the above that MiR-224 has different and similar targets in the diseases caused by viral infections and in the end stages of the disease to cancer, the ultimate goal of the study is to use the indicator as a prognostic and diagnostic biomarker. Gene silencing is another important roles of ncRNAs at different stages of transcription and post- transcription, which function mainly through miRNAs and siRNAs pathways. mRNA is formed in the post-transcriptional silencing, but for some reasons, such as rapid analysis of transcription or delay in translation, gene expression cannot be seen (25). At least 700 unique miRNAs and hundreds siRNA to thousands of miRNA have been sequenced in humans (5). Liver cancer is the fifth most common cancer worldwide and the third leading cause of death among cancers. Although there are various viral and non-viral causes for disease and about 80 percent of the diseases is associated with hepatitis B. Liver cancer is often diagnosed at the stage of progression of the disease (9). Micro RNAs are small non-coding RNA molecules containing 24-18 nucleotides which play an important role in regulating many biological processes, including cell proliferation, differentiation and cell death. By binding to region 3 / UTR, mRNA, these molecules would lead to translational repression and destruction (10).

Method

This study includes three groups: control, chronic hepatitis B and that cirrhosis caused by hepatitis B. Serum samples were taken from each group at a rate of 2-3 ml. Total RNA was extracted using RNX-PLUS kit. To evaluate the concentration of extracted RNA, the OD was measured using a Nanodrop. The ratio of light absorption at a wavelength of 260/280 was between 1.8 to 2.2. Then with MiR-Amp kit, ParsGenome was carried out according to the kit protocol including three stages. Including:

Adding a poly-A tail, Synthesis of the first strand cDNA and Real Time PCR amplification.

The Ct level of miR-224 and U6 were evaluated as an internal control using the Relative Real time PCR. The results were evaluated by comparing Ct level in the markers studied and U6 using statistical analysis.

Results And Discussion:

Studied groups include healthy subjects and patients with chronic hepatitis and hepatitis-related cirrhosis and demographic variables included gender (male and female) and age in years. Measured variables include Ct (miR-224) variable, Ct (U6) variable, Δct variable and ultimately $2^{-\Delta Ct}$ variable. In this section, ΔCt variable results from the difference between two variables, as follows: ΔCt=Ct (miR-224) – Ct (U6) In this study, the population of the study consisted of chronic hepatitis patients and cirrhosis caused by hepatitis, who among them 30 patients were randomly selected according to the study, and access to the patients in each group. The total sample size is equal to 90.

Evaluation of studied gender

In this study, 62 patients (68.9%) were male and 28 (31.1%) were female. The sex ratio in the studied groups was equal, as in healthy subjects, 22 (73.3%), in patients with chronic hepatitis B, 28 patients (93.3%) and in subjects with cirrhosis due to hepatitis B, 12 subjects (40.0 percent) were male. The results are in table and graph below:

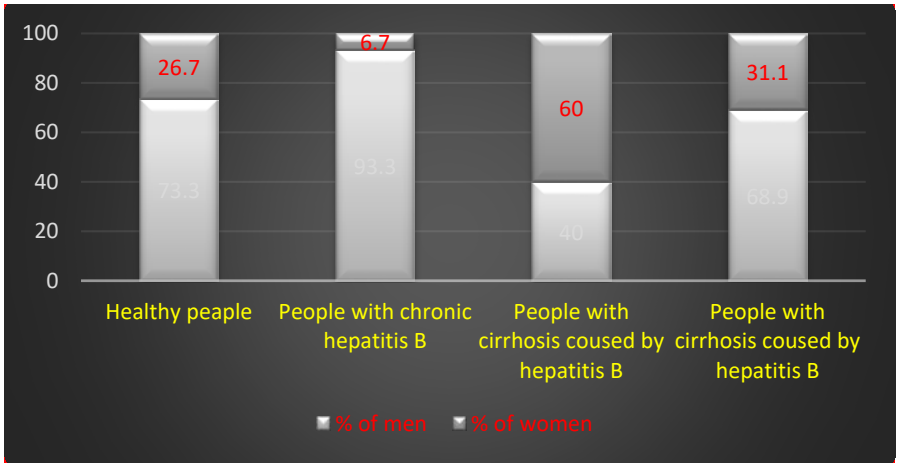

Figure 1. Comparing the average percentage by sex

The participants in this study had different age ranging from 18 to 59 years old and the average age of the participants was (39.11±10.04) in years. Healthy subjects were aged 18 to 55 years and had an average age of (34.9±37.92) years. The group of patients with chronic hepatitis B aged 22 to 59 years and their mean age was (36.00 ± 9.37) years and people with cirrhosis caused by hepatitis B aged from 32 to 65 years and their mean age was (38.9± 9.24).

The results are in the following graph:

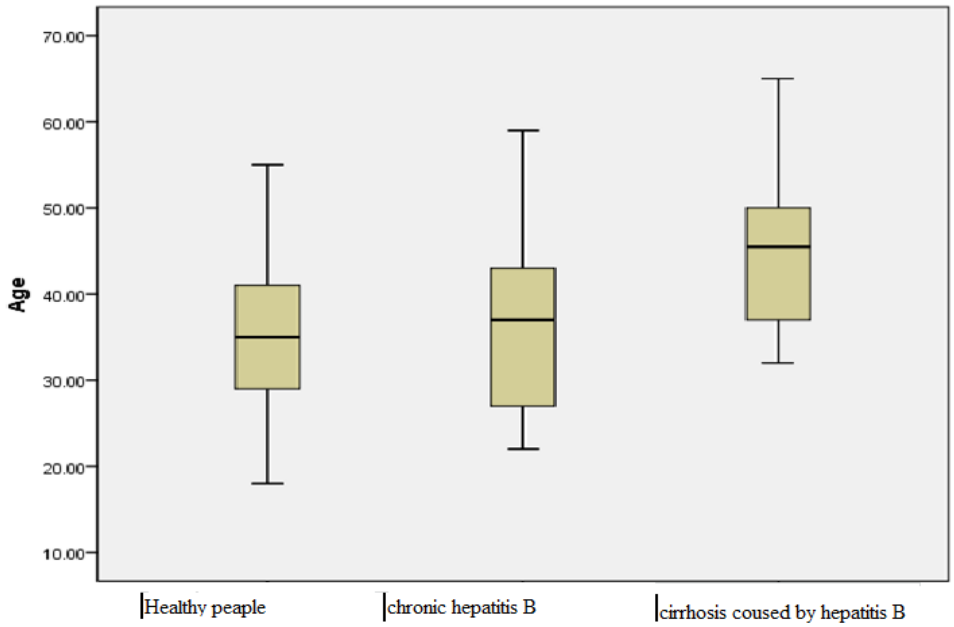

Figure 2. Average of age changes in study groups

Data normality test

According to the normality of the data, parametric tests such as ANOVA, independent sample t test can be used which is based on the normal distribution and statistics based on the mean and standard deviation. In case of non-normal data, nonparametric Kruskal-Wallis and Mann-Whitney tests can be used which are independent of the distribution and are based on the average data ratings. According to Shapiro-Wilk test, Ct (miR-224) and Ct (U6) and ΔCt variables had a normal distribution, but ΔCt and $2^{-\Delta Ct}$ did not have the normal distribution. Thus, normality of data is essential in test selection.

Figure 3: Resulte of dataes normality analysis of variations

variable	P-Value Kolmogorov-Smirnov	P-Value Shapiro-Wilk
Ct(miR-224)	0.200	0.087
Ct(U6)	0.200	0.305

ΔCt=(CtmiR-224) – (CtU6)	0.056	0.025
$2^{-\Delta Ct}$	< 0.001	< 0.001

Evaluation of Ct (miR-224) variable

In the evaluation of Ct (miR-224) variable of the participants in this study, it was observed that the total amount of this variable ranged from 29.32 to 39.06 and its mean was (35.64 ± 1.78). In healthy subjects, this amount was varied from 29.32 to 38.73 and its average was (35.13± 2.32). Also, in the group of patients with chronic hepatitis B, this amount has varied from 33.56 to 39.6 and its mean was (35.51 ± 1.49) and finally in people with cirrhosis caused by hepatitis B, it was varied between 33.99 to 38.70 and its mean was (36.29 ± 1.18).

Results are as follows:

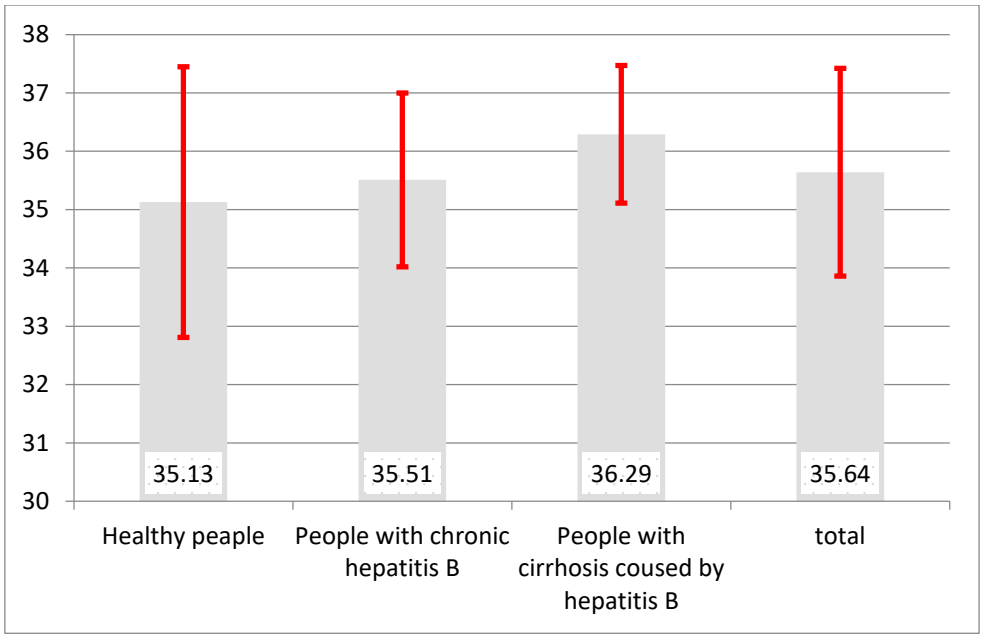

Figure 4. Comparing the main of Ct (miR-224) in study groups

According to data normality test, this variable is normal and then we have the permission to use parametric tests.

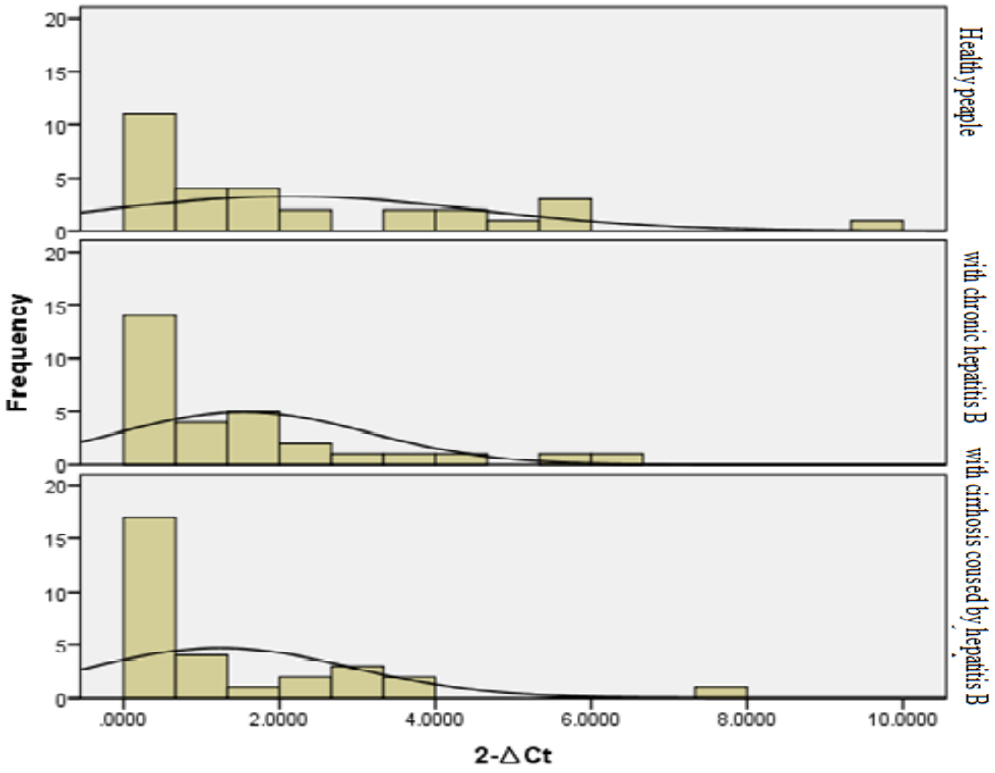

Figure 5. Normal distribution of Ct (miR-224) in study groups

In the initial study analyzed by ANOVA P_{Value}= 0.033, it can be seen that the average Ct (miR-224) in the three groups was not equal to each other and at least average of one group has significant difference with the rest.
By doing comparison tests 2 to 2 of LSD, it becomes clear that:
• There is no significant difference between groups of healthy subjects and patients with chronic hepatitis B (P-Value = 0.398)
• There are statistically significant differences between groups of healthy subjects and those with cirrhosis caused by hepatitis (P-Value = 0.011)
• There are significant differences between the groups of patients with chronic hepatitis and those with cirrhosis caused by hepatitis (P-Value = 0.082)
Additionally, generalized linear model (GLM) shows that:
• Variable Ct (miR-224) does not have a significant relationship with gender. (P-Value = 0.050)
• Variable Ct (miR-224) does not have a significant relationship with age (P-Value = 0.706)

Evaluation of changes in variable $2^{-\Delta Ct}$
In the evaluation of $2^{-\Delta Ct}$ variable of the participants in this study, it was observed that the total amount of this variable ranged from 0.03 to 9.93 and its mean was (1.64 ± 1.97). In healthy subjects, this amount has varied from 0.03 to 9.93 and its average was (2.13± 2.45). Also, in the group of patients with chronic hepatitis B, this amount has varied from 0.03 to 7.76 and its mean was (1.24 ± 1.71).
The results are in the following graph:

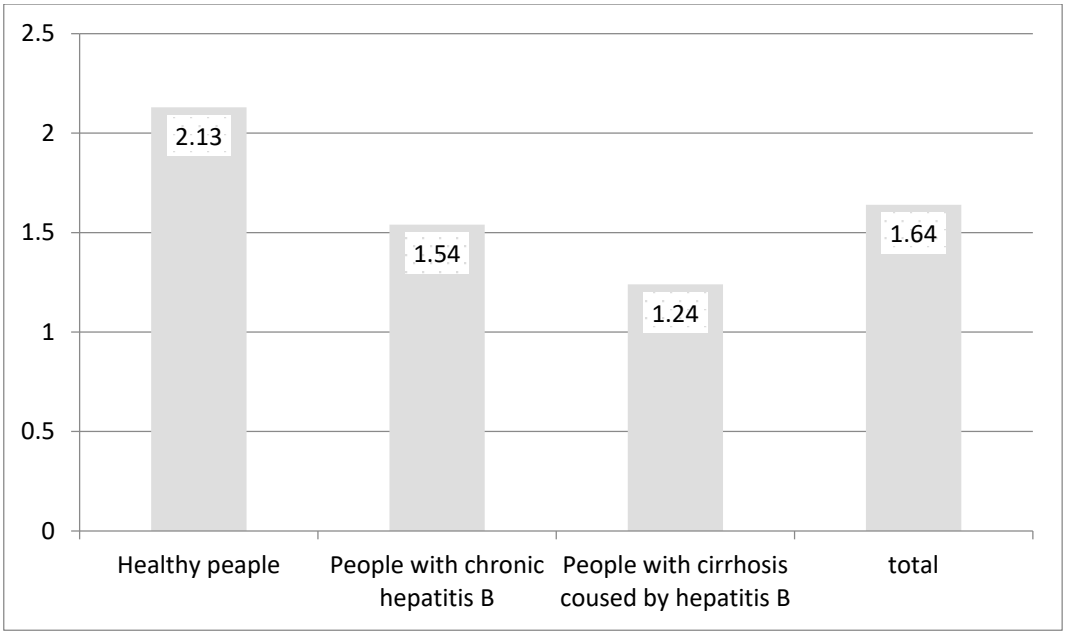

Figure 6. Comparing the main of $2^{-\Delta Ct}$ in study groups

According to data normality test in both Kolmogorov-Smirnov and Shapiro Wilke tests, this variable is not normal and then we do not have the permission to use parametric tests. In the initial study by Kruskal-Wallis nonparametric statistical analysis, which is equivalent to parametric test ANOVA, Value = 0.242 showing that average $2^{-\Delta Ct}$ rating in three groups was equal and there was no significant difference between them.

By doing comparison tests 2 to 2 of LSD, it becomes clear that:

• There is no significant difference between groups of healthy subjects and patients with chronic hepatitis B (P-Value = 0.244)

• There are no statistically significant differences between groups of healthy subjects and those with cirrhosis caused by hepatitis (P-Value = 0.082)

• There are significant differences between the groups of patients with chronic hepatitis and those with cirrhosis caused by hepatitis (P-Value = 0.082)

• There are significant differences between the groups of patients with chronic hepatitis and those with cirrhosis caused by hepatitis (P-Value = 0.558)

Additionally, generalized linear model (GLM) shows that:

• Variable $2^{-\Delta Ct}$ does not have a significant relationship with gender. (P-Value = 0.262)

• Variable $2^{-\Delta Ct}$ does not have a significant relationship with age (P-Value = 0.821)

Study of Florin M. Selaru and et al (2015) has shown that miRNA-224 has a high expression in collangio carsinima and liver cancer. They found that miR-224 is a skilled cell cycle regulator that will be release G1 to S by increased expression. Followed increased cell proliferation..(14)

Researchs of Xiaoyan He and et al (2015) showed that miRNA- 224 have been a significant expression for Neoplasia of esophageal epithelial and ESCC tissues. While expression of $PHPPL_1$ and $PHPPL_2$ Proteins and miRNA-224، s target gens have a low expression. In this conditions m iR-224 targeting $PHLPP_1$ and $PHLPP_2$ act to miRNA oncogene. (1)

Zhuang and et al (2014) found that Serum level miR-224 has a significant relation with liver damage parameters and Serum AFP . (16)

Conclusion

In this study, the miR224 gene expression was measured in three groups of healthy individuals, hepatitis B and hepatitis-related cirrhosis B. Previous studies conducted on cell lines and in patients with liver cirrhosis and liver cancer have shown increased expression levels of miR224 (11). However, in the serum samples investigated in this study, statistical analysis shows that there is a significant correlation between the expression levels of miR224 (CtmiR-224) with (P> 0.05) compared between healthy subjects and hepatitis B. However, the comparison between healthy subjects and those with cirrhosis caused by hepatitis B (P = 0.011) indicates that the relationship is meaningful. Since in the statistical analysis on variable $2^{-\Delta Ct}$, there is an exponential function, so the numbers were abnormal and non-parametric statistics were used in the statistical analysis. In the study, the relationship between healthy subjects with chronic hepatitis is no significant (P = 0.244). Also, in the comparison between healthy subjects and patients with cirrhosis resulting from hepatitis B, there was no significant difference (P = 0.082). Moreover, this factor is not significantly associated with gender and age (P = 0.262 P = 0.821). According to the

low P Value compared between healthy subjects with cirrhosis, perhaps it can be said that miR-224 could be effective in the course of the disease from hepatitis to cirrhosis and there is a need for further research and more samples are also necessary to make definite statements about it. As a result, according to the increased expression levels of miR-224 in non-cirrhotic, this factor can be considered as a marker to discriminate between healthy individuals and liver cirrhosis, which is consistent with research conducted by previous researchers on cirrhosis and hepatocellular carcinoma, and confirms previous research.

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