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Investigation of LncRNAs expression in patients with hepatitis B virus

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Abstract

Aim: Patients infected with the hepatitis B virus (HBV) are at a higher risk of cirrhosis and hepatocellular carcinoma. Despite the recent advancement of antiviral therapy, many patients still cannot respond to existing therapies. Hence, to detect the changes in liver function earlier, non-invasive methods are needed. Long non-coding RNAs (lncRNAs) play important roles in essential biological process as well as human cancer. LncRNAs may be used as biomarkers in human diseases. Thus, in this study, we purposed to analyze the expression levels of lncRNAs (HOX transcript antisense RNA (HOTAIR), maternally expressed 3 (MEG-3), highly upregulated in liver cancer (HULC)) in patients with hepatitis B virus and healthy volunteers.

Methods: We selected three lncRNAs as candidate lncRNAs based on their association with liver disease. Whole blood samples were collected from 40 patients with HBV and 48 healthy volunteers. The expression levels of all the samples were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Statistical analysis was implemented using GraphPad Prism software. A p-value lower than 0.05 was statistically meaningful.

Results: The expression levels of HOTAIR and HULC were remarkably upregulated in the plasma of the patients with HBV compared with healthy control ($p<0.05$). In contrast, no significant difference in MEG-3 expression levels was observed between groups.

Conclusion: Our findings showed that the expression of HOTAIR and HULC in plasma might be new promising diagnostic and/or prognostic biomarkers for HBV.

Keywords: Long non-coding RNA, Hepatitis B Virus, HOTAIR, HULC

Introduction

Hepatitis B is common worldwide and is a severe health problem. Approximately 257 billion people have a past or present hepatitis B virus (HBV) infection, and chronic HBV carrier is more than 350 million [1]. In patients infected with HBV, it has been reported that there is a risk of death from cirrhosis, liver failure, hepatocellular cancer (HCC) and HBV-related liver disease, and this group constitutes 15-40% of patients with HBV-infected [2]. More than 887,000 people die yearly because of chronic and acute HBV-related diseases [3]. According to hepatitis B surface antigen (HBsAg) positivity in Türkiye, the prevalence of HBV is between 2-8%, and it is in the middle endemicity region [4].

Despite the improvement of antiviral therapy, many patients still cannot respond to novel treatments. Thus, the determination of non-invasive methods to detect changes in liver functions is urgently required to improve the clinical outcomes of HBV infection [5].

Non-coding RNAs (ncRNAs) are regulators of complex biological processes, and their dysregulation plays a role in the pathogenesis of many diseases, including HBV [6]. The ncRNAs involved in gene regulation are basically classified into two classes based on their length. Long non-coding RNAs (lncRNAs) are single-stranded RNA molecules that are not translated into proteins at least 200 nucleotides long [7]. LncRNAs play important roles in the essential pathophysiological processes including apoptosis, inflammation, cell cycle, differentiation, and proliferation by modulating gene expressions of their target at the transcriptional and translational level. Further, lncRNAs act as strong regulators for metabolic activity by modifying protein molecules [8-10,13]. Moreover, the lncRNAs take part in

antiviral defense and regulates cell development in the immune system. Mutation or abnormal expression (up-down regulation) of lncRNAs is closely associated with human pathologies. Expression of cellular lncRNAs can change in response to viral replication or viral protein expression. lncRNA functions as negative or positive regulators of the antiviral response in the innate immune response. It regulates the cellular activities (macrophages, NK cells) involved in the innate immune response [11, 12, 13, 14]. Recently, many studies have shown that lncRNAs, such as HOX Transcript Antisense Intergenic RNA (HOTAIR), Highly upregulated in liver cancer (HULC), and maternally expressed gene 3 (MEG-3), have a role in the development of HBV-associated HCC [9, 20]. HBV-mediated interference with HOTAIR represents a crucial mechanism by which the virus can promote tumor development by deregulating fundamental metabolic and cell cycle regulatory processes that are needed to maintain the hepatocytes highly differentiated. HULC has been implicated in lipogenesis and angiogenesis in hepatoma cell lines, and siRNA knockdown of HULC has been shown to deregulate proliferation-related genes. Furthermore, some studies have reported that the expression of HOTAIR and HULC were upregulated in inflammatory processes and their upregulation inhibits proliferation and promotes apoptosis and inflammatory responses [6, 24]. Based on the aforementioned studies, in our study, we aim to investigate the expression levels of HULC, HOTAIR and MEG-3 in peripheral blood samples of patients with HBV.

Material and methods

Patient and control groups

Forty patients were randomly selected (21F, 19M) with untreated chronic patients with HBV who had been applied to the Mustafa Kemal University, Health Practice and Research Hospital, the Department of Gastroenterology. Patients under 18 years old, pregnant women, active infection, chronic diseases (DM, COPD, CRF, CHF), cirrhotic patients and malignancy were excluded from this study. The control group consisted of healthy volunteers (25 F, 23 M) compatible with age and gender.

In our study, demographic and clinical features of HBV and control groups were considered age, gender, HbsAg (Hepatitis B surface antigen), Anti-HBs (Antibody for HBsAg), Anti-HBe (Antibody for HBeAg), HBeAg (Hepatitis B envelope antigen), Albumin, ALT (Alanine aminotransferase), and AST (Aspartate aminotransferase), TBil (Total bilirubin), DBil (Direct bilirubin), AFP (Alpha-fetoprotein), Hb (Hemoglobin), Plt (Platelets), and PT/INR (Prothrombin Time and International Normalized Ratio).

Ethical approval

An informed consent form was obtained from all the participants who participated in this study. This study was approved by the Clinical Ethics Committee of Hatay Mustafa Kemal University (ethical approval number 2016/63). Further, the present study was conducted according to the Declaration of Helsinki.

Sample collection and gene expression analyses

Peripheral blood samples from patients and healthy controls at diagnosis were collected. Peripheral blood samples were transferred into a tube containing EDTA, and all tubes were centrifuged at 3000 rpm, + 4 °C for 15 minutes and then tubes were stored at – 70 ºC for further gene expression analyses.

Measurement of lncRNA levels by Real-time PCR

Total RNA isolation from plasma was performed using an extraction kit (QIAGEN RNeasy Mini Kit Cat No: 74104), and total RNA quality was determined by A260/A280 ratio and 1.5 agarose gel electrophoresis. Subsequently, total RNA was converted into complementary DNA (cDNA) using the cDNA synthesis kit (QIAGEN, RT2 HT first strand kit) according to the manufacturer's instructions. The expression levels of lncRNAs (HOTAIR, HULC and MEG-3) were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) using the Rotor-Gene 600 device (Corbett Research, Australia). Specific amplification was confirmed by melting curve analysis and The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of lncRNAs.

Statistical analysis

Using https://www.qiagen.com/tr/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo=off site, the results were uploaded to the analysis system as an excel table. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of lncRNAs. GraphPad Prism 6.0 (GraphPad Software) was used for statistical analyses and plotting the graphs. A p-value lower than 0.05 was accepted statistically significant.

Results

Plasma samples were taken from 48 healthy controls. (23 males and 25 females,), 40 patients with HBV (19 males and 21 females). No significant difference was observed in age and sex ratio among the two groups ($p>0.05$).

Analyses of lncRNAs expression in patients with chronic HBV infection

In 40 patients with chronic HBV infection, lncRNA expression was 2-fold higher than the control group during initial diagnosis ($p<0.05$). In other words, our results showed that the expression levels of HOTAIR and HULC were notably up-regulated in the plasma of patients with HBV compared with healthy control and LncRNAs exhibited more than two-fold increased ($p<0.05$). In contrast, we did not observe any significant difference in MEG-3 expression levels between the groups (Figures 1, 2, 3).

Other biochemical parameters were analyzed using routine techniques, and our data showed that the levels of some parameters, such as AST, ALT, TBA, Crea, UA, GGT, and ALB were significantly different between healthy controls and patients with HBV ($p<0.001$). Further, no significant difference was determined for the level of TP and TBIL among two groups ($p>0.05$) (Table 1).

Discussion

ncRNAs play a major role in the detection of immunomodulators involved in the host's resistance to hepatitis viruses and determine the prognosis. Moreover, there is a need to identify novel non-invasive biomarkers that allow early detection of changes in liver functions.

Some studies have shown that lncRNAs might take part in many different biological processes, such as cell proliferation, differentiation, cell cycle, apoptosis, and invasion. It has also been shown that lncRNAs can play an essential role in the regulation of the eukaryotic genome [12-15].

Figure 1 - Comparison of expressions of HOTAIR, HULC and MGE-3 in healthy control and patients with HBV.

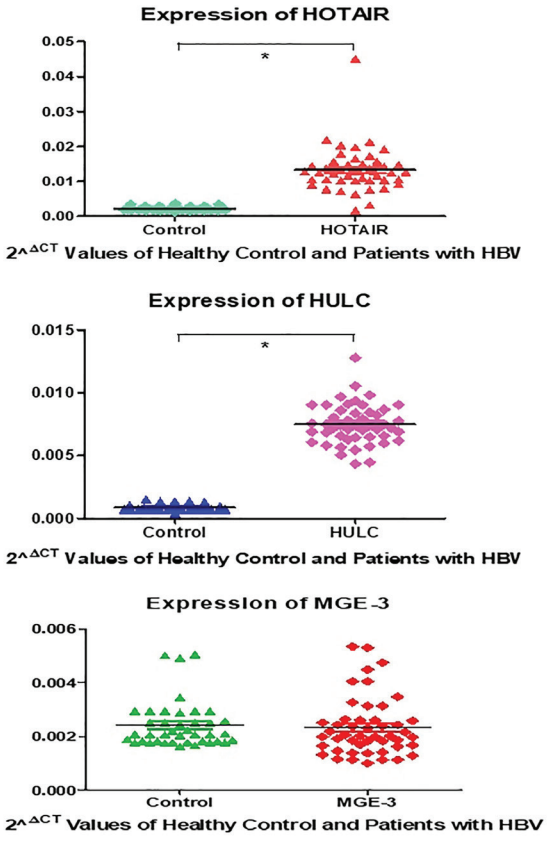


Figure 2 - Magnitude of gene expression

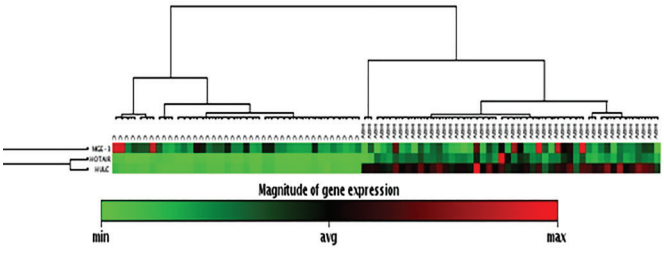
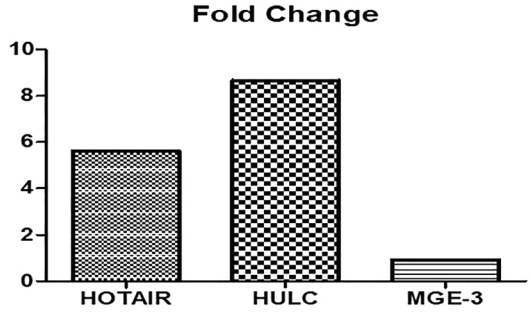


Figure 3 - The relative expression levels of HOTAIR (a), HULC (b) and MGE-3 (c) in healthy control and patients with HBV



Especially, dysregulation expression of lncRNAs has been associated with many diseases in humans, including cancer [16-17].

In response to the viral replication or viral protein expression, the expression of cellular lncRNAs can alter, whereas many cellular lncRNAs make a response to antiviral pathways induced by infection. Indeed, many lncRNAs can act as the innate antiviral response's positive or negative regulators. Our current knowledge of the identities and functions of lncRNAs in infected cells is very limited. However, identifying new cellular pathways has already been assisted by new research in this area and that could help for the improvement of therapeutic tools in the treatment of viral infections, autoimmune diseases, neurological diseases, and cancer [18].

Table 1 Demographic and clinical features of HBV and control groups

Parameter	Chronic Patients with HBV	Healthy Control	*p*-value
Age Median (range)	±35	±34	$p>0.05$
Gender	19 F 21 M	25 F 23 M	$p>0.05$
AST Median (range)	32.00	21.00	$p<0.001$
ALT Median (range)	30.00	17.00	$p<0.001$
TBA	3.20±2.42	2.8±2.32	$p<0.001$
Crea	35.40±31.02	62.12±12.4	$p<0.001$
GGT	35.40±31.02	18.06±6.84	$p<0.001$
ALB	45.60±2.42	43.21±2.05	$p<0.001$
TP Median (range)	1.07	1.03	$p>0.05$
TBIL Median (range)	0.60	0.60	$p>0.05$

HOTAIR is a kind of lncRNA associated with human HOX loci and contributes to metastasis in HCC [15]. Functional analyses indicated that HOTAIR supported HBV transcription and replication by elevating the activities of HBV promoters. In our study, we investigated the clinical importance of HOTAIR in patients with HBV and detected that HOTAIR expression is upregulated in patients with HBV, which is consistent with previous studies. A recent study revealed that HOTAIR expressions were increased in patients with chronic hepatitis B and its elevated levels are relationship with liver biomarkers [6]. In another study, *Zhong et al.* reported that HOTAIR expression is increased in patients with HBV compared to the control group [21]. Furthermore, these results highlight that the upregulation of HOTAIR plays a crucial role in the inflammatory process. [6].

HULC is another lncRNA which is highly expressed in liver cancer and plays a significant role in liver carcinogenesis [19-20]. *Panzitt et al*. reported that HULC was extremely tissue specific [20]. *Zhong et al.* found that expression of HULC is elevated in patients with HBV compared to the control group [21]. Moreover, *Wang et al.* showed that the expression of HULC was upregulated in inflammatory processes [25]. In our study, we found that HULC expression was upregulated in patients with chronic hepatitis B compared to the control group, which is consistent with previous studies.

MEG-3 is located on human chromosome 14q32.2 and is widely expressed in normal tissues [22]. It was reported that MEG-3 is down-regulated in cancerous tissues [23]. In addition, *Chen et al.* unveiled that MEG-3 is lowly expressed in patients with chronic hepatitis B [24]. In contrast, we did not observe a significant difference in MEG-3 expressions between healthy controls and patients with HBV.

In favour of its capability to enable HBV transcription and replication, HOTAIR and HULC may function as new HBV diagnostic and therapeutic biomarkers. Therefore, elevated lncRNA levels in patients on antiviral therapy may be associated with HCC. In addition, we thought that the altered levels of gene expression in patients receiving antiviral treatment might be interesting because the current treatment may prevent the development of HCC through the regulation of gene expressions, and it is a subject worth investigating. Therefore, in our following study, we plan to examine lncRNA expression levels in patients with HBV receiving antiviral therapy.

In brief, lncRNAs play an essential role in the regulation of gene expression and are involved in various biological processes, including inflammatory response and apoptosis. Thus, it is not surprising that altered lncRNA expression is linked to a variety of human diseases, including diabetes, infection, autoimmune diseases, and most notably, cancer. In this regard, growing evidence suggests that lncRNAs may play a role in the pathogenesis of various cancers, including gastric, colon, lung, and pancreatic cancers, as well as glioma, melanoma, and hepatocellular carcinoma (HCC). It can be used as a biomarker in at least some patients with HBV and HBV-associated HCC, especially in connection with other risk factors, such as age, alcohol, smoking, obesity, and metabolic syndrome. However, we should note that we need advanced studies for this.

Limitations

There are some limitations in our study. Our study included a small sample group. Further research with larger samples and patients with HBV-related HCC can validate the findings and produce more reliable results. Possible confounding factors of our study may be that the participants did not have similar characteristics (e.g., age, gender, other chronic diseases). Despite these limitations, the research described above provides useful information for further screening circulating lncRNAs that may serve as HBV biomarkers.

Conclusion

As a result, the plasma level of HULC and HOTAIR in patients with HBV could be used as a biomarker for disease progression. Further research is needed to verify the prognostic role of HULC and HOTAIR in patients with HBV and to better understand the relationship between candidate lncRNAs and the hepatitis B virus infection.

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