The mutations frequency of enhancer II/HBx regions of hepatitis B virus in acutely infected Iranian patients: a cross-sectional study

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ABSTRACT

Background and Objectives: The viral transactivator HBx protein affect cellular, viral and pregenomic factors pathway. Mutations in this protein can produce new viruses with new antigenic determinants that are generally related to developing cancerous.

Materials and Methods: In this cross-sectional study, 33 serum samples of patients diagnosed with acute HBV infection were investigated for HBeAg and HBV DNA viral load and HBx gene mutations. mutation in the HBx protein detected by sequencing analysis.

Results: Out of the 33 samples, 19 samples were males (57.6%), and 14 samples were females. 15 (45.5%) were positive for HBx DNA and 18 patients were negative for HBx DNA (54.5%). After sequencing, three mutations were recognized in HBx at nucleotide positions 147, 148, and 391 that were stationed to G1524A, G1525A, and G1767C mutations.

Conclusion: The analysis result of this study shows G1524A and G1525A mutations that an important role in altering the inhibition function of the HBx activity domain. The G1767C mutation inactivates HBx transactivation activity. These mutations have a critical role in the pathogenicity of the virus, and the intensity of hepatic tissue demolition and the development of cirrhosis or carcinoma in patients can be understood.

Keywords: Hepatitis B virus; X protein; Infections; Enhancer II; Mutation; Liver diseases

INTRODUCTION

Hepatitis B virus (HBV) is the prototype member of a family of hepatotropic DNA viruses that can cause acute and chronic infection of the liver. HBV is an important agent for intense diseases including Hepatocellular carcinoma (HCC). The prevalence of HBV infection in Iran was reported between 2.5%-7.2%, in comparison to the global average (1). HBV belongs to the genus Orthohepadnavirus of the fam-

ily Hepadnaviridae. The genome of HBV is around 3.2 kb in length and tendentiously relaxed circular double-stranded DNA genome with four overlapping Open Reading Frames (ORFs) that replicate via reverse transcription (2). The ORF X encodes Hepatitis B virus X protein (HBx), an essential for virus replication (3). The ORF-X encodes a multifunctional non-structural protein X with 465 bp (154 amino acids) (4). Studies with knockout mutants have shown that some (HBx) domains have a functional role, such as the HBx domain encoded by amino acids 1-50 and a domain between amino acids 52-148 that has a transient activity function (5). Domains of amino acids 120-140, 58-119, 54-70, and 109-131 have nuclear activation function, and transactivation signal and are essential for HBx insertion into mitochondria respectively (6). The amino acids 82-154 and 10-136 are functional domains that can be inhibitory to potease and P53 (A tumor repressor protein) activation (7). HBx has transcriptional activity in both nuclear and cytosolic fractions. HBx can activate Enhancer I (Enh I) and cellular RNA polymerase I, II, and III in the nuclear site. HBx is also involved in the activation of Enh II and the HBV pregenomic promoter. HBx plays a crucial role in this HBV-induced autophagy and activation and blockage of cellular calcium and tyrosine kinase signaling pathways (8). Due to the loss of proofreading activity of the viral polymerase; base substitution and frameshift mutations can occur in each of HBV ORFs, such as ORF-X (9). HBx contributes to the durability of virus-infected cells and the pathogenesis of chronic liver diseases by stimulating multiple distinctive features that are characteristic of cancer. Understanding the role (s) of HBx in the virus life cycle and the development of different forms of the disease remains a significant challenge (10).

Full-length HBx sequences are required to investigate the different effects of HBx on cellular pathways and viral pathogenesis. On the other hand, the Enh II region of the HBx is associated with increased transcription of viral and cellular genes, it may be associated with accelerated viral pathogenesis and malignancy in the event of a mutation. Therefore, the duration of the chronic phase may be reduced. The aim of this was study to investigate mutations in the HBx region in patients with acute infection conducted after obtaining approval from the Research Ethics.

MATERIALS AND METHODS

Patients. A cross-sectional study was done in the Virology Department of Kerman University of Medical Sciences from April 2018 to May 2019. Out of 65 HBV patients that were identified, 33 patients with acute infection were included in our study before antiviral treatment. Patients with co-infection with HCV or HIV or immune system defects or genetic disorders were excluded from this study. Out of 65 HBV patients, peripheral blood from 33 patients was collected in a sterile tube -containing EDTA. Plasma separation was provided by centrifugation under (3000 rpm for 5 minutes), and the plasma samples were stored at -80°C for further experiments. All patients imputed written consent informed for participation in the study. The study was approved by the ethics committee of Kerman University of Medical Sciences (IR.KMU.REC.1397.009).

Real-time PCR assays for hepatitis B virus DNA quantification. HBV DNA and HBx RNA were extracted from 100 μl of plasma with the RiBo-Prep Extraction kit (Inter Lab Service, Russia). Extracted viral DNA, and RNA pellets, were re-suspended in 100 μL of pre-warmed elution buffer and stored at -20°C until use. (HBV–FRT Real-Time kit, Amplisense, Russia) were used for quantitative detection of hepatitis B virus DNA using real-time PCR. (HBV–FRT Real-Time kit, Amplisense, Russia). Quantitative designation of the amplified products was done with the Rotor-Gene Q (Qiagen, Germany).

Detection of HBx. Specific primers were designed to detect HBx DNA. The position of 265 in the HBx region was designed using Beacon Designer Software. The primer pairs are stated in Table 1.

Primers were synthesized by Metabion Company (Metabion international AG, Germany). A 15 μl reaction Master Mix (FirstAid cDNA synthesis kit, ThermoFisher) was set up containing 5μl of RNA under the following conditions: 25°C for 5 min, 42°C for 60 min, then 70°C for 15 min. Next step 5 μl of cDNA (complementary DNA) was added to a 20 μl reaction mixture of RealQ Plus Master Mix Green (Amplicon, Denmark) containing specific primers and were subjected to Real-time PCR. The condition for the PCR assay was at first 10 min with hot start Taq DNA polymerase at 95°C followed by 40 cycles at 95°C for 10 sec and 60°C for 30 sec.

Table 1. Specific primer sequence for detection and sequencing of HBx

Name	Real-time PCR primers	Location	Product size (bp)
HBXF:	GTCTGTGCCTTCTCATCTG	265-284	168 bp*
HBXR:	GTTCACGGTGGTCTCCAT	433-415	
Name	Sequencing Primers	Location	
HBXSF:	ATGGCTGCTAGGCTGTGCT	-19-0	506 bp
HBXSR:	GGCAGAGGTGAAAAAGTTGC	487-465	

* A 168 bp product was used to identify HBx and a 506 bp product size were used for sequencing

Sequence alignment and mutation analysis. Real-time PCR permits to determine HBx gene mutation, PCR products were analyzed using 1.5% agarose gel in 1×TBE buffer. DNA Ladder (100 bp) was used as the molecular standard to verify the suitable size of the HBx gene. The electrophoresis was done at 80 V, and bands were visualized with Red Safe staining under a UV detector. DNA amplified by PCR, subjected to sequencing in the two directions of the HBx gene using an automated DNA sequencer (Applied Biosystems 3730/3730XL DNA Analyzer sequencing), Iran. The results of sequencing compared with the respective sequences HBx of HBV obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). The sequences of mentioned above deposited in GenBank database under accession numbers: KX196232, KX196232, KU668446, MF618339, KU668445, KU668443, KU668442, KU668437, KU668435, KU668433, JN664938, HE805982, HE805981, HE805980, HE805979, GQ253319, GQ253318, GQ253317, GQ253316, GQ253315, GQ253314, GQ253313, GQ253312, JX558098, GQ253297, GQ253298, GQ253304, GQ253308, GQ253309. Sequence analysis of the gene and amino acids was managed using the Basic Local Alignment Search Tool. The sequencing results of these genes compared with the deduced amino acid sequences available in GenBank. The de novo assembly of derived paired-end sequence reads was by contacting CLC Genomics Workbench version 8.5.1 (CLC bio, QIAGEN, Aarhus). The amino acid substitution was compared By HBx of HBV reference sequence in the NCBI database.

Serological tests. Serum liver enzymes (SGOT, SGPT, ALK) were assayed in patients with acute Hepatitis B at our clinical laboratory using an automated analyzer. The presence of HBsAg, HBeAg, Anti-HCV, and Anti-HIV was resolved using commercial Enzyme immunoassay kits (Diapro, Italy).

Statistical analysis. Statistical analyses were accomplished using the SPSS version 16.0 Normality test was used by, the Kolmogorov-Smirnov test. Analysis of continuous variables was carried out using a non-parametric equivalent to independent samples t-test. The Chi-square test was used to compare the distribution of classified variables. The result of the statistical test was statistically significant if the p value was ≤ 0.05. Statistical analysis was processed with SPSS statistical package (SPSS Inc., Chicago, IL, USA). Statistical analyses were based on population, defined as subjects who gave a blood probe and completed the questionnaire. The results are presented as frequencies. Differences between groups were analyzed by the χ^2 test. P-values <0.05 were interpreted as statistically significant. The summary of material methods and results in this research on 4 sections of patients, Real-time PCR, HBx of detection, and mutations of detection are shown in Fig. 1.
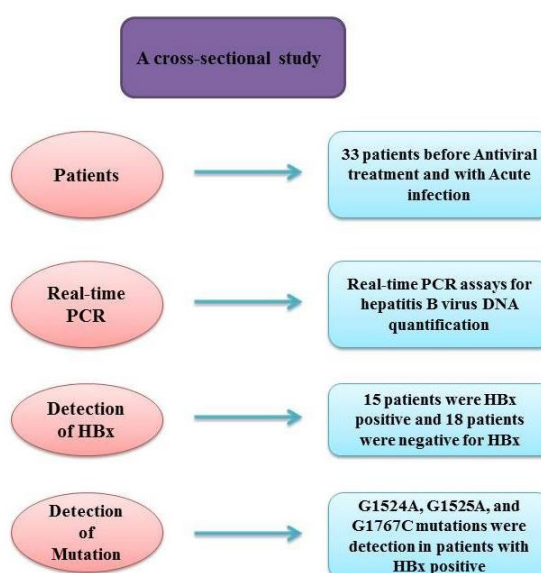
Fig. 1. Presentation diagram of methodology and results

RESULTS

Clinical, demographic, and epidemiological characteristics of patients with HBV infection are presented in Table 2.

Out of 33 patients, 19 (57.6%) were, men with a mean age of 50.42 ± 6.9, and 14 (42.4%) were women with a mean age (31.74 ± 6.6) with acute HBV infection. The youngest and oldest patients were 21 and 63 years respectively. The real-time PCR based on SYBR green showed 15 (45.5%) patients were HBx positive and 18 (54.5%) patients were negative for HBx. There were 10 (30.1%) male, and 5 (15.2%) female. Out of 15 HBx positive, there were 9 (27.3%) male and 9 (27.3%) female out of 18 HBx negative. The most positive (24.2%) and negative (27.3%) HBx cases were detected, respectively in the age group of 20 to 40 years old. There was no considerable difference between the age group and HBx results (P = 0.406). The standard curves obtained by Real-time PCR of HBx in acute patients with HBV are shown in Fig. 2.

The results of liver biochemical tests and HBV-DNA viral load levels were evaluated with the results of PCR-HBx (Fig. 3a). The maximum frequency of HBx positive samples (30.30%) at HBV viral was 10^3 to 10^5 (copies/ml) and the maximum frequency of HBx negative samples was (39.39%) in the viral loads less than 10^2 (copies/ml). Finally, statistical analysis showed that there was a considerable relationship between the HBx gene and HBV viral Load (PValue = 0.01). The mean levels of serum ALK and a maximum frequency of HBx gene-positive/negative showed that there were HBx positive and negative equal to (30.30%) and (27.27%) respectively in ALK level > 200 (U / L). So there was no significant association between HBx and serum ALK level (P = 0.345) (Fig. 3b). The mean SGPT and maximum positive and negative HBx patients showed that there were HBx positive (30.30%)

and HBx negative (24.24%) in the SGPT level between 40-200 (U / L). There was no remarkable correlation between HBx and SGPT (P = 0.348) (Fig. 3c). Finally, the mean levels of SGOT in HBx positive and negative patients were between 40 and 200 (U / L). There was no substantial correlation between HBx and SGOT (P = 0.544) (Fig. 3d).

Evaluation of the HBeAg marker of HBV replication and infectivity agent in 33 patients showed 8 (23.5%) samples were positive that 4 (12.12%) samples were male and 4 samples (12.12%) were female. Out of 25 (73.5%), HBeAg negative samples 15 (45.45%) samples were male and 10 (30.30%) samples were female. Statistical analysis showed there was no significant relationship between HBeAg and sex (P = 0.618). HBeAg is important for natural course infection in vivo. Statistical analysis of the results revealed a significant correlation between HBeAg and HBx (P = 0.044) (Table 3).

Sequencing, phylogenic and mutation analysis. In this study, SYBR Green I real-time RT-PCR assay showed out of 15 HBx-positive patients from point difference, and their melting curves were compared with reference sample; gel electrophoresis at 2% agarose identified 2 samples doubtful of mutation. Suspected specimens were extracted from gel agarose (Accu-Prep® PCR/Gel Purification Kit, Bioneer) and subject to sequencing. Nucleotide BLAST and multiple Sequence alignment showed that transversion mutations occur at position 136 N→K at sample No.1 although there was no mutation in the other sample (No.2A). We also identified single point mutations in the position of G1524A, G1525A, and G1767C in sample No.1A. NCBI GenBank provided accession number type ID: MT538222 for submitted sequences. A phylogenic tree was constructed by the neighbor-joining (NJ) method of MEGA X (Fig. 4).

For possible effects of mutations on proteins, the

Table 2. Distribution of demographic characteristics of patients with acute hepatitis B infection

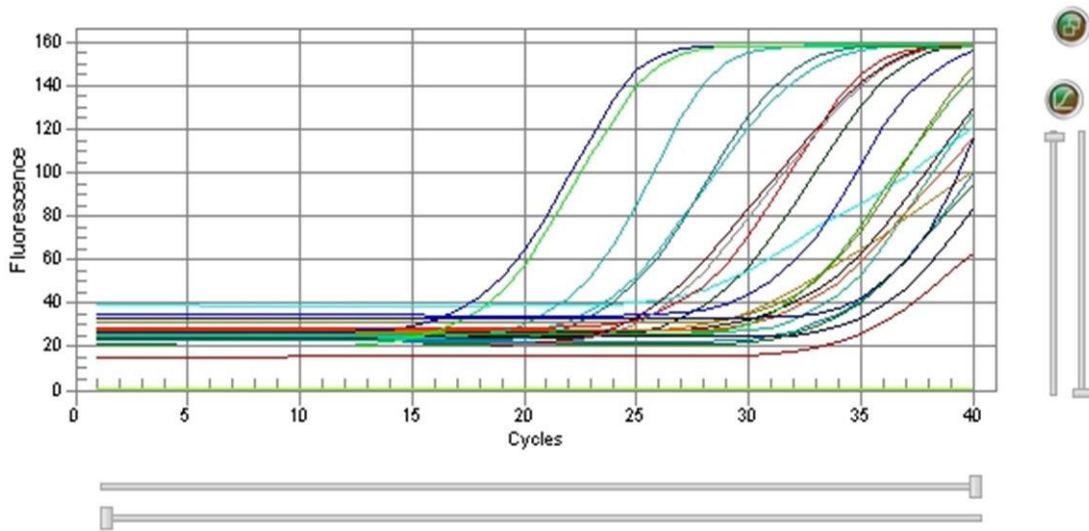| Test	Age (yrs)	HBV load (copies/ml)	ALK (U/L)	SGPT (U/L)	SGOT (U/L)
Mean	41.54	2.6 + 1E5*	235.56	185.32	200
Std. Deviation	12.359	1.2 + 1E2	18.46	12.25	13.45
Range	42.00	7 + 1E7	342	91	133
Minimum	21.00	0.00	123	138	156
Maximum	63.00	7 + 1E7	465	229	289

*(+1E5): Means ×100.000 copies/ml

Fig. 2. Quantitative HBx represents the standard curve with reaction efficiency.
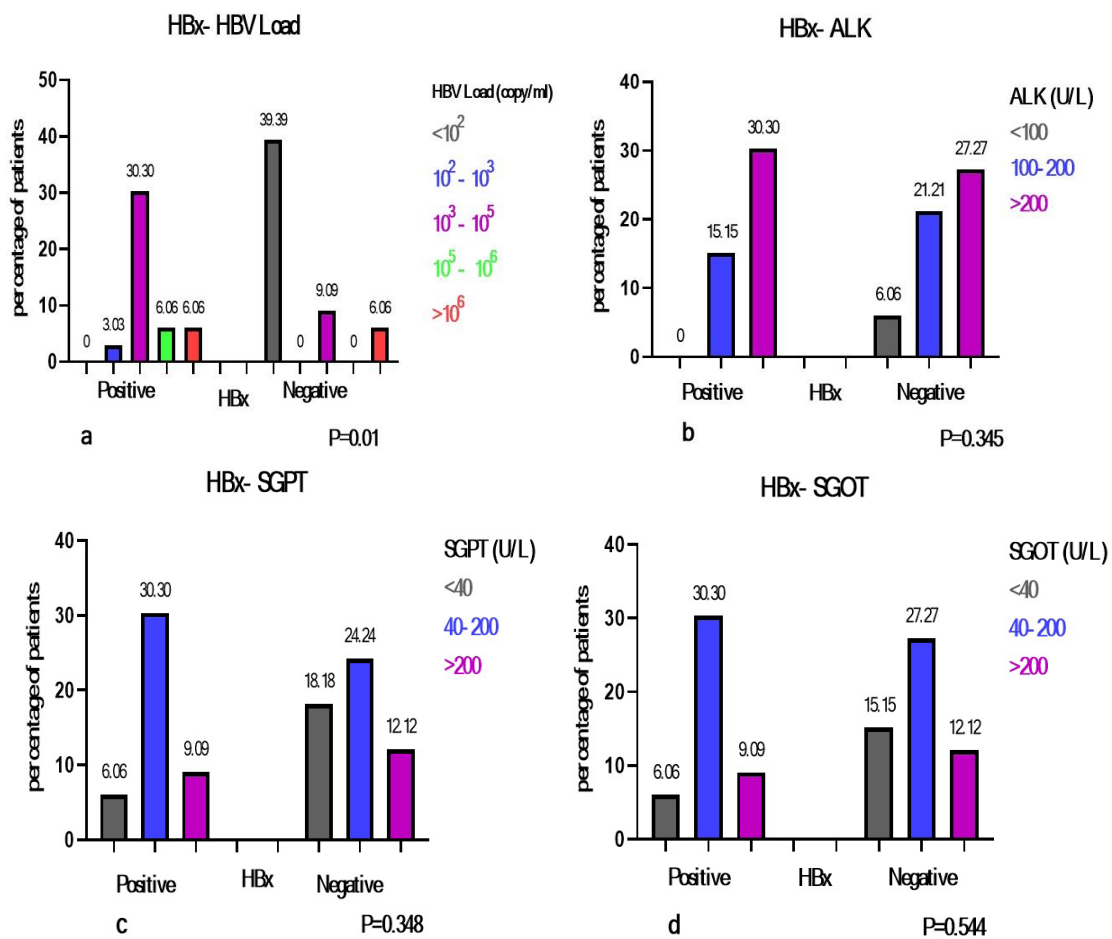

Fig. 3. Comparison of the liver function tests in positive and negative HBx cases in patients with acute HBV infection. a) comparison between the mean of HBV Load mean and HBx positive/negative b) comparison between mean of ALK and HBx positive/negative, c) comparison between the mean of SGPT and HBx positive/negative, d) comparison between the mean of SGOT and HBx positive/negative.

Table 3. Comparison between sex, HBeAg, and HBx in patients with acute HBV infection

Sex								P.value
Male: 19 (57.6%)				Female: 14 (42.4%)				
HBeAg				HBeAg				0.618
Positive: 4 (12.1%)		Negative: 15 (45.5%)		Positive: 4 (12.1%)		Negative: 10 (30.3%)		
HBx		HBx		HBx		HBx		
Positive: 4 (12.1%)	Negative: 0 (0%)	Positive:6 (18.2%)	Negative: 9 (27.3%)	Positive: 2 (6.1%)	Negative: 2 (6.1%)	Positive: 3 (9.1%)	Negative: 7 (21.1%)	0.044
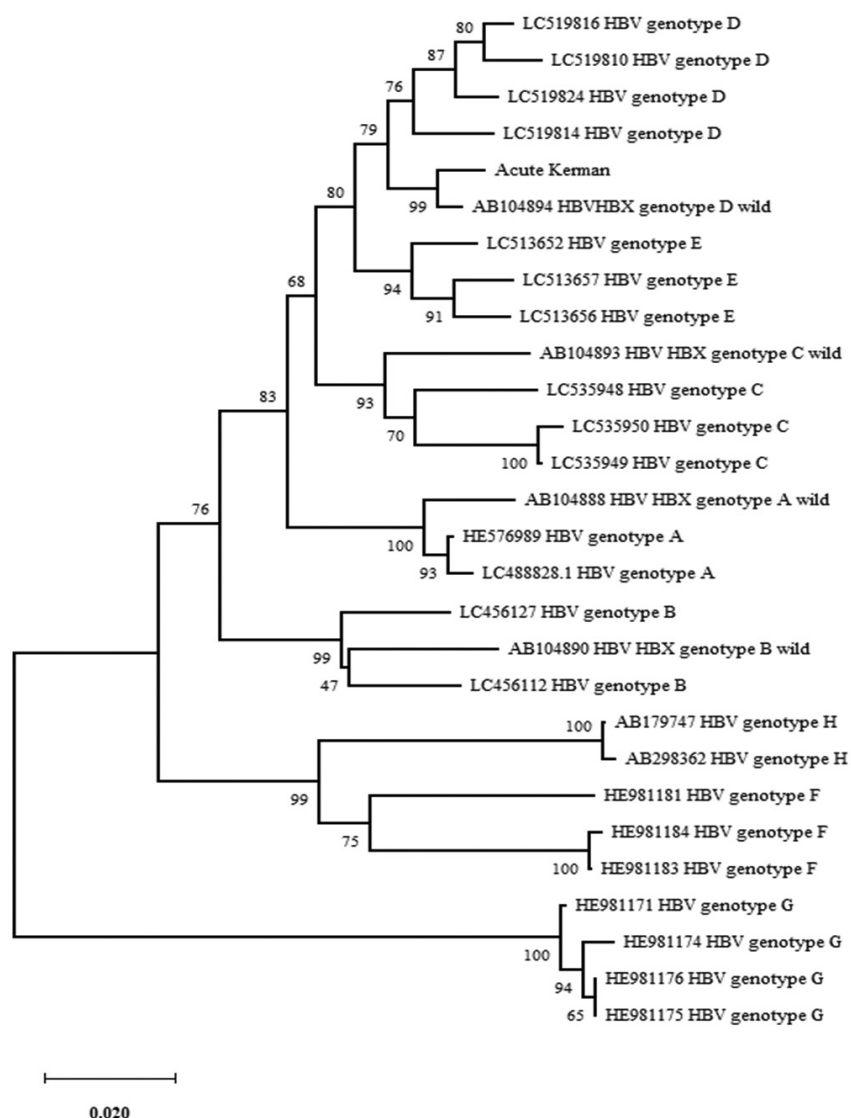
Fig. 4. Phylogenetic analysis of a 465 bp product of HBx gene extracted from 1 isolate of HBV genotype D and other genotype reference sequences. After analysis sequencing 10 HBx region positive. Only one sample had a mutation in several locations. The phylogenetic tree was constructed by MEGA. X using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Swiss-model database predicts peptide structures from amino acid sequences (Fig. 5).

A comparison of our nucleotide sequences with reference sequences revealed these mutations can cause changes in the arrangement of HBx amino acids, including conversion at position 50 G50K (glycine) G→K (lysine) and position 130 V130L (Valine) V→L (Leucine).
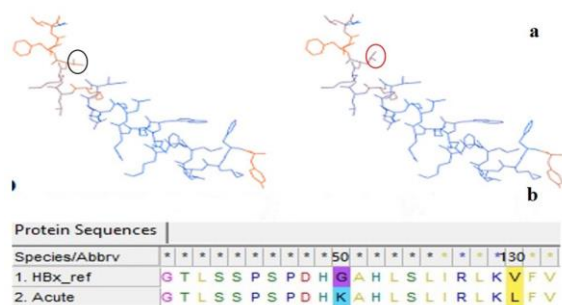
Fig. 5. Drawing of a linear structure with HBx mutant sequences in the SWISS-MODEL database https://swissmodel.expasy.org/ a) Linear structure of HBx with the reference sequence (left), and mutant sequence (right). b) Amino acid substitution in mutated sequence at positions 50 and 130 of HBx.

DISCUSSION

HBx is essential for the virus replication cycle, by acting on viral promoters embedded within protein-coding. The HBx gene has 1376-1840 nucleotide lengths and Basal Core Promoter 1613-1744 nucleotide lengths. Because of these overlapping genes, and mutations identified in the Basal Core Promoter region with increased HBV DNA, especially with an average viral load equal to or less than 10^5 copies/ml. Mutations in the X genomic region can occur in patients with HBV infection, these markers were linked with an increased risk of developing cancer (11). In this study, HBV viral load and HBx-PCR in 33 patients with acute HBV infection were measured using real-time PCR methods. The sequencing was performed, next step phylogenetic trees and linear protein structure mutation samples were constructed. HBeAg is a marker of HBV viral replication that may be related to increased HBV viral load. This study evaluated the significant correlation between HBeAg and HBVviral load (P = 0.011). Report data obtained at Hirayuki Enomoto et al. in Japan on 198 patients

with HBV infection DNA levels were significantly higher comparing HBV DNA and HBeAg levels in HBeAg positive patients. Thus, HBeAg-positive patients had higher HBV DNA than HBeAg-negative patients. Increased levels of Viral Load in patients with chronic HBV infection were associated with HBeAg positive (12). In this study, there was a considerable relationship between increased HBV load (copies/ml) and HBx (P = 0.01) particularly the mean viral load between 10^3 and 10^5 copies/ml, which increased the presence of the HBx gene. Chun-Jen Liu et al. (Taiwan, 2006) investigated the association between HBV viral Load and basal core promoter (BCP) mutations in a patient with hepatocellular carcinoma. There was a relationship between mutations and HBV DNA with an increased risk of HCC (13). The increased viral load associated with increased viral polymerase activity can produce mutations by causing incorrect nucleotides. The high rate of mutation gets from the lack of proofreading ability in RNA polymerases. Depending on their function, such silent mutations can be an important role to play in the course of the disease process, such as the T1800C mutation, causes of change in C143R amino acid in the HBx protein that are the most common risk factors for HCC (14). Mutations were shared between the HBx and the blind P53 activity through the SKp2 (S-phase kinase-associated protein 2) pathways and gene regulation which ultimately led to cell proliferation and cancer (14). The two most important mutations in these regions are A1762T, and G1764A, which change the codon to two amino acids at positions 130 (Lysin) L→K (Methionine) and 131 Valine (V)→(I) Isoleucine, respectively, most often linked to HCC (11). Xie et al. reported mutations in eight different nucleotide positions (1383 to 1753), (1653-1719) both of which were in the enhancer II region (1653-1719) (15). In this study, three mutations G1524A, G1525A, and G1767C were identified at the amino acid positions of 50 and 131 HBx genes (Fig. 3). Whereas aa 1-50 encodes the second inhibitor of HBx activity, the mutation created in amino acid 50 (G1524A, G1525A) probably changes the function's domain, and the inhibition of HBx activities in this type of mutation. Murakami et al. (1994) in a study of HBx deletion mutants, showed that pS-G5UTPL plasmid and using polymerase chain reaction cloning that lack 1 to 50 amino acids made HBx active (this domain perhaps had HBx inhibitor function). Also in this research, the transactivation

domain in HBx was detected between amino acids 52-148 (16). Lizzano et al. (2011) studied the C-terminal region of the HBx gene, suggesting that this region is essential for HBx function and stability. They show that cloning using pcDNA3.1(-) plasmid containing the HBx gene and deletion mutants of this region revealed that there is a domain containing 120-140 amino acids involved in the activation of nuclear mechanisms (17). Also, Kumar et al, 2004, reported that amino acids at the 109-131 domain are involved with HBx-associated mitochondrial (18). Thus, amino acid 131 is implicated in transactivation, activation of nuclear mechanisms, and mitochondrial localization of HBx. The mutation at this position (G1767C) will probably be made to disable transactivation activity, activation of nuclear mechanisms, and HBx localization. Also, in mitochondria, inactivation of these domains may alter acute or persistent to severe infection. Limitations of our study: We looked for mutations in the Enh II region of the HBx gene, but we did not find any mutations in this region, so we investigated the 3 mutations identified in the HBx region. More studies are needed, in particular, new approaches, such as NGS, have investigated the mutations that occur after treatment for patients to prevent the continuation of the disease and especially cancer.

CONCLUSION

HBx mutations present in different phases of HBV infection, often associated with prevalence rates of liver cancer. Identification of these mutations' importance on protein function, understanding of the course of the disease antiviral therapy. Certain the recognition and function of these mutations and alterations in the HBx gene can be predicted the pathogenicity of the virus and the severity of hepatic tissue damage and cirrhosis or carcinoma. This study is the first study in this region (southeastern Iran, Kerman) to investigate mutations in the HBx gene in the acute phase of HBV infection.

ACKNOWLEDGEMENTS

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