Original Article

Molecular Evaluation of HBV Core Gene Mutations in Asymptomatic HBV Infected Blood Donors in Iran

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Abstract

Mutations in the core promoter and precore regions of HBV cause down-regulation of HBeAg. These mutations are associated with chronic hepatitis, cirrhosis and Hepato Cellular Carcinoma (HCC). This study was carried out to sequence analysis of HBV core gene in HBsAg- positive blood donors in Iran. A total of 50 HBsAg- positive blood donor samples were examined in this study. Serological markers of hepatitis B including: HBsAg, HBeAg, HBeAb and HBcAb were measured by ELISA method. HBV-DNA was extracted from the sera, and then PCR was performed on extracted HBV-DNA using specific primer of gene C. After direct sequencing, the nucleotide sequences from 50 blood donors were analyzed using a reference sequences and then phylogenetic analysis was performed. Also, the line probe assay was used to detect mutations. The majority of donors (62.5%) were in the age group of 29 – 40 years old. Among all the HBV DNA positive cases, 87.8% were HBeAg negative. The prevalence of PC and BCP mutants were 12% and 55% respectively, among asymptomatic HBV infected blood donors by direct sequencing method. The results of this study showed that some of HBV infected blood donors had mutation in core gene of HBV and amino acid changes in B cell, T helper and CTL epitopes that can cause reducing HBe and HBc antigenicity in asymptomatic HBV infected blood donors and the development of escape mutants from host immune.

Keywords: Blood donors, core, epitopes, hepatitis B virus, mutations, precore

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Introduction

Hepadnavirues employ an unusual strategy for production of enormous number of virions during replication which makes rapid and substantial genetic sequence changes and alternations.[1] HBV infection can cause abroad spectrum of clinical outcomes ranging from asymptomatic carriage, self- limiting acute hepatitis, fulminant hepatic failure, chronic liver disease, and cirrhosis to Hepato Cellular Carcinoma (HCC).[2]

Previous studies from Iran have shown that prevalence of HBsAg has been reported as 1.7% population and 0.4% in blood donors. Over 35% of Iranian populations have been exposed to HBV.[3,4]

Mutations in the precore and core promoter regions of the HBV genome have been reported in many HBeAg- negative CHB patients. The most common precore variant is a point mutation from a G to A at nucleotide (nt) 1896 (A1896), that creates a premature stop codon at codon 28 and abolishes the synthesis of HBeAg.[5,6]

Basal core promoter mutants are characterized by point mutation in the promoters of both HBeAg mRNA and the core protein mRNA. The most common core promoter mutation involves two-nucleotide substitution at nucleotides 1762 and 1764.[7]

The reason for selection of mutations in the HBV genome is not fully discovered. However, viral and host factors, in addition to exogenous selection pressure, many partly be responsible for the predominant HBV species in an infected individual.[1,7]

The genotypic classification of HBV has been extended to eight genotypes (A to H) based on the complete genome sequences and/or S gene.[8–10] The most common pre core mutation, a G to A substitution at nucleotide 1896 (G1896A) is found in association with HBV genotype B, C, and D but not genotype A.[9,11] Absence of pre core mutation in HBV/A was expected due to the presence of C1858 instead of T1858, therefore no G-T wobble base pairing occurs.[2,12]

The extremely high immunogenicity of HBc particles has been known for a long time, in contrast to the relatively low immunogenicity of HBV envelope proteins. Thus, HBV infected patients develop a strong and long-lasting humoral anti-HBc response.[13,14] Among the HBV polypeptides, HBc induces the strongest B cell, T cell and cytotoxic T lymphocyte (CTL) response.[15]

There are limited studies available on asymptomatic HBV infected blood donors in our country. This study was designed to investigate molecular evaluation of HBV core gene in asymptomatic HBsAg-positive blood donors from Iran.

Materials and Methods

Samples

This was a cross- sectional study on fifty sera samples. These sera samples were collected between 2008 to 2012, during the routine screening of volunteer blood donors from all the blood transfusion centers in Iran. All serum samples were selected and stored at -70 °C for further works. The study population included 96% males and 4% females, aged 18 to 60 years old. The mean age of males was 35 ± 9.16 years old and that of females was 30.5 ± 0.7 years

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old. The majority of donors (62.5%) were in the age group of 29 – 40 years old. This study was approved by the medical ethics committee of Iranian Blood Transfusion Organization (IBTO).

Serological markers

Hepatitis B serological markers on the serum samples including HBsAg, HBeAg, HBeAb, and HBcAb were carried out by Enzyme Linked Immuno-sorbent Assay (ELISA), (DiaPro Diagnostic Bioprobes Kit; Milano, Italy) according to the manufacturer's instructions.[18]

HBV-DNA extraction and amplification

For amplification of core gene, HBV DNA was extracted on serum samples according to the manufacturer's instructions, QIAmp DNA mini-extraction Kit (Qiagen, Hilden, Germany) and amplified by PCR method. For performing HBV-DNA PCR, two oligonucleotide primers for core region were used. PCR mix was prepared in a total volume of 50 µl containing 25 µl Master mix (Takara Bio Inc, Shiga Japan), 1 µl of each (10 µM) primers and 5 µl DNA template. The amplification was carried out under the following condition; 3 minutes initial step at 95 °C, followed by 35 cycles of amplification including denaturation at 95 °C for one minute, annealing for 45 seconds at 60 °C and extension for one minute at 72 °C, one cycle including final extension at 72 °C for 10 minutes and final hold at 4 °C.

Sequencing and phylogenetic analysis

All PCR products were purified and sequenced with the dideoxy method (Takapozist Company) using the PCR primer (Forward 5'-GCATGGARACCACCGTGAAC-3' and Reverse 5'-GATACAGAGCWGAGGCGGT-3'). The BACKMAN COULTER software (CEQ 8000 Genetic Analysis System) was used for sequences analysis. Using the CLUSTAL W 1.8 software, the nucleotide sequences of HBV strains were aligned with a reference panel of sequences representative of each subtype retrieved from the Gen Bank database. Phylogenetic tree was drawn using the neighbor- joining (NJ plot software) method. The reliability of the phylogenetic classification was evaluated by a 1000- cycle bootstrap test.

INNO-LiPA kits

The detection of gene promoter polymorphisms in nucleotides 1762 and 1764 (BCP) as well as nucleotides 1896 preccore HBV by INNO-LiPA HBV precore assay (Innogenetics; Ghent, Belgium) was performed. After purification of viral DNA from the sample of blood donors, purified DNA was amplified in two stages with biotinylated primers. After amplification, the biotinylated DNA material generated from the basal core promoter and precore region of HBV was hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. After hybridization, unhybridized DNA was washed from the strip. Streptavidin labeled with alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen resulted in purple/brown precipitate.

Statistical Analysis

The results are expressed as mean ± SD. Chi-square and Fisher's exact tests were used to compare qualitative data. *P*-values of < 0.05 were considered statistically significant. All analyses were done using the SPSS software (version 20).

Results

HBV DNA was detected in all subjects. Among all the HBV DNA positive cases, 12.2% were HBeAg positive and 87.8% were HBeAg negative. HBcAb was detected in all cases.

The results of INNO-LiPA precore assay showed that from examined 50 samples, 11% had mutation from G to A in the precore region at nucleotide 1896 and 8% of cases had mixed infection including two types of wild type and mutant viruses. With direct PCR sequencing method, about 12% had mutation in precore mutation. Besides, 25% cases studied had precore variant T1858.

The most prevalent precore mutation, A1896, was frequently detected in HBeAg negative/anti-HBe positive status (66%). Among the HBeAg positive donors, 5% of cases had mixed precore mutation. There was no significant difference observed between the sex, age, and presences of each mutation ($P > 0.05$).

The basal core promoter mutant phenotype determination was 55% using direct PCR sequencing method and 53% INNO-LiPA precore assay. BCP mutation was more common than PC mutations in this study.

The results of sequence analysis showed that there were amino acid changes in HBV isolates in 14 donors, while the remaining of HBV isolates had wild type amino acid sequences (Table1). All 14 samples with amino acid changes in the core protein were anti-HBe positive. Of the cases having amino acid mutations, all had changes in T helper and B cell epitopes also 42% of them had changes in CTL epitopes. The most prevalent of these changes were found in the amino acids at the positions 21 (from T/A to S), 23 (from Y to F), 25 (from A/Q/T to P), 26 (from T to S) in CTL epitopes , 36 (from S to A), 40 (from D/P to E), 43 (from D to E), 44 (from N/P/T to S), 62 (from T/N to W), 64 (from D to E) in T helper epitopes and 79 (from N/R to P), 80 (from T to I), 81 (from G/A to S), 83 (from E to D), 109 (from A/ I /M to T), 112 (from F/N/T to R), 113 (from P/Q to E), 114 (from I/N to T) in B cell epitopes, (Table1).

Discussion

In this study, samples collected from asymptomatic HBV infected blood donors from all of 29 blood transfusion centers in Iran, and HBV C gene were amplified by PCR with specific primers. All PCR positive cases were analyzed by two methods: INNO-LiPA precore and nucleotide sequencing. The results showed that the INNO-LiPA precore assay is a rapid, sensitive and specific means of identifying commonly HBV precore variants, and showed good agreement with results of nucleotide sequence analysis. The INNO-LiPA assay is also easier to perform and analyze than nucleotide sequencing. Mixed populations of core wild-type and variants are frequently observed in serum, such mixtures can be difficult to detect by direct PCR sequencing. While the INNO-LiPA had high sensitivity in detect mixed variants.[16,17] In this study, 8% of cases had mixed mutations in the precore and core regions. Among donors, 12.2% were HBeAg positive that 5% of these cases had mixed mutation in precore regions.

Previous reports have shown that mutations in the HBV core are in HBeAg-negative and anti-HBe positive chronic patients with wild type precore sequences, suggesting that absence of HBeAg

Table 1. Amino acid substitutions in CTL, T helper and B cell epitopes of the hepatitis virus Core gene from blood donors with hepatitis B infection.

Blood donor	Amino acid change
IBTO-7.541	I66M, L116I
IBTO-24.16	S19L, T20P, T21S, T25P, D64E, E78D, A109T
IBTO-27.67	Q47H, S114T
IBTO-28.81	N18F, A21S, F38Y, L58A, T62W, T80I, I108L, F112R
IBTO-12.71	I42L, M109T
IBTO-16.116	R20P, Y23F, T26S, S36A, Q43E, N44S, D64E, I68L, Y110F, T112R
IBTO-16.85	D43E, N112R
IBTO-28.42	P44S, P113E
IBTO-26.42	Y23F, A25P, P40E, Q43E, T56R, N62W, T80I, Q113E, L116I
IBTO-22.4	Q25P, T26S, S36A, I42L, E57Q, I65L, G81S, T82R, N112R, L116I
IBTO-27.92	Y24F, D40E, T44S, A51H, S58A, S63G, Q84E, I86V, M109T, V115L
IBTO-28.76	S58A, D64E, I114T
IBTO-24.53	S41A, D43E, T49S, I53T, V58A, F59I, N78D, R79P, T80I, E83D, I109T, N112R, L115V
IBTO-24.54	P52H, E57Q, E78D, N79P, A81S, E83D, A106S, A109T, Y110F, N114T

is never solely due to a high proportion of precore mutants.[18–20] Core region mutations have frequently been identified in patients with chronic HBV infections and these mutations are even more common in HBeAg-negative patients, which coincide with the present results.[21,22]

The Sequencing results were compared to GenBank sequences in a BLAST search. The best matches and high-scoring matches were from Turkey (JF754630), China (FJ386590), and Syria (JN257198). Afterwards, the sequences from Iranian isolates with isolates from these countries were aligned and a phylogenetic tree was drawn. High similarly between these sequences was shown and the results of the BLAST search confirmed. This resemblance was due to pilgrimage and tourist trips to neighboring countries.

The genotypes of HBV are defined by an intergroup divergence of more than 8% in the complete genome sequence. So far, eight genotypes (A to H) have been identified.[23,24] There are some differences between the genotypes of HBV in the length of the Open Reading Frames (ORFs) and the size of protein products translated. The distribution of HBV genotypes found to be varied in isolates depends on geographical areas.[19,25] This study has also shown that genotype D, of HBV was dominant in Iranian HBV-infected blood donors. Our finding is consistent with the predominance of genotype D in the Mediterranean basin and in the Middle East. The predominant genotype of HBV in our neighboring countries such as Turkey and Syria is genotype D.

In a study carried out in 2007 on 100 HBsAg positive voluntary blood donors in France, it was reported that prevalence rate of mutation in precore region is higher than mutations in basal core promoter region.[12] In contrast, in our study the prevalence of PC mutation is lower than BCP regions. The discordance between our results and this study may be due to high relatively percentage of T1858 variant among blood donors in the present study and prevents incidence of mutation in precore region. This finding is consistent with those of other studies and suggests that the selection of a mutation may influence the subsequent selection of another substitution, thus conditioning the evolutionary pathway of the virus. The generation of multiple variant transcripts from a single template and the formation of a quasi-species pool provide the source material for the emergence of a mutant when selection pressure is applied.[13,20]

By analyzing the relationship between the different nucleotides that affect HBeAg expression, it was possible to observe that mutations in the BCP region did not affect the prevalence of substitutions in the PC region and vice-versa ($P > 0.05$). The substitution at position 1762 was usually present concurrently with the substitution at position 1764A and vice versa.[25]

The hepatitis B core protein (HBc) is highly immunogenic whereas HBV envelope proteins have comparatively low immunogenicity.[26] This means HBc is an important target for immune-mediated viral clearance via inducement of B cell, T helper cell and cytotoxic T lymphocyte (CTL) responses.[15] Important B cell epitopes of the HBc protein are located near amino acid sequences 74 – 89 and 126 – 135.[14,15] While important HBc protein immune recognition sites for T helper epitopes are at sequences 1 – 20, 28 – 47, 50 – 69, 72 – 105 and 108 – 165, as well as those for CTL epitopes are at 18 – 27, 88 – 96 and 141 – 151.[26,27] Studies showed that amino acid substitutions in positions 77 and 80 can cause to reduce HBe and HBc antigenicity.[14,23]

In the present study, mutations in immunodominant epitopes were identified in 14/50 (28%) of the analyzed asymptomatic HBV infected blood donors, while results for OBI donors in Mexico was 9/23 (39.1%).[3] A review study on Iranian patients, showed that the amino acid substitutions found in the five epitopes (CTL recognition epitope from amino acids 18 – 27, T helper recognition epitopes from 35 – 45 and 49 – 69 and B cell recognition epitopes 76 – 87 and 105 – 116) of the Hepatitis B core antigen.[28] In the present study, changes in amino acids were observed in CTL recognition epitope from amino acids 18 – 27 in positions 19, 21, 23, 24, in T helper recognition epitopes from 35 – 45 and 49 – 69 in positions 38, 40, 59, 64 and in B cell recognition epitopes 76 – 87 and 105 – 116 in positions 80, 109, 113, 114, 116 that our results are consistent with those of previous studies in Iran.[28]

Therefore, the accumulation of these mutations due to host immune pressure in the course of viral persistence could lead to the progression of liver damage and the development of mutants that escape recognition by host immune.

Previous reports have shown the presence of a higher viral load in infection with BCP/ PC mutations in cases with severe liver disease.[6,29] In this way, blood donors with these mutations should be followed up for presence of these mutation and development of liver complications.

In conclusion, the D genotype is the main circulating HBV strain in Iranian blood donors with asymptomatic status. Molecular

evaluation of core gene mutations in CTL, T helper and B cell epitopes in a population of asymptomatic HBV infected blood donors would give further insight into the role of mutations of these regions in viral persistence. For determining a relationship between mutations in these epitopes and the disease outcome in asymptomatic blood donors, it is necessary to perform these studies on bigger sample size with the complete sequence of hepatitis B core gene.

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