

## Mutations in the Basal Core Promoter and Precore/Core Regions of Hepatitis B Virus in Patients Co-Infected With Human Immunodeficiency Virus

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### ABSTRACT

**Background and objectives:** Globally, about one third of the population has been infected with Hepatitis B virus (HBV) and more than 400 million people have become chronically infected. Nearly, 20-25% of all carriers develop serious liver diseases such as cirrhosis, chronic hepatitis and hepatocellular carcinoma (HCC). According to the World Health Organization, HBV infection causes more than one million deaths every year. Co-infection with Human Immunodeficiency virus (HIV) and HBV is common, since both viruses have the same routes of transmission. Approximately 10 -15% of HIV-infected individuals develop chronic hepatitis B. The risk of liver diseases-related deaths is also higher in the co-infected patients. According to previous studies, mutation of the pre-core (PC) and basal-core promoter (BCP) regions may play an important role in development of HBV-related HCC and severe liver disease. The aim of this study was to investigate mutations in the BCP, PC and core regions of HBV in HIV-positive patients.

**Methods:** DNA was extracted using commercial kits to determine the BCP, PC/core mutations in 124 HIV/HBV co-infected patients (32.4% female and 67.6% male). Polymerase chain reaction (PCR) was performed using specific primers. The positive PCR products were subjected to automated sequencing. Then, nucleotide sequences were aligned with the standard hepatitis B sequence [Gene bank, accession number: AB033559] for mutation detection and analysis.

**Results:** In this study, three patients (8.1%) were HBeAg-positive and all of them were HBsAg-positive. The mean of CD4 cell count was 120 cells/mL. The mean age of the patients was 36.16 years. The important pathological mutations in HBV patients including 1752A (73%), 1773C (70.3%), 1753C (10.8%), 1896A (8.1%) and 1762T/1764A (2.7%) were detected in this study.

**Conclusion:** Identification of mutations in co-infected patients is of greater importance compared to mono-infected patients, because it can be useful for prediction of HCC-related mutations. Co-infection with HIV has important effects on the natural history of HBV infection, and creates different mutational patterns compared to mono-infected patients.

**Keywords:** HBV, HIV, Mutation.

## INTRODUCTION

Hepatitis B virus (HBV) infection is an important health problem in the world. About more than one third of the world's population has been infected with HBV. While 2-10% of the world's population are chronic carriers of HBV, nearly 25% of them develop serious liver diseases such as cirrhosis, chronic hepatitis and hepatocellular carcinoma (HCC). Hepatitis B infection leads to more than one million deaths every year (1-3). Polymerase chain reactions (PCR)-based assays and genome sequence analysis have shown remarkable genetic variability in HBV. Currently, 10 genotypes (A–J) and several sub-genotypes have been identified, which are scattered across different geographical regions (4). Although Iran is considered a low endemicity country, the prevalence of HBV is increasing in the country. The co-infection with human immunodeficiency virus (HIV) is another important challenge in the healthcare system (5). Co-infection with HIV and HBV is common, since both viruses are usually transmitted through blood-to-blood contact and sexual contact. Approximately 10-15% of HIV-infected individuals are chronically infected with hepatitis B. In highly endemic areas like Africa and Asia, the prevalence of HBV is even higher (6). It is well demonstrated that HIV infection can accelerate HBV-associated liver diseases, such as the progression of cirrhosis in co-infected individuals (4). The risk of death caused by liver disease in HIV–HBV co-infected patients is 14 times higher than in mono-infected patients (7). Different HBV genotypes may have distinct impacts on progression of acquired immune deficiency syndrome. Increasing evidence suggests that HBV genotype is an important factor in determining HBV disease progression and response to antiviral treatment. In addition, certain mutations that emerge under medical pressure (vaccine or antiviral therapy) made the management of HBV infection more difficult (7). Mutations in the pre-core (PC)/core promoter are not HBV genotype restricted and there is limited information available on the association between HIV infection and HBV mutations in co-infected patients. The route of transmission and prevalence of HBV in HIV-positive patients are different. The highest incidents of HBV transmission in HIV co-infected individuals are observed in

homosexual men followed by intravenous drug users, especially in Iran (8, 9). The rate of HBV virion production (up to  $10^{11}$ /day) is higher than HIV and approximately 10 times higher than other DNA viruses. The HBV reverse transcriptase does not have proofreading activity, and with enormous daily virion production, errors occur during replication. The error rate of the HBV pol has been estimated as  $10^{-7}$  per nucleotide per day. Thus, approximately  $10^{14}$  nucleotides are replicated daily with potentially  $10^7$  base pairing errors (10). The core promoter (CP, nt1575-1849) of the viral genome plays a significant role in HBV replication as it directs initiation of transcription for the synthesis of both the PC and pre-genomic (pg) RNAs. The CP consists of the basal core promoter (BCP, nt 1743-1849) and the upper regulatory region (nt 1613-1742). The BCP has both positive and negative regulatory elements that modulate promoter activity (10). It is sufficient for precise initiation of both PC mRNA and pg RNA transcription *in vivo*. BCP contains a major nuclear binding site, which is recognized by different members of the nuclear receptors superfamily (including PPAR $\alpha$ -RXR $\alpha$  heterodimer, HNF4) and a series of transcription factors (such as C/EBP) to regulate the transcription of the PC and core RNAs. The CP overlaps with the 3'-end of the X ORF (nucleotide 1374-1836) that affect mutations in this region. The 5'-end of the PC region also contains *cis*-acting elements, and its transcription regulation is controlled by cellular and viral factors. This regulatory region exhibits high sequence conservation but when variations occur, they may contribute to the persistence of HBV within the host, leading to chronic infection and cirrhosis. Different biological functions of the PC and pg RNA, and differential regulation of BCP may affect HBV replication and pathogenesis (11-13). G to A change at nucleotide 1896 is the most frequently observed mutation in the PC region, which results in a stop codon and ultimately leading to premature termination of the PC/core protein (precursor of HBeAg). This mutation is located within the epsilon ( $\epsilon$ ) structure, a highly conserved stem-loop that is crucial for initiation of encapsidation within the viral replication cycle. However, this mutation pattern in co-infected patients is slightly different and less frequent (13, 14).

Isolates with transversion at A1762T together with G1764A mutations in the BCP are often present in hepatitis B carriers with chronic hepatitis, fulminant hepatic failure (FHF) and HCC patients but less frequent in inactive carriers and immune suppressed patients. The 1762 and 1764 mutations reduce transcription of the PC mRNA by interfering binding of transcription factors, which in turn down-regulates HBeAg synthesis. Some studies have shown that genomic mutations at positions 1727G, 1741C, 1761C, 1757A /1764T /1766G, 1773T, 1773T /1775G and 1909C are associated with HCC in HBV-infected patients (15-19). Several studies have reported an association between CP mutations and increased risk of liver inflammation, cirrhosis (20) and HCC (21). Some studies have reported an association between PC mutations and fulminant hepatitis (22-24). To our knowledge, there are limited reports on HBV BCP, PC/core mutations in patients co-infected with HIV in Iran. The current study investigated the mutational patterns of HBV in HIV-HBV co-infected patients.

#### MATERIAL AND METHODS

A cross-sectional study was performed to identify patients with HIV and HBV co-infection. Blood samples were collected from 124 patients with confirmed HIV/HBV infection who were referred to HIV Prevention Centers in cities of Kermanshah, Khorramabad, Tehran and Shiraz. Serum samples were collected to identify and confirm hepatitis B by serological testing using HBV serological markers, enzyme immunoassay kits (HBsAg, anti-HBc, and HBeAg) (Dade Behring Inc., Delaware, USA) and virological analysis. Medical records and demographic data including gender, age, CD4 count and probable route of HIV transmission were collected. The most common route of HIV transmission (59.40%) was via intravenous drug use along with risky sexual behavior. Two hundred  $\mu$ l of serum were used for HBV-DNA extraction using commercial high pure viral nucleic acid kits (Roche, Hamburg, Germany), according to manufacturer's instructions. The DNA was amplified by polymerase chain reaction (PCR) using the following pair of primers (GeNet Bio, Korea): Forward 5'ACCTTGAGGCATACTTCAAA- 3' Reverse 5'-CAGAATAGCTTGCCTGAGTGC-3'

The length of amplicon was 390 bp. Briefly, 100 ng of the extracted DNA was added to an amplification mixture containing 1X PCR buffer, 1.5Mm  $MgCl_2$ , 0.15 Mm dNTP mix, 2.5 U Taq DNA polymerase (QIAGEN, Hamburg, Germany), 0.2pmol/ $\mu$ l of each primers and distilled water in a total volume of 50  $\mu$ l. Thermal cycling conditions for PCR were as follows: initial denaturation at 95° C for 5 min; 35 amplification cycles (denaturation for 1min at 95°C; annealing of primers for 1 min at 55°C; extension for 2 min at 72°C); and a final extension step for 5 min at 72°C.

Direct sequencing of positive PCR products (Macro gene, South Korea) was done to determine the BCP and PC/core mutations. The PCR products were compared to the reference sequence (GenBank, Accession number: AB033559) using a BLAST search analysis. The nucleotides were translated into amino acid sequences using the translation tool on NCBI (National Center for Biotechnology Information) website and Gene Runner software to determine amino acid mutations. The data was analyzed using SPSS software 17.0 (SPSS, Inc., Chicago, IL). P-values < 0.05 were considered as statistically significant. The study was approved by the Research Ethics Committee of Golestan University of Medical Sciences.

#### RESULTS

The mean age of patients was 36.16 (43.2% were <32 years). All patients were HBsAg-positive and three (8.1%) were HBeAg-positive. The mean of CD4 cell count was 120 (cells/ $\mu$ L). There were 70 point mutations consisting of 42 (60%) missense mutations and 28 (40%) silent mutations, which had no effect on the amino acid sequence. There were 17 BCP mutations at the amino acid level and 25 amino acid changes in the PC/core region.

Some of the high-level mutations such as 1912T (94.6%) and 1978A (86.5%) were silent. The important missenses mutations that altered the amino acid were 2003T/2005A [(100%) that changes A64S (100%) amino acid] and 2020A [(86.5%) that changes D69E amino acid]. The main pathologic mutations in patients were 1762T/1764A (2.7%), 1896A (8.1%), 1753C (10.8%), 1773T (70.3%) and 1774T (13.5%).

Figure 1- The most common amino acid mutations

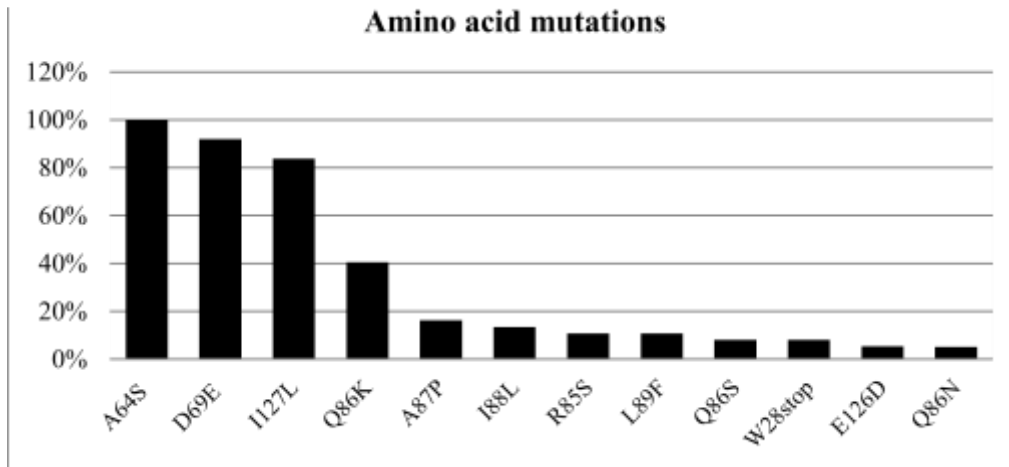


Figure 2- The most common nucleotide mutations

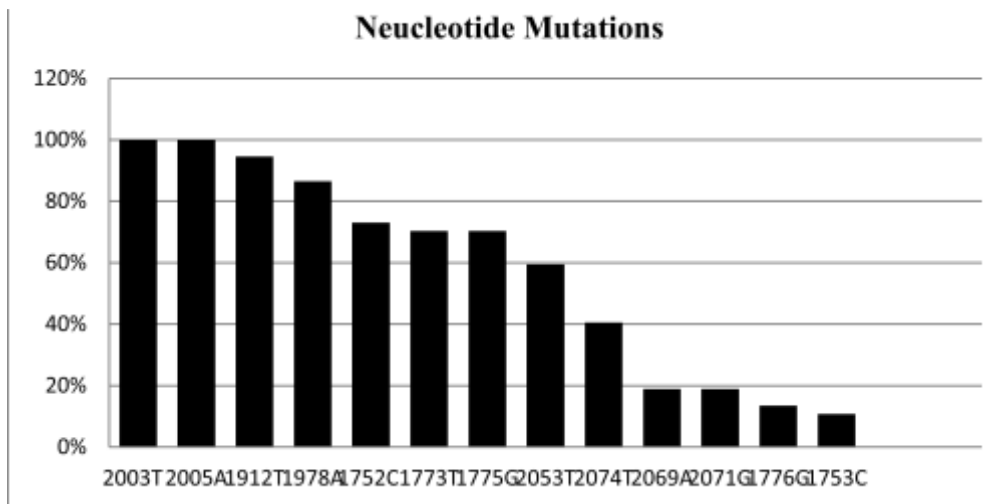
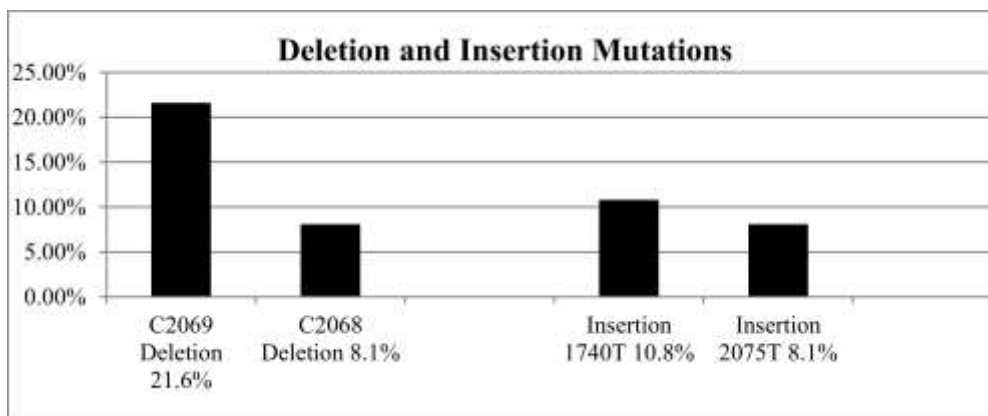


Figure 3- The most common deletion and insertion mutations



There were 15 insertion and seven deletion mutations. The most common insertion and deletion mutations were observed at nucleotide 1740T (10.8%) and 2069 (21.6%), respectively. There was no significant association between the age, gender and HBeAg of patients, and the patterns of amino acid substitutions.

## DISCUSSION

The co-infection with HIV changes intracellular microenvironment and provides suitable conditions for HBV that leads to more stability and affects mutational patterns. The high rate of mutation in HBV compared to other DNA viruses is due to lack of HBV polymerase proofreading and production of large amounts of virus particles. The patients co-infected with HIV have higher level of hepatitis B viremia, rapid progression to chronic hepatitis B and increased risk of cirrhosis and HCC compared to mono-infected patients (12). The HBV genotype, CP mutations, and high viral load are viral factors associated with increased risk of HCC. The other hotspot CP mutations are 1753A/C/G and 1768A, which are also associated with the increased risk of HCC. BCP mutations are common in HCC patients and there is a high rate of point mutations in this region (particularly dual V131L and K130M) among patients with cirrhosis and HCC (18, 25-27). In our study, we identified these mutations in two patients that can be indicator of HCC progression. Some studies have shown that mutation 1762T/1764A suppresses mRNA transcription and enhances HBV replication. Although CP mutations may enhance HBV replication(28), several clinical reports have revealed that the presence of 1762T/1764A mutation is not always associated with the high HBV-DNA levels (29). In the study of Chilo L. et al., CD4 counts of patients with HIV/HBV infection were significantly lower than mono-infected patients (30). Another study also demonstrated low amount of CD4 cells in co-infected patients (31). In the present study, the mean number of CD4 cells in patients was 120 (cells/ $\mu$ L). This indicates that HBV infection has caused a substantial decrease in CD4 of co-infected patients. The most common route of HIV transmission is sexual contact, intravenous drug use and perinatal transmission. The most common

modes of HIV transmission in Iran are intravenous drug use and risky sexual behaviors (32, 33). Similarly, the main modes of transmission in this study were intravenous drug use and history of risky sexual behavior. The high rate of HIV infection in married patients in the present study highlights the need for measures to prevent HIV and HBV infection in family of patients.

Mutation at nucleotide 1762T/1764A increased the level of HBV DNA replication and pg RNA transcription compared to the wild type strain. The serum level of HBeAg in the 1762T/1764A mutant group is lower than that of the wild type strain. The mutation is located in hepatocyte nuclear factor 4 binding site. A number of liver transcription factors are involved in the regulation of HBV transcription and replication (34). The mutations at nucleotides 1766T/1768A and 1896A/1899A are associated with high replication levels. Moreover, 1753C and 1766T mutations are related to high replication levels. Apart from the 1753C mutation that has been observed in HCC patients, other mutations in this study were at low level. HBV replication level in HBV/HIV co-infected patients is lower compared to mono-infected patients. Cassino et al. showed that BCP/PC mutations in HIV/HBV patients is lower compared to HBV patients (35), which is consistent with the findings of the present study.

Some studies determined whole genome HBV mutations in co-infected patients. Tangkijvanich et al. found no difference in mutations, age, gender, and HBeAg and alanine transaminase between co-infected and mono-infected patients. They reported 1762T, G1764A, 1896A and 1753C mutations in 45.8 %, 50%, 25% and 20.8% of co-infected patients, respectively. The rate of 1762T, 1764A and 1896A mutations in co-infected patients in the present study was lower than the values reported by Tangkijvanich et al. This could be due to geographical differences in HBV mutational patterns, since different HBV genotypes are associated with various mutations in the HBV core region(36). Fernando Gallego et al. reported no mutations at nucleotides 1762, 1764 and

1896 in HIV/HBV co-infected patients (37). However, Yousif et al. reported low frequency of these mutations in HIV/HBV patients and 88% frequency rate for HBeAg-negative patients (38), which is in agreement with the findings of the present study. Mutations at nucleotides 1762, 1764, 1812, 1809, 1814-1816 and 1809 affect the production of HBeAg, which is highly immunogenic (39). Since co-infected patients with HIV have reduced immune pressure, a reduction can be seen in the production and secretion of HBeAg. Taffon et al. showed the high frequency of BCP and PC pathobiological mutations in Italian co-infected patients, while several corresponding sites showed amino acid variations. Similar to the present study, the rate of mutations in the mentioned study was not correlated with age and gender. However, high rate of 1762, 1764, 1766 and 1809 mutations is related to different geographical distribution of HBV mutations. Different HBV genotypes are associated with mutations in different regions (40-42). Survival of an organism depends on its capacity to cultivate variety. While mutations can cause this variety

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in viruses, the virus's genome remains unchanged in suitable host environment. Co-infection of patients with HIV provides better conditions for HBV, which results in low rate of mutations in the HBV genome.

It is recommended to conduct a large-scale study in countries with high prevalence of HBV/HIV infections, to evaluate clinical and virological differences between mono-infected and co-infected patients.

## CONCLUSION

Detection of the mutations in the BCP and PC/core regions of HBV in co-infected patients could be useful for development of better preventive and therapeutic strategies, and help predict the progression of liver diseases.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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