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Original Article

Internal Medicine Section

Mutations in Hepatitis-B X-Gene Region: Chronic Hepatitis-B versus Cirrhosis

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ABSTRACT

Introduction: Specific mutations in Hepatitis-B Virus (HBV) genome would proceed the development of chronic hepatitis B to more serious consequences like cirrhosis and end-stage liver disease.

Aim: This study was designed to detect deletion and insertion mutational patterns in the X-gene region in a population of chronic HBV and related cirrhosis patients.

Materials and Methods: Sixty eight chronic HBV patients and 34 HBV-related cirrhotics were recruited from the eligible cases (N=50) referred to the academic hospitals of Gorgan city, Northeast of Iran, between Jan 2011 to Dec 2013. The HBx region was amplified by semi-nested PCR using serum samples and analyzed by sequencing.

Results: Our findings showed deletions and insertions in the C-terminal of HBx of the cirrhotic group and 8 bp found in two chronic HBV cases (2.9%). We detected 15 types of deletions in cirrhotic cases such as 1762-1768, 1763-1770, 1769-1773 and T1771/A1775.

Conclusion: We found that the frequencies of deletion and insertion mutations in C-terminal of X-gene were more seen in cirrhotic patients comparing to chronic HBV cases in our area of study.

Keywords: Deletion mutation, Genotype D, Hepatitis B-virus, Hepatitis-B X-region, Insertion mutation

INTRODUCTION

Hepatitis B Virus (HBV) plays a critical role in the development of Hepatocellular Carcinoma (HCC). There are about 400 million people at risk of developing liver cirrhosis and other non-favourable consequences of HBV in the world [1].

Random genetic alterations in the viral genome such as point mutation, insertions and deletions would essentially affect the HBV natural course [2]. X gene is the one of the four partially overlapping Open Reading Frames (ORF) of HBV and highly conserved among all genotypes of this virus. HBx region encodes a 154-amino acid regulatory protein with a molecular weight of 17 kDa [3]. HBx protein has two functional domains, the N-terminal third (residues 1–50) is a negative regulatory domain, and the C-terminal two-thirds acts as a transactivation domain [4]. It comprises 456 nucleotides that contain main regulatory region such as TA rich sequences: TA1 (nts 1750–1755); TA2 (nts 1758–1762); TA3 (nts 1771–1775); TA4 (nts 1788–1795); Basal Core Region (BCP) and the Enhancer II (EnII) [5,6].

Mutation in these regulatory regions can lead to amino acid substitution in HBx protein and influence the function of this protein [7]. HBV gene replication and expression would be augmented by HBx as the transcriptional co-activator collaborating with host transcription factors. HBx would prepare a better cellular environment for HBV replication by stimulating the transcription of host genes related to cell proliferation and inflammation [3].

In addition, any mutational pattern including point mutation, combination patterns, deletion and insertion mutations in the HBx region are associated with severe liver disease. Furthermore, deletion and insertion mutations would lead to frame-shift mutations and abnormal expression of X-gene [8,9].

This study was designed to detect and compare the deletion and insertion mutational patterns of X-gene in a population of chronic Hepatitis B and cirrhotic patients in Gorgan city, Northeast of Iran.

MATERIALS AND METHODS

In this cross-sectional study a total of 118 serum samples were obtained from cases visiting the academic hospitals and private clinics in Golestan province between January 2011 to December 2013. Blood samples were collected from 68 HBsAg positive chronic cases and 50 HBV-related cirrhotic cases diagnosed by Magnetic Resonance Imaging (MRI), clinical and pathological analyses. HBV-DNA was just been detected in 34 of the cirrhotics. All statistical analysis was done for 102 HBV DNA positive-samples.

Exclusion Criteria

Patients were excluded if they were positive for HIV antibody, HCV antibody, HDV antibody, or negative (undetectable) for HBV DNA.

The study was approved by the Ethical Committee of Golestan University of Medical Sciences. Informed consent was taken from each case after a complete interview to explain the study goals and answering the possible questions for participants. Entering into the study was voluntarily and had no effect on the treatment course of patients.

HBV DNA Extraction: The sera were collected separately and stored at –70ºC until used. HBV-DNA was extracted from 200 µl of plasma using QIA amp DNA Mini Kit (QIAGEN, Hamburg, Germany) following the manufacturer's instruction. Extracted DNA was stored at –20°C for the next steps.

Primer Selection and Semi-Nested PCR: A primer pair (F1 and R1) was used for amplification of the HBV sequences spanning nucleotides 1365 to 2078 fragment, hence produced a 713-bp amplicon [Table/Fig-1] [10,11].

In the first round of semi-nested PCR, HBV DNA isolated from serum was amplified in 25 µl reaction volume containing 2.5 µl of 10X PCR buffer, 1.5 µl $MgCl_2$ (25 mMol/L), 0.5 µl of dNTP (10 mM),

Primer name	Sequence (5' to 3')	Target sequence
F1	ATCGTATCCATGGCTGCTAGGCT	1365-1387
R1	CAGAATAGCTTGCCTGAGTGC	2058-2078
R2	CACAGCTTGGAGGCTTGAACA	1881-1861

[Table/Fig-1]: Oligonucleotide primers used for semi-nested PCR and sequencing of X-region of hepatitis B [10,11].

0.5 µl of Taq DNA polymerase (QIAGEN, Hamburg, Germany) (5 U/µl) and 1 µl of each primers (10 pmol) in a total volume of 25 µl with distilled water. This mixture was heated to 94°C for five minute. The amplification was performed for 35 cycles 94°C for one minute, 55°C for one minute, and 72°C for one minute and final extension step consisted of 72°C for seven minute.

In the second round 713 bp used as template to amplify nucleotides 1365 to1881of HBV genome in with primer F1and R2. The second round semi-nested PCR was pre-ceded at 94°C for five minutes, followed by 35 cycles at 94°C for 30 seconds, 57.5°C for 30 seconds, and 72°C for 45 seconds, during which five seconds additional elongation time was added after each cycle, and at 72°C for five minutes finally. The 516 bp fragments as the second round PCR products were analyzed by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide to determine HBV DNA positive and negative samples.

DNA Sequencing and Mutation Analysis: The positive PCR products were examined by automated one direction sequencing (Macrogen Inc., Korea). For detection of mutation and analysis, nucleotide sequences were aligned with standard hepatitis B sequence (14, Accession number: AB033559) from Gene Bank database, and also with standard hepatitis B sequence of Iran in Gene Bank (8, Accession number: GU938305).

STATISTICAL ANALYSIS

Data were analyzed using the SPSS version 11.5 statistical software package (SPSS Inc., Chicago, IL, USA). For comparisons of the collected data, student's t-test was performed. The differences between the groups with p-values <0.05 were considered statistically significant.

RESULTS

In the present study complete HBx gene were examined by sequencing 102 serum samples consisting of 68 chronic hepatitis B and 34 cirrhotic patients.

[Table/Fig-2] shows the basic characteristics of chronic Hepatitis B carriers and cirrhotic cases. HBx was 3-truncated in 2 (2.94 %) HBeAg-negative chronic HBV cases. Furthermore, HBx was complete in 20 (58.82 %) cirrhotics, 3-truncated in 13 (38.23 %) and 5-truncated in one cirrhotic case (2.94%). A 3-truncated cirrhotic patients included 9 (69.2%) HBeAg negative and 4 (30.7 %) HBeAg positive cases. Also, the only 5-truncated cirrhotic case was HBeAg positive [Table/Fig-2].

Apart from the point mutations, in total, we found 15 different patterns of deletion and 10 patterns of insertion. No deletions or insertion, apart from 8bp deletion, were found in the X gene in 68 chronic HBV patients.

In this study 1763/1764 and 1766 deletion characterised as high frequency of deletions.

We also found a Basal Core Promoter (BCP) deletion mutation at position (1771-1775) in four cirrhotic cases (11.76%) with an especial pattern. In addition; the TA3 deletion mutation concurrently occurred with G1763/G1764 and C1766 deletions.

In one cirrhotic case, six types of insertion contained two 1-bp, two 2-bp, 10-bp and 12-bp were detected spanning from nt1623 to nt1660. Two large insertions of nucleotide sequences were TTCTTGCCCAAG (12-bp) and CTCTCTGTTG (10-bp) that respectively inserted at nt1623 and nt1651 of the X gene. This cirrhotic patient had insertion of A at nt1741 and insertion of G at nt1744.

In another cirrhotic case, G insertion at nt1505 and 1-bp nucleotide deletion (G) concurrently occurred at position 1512. A 1-bp deletion (G) at nt1512 counteracts effect of G insertion at nt1505 resulted in no alteration of ORF but amino acid sequence at 45 to 50 (VPADHG) showed no similarity with HBx (GSTADG) [Table/Fig-3].

Basic characteristics	Patients with chronic hepatitis	Patients with cirrhosis	p-value
Age (Mean±SD)	38.3±11.5	54±9	0.02
Sex			
Male Female	51 17	27 7	0.62
HBeAg			
+ -	18 50	9 25	0.87
Anti-HBe			
+ -	50 18	25 9	0.87

[Table/Fig-2]: Main characteristics of chronic HBV carriers and cirrhotic patients.

Deletion types mutation	Length of fragment	No.	%
G1743-T1745	3 bp	1	2.9
T1747&T1748	2 bp	1	2.9
A1762-G1764	3 bp	1	2.9
G1763- G1764	2 bp	5	14.70
G1763-T1765	3 bp	2	5.8
C1766	1 bp	6	17.34
T1767-T1768	1 bp	1	2.9
T1769-C1773	5 bp	1	2.9
G1770	1 bp	3	8.82
G1770-T1771	2 bp	1	2.9
T1771-A1775	5 bp	4	11.76
A1772-C1773	2 bp	2	5.8
C1773-A1775	3 bp	1	2.9
A1762-T1768	6 bp	1	2.9
G1763-G1770	8 bp	2	5.8

[Table/Fig-3]: Following deletion in X gene of cirrhotic patients.
*No deletions or insertion, apart from 8 bp deletion, were found in the X-gene in 68 chronic HBV patients

DISCUSSION

Most studies reported different types of point mutation in the HBx gene, and rarely published details about the rearrangement of X gene including deletions, insertions and duplication. Therefore, we tried to investigate the patterns and frequency of deletions and insertions in the X region in Iranian patients with chronic hepatitis B/genotype D and a group of HBV-related cirrhosis. Findings of the previous studies indicated that the C terminal truncated X protein often occurs in the progressive stages of chronic HBV infection, lead to severe liver disease such as cirrhosis or HCC [12-14].

C-terminal truncated HBx proteins lack C domain that is necessary for the suppressive effects of HBx on cell growth, proliferation, transformation and transactivation activity but this protein inhabited p53-mediated apoptosis, thus contributes to hepatocarcinogenesis [13,15,16].

In this study all deletions occurred in the C-terminal of X gene. Also, we described some novel types of deletions and insertions in the HBV/D-related cirrhosis patients.

One of the deletions was an 8 bp pair deletion presented in the chronic hepatitis B and cirrhotic cases. The 8 bp deletion found in the BCP region (nt1763 to 1770).

This region contains binding site of TATA box Binding Protein (TBP), liver-enriched transcription factors and regulated pre-C promoter activity [17]. Previous studies have shown that 8 bp deletion occurs in various clinical statuses of chronic hepatits B, cirrhosis and HCC patients [18-21]. In an experimental study, Biswas A et al. reported that 8 bp deletion results indown-regulation of pre-C promoter

activity and reduction of HBeAg synthesis by deleting the binding site of transcription factors [6].

TA-rich sequences affect the efficiency of pre-C mRNA transcription and span in the BCP sequence from nt1760 to nt1780. TA-rich sequences divide into TA1 (nts 1750–1755); TA2 (nts 1758–1762); TA3 (nts 1771–1775); TA4 (nts 1788–1795) regions [5,6].

Also, TA-rich region overlaps the TBP binding site and pre-C mRNA transcription initiation site. In the present study, 13 out of 15 types of deletions found in TA-rich region and some of them were 2 or 3 bp nucleotide sequences.

Deletion of this sequence may influence the core promoter function and pre-C mRNA, pregenomic RNA transcription leading to stop codon or frame shift; truncated HBx protein. The pre-C mRNA is translated into HBeAg, a secretary protein and pregenomic RNA is translated into the core protein (HBcAg) [22].

According to the previous studies, C-terminal truncated HBx is more oncogenic and one of the logical reasons can be deletion of the Sp1 binding site (nts 1731–1752) which negatively regulates the expression of X gene. X gene expression increases the deletion of Sp1 binding site [23]. In accordance with these published reports, we found G1743/T1745, T1747 and T1748 deletion in Sp1 binding site in a cirrhotic case which disrupted the Sp1 binding site.

P53-dependent transcriptional repression binding site of HBx mapped at nt1637–1667 and overlaps with the En-II region (nts 1627-1774) [24,25]. This binding site negatively regulates the replication of HBV; so deletion of this region may enhance the HBV replication. Furthermore, nts 1644-1666 of En-II considered as alpha box [26], which contains binding site for liver-specific transcription factors, such as HNF4, C/EBP, and/or C/EBP-like factors [27-29], and plays a central role in functions of En-II.

Our findings showed insertion of 12bp disrupting alpha box sequences in one cirrhotic case that might create new binding site (important site). Another 10bp sequences inserted in the Negative Regulatory Element (NRE) and P53-dependent transcriptional repression binding site disrupt these sequences.

Results of recently reports showed that deletion and insertion mutations at C terminal of HBx were more common in HBeAg negative, compared to HBeAg positive patients, which is similar to our findings [8,30] whereas, in other study deletion mutation were found at HBx C end of HBeAg negative patients.

In the present study, some C-terminal X gene deletions and insertion observed in Iranian patients with HBV-related cirrhosis. No similar mutations were found in other studies except 8d and TA3d [18-20]. These deletions and insertion vary in location and length; however, causes X protein truncation. Many studies claimed that BCP deletion causes reduction of HBeAg expression and might enable virus escape from the host immunity system. Furthermore, these deletions and insertions made a frame-shift in the HBx ORF and create a truncated HBx- protein associated with advanced liver disease [6,18,31].

Findings of the present study may shed a light for future studies to detect specific deletions and insertions of X gene and suggesting more follow ups of patients prone to the progression of liver disease at very early age.

LIMITATION

We did not detect enough cases of HCC to compare them with the studied population.

CONCLUSION

We found that the frequencies of deletion and insertion mutations in C-terminal of X-gene were more seen in cirrhotic patients comparing to chronic HBV cases in our area of study.

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