

Mutations in pre-core and basal-core promoter regions of hepatitis B virus in chronic HBV patients from Golestan, Iran

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ABSTRACT

Objective(s): It has been reported that the mutation of the pre-core (PC) and basal-core promoter (BCP) may play an important role in the development of HBV-related hepatocellular carcinoma (HCC). In this study the PC and BCP mutations were investigated in chronic HBV patients.

Materials and Methods: In this study, 120 chronic HBV patients from Golestan, Northeast of Iran who were not vaccinated against HBV, were recruited from the year 2008 to 2012. HBV-DNA extraction from plasma and PCR were performed and positive PCR products were subjected to automated sequencing.

Results: One hundred out of 120 (83.3%) patients were HBeAg negative. Comparison of our nucleotide sequences with reference sequence showed high rate mutation in BCP and PC region (96.66%). Frame shift mutation was found in 78 (65%) of patients in BCP region, among them 8 (6.6%) patients showed mutation in PC region.

Conclusion: Our results demonstrated high rate of mutations in BCP and PC regions among HBV chronic patients in Northeast of Iran.

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Introduction

Currently, Hepatitis B Virus (HBV) infection is a global health problem, and more than 350 million people around the world are chronic carriers of this virus (1). Because of the spontaneous error rate of viral reverse transcriptase, the hepatitis B virus (HBV) genome evolves during the course of infection under the antiviral pressure of the host immunity (2). These HBV variants could display alterations in epitopes important to the host immune recognition, resulting in enhanced virulence with increased replication of HBV, resistance to antiviral therapies, or facilitated cell attachment and penetration (3). HBV genome has 4 open reading frames (ORFs), including envelope genes coding region (pre-s₁, pre-s₂ and s), pre-core (pc) and core (c), polymerase (p), x gene coding region (4). The HBV x gene (HBx) is a 16–18 kDa protein, a transcriptional transactivator and can positively regulate the transcription of a wide variety of viral and cellular promoters (5). The observation which reported that HBx transactivates

several cellular genes regulating cell growth suggests that HBx may induce uncontrolled cell proliferation (6). HBx is a multi-functional protein that plays a role in the development of hepatocellular carcinoma (HCC). The basal-core promoter (BCP) is the minimal essential promoter sequence accurately mapped from nucleotide 1742 to 1849 of HBV complete genome and overlaps with the x gene region (nucleotide 1374-1836). Since the x gene overlaps with the BCP region, mutations in the x region will also have specific effects on BCP region (7). T-A mutations in BCP are frequent in all genotypes while other mutations seem to be more related to specific genotypes (8). Pre-core (PC) variants are more common among patients with genotype D (65-75%) than genotype A (9-18%) (9,10). Isolates with transversion at A1762T together with G1764A mutations in the BCP are often present in hepatitis B carriers with chronic hepatitis, fulminant hepatitis, HCC and less often in inactive carriers and immune suppressed patients (11).

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The most frequently observed mutation in the PC region is a G-A change at nucleotide 1896 resulting in a stop codon and hence, leading to premature termination of the PC/Core protein construction, which is the precursor of HBeAg (12). However, this mutation is located within the epsilon (ϵ) structure, a highly conserved stem-loop essential for initiation of encapsidation within the viral replication cycle. In order to stabilize the ϵ structure, the nucleotide at position 1896 is paired with the nucleotide at position 1858, which naturally is a thymidine (T) in genotypes B, D, E, and G and a cytosine (C) in genotype A (13). In an experimental study, the 1762 and 1764 mutations reduced the transcription of pre-core mRNA by interfering with the binding of transcription factors, thus, supporting the assumption that these mutations down-regulate HBeAg synthesis (14). Moreover, it was also frequently found in both HBeAg-positive and HBeAg-negative chronic hepatitis (15). Iran is considered as a country with low endemicity of chronic HBV infection (16). Our previous study in Golestan province showed higher prevalence of HBV infection and HBV S gene mutation comparing to other parts of Iran (17, 18). In this study, the PC and BCP mutations in chronic HBV patients in Golestan province of Iran was investigated.

Materials and Methods

Sample selection

A total of 120 patients with proven chronic HBV infection for more than two years, were chosen according to clinical and paraclinical evidence from Golestan province, Iran from the year 2008 to 2012.

None of the patients were vaccinated for HBV or had antiviral therapy; all patients had negative results for antibodies against hepatitis C, hepatitis D and Human Immunodeficiency Virus. The research project received the confirmation of the Ethics Committee and all the patients signed the testimonial letter. Blood samples were collected in the EDTA 5% anti-coagulant and plasma was separated for further examination.

HBV infection marker detection

The HBV serological markers (HBeAg) were tested using commercially available enzyme-linked immune sorbent assay kits (DIALAB GmbH, Germany).

DNA extraction

HBV-DNA was extracted from 200 μ l of each plasma sample using QIAamp DNA Mini Kit (QIAGEN, Hamburg, Germany) following the manufacturer instruction and extracted DNA was stored at -20°C for PCR process.

Table 1. Oligonucleotide primers used for PCR and sequencing of BCP and Pre core region of Hepatitis B (19)

Name	Sequence 5' to 3'	Base position
HBc-F	ACCTTGAGGCATACTTCAA	1689-1708
HBc-R	CAGAATAGCTTGCTGAGTGC	2058-2078

Primer selection

Suitable primers were selected to achieve optimum PCR sensitivity and the BCP and PC region amplification. Following primers listed in Table 1 yielded 390 bp amplification according to nucleotide position 1689 to 2078.

PCR and DNA amplification

Amplification mixture contained: 100 ng of extracted DNA 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 U Taq DNA polymerase (QIAGEN, Hamburg, Germany) and 0.4 pmol/ μ l of each primer in a total volume of 50 μ l with distilled water. The PCR profile started with an initial 5 min denaturation at 95°C, followed by 30 cycles of amplification including denaturation for 1 min at 95°C, primer annealing for 1 min at 55°C and extension for 2 min at 72°C, with a final extension at 72°C for 5 min. The 390 bp amplification products were analyzed by gel electrophoresis on 1% agarose gel stained with ethidium bromide to determine HBV-DNA positive and negative samples.

DNA sequencing and mutation analysis

The positive PCR products were subjected to automated sequencing (Macrogen Inc., Korea). Then, nucleotide sequences were aligned with standard hepatitis B sequence, [Accession number: AB033559] from Gene Bank database, for mutation detection and analysis (20). All data that were collected from the study were analyzed using SPSS.16 software.

One of the samples with critical mutations in BCP and PC region was registered in NCBI under the accession number: KC928094

Results

The mean age of patients was 36.8 year, 74% of them were male and 26% were female. All of the patients were HBsAg positive and 100 out of 120 (83.3%) patients were HBeAg negative. Isolates belonged to genotype D subgenotype D₁, subtype *ayw*₂ according to our previous study (18). Comparison of our nucleotide sequences with reference sequence showed high rates of mutations with a frequency of 97.5% and 99.2% in BCP and PC regions, respectively. In BCP region A1762T and A1762G mutations were detected in 25 (20.83%) and 5 (4.16%) of patients, respectively. The most important mutations detected in BCP were the 8 bp nucleotide deletion from 1763 to 1770 which were observed in 7 (5.83%) patients. In the case of

Table 2. Frequency and position of point mutations in pre-core (PC) and basal-core promoter (BCP) of HBV

HBV PCregion			BCP region		
No. (%)	Frequency of point mutation	position	No. (%)	Frequency of point mutation	position
49 (40/83%)	2	A64S, D69E	5 (4/16)	1	C148W, R128D, L141W, C137G, V131L
21 (17/5%)	3	G29D,A64S, D69E, C12stop,P15A,E37Q, A87P, Q2E, W28stop	4 (3/33)	2	V142G,N149G, L134G,L141W, E122G,V131L, K140F,V142G
20 (16/66%)	4	S13T,V17I, Q2stop, A64S,Y67F,D69E, G29D, M1L,Q2R, W28stop, P79A,T41S, ,I32L, S50H, Q86K, A87P	9 (7/5)	3	C137G,L141W,C143stop, K130E,V133L,L134W, K140F, W120G,V131L, E126C,I127S,R128S, W120G,E121R,E122S, L123V W120D,E121G,E122R,L123V, C137G,K140F,L141W,N149D, R128G,V131L,I127L,K130M,, D119A,V142G
14 (11/66%)	5	M1L,S13T, V17I, W28stop, A64S, D69E, T41S,S78T,Y67F,G29D, A87P,S50A, V56L,L60Q, E72K,P79A, S78T, V42L	6 (5)	4	E122R,L123V,I127S,K130M,V131I, W120stop,A146S, E125G,E126G,I127D
5 (4/16%)	6	G29D,V42L,A64S,D69E,A87P, M1L, Y67F, P74Q,G29D,S50Q, S78T, T41S, S50T, S13T,V17I,W28stop,E37K	4 (3/33)	5	E126R,I127R,R128stop,L129V,K130M, V131I, E122R,L123V, K130M,V131L,A146S, E121R,E125G, W120D
2 (1/66%)	7	S13T,V17I,W28stop,S78T,A87P,T41S, A64S,Y67F,D69E,Q86K,A87P	4 (3/33)	6	E121G,R128D,L129stop,K130I, C137E,R138W, W120D, E122R,L123S,E125G,E126G,I127D, L134stop
101 (100%)			36 (100)		

mutations in PC region, G1896A was detected as the most prevalent mutation in 44 (36.66%) patients leading creation of stop codon at the 28th amino acid of PC region in 39 (32.50%) of patients which 41 (93.18%) of them were HBeAg negative. Frame shift mutation was found in 78 patients (65%) in BCP region, but this rate of mutation in PC region was seen only in 8 patients (6.6%).

HBeAg positivity and BCP and PC mutations

Our results showed that among 31 cases with A1762T and 44 cases with G1764A point mutations in BCP region of HBV, 24 (77.4%) and 34 (77.27%) were found in HBeAg negative patients, respectively. In the case of mutations in PC region, G1896A was detected as the most prevalent mutation in 44 patients from which 41 (93.18%) were HBeAg negative (Table 2).

Point mutations in basal-core promoter (BCP) region

Our findings demonstrated the occurrence of 59 point mutations including 21 (35.6%) cases as missense mutation (with amino acid altering) and 38 (64.4%) cases as silent mutation (without amino acid altering) in BCP region. These mutations occurred in area of BCP which have overlap with X region of HBV (HBx). Distribution of single, double, triple, quadruple, and more than quadruple point mutations in the BCP region were 5 (4.2%), 4 (3.3%), 9 (7.5%), 6 (5%) and 8 (6.7), respectively. Mutations at T1753G and T1753C of BCP region were seen in 10 (8.33%) and 12 (10%) cases, respectively, which caused I127C amino acid mutation in 27 (22.5%) of

patients. A1762T and A1762G mutations were detected in 25 (20.83%) and 5 (4.16%) of patients, respectively, which affected the amino acid L130M in 10 (8.33%) patients. G1764A, G1764T and G1764C substitutions were seen in 24 (20%), 16 (13.33%) and 4 (3.33%) cases, respectively, which caused V131I change in 9 (7.5%) patients (Table 3).

The most important mutations detected in BCP were the 8 bp nucleotide deletion from 1763 to 1770 which was observed in 7 (5.83%) patients. Figure 1 shows the distribution and frequency of amino acid substitutions in BCP and x region of HBV in chronic patients.

Point mutations in precore (PC) region

Among 78 point mutations in PC region, 26 (33.3%) occurred as silent mutation and 52 (66.7%) as missense mutation. In the case of point mutation distribution, 49 (40.8%), 21 (17.5%), 20 (16.66%) and 21 (17.4) cases showed double, triple, quadruple and more than quadruple mutations in the sequenced region of PC region, respectively. Substitution at G1896A was seen in 44 (36.66%) leading to the creation of stop codon in the 28th amino acid of PC region in 39 (32.50%) of patients. The most prevalent mutations in PC region at the level of amino acid substitution, were A64S and

Table 3. The rate of basal-core promoter (BCP) and pre-core (PC) mutations in chronic patients and their HBeAg result

Total mutation	HBeAg-	HBeAg+	Mutation
31(100%)	24 (77.4%)	7 (22.6%)	A1762T
44 (100%)	34 (77.27%)	10 (22.73%)	G1764A
44 (100%)	41 (93.18%)	3 (6.82%)	G1896A

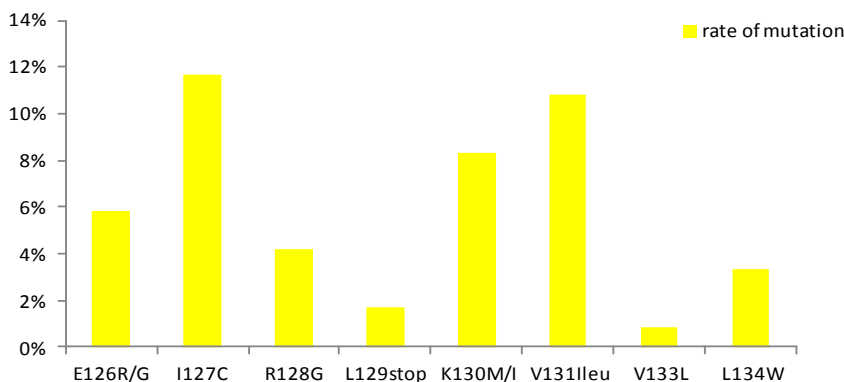


Figure 1. Frequency and distribution of amino acid substitution in X gene

D69E as they were detected in 102 (85%) and 99 (82.5%) of patients, respectively. Figure 2 illustrates the rate of mutation in different amino acids of PC region of HBV in chronic patients.

Discussion

HBV core promoter overlaps partially with HBx coding sequence, so, the nucleotide mutations in core promoter induce HBV X protein substitution (21). Since HBV genotypes are not uniformly distributed around the world, pre-core mutants also have a particular distribution, being more frequent in some regions such as Asia and the Mediterranean basin, where genotypes B, C, or D are predominant, and rarely in North America and Europe, where genotype A is commonly found (22). This study was designed to determine the BCP and CP mutations in patients infected with HBV genotype D and its distribution in HBeAg negative cases using sequencing and alignment with standard sequence.

As it was shown in the results, genotype D was dominant type of HBV in our study and overall prevalence of BCP and PC mutation was 99.2% and 97.5%, respectively. It has been shown that the prevalence of PC and BCP mutations depends on HBV

genotypes, and these mutations are related to fulminant and severe hepatitis and hepatocellular carcinoma (23-26).

The high rate of mutations in PC and BCP region of HBV genotype D patients in compare with other genotypes has been reported from different studies in Brazil (22, 27, 28) and Netherland (29). In contrast with our data a report from UAE revealed low rate of mutation in precore (58.0%) and Basal Core Promoter (25.3%) regions (30).

The G1896A mutation in PC has been found to be the most common mutation in chronic HBeAg-negative patients (31). This mutation creates a stop codon that prevents translation of the pre-core protein and terminates the production of HBeAg. However, these patients continue to synthesize HBV DNA at sufficient levels to continuously damage liver and progress toward cirrhosis (32). Median prevalence of the 1896 PC mutation in HBeAg-negative adults was reported to be 50% in Asia, 92% in the Mediterranean, and 24% in America (33). The frequency of the 1896 PC mutation varies geographically and primarily depends on the genotype of HBV (34, 35). The present study showed substitution at G1896A in 44 (36.66%) patients from which, 41 (93.18%) were HBeAg negative. In

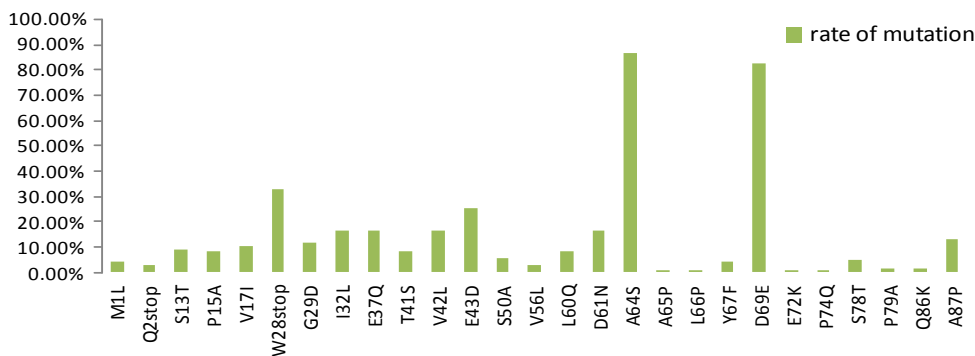


Figure 2. Frequency and distribution of amino acid substitution in PC region

agreement with our results, a study done in Brazil showed that the G1896A pre-core mutation occurred in 36% of Brazilian patients with chronic hepatitis B, among which, 58.6% were HBeAg-negative (36). Higher rate (95.3%) of the G1896A mutation was also reported in the HBeAg negative group (37). The *e* antigen is an important target for cell-mediated and antibody-mediated immune responses, so that the inhibition of *e* antigen production by pre-core mutants may help the virus to evade the host immune response (38). Hence, the occurrence of the pre-core mutation seems to be a strategy of viral selection secondary to the immunological pressure against HBV (39). In contrast with our results the study from Sweden determined that none of six genotype D isolates showed the PC stop codon mutation at nt 1896 during 17 month follow-up of after anti HBe seroconversion (40).

The most common BCP mutation is the double A1762T and G1764A nucleotide exchange, which results in a decrease of up to 70% of HBeAg expression (41). These mutations reduced the transcription of pre-core mRNA by interfering with the binding of transcription factors, thus, supporting the assumption that these mutations down-regulate HBeAg synthesis (42). Results of present research showed both BCP mutations at A1762T (36.66%) and G1764A (25%) of patients and 19.16% showed A1762T/G1764A double mutations from which 24 (77.4%) and 34 (77.27%) were seen in HBeAg negative patients, respectively. These are in agreement with a study from China which reported that the rate of T1762/A1764 mutation was 35% in genotype D of hepatitis B (43). A total of 77.3% HBeAg negative mutants of A1762T and G1764A in the present study corresponds to the Poovorawan study that shows paired mutations from A to T at nucleotide 1762 and from G to A at nucleotide 1764, in 75% of HBeAg-negative patients (43). Changing the core promoter from T1762A to G1764A not only has a bearing on the HBeAg anti-HBe phenotype, but also will affect the function(s) of the X protein. These nucleotide changes cause K130M and V131I conversions. The wild-type sequence is part of a region which confers a serine protease inhibitor function of the x protein (44), which has been suggested to play an important role in transcriptional transactivation activity (45).

Present study showed accompaniment of mutation at G1896A in 44 (36.66%) patients, A1762T, G1764A in 36.66% and 25% of patients infected with genotype D HBV respectively, that is similar to a recent report from Iran (46), as well as a study from Thailand (47). In agreement with our results, a study from Germany which was done on HBeAg negative chronic hepatitis B patients, showed that the overall prevalence of the precore stop codon mutant and basal core promoter mutant was 46%

and 59%, respectively. Genotype A and D are the major strains in Germany (48). It has been also determined in China that 38% of the HBeAg-negative patients harbored the pre-core stop codon (G1896A), 42% had the BCP mutation of A1762T/G1764A, while 12% had both the BCP double mutation and the PCG1896A mutation after HBeAg seroconversion (49). Similar results have been observed in Thailand (50). Lower rate of mutation in pre-core (58.0%) and basal core promoter (25.3%) regions were reported from the UAE in which 94.3% of patients were HBeAg-negative (30).

The 8bp deletion mutation in nucleotide 1763-1770 was observed in 7 (5.8%) of HBeAg negative patients. This mutation has been reported by Moriyama *et al* (51). This 8 bp deletion was shown by Unchida *et al* (52) and Kohno *et al* (53) at 1770-1777 and 1768-1775, respectively. It is suggested that these mutations create a C-terminally truncated X protein, and probably mutation of the enhancer II/core promoter element (52). The reduction of HBeAg is due to both the reduced pre-core promoter activity and the defect in HBx (53).

Deletions in core promoter (CP) region have been found in patients with chronic hepatitis B, in asymptomatic carriers, anti-HBe positive infection and HCC (54-56). The core promoter deletion mutant is characterized alone by low-level viremia (57). Most CP deletions are around 18bp to 21bp and have been found to be in BCP region. We did not see any mutation in our patients. These deletions usually involve the first and second AT rich regions and overlap some transcription factor binding sites; therefore, these mutations may affect the expression and function of HBx protein, and mostly result in a frame shift and truncation of the HBx at its C-terminal end which is essential for transactivating activity (57).

Amino acid substitutions in the core gene at amino acid 64 and 69 were much more common in anti-HBe positive samples with the pre-core stop-codon mutation. Most of reports show low rate of mutation at these positions (58). However, we have shown high rate amino acid substitution as it was observed in 102 (85%) and 99 (82.5%) patients at A64S and D69E, respectively.

The results of this study indicate that the average rate of stop codon mutations in PC and double mutations in BCP in Golestan province of Iran were similar to the other reports from Iran (43) and other countries (22, 27, 28, 59). These mutations were specially found in HBeAg negative patients, as shown in the results of this study, which may help HBV to expand its viral proteins, and promote cancer development.

Conclusion

The average rate of stop codon mutations in PC and double mutations in BCP in Golestan province of Iran were similar to the reports from Iran and other

countries. These mutations were specially found in HBeAg negative patients. X gene protein was found to display various types of biological activities, but its specific role of wild and mutant forms in the pathogenesis of liver cancer has yet to be elucidated. The clinical and virological significance of PC and BCP mutations are yet to be fully understood. Further studies are necessary to elucidate the exact role of these mutations in the clinical course of HBV infection.

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