Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Importance of miR-141-5p and miR-501-5P expression in patients with HBV infection

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Received February 6, 2020; **Accepted** December 26, 2020; **Published** November 22, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.3.29

Abstract: MicroRNAs (miRNAs) as small RNA and post-transcriptional modulators are shown to have regulatory effects for different cellular activities and pathways, such as metabolism, virus replication and also cell growth. In addition, miRNAs can regulate the replication of the hepatitis B virus (HBV). Therefore, the expression profile of miRNAs was evaluated in HBV-infected patient groups and healthy controls. The expression levels of the following microRNAs (as noninvasive biomarkers) were compared in two experimental (those with various stages of HBV infection) and control groups to evaluate their diagnosis ability: mir141-5p and mir501-5p. RNA extraction was performed for 45 serum samples. The miRCURY LNA™ Universal RT-miRNA-PCR system and miRNA PCR panels were used for measuring microRNA expression profiles. To normalize quantitative values, the endogenous reference by UniSp6 expression was used. Serum mir141-5p and mir501-5 were significant None in patient in different stages of HBV infection($p<0.001$) than in controls($p<0.01$). Receiver operating characteristic (ROC) curve analyses suggested that serum has mir141-5p and mir501-5p none significant diagnostic value for HBV infection. Results suggest that mir141-5p and mir501-5 can not be used as diagnostic biomarkers for monitoring of HBV infection and other biomarkers in this disease need to be investigated.

Key words: MicroRNAs; Hepatitis B virus; Biomarker.

Introduction

Studies have shown that one-third of the world's population is influenced by Hepatitis B virus (HBV) infection, which is considered a certain health concern all over the world, of whom over 350 million people have been diagnosed with chronic HBV (CHB) infection (1). HBV infection can be associated with several complications, such as asymptomatic carriers, liver cirrhosis and hepatocellular cancer (2,3). Therefore, understanding the mechanism underlying chronic HBV infection at the molecular level is of great importance for the optimal management of chronic HBV infection. MicroRNAs (miRNAs) have been shown to possibly be associated with HBV infection regulation and relevant disorders (3, 4). It has indicated that miRNAs as small endogenous non-coding RNAs containing 21–25 nucleotides are capable of post-transcriptional gene expression regulation via target mRNAs degradation induction or inhibition of their translation into protein (5,6). Recent several studies indicated that miRNAs might affect virus-host interactions and play a crucial role in the viral life cycle and pathogenesis (7, 8). Indeed, HBV replication can be regulated by miRNAs directly through linking to the HBV genome or indirectly via modulating transcriptional factors linked to HBV for the regulation of HBV enhancer/promoter activities (9, 10). Zhang et al. were first studied miRNAs, which directly target HBV transcripts. They found that HBsAg expression and HBV replication were appropriately decreased by miR-199a-3p and miR-210-5p (11). In another study, the authors stated that HBV replication is promoted by miRNA-1 via an elevation in transcriptional activity of HBV core promoter (12). MiRNAs not only are interacted with HBV transcripts, but they have also regulated immune responses and signaling pathways, so they are essential for HBV replication and pathogenesis (13, 14). MiR-155-5p has been shown to increase the expression level of Interferoninducible antiviral genes against HBV via direct prevention of suppressor of cytokine signaling 1 (SOCS1) expression levels, which results in an elevation in the JAK/ STAT signaling pathway (14). Recognizing the appropriate non-invasive biomarkers for liver disease caused by the HBV virus is challenging, which can lead to early effective treatment and also desirable treatment outcomes (15). Novel methods have emerged to use miRNAs as biomarkers for liver disease by recognition of miRNAs capable of indicating pathophysiological conditions of human bodily fluids (HBF). In addition, they are considered suitable biomarkers for early and pre-symptomatic diagnosis due to their availability, great stability in the circulation and also expression patterns consistent with the tissues (16). Serum miRNA profiles from HBV carriers for recognizing appropriate biomarkers have been widely investigated (3,13–17). Therefore, in this study, the expression profile of a panel of miRNAs was analyzed in sera of HBV infected patient groups and healthy controls.

Materials and Methods

Study population

The present research was performed at Pars Hospital Laboratory, in Tehran, Iran from January 2015 to January 2017. All admitted untreated patients with CHB (n=45) based on immunological results divided into three groups: HBeAg positive, HBeAg negative, OHB and healthy controls (n=15) were recruited. CHB diagnosed by serum HBs antigen for more than 6 months was considered as the inclusion criterion. Patients exhibiting the following were excluded from the study: alcoholic liver disease, organ transplantation, immunosuppression and malignant comorbidities within the last 5 years. All participants gave written informed consent.

Serum sample collection

Blood samples were collected from each participant and kept on ice followed by centrifugation at 1500× g for 10 min at 4°C. The resulting supernatant was sent to three RNase/DNase-free tubes separately (Immunological-biochemical, HBV Molecular testing and miRNA study) and was stored at -80°C until analysis.

Clinical Chemistry and immunological markers

Cobas Integra 400 Plus (Roche Products Ltd., Switzerland) was used for serum ALT and AST levels according to the manufacturer's instructions. The levels of HBsAg, HBeAg, anti-HBeAg, anti-HBs, anti-HBc, and AFP were tested by automated available immunoassays (Roche Cobas e 411 analyzer).

DNA isolation and RT-q-PCR for HBV

Magcore Viral Nucleic Acid Extraction Kit was used for HBV DNA extraction from 400 µl of each serum sample. Flurion HBV QNP 2.0 PCR kit(Iontek, Turkey) was also employed for real-time PCR on Bio-Rad CFX96. The following steps were considered for amplification: Initial hot-start denaturation (95°C/15 min), followed by 50 cycles (95°C/30 s), annealing and extension (54°C/90 s). Genotypes of HBV were determined using AmpliSens® HBV genotype-FRT PCR kit variant FRT by real-time PCR. The following steps were considered for amplification: Initial hot-start denaturation (95°C/2 min), followed by 40 cycles of denaturation (95°C/25 s), annealing (58°C/25 s), extension (72°C/1 min), and final extension (72°C/5 min).

miRNA analysis

RNA extraction and cDNA synthesis

200 µL of the serum was used to extract miRNA using a miRCURYTM RNA Isolation Kit-Biofluid (Exiqon, USA) based on the manufacturer's protocol. The optical density in 260/280nm was measured for assessing the purity and concentration of RNA. Agarose gel electrophoresis (1%) was applied in order to measure RNA integrity. cDNA synthesis was performed by miRCURY LNA™ Universal RT-cDNA-Synthesis (Exiqon, USA), based on the provided protocol by the manufacturer.

SYBR-Green Real-Time PCR

The level of gene expression was determined via CFX96 Real-Time thermal cycler (Biorad, USA). Real-time PCR for the 2 miRNAs (miR141-5p, miR-501-p) was performed using miRNA PCR panels, miRCURY LNA™ Universal RTmiRNA-PCR system. The specific microRNA LNA™ PCR primers (Exiqon, Denmark) were used. To normalize the results, we were used UniSp6 as an internal standard control and all samples were run in duplicate. The reaction mixture was prepared by SYBR Green Premix (5µl), water (1µl) and 4µl of the control cDNA. The amplification of the reaction mixture was performed as follows: one cycle (95°C/10 min) followed by 40 cycles (95°C/10 s, 60°C/60 s, and 95°C/60 s, respectively). Melting Curve Analysis was used to prove the specificity of the amplified product.

Statistics

Results are represented as the mean ± standard deviation (SD). One-way ANOVA and the Kruskal-Wallis test were used for comparisons between groups. Data were analyzed by SPSS 23 (IBM Corporation, USA). The p values of less than 0.05 were considered significant. For estimation of diagnostic ability of miRNA receiver operating characteristic (ROC) curve analysis was used via medcalc statistical software (Belgium).

Results

Clinical details of patients

The study included 45 patients with Hepatitis B infection and 15 healthy control. Patients with HBV infection were divided into 3 groups based on immunological markers. 15 patients were HBeAg positive chronic hepatitis, 15 patients were HBeAg negative chronic hepatitis and 15 patients were occult hepatitis B. Mean age of the patient's group was 38.04±8.9 SD (range 22-56 years) and the distribution of gender in the patient group was 36 males (73.3%) and 9 female (%26.6). The mean age of the control group was 33.74±5.7 SD (range 2644 years) and the distribution of gender in the control group was 9 male (%60) and 6 females (%40). Separately in different patient groups, the mean age of ENCH Patient was 36.42±7.9 SD (range 27-49 years) and the distribution of gender in the ENCH group was 12 males (80%) and 3 females (%20). The mean age of EPCH patients was 36.63±8.3 SD (range 22-47 years) and the distribution of gender in the EPCH group was 11 males (73.3%) and 4 females (%26.6). The mean age of OHB patients was 41.5±7.8 SD (range 29-56 years) and the distribution of gender in the OHB group was 12male (80%) and 3 females (%20). The crowding index was successfully determined in patients and healthy groups. In index value <0.5; just 13.3% of control groups. In index value 0.5-0.99; 1.5% were control group, 6.6% were EPCH group, 13.3% were ENCH group and 6.6% were OHB. In index value >1; 73.3% were control group, 93.3% were EPCH and 93.3% were OHB.

Biochemical and molecular markers and HBV groups

All patients were negative for anti-HCV, antiHIV+Ag and anti-Hbc-IgM. Mean ALT, AST and AFP of patients were 29.24±18.3 SD, 26.18±15.3SD and 7.17±15.0SD respectively. Whereas mean ALT,

AST and AFP OF Controls were 25.80±7.94SD, 23.70±5.78SD and 2.12±0.56SD respectively. There was no statistically significant difference between the patients and the control group in this value. But mean ALT, AST and AFP of ENCH group was: 27.2±14.46 SD, 24.60±11.4SD and 5.97±10.4SD, EPCH group: 45.6±25.5SD, 37.53±21.71SD and 6.35±11.3SD, OHB group: 20.93±3.12 SD, 19.40±2.4 SD and 2.4±0.69. The means of ALT and AST were significantly higher ($p<0.001$) in the EPCH group than in ENCH and OHB and healthy controls. The positive cases of HBsAb in control, EPCH, ENCH and OHB were 66.7%, 0%, 0% and 53.3% respectively. The positive cases of HBsAb in control, EPCH, ENCH and OHB were 5%, 100%, 100% and 100% respectively. The positive cases of HBeAg were 0%, 100%, 0% and 0%, in HBeAb were 0%, 0%, 100% and 100% and the positive cases of HBsAg were 0%, 100%, 100% and 0% respectively. All patients were positive for HBV Nested PCR and Taqman real-time PCR Methods simultaneously. HBV genotypes were successfully determined in all of the patients and 100% were genotype D. The EPCH cases had high HBV viral load (3.5±0.7) than in ENCH patients (Mean 7.1E+05) and OHB cases (mean 8.3.E+02). The results of many clinical tests are quantitative and are provided on a continuous scale. To help decide the presence or absence of disease, a cut-off point is chosen and results that are below the cutoff are regarded as normal. The receiver operating characteristic (ROC) curve is widely accepted as a method for selecting an optimal cut-off point and for comparing the accuracy of diagnostic testing. The ROC curve is also important because the area under the curve (AUC) is a reflection of how good the test is at distinguishing between the patient with the disease and without the disease. A perfect test will have an AUC of 1.0, while a completely useless test has an AUC of 0.5. The AUC of many clinical tests falls between these two values one way of interpreting the AUC is that a test with an area greater the 0.9 has high accuracy, while 0.7-0.9 indicates moderate accuracy, 0.5-0.7 low accuracy and 0.5 a chance result. In order to check the accuracy of the tests done in the study and their ability to detect hepatitis B infection, we used the ROC curve analysis. The result of different performed diagnostic tests has been shown in Figures 1 and 2.

MiRNA profile and HBV groups

The serum levels of miRNA including miR-141-5p and miR-501-5P were analyzed in different samples from ENCH, EPCH, OHB and healthy control groups. The serum miRNA levels in different Patient groups including ENCH, EPCH and OHB was not correlate significantly with each other. The serum level of miR-141-5p and miR-501-5P and residual miRNAs was not significantly different between all patient groups and healthy control ($p<0.001$) (Figures 3 and 4).

Data with increase/decrease of expression are highlighted red/blue, respectively and unchanged data are displayed white. As shown on the side of the heat map, color intensity was calibrated to the expression level (Figure 5). 2 miRNAs assessed by miRNAs did not show differential expression in HBV infected patients compared to the control groups. The up-regulated miRNAs included miR-141-5p and miR-141-5p which

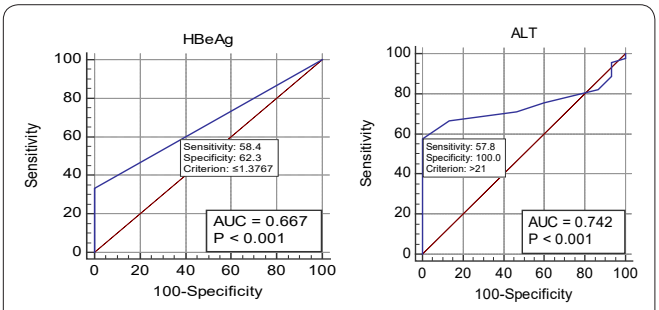

Figure 1. ROC curve analysis of immunological and molecular testing of HBV (Medcalc software).

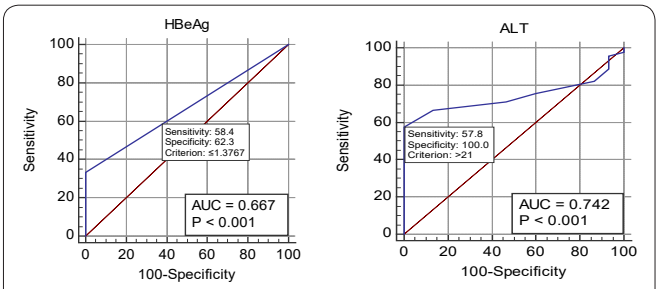

Figure 2. ROC Curve analysis of serum miR levels for diagnosis of HBV disease.

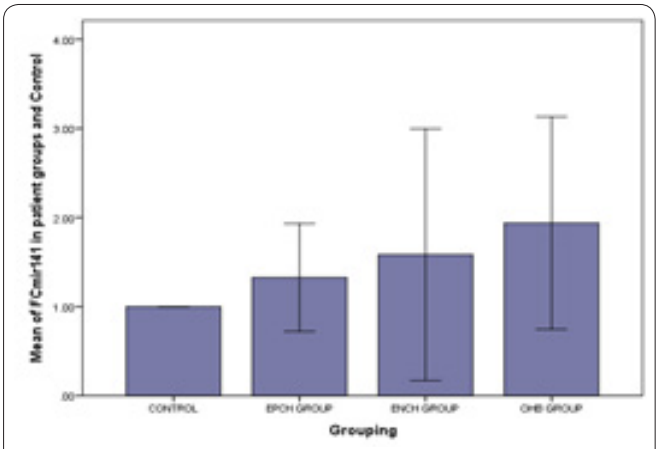

Figure 3. Mean of FC miR-141-5p in the different patient groups (EPCH=HbeAg Positive Chronic Hepatitis, ENCH=HbeAg Negative Chronic Hepatitis, OHB=Occult Hepatitis B infection) and healthy control. *: $P<0.05$.

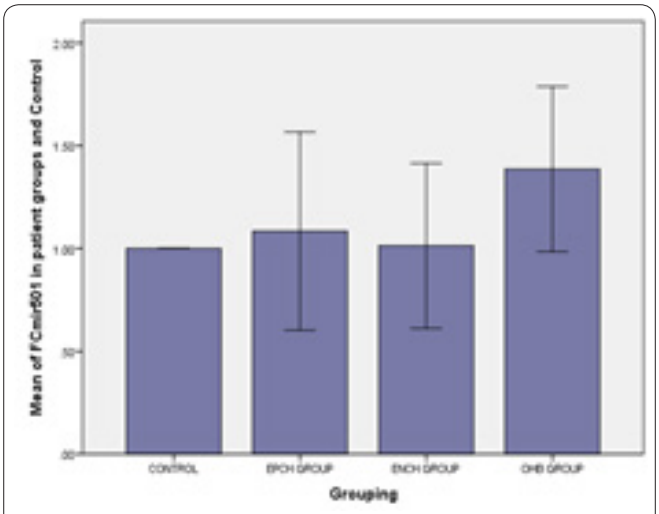

Figure 4. Mean of FC miR-501-5p in different patient groups (EPCH=HbeAg Positive Chronic Hepatitis, ENCH=HbeAg Negative Chronic Hepatitis, OHB=Occult Hepatitis B infection) and healthy control. *: $P<0.05$.

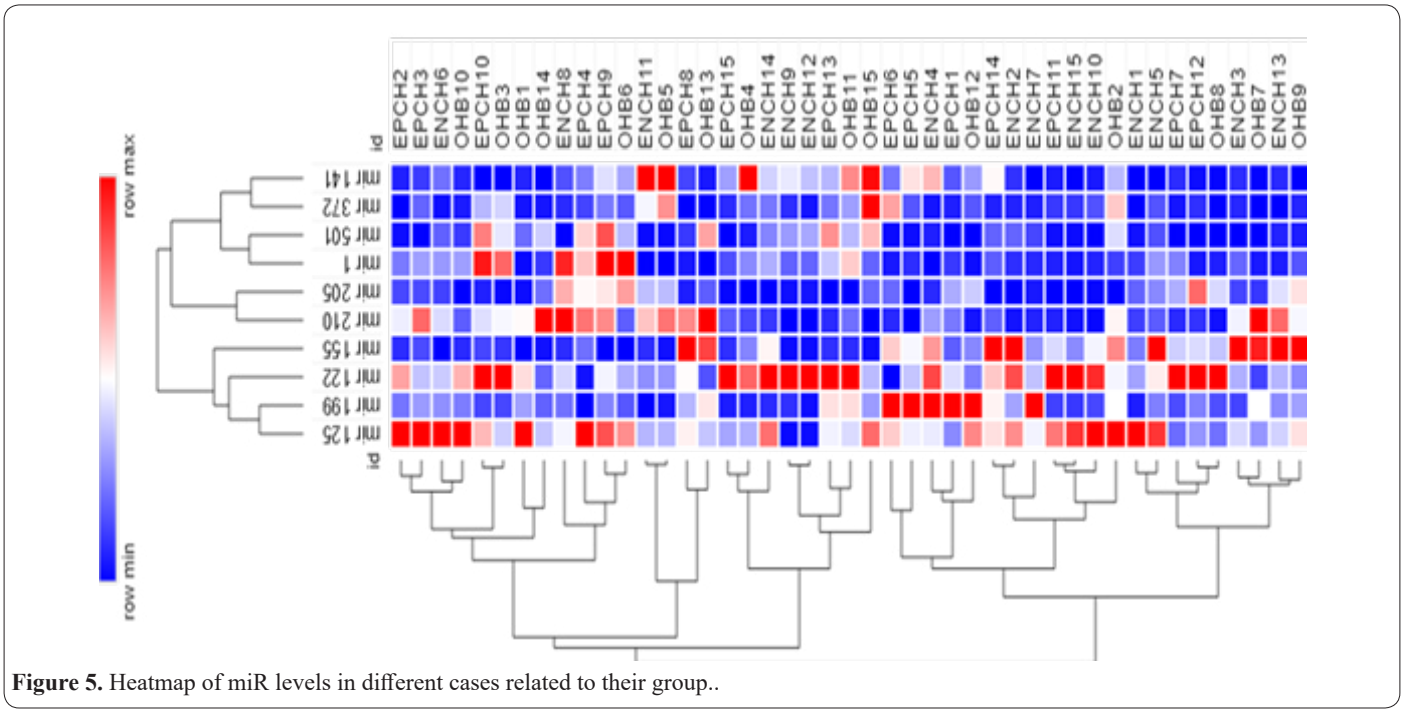

Figure 5. Heatmap of miR levels in different cases related to their group..

implies the not important role of their binding sites in aberrant miRNA expression caused by HBV in the host target genes.

Discussion

The role of miRNAs in HBV infection has attracted great interest in recent years, since accumulating evidence has suggested that miRNAs play crucial roles in HBV pathogenesis (3, 4, 9). Cirrhosis and hepatocellular carcinoma (HCC) known for their high mortality rate are among the complications of HBV infection (18,19). The present study aimed at assessing the serum levels of two miRs miR-141-5p and miR-141-5p in patients infected with HBV in clinical different stages compared with the healthy control group. The between studies, which supported the results reported by previous studies, and between MiRSs, up-regulated expression of miR-125a-5p in HBV patients compared.

The prediction of target sites for human microRNAs within the HBV genome was first made by Potenza et al. and they indicated that miR-125a-5p can bind to the target sequences in the viral genome. Indeed, miR-125-5p can affect HBsAg expression, as miR-125a mimic or inhibitor transfection leading to secretion of HBsAg by PLC/PRF/5 cells induced a noticeable reduction or increase in HBsAg expression, respectively (20). In the HBV-producing cell line HepG2.2.15, hsa-miR-125a-5p expression was indicated at least three times more than parental HepG2 cells (21). Moreover, the authors show that treatment with iron can lead to a reduced level of hsa-miR-125a-5p expression via an enhancement in HBV replication, whereas by TGF-b treatment, an elevation can be seen in the expression level of hsa-miR-125a-5p due to the decreased HBV replication (22). Accordingly, HBV replication can be prevented by miR-125a-5p via direct binding to viral transcripts and the HBV sequence targeted by hsa-miR125a-5p encodes amino acids falling within an important segment (for cell binding) of the extracellular pre-S1 domain of HBsAg (23). Recently, miR-122, miR-130, miR-183, miR-196, miR209 and miR-96 were presented as potential biomarkers to distinguish different stages of hepatitis and liver injuries (24). However, there are no conflicting results regarding the relationship between miRNA-141-5p and miRNA501-5P expression levels and HBV replication. In this study, we have not found a significant difference in the serum level of miRNA-122-5p among all patient groups and healthy control ($p<0.05$). Consistent with our results. Jin J et al indicated that the Survey of miRNA-501-5p expression in patients HBV is no diagnostic value (25). In contrast, Ebrahimi-fard et al indicated that the rate of miRNA-122-5p expression in patients with CHB and HBV-related cirrhosis was 1.8 times significantly more than the control group ($P < 0.05$) and it is a diagnostic value (26). The study HuW et al indicated that miR-141 expression PPARa in two effects both translation and transcription levels and finally reduces HBV proliferation. The result of the survey of miRNA 141-5p expression in patients HBV like miRNA 501-5p It is not diagnostic value (27). In the study like we found no significant difference between the six miRs (miR-199a-3p, miR-210, miR-205, miR155, miR-372, and miR-1) in the serum of patients infected with HBV compared to the control group (28). Our data indicated that mean ALT and AST were significantly higher ($p<0.001$) in the EPCH group than in ENCH and OHB and healthy controls, suggesting a positive correlation with hepatic necroinflammation. ROC curve analysis was employed to assess whether serum miRs can be used as a diagnostic marker for CHB and OHB. Its findings indicated that miRNA-501-5p, miRNA141-5P serum levels were not useful markers for discriminating against patients infected with HBV. An AUC of ROC curve for miRNA141-5P0.522 and miRNA 501-5p 0.533 with the sensitivity and specificity 522 and 533 respectively. This suggests that miRNAs can not clearly improve the diagnostic accuracy of efficacy evaluation in HBV infection. In addition to, The AUC for HBcAb (1.00, $P<0.001$) was equal to HBV viral load (1.00, $P<0.001$) and markedly lower than that of HBsAg (0.52, 0.53, $P<0.001$) ALT (0.74, $P<0.001$) HBeAg

(0.66, P<0.001). Cellular miRNAs can influence HBV replication in the following ways; 1) they can bind to HBV transcripts, 2) via affecting cellular factors related to the life cycle of HBV, 3) through regulating genes and signaling pathways essential for the pathogenesis of HBV, 4) via adjusting epigenetic reformations, including histone modification and methylation(15,29) MiRNAs can directly interact with viral components, which has empirically been approved, including miR199a-3p (affecting HBsAg coding region) (11), miR210 (affecting HBV preS1 region) (11) and miR205 (affecting X gene) (30) Replication of HBV is effectively suppressed by MiR-141 and miR-155 directly by affecting peroxisome proliferator-activated receptor alpha (PPARα) as well as CAAT Enhancer binding protein β (C/EBP-β), respectively(31) However, it has indicated that the replication of HBV is promoted by miR-1, miR-372, and miR-501 by influencing host gene expression. MiR-1 as an example is able to alter the expression level of several genes via affecting histone deacetylase 4 (HDAC4), including up-regulation of farnesoid X receptor (FXR) leading to an increase in transcription and replication of HBV via binding to the HBV core promoter (12). MiR-372 is capable of increasing the expression level of HBV via the NFIB transcription factor. NFIB levels were lower in a stable HBV-producing cell line (HepG2.2.15 cells) with elevated endogenous expression of miRs-372 in comparison with the control cell line (HepG2 cells) (32). There are limitations to research on gene expression that researchers need to consider (33-45). In these regards immunogenicity of the multi-epitopic recombinant glycoproteins virus: implications may be concerned (46). A lot of information needs to be analyzed for a closer look (47-49). Information on DNA and protein structure and sequences can be very useful (50-54).

Acknowledgments

This study was supported by Islamic Azad University. We appreciate all patients who participated in this study and thank also Dr. Afsson Afshari for her scientific and statistical advice. Thanks to Bistoon Shafa Co., Iran (website: www.camelina.info, Instagram: @camelina .ir) for all supports

Interest conflict

The authors have no conflict of interest to declare.

Author contributions

Study concept and design: Lak R, Yaghobi R, Garshasbi M, Performed the experiments: Lak R, Drafting of the manuscript: Lak R, Yaghobi R, Garshasbi M. Statistical analysis: Lak R, Yaghobi R, Garshasbi M.

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