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Original paper

Comparison of pre-S1/S2 variations of hepatitis B virus between asymptomatic carriers and cirrhotic/hepatocellular carcinoma-affected individuals

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Abstract

Aim of the study: Host and viral factors can influence the clinical course of chronic hepatitis B virus (HBV) infection. Mutations in pre-S1/S2 gene regions are among the most important viral factors determining the HBV infection outcome. The aim of this study was to investigate the role of pre-S1/S2 mutations in HBV infection outcome.

Material and methods: A total of 52 samples from 26 asymptomatic carriers (ASCs) and 26 liver cirrhosis/hepatocellular carcinoma (LC/HCC) patients were enrolled. The HBV DNA genome was extracted from the sera, and pre-S1/S2 regions of the samples were amplified by nested-polymerase chain reaction, prior to being subjected to sequencing, sequence investigation and phylogenetic analysis.

Results: Certain deletions were detected mostly located at the boundary of the pre-S1 and pre-S2 regions. These deletions were detected more frequently in ASC cases than in LC/HCC patients ($p < 0.007$). The rate of critical point mutations, including L11Q, N37S and K38R, was significantly higher in the ASC group, whereas the A49V substitution rate was significantly higher in the LC/HCC group ($p < 0.05$). The phylogenetic analysis indicated that all the sequences belonged to genotype D.

Conclusions: According to the results, point mutations such as L11Q, N37S, K38R and A49V, as well as certain deletions, may be associated with HBV infection outcome, among an HBV genotype D pure population.

Key words: hepatitis B virus, genotype D, pre-S1/S2 variations, asymptomatic carrier, cirrhosis, hepatocellular carcinoma.

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Introduction

Despite the effectiveness of global vaccination, nearly 250 million individuals are persistently infected with hepatitis B virus (HBV) worldwide [1]. In Iran, approximately 2% of people are estimated to be chronic HBV carriers [2]. The clinical outcomes of chronic HBV infection include the asymptomatic carrier (ASC) state, chronic hepatitis (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [3]. Among the factors contributing to HBV disease progression, variation in viral genome sequences has a particular role [3].

The mutations of surface proteins are the most impressive candidates to determine the outcome of HBV infection. These envelope proteins including small (S), middle (pre-S2/S) and large (pre-S1/S2/S) HBsAg, are encoded by a single open reading frame. The small, middle and large proteins are made of 226, 281 and 389 amino acids in genotype D, respectively [4]. In the pre-S1/S2 protein, different motifs that are responsible for attachment, virus production, HBs secretion and

more importantly antigenicity are present [3]. Different types of variations including substitutions, deletions and recombination have been reported in this part of the virus genome [5].

As the pre-S1 and pre-S2 regions are exposed at the surface of HBV particles, they evolve under selective immune pressure and exert some drastic effects on virus replication. Genetic variation in pre-S1/S2 specifically in T-cell or B-cell epitopes helps the virus to escape from the host immune response and establish a persistent infection [3, 6]. This region encompasses the binding site for hepatocyte and serum albumin that is responsible for attachment and infectivity. As a result, its variation leads to destruction in the entry step of viral replication [6].

Moreover, some studies have shown that deletion mutations in pre-S1/S2 might occur and influence the outcome of HBV infection [7]. It has been reported that these deletion mutants might contribute to endoplasmic reticulum stress, which ultimately accelerates the infection progression toward cirrhosis and HCC [8, 9]. In this regard, Qu *et al*. reported the occurrence and association of pre-S deletions with HCC among patients with genotype C in China [10]. Also, Yeung *et al*. showed that pre-S deletion mutations have been detected more frequently in LC/HCC cases as a determining factor of HCC development in patients infected with genotype B and C [9, 11]. On the other hand, Mohebbi *et al*. demonstrated that the frequency of HBV mutations, particularly deletions in the pre-S1/S2 region, was not statistically significantly different between patients with chronic active hepatitis and those in LC/HCC stage [12]. Therefore, to develop a conclusive theory on the role of pre-S variations in HBV genotype D progression, further investigations are required.

Furthermore, it has been suggested that the frequency of mutations in the pre-S1 and pre-S2 region might be dissimilar in different HBV genotypes, meaning it follows a genotype-dependent trend [13, 14]. Although these types of mutations were detectable at the pre-S2 site of genotypes A, B and C viruses, they were diagnosed commonly alongside the pre-S1 when evaluated among genotype D viruses.

The reports demonstrated the dominant prevalence of genotype D among Iranian HBV-infected patients [12, 15]. As studies on a pure genotype D-infected population are rare, the aims of the present study were to investigate the mutation patterns of the pre-S1/S2 region in ASC and LC HCC-affected patients and to evaluate their association with disease progression.

As the study on a pure genotype D infected population is limiting, the aims of this study were to investigate the mutations patterns of the pre-S1/S2 region in ASC and LC HCC-affected patients and to evaluate their correlation with disease progression.

Material and methods

Patients

A group of patients with chronic HBV infection were primarily selected from the Liver Clinic of the Gastroenterohepatology Research Center at Nemazee Hospital (Shiraz, Iran), according to their stage of disease. All the patients were HBsAg positive, as determined by ELISA test. All the cases had no co-infection with hepatitis C virus, hepatitis D virus and human immunodeficiency virus recorded in their medical records. Three groups of patients were selected based on their medical records as follows: i) ASC group individuals presented with the absence of or low liver damage represented by a repeatedly normal liver function test (LFT), and anti-hepatitis B e antigen (may or may not be due to absence of HBeAg in our HBV-infected patients, from the outset) and a low HBV DNA level (< 2,000 IU/ml); ii) the LC group was determined on the basis of histology grading, ultrasound scanning and abnormal liver function tests; and iii) HCC patients were enrolled based on either histological reports or elevated α-fetoprotein levels (> 400 ng/ml, compared with the normal adult level of 10-50 ng/ml). They did not receive therapy for at least 1-2 months before sampling time. However, they were receiving a nucleoside analogue treatment based on the disease stage, with the exception of the ASC group.

The Ethics Committee of Shiraz University of Medical Sciences approved the study, and written informed consent was obtained from all participants prior to sampling.

DNA extraction and amplification of pre-S1/S2 region by PCR

Blood samples (~5 ml) were obtained from the participants, and then the sera were separated and stored at –70°C until use. DNA was extracted from 200 µl of sera using a viral DNA extraction kit (Invitec GmbH and Co. KG, Duisburg, Germany) according to the manufacturer's protocols. Pre-S1/S2 regions were amplified by in-house nested-polymerase chain reaction (nested-PCR). Two pairs of primers were employed in the nested-PCR method, as shown in Table 1. The primers were designed regarding the validated genotype B, C and D sequences with the assistance of the Primer Designing Tool from the National Center

for Biotechnology Information (NCBI) homepage (https://www.ncbi.nlm.nih.gov/) and MEGA7 software package, version 7.0 (20), and then employed in two subsequent rounds of PCR. The amplification mixture contained standard PCR ingredients as well as Taq DNA polymerase (CinnaGen Inc., Tehran, Iran), 6 µl DNA templates and 0.2 pmol of each primer in a total volume of 50 µl. The first round of PCR was performed with a first denaturation at 94°C for 4 min and then 30 cycles of 94°C for 40 s, 56°C for 35 s and 72°C for 40 s, followed by a final extension step of 72°C for 4 min. The second round of PCR was performed under the same conditions, but for 35 cycles and with an annealing temperature of 57°C.

Sequence analysis and detection of pre-S1/S2 variations

After agarose gel slicing, the PCR products were purified using PCR Product Purification Kit (Biooneer Inc. South Korea) according to the manufacturer's instructions. Then the purified PCR products were subjected to bi-directional sequencing with previously mentioned inner primers (pre-S1 and pre-S2). The sequencing results were retrieved by MEGA7 software and then they were multiply aligned and compared with some selected sequences. The reference sequences belonged to genotype D were selected from the HBV databank (https://hbvdb.ibcp.fr/HBVdb/HBVdbNomenclature) or the HBV genotyping tool (NCBI Homepage), which included X65259.1, AF121240.1, FJ904433.1, M32138.1 and X85254.1.

Phylogenetic analysis and genotyping

After sequence analysis, the retrieved sequences were multiply aligned with some defined sequences in MEGA7 software using the default parameters of the ClustalW method. The selected reference sequences were AB033559.1, X85254.1 and M32138.1 (genotype D); AM282986.1 (genotype A); AF282918.1 (genotype B); NC-003977.1 (genotype C); AB032431.1 and X75657.1 (genotype E) and AB275308.1 (genotype H).

After that, the phylogenic tree was constructed by the neighbor-joining method regarding the Kimura-2 parameter based distance estimation model using MEGA7 software. Finally, the designed tree was evaluated by 500-replicate bootstrapping to ensure fidelity.

Statistical analysis

Statistical analysis was performed using Epi Info version 7.1.5 (Center for Disease control, Atlanta) using the chi-square test. A p-value of ≤ 0.05 was considered as significant.

Table 1. Sequences of primers used in this study

1st Round PCR	Forward Primer 5′-GTCACCATATTCTTGGGAAC-3′ (nt 2816-2835) Reverse Primer 5′-AGAAGTCCACCACGAGTCTA-3′ (nt 249-268)
Nested-PCR	Forward Primer 5′-GAACAAGAGCTACAGCATGG-3′ (nt 2832-2851) Reverse Primer 5′-ATCCTGATGTGATGTTCTCC-3′ (nt 157-176)

Results

Patients data

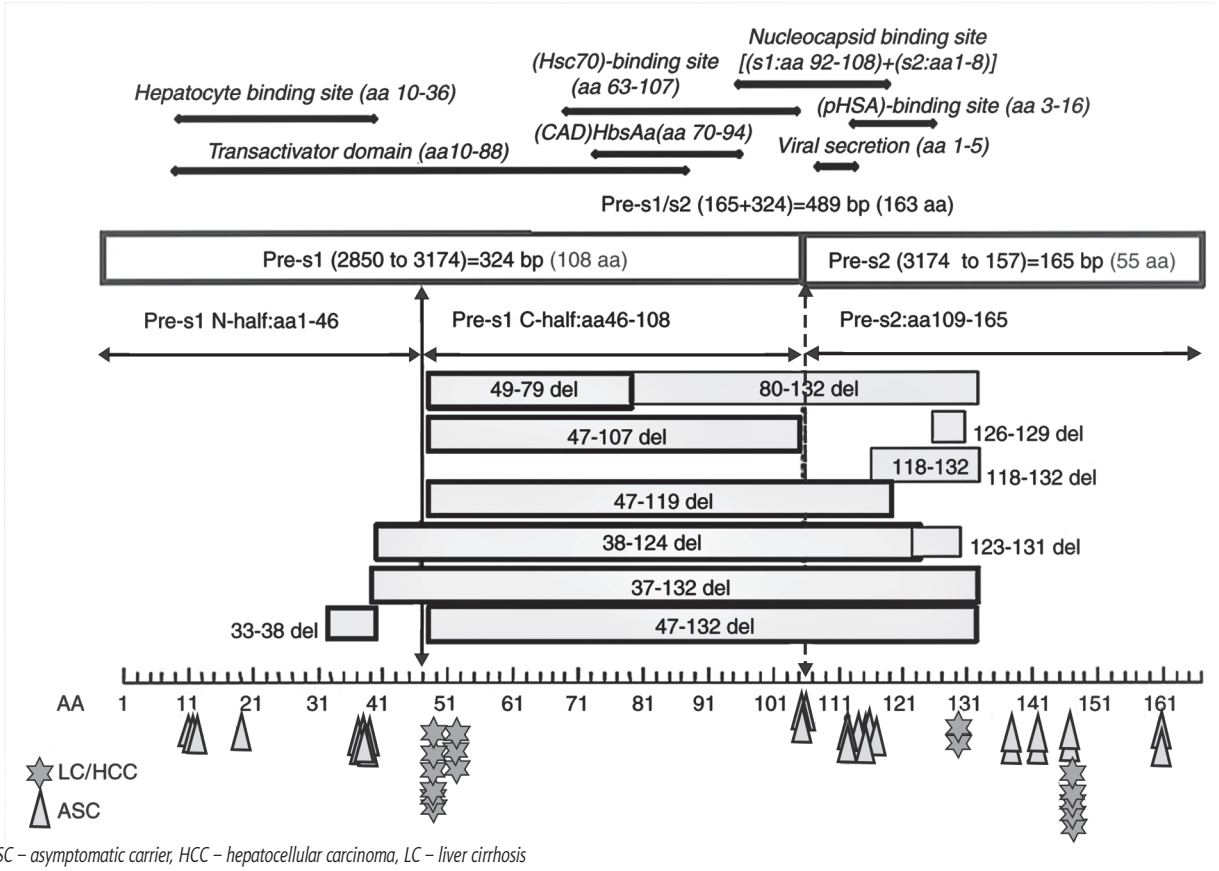
Out of 70 patients, 52 cases with positive PCR results were enrolled in further sequence analysis. In total, 26 ASC and 26 LC/HCC subjects were included. Out of 26 ASC individuals, 22 (85%) were male and 4 (15%) were female. In the LC/HCC group 22 patients (85%) were male and 4 (15%) were female. The mean age of ASC and LC/HCC groups was 51 and 53 years old, respectively.

DNA sequence analysis

After comparing the amplified sequences with HBV genotype D references, the results indicated that point mutations were dispersed along the coding and regulatory sequences of the pre-S1/S2 region. Despite this, deletion events were mostly detected at the pre-S1 region and at the boundary of the pre-S1 and pre-S2 regions, as depicted in Figures 1 and 2.

Comparing the sequence of the pre-S1/S2 region between ASC and LC/HCC samples

Overall, the critical point mutation rate in the ASC group was approximately four times higher than that of the LC/HCC group. The frequency of certain mutations within B-cell epitopes of the pre-S1 region, including L11Q (p = 0.02), N37S (p = 0.02) and K38R (p = 0.04), was higher in the ASC group compared with that in the LC/HCC group. Moreover, in the hepatocyte binding site (amino acid residue 10-36 in pre-S1), the L11Q point mutation was detected in 6 ASC samples, which was significantly different from that in the LC/HCC sample (p = 0.02). By contrast, the A49V point mutation at the transactivation domain was detected significantly more frequently in the LC/HCC samples (26.92%; 7/26) than in the ASC cases (p = 0.01). In ad-



ASC – asymptomatic carrier, HCC – hepatocellular carcinoma, LC – liver cirrhosis

Fig. 1. Scattering pattern of pre-S1/S2 mutations in the ASC and LC/HCC samples. The different regions of pre-S1/S2 are shown in the upper section and mutations are indicated in the lower section of the figure. Rectangles, vertical triangles and asterisks stand for deletions (del), critical point mutations in ASC and point mutations in LC/HCC, respectively

Fig. 2. Protein alignment of the sequenced asymptomatic carrier samples. The differences are considered as mutations. The deletion patterns among sequences were indicated when compared with reference sequences

dition, although there were 6 mutations (2 point and 4 deletion mutations) in the nucleocapsid binding site of ASC cases, none of the LC/HCC patients had a mutation in this region. Furthermore, point mutations attributed to the blocking of middle HBsAg production (Met1R/T) were detected at a similar rate in the ASC and HCC cases. No signs of deletion were distinguishable among the LC/HCC samples (Fig. 1).

Sequence analysis of pre-S1/S2 region in ASC subjects

In the ASC samples, more prevalent substitutions were located within B-cell epitopes (18 mutations in 22 ASCs), T-cell epitopes (14 mutations in 13 ASC) and the transactivation domain (10 mutations in 9 ASC) (Figs. 1 and 2). Notable point mutations and deletions in the ASC samples were located in the C-terminal of pre-S1 that stretched to the N-terminal of pre-S2. From them, L11Q, N37S and K38R were more prevalent, but L19* (stop codon), M1T, T5P/A, G29A and N32D were also observed with lower prevalence. Nucleotide substitutions, including 3,015 GT>A and 3,138 G>T, were found significantly more frequently in 14 and 18 ASC individuals, respectively. The substitutions were located in the S-promoter (nt 3012 to 3147) and in a CCAAT binding factor (CBF)-binding site (nt 3,104 to 3,114), which is necessary for expression of the S gene. In addition, deletion mutations with > 200, > 180, > 150 and < 100 bp fragment lengths were detected in 2, 4, 1 and 1 cases, respectively. The three other deletions were < 40 bp in length. All the mentioned deletions created a frame-shift and consequently hindered pre-S1/S2 production. Hence, an impressive substitution at the start codon of pre-S2 (M109T) in 2 ASC samples and a stop codon (L19*) in the pre-S1 region of 3 ASC samples were detected (Fig. 1).

Sequence analysis of pre-S1/S2 region in LC/HCC patients

In this group, 76.92% (20/26) of the samples, including 88.89% (8/9) of the LC samples and 70.59% (12/17) of the HCC samples, harbored critical point mutations, as depicted in Figure 1 and Table 1. In the LC/HCC samples, a transactivator domain in the pre-S1 site, and an Ab-neutralizing sequence in the pre-S2 region, with 2 point mutations in 10 samples and 2 point mutations in 4 samples, respectively, exhibited the maximum redundancy. The most prevalent substitution event in this group was A49V at the transactivation domain, which was not significantly different between LC (11.11%; 1/9) and HCC (35.29%; 6/17) groups ($p = 0.29$). Moreover, an S130F/L mutation in the pre-S2 region of the LC samples (22.22%; 2/9) and a substitution at the start codon of the pre-S2 (Met1R) region in 11.76% (2/17) of the HCC cases were also detected, with a non-significant difference ($p = 0.46$) (Fig. 1).

Phylogenetic analysis and genotype determination

The survey of sequences alignment revealed that in comparison to non-D genotypes, none of the 52 samples have deletions at the beginning of the pre-S1 region, p2-13, which is a unique feature of HBV genotype D. This was indicative of the dominant prevalence of genotype D in all of the investigated samples. Furthermore, phylogenetic analysis of samples also supports this finding, as it revealed that they belong to genotype D in the constructed tree (Fig. 3). Furthermore, the online recombination test revealed no sign of a recombinant isolate in all cases.

Discussion

The natural history and clinical course of chronic HBV infection vary significantly among infected subjects. The infection fate depends on the host and viral factors, including the age of acquisition, gender, genetic background and HBV genotype, as well as point/deletion mutations in different regions of the HBV genome [16]. From previous results, a genotype-dependent pattern is indicated for the frequency of mutations in pre-S1 and pre-S2 regions [13, 14]. However, there are limited data regarding this point in a pure HBV genotype D population. Therefore, the present study investigated the difference in pre-S1 and pre-S2 variations among ASC subjects versus LC/HCC patients to find their possible association with the clinical course of HBV infection.

In the case of point mutations, the data showed a higher prevalence of mutations in epitopic and functional areas of the pre-S1/S2 region of the ASC group. Certain substitutions, including L11Q, A28T and K38R, at the pre-S1 region, and S130F/L in the pre-S2 region, were detected significantly more frequently in the ASC individuals than in those with LC/HCC. The pre-S1 and pre-S2 regions encompass well-described B-cell and T-cell epitopes [17]. The majority of these mutations lead to viral escape from the immune response and establishment of a persistence state [7, 9], as well as less liver damage despite the existence and replication of the virus [18]. Based on the role of T cells in immunopathogenesis, mutations in these epitopic sites may alleviate disease progression [5, 19], which may explain the higher prevalence in the ASC group, although this requires confirmation. Recently, studies from China and Korea on non-D genotype viruses demonstrated that certain pre-S1/2 mutations may accompany HB variations for development of occult HBV infection [20, 21]. However, whether the variations in the ASC group of the present study are associated with reducing the pathogenicity and as a result are involved in the development of the asymptomatic stage requires further investigation.

As an expression regulatory region, the pre-S1 region containing S-promoter and CBF showed more

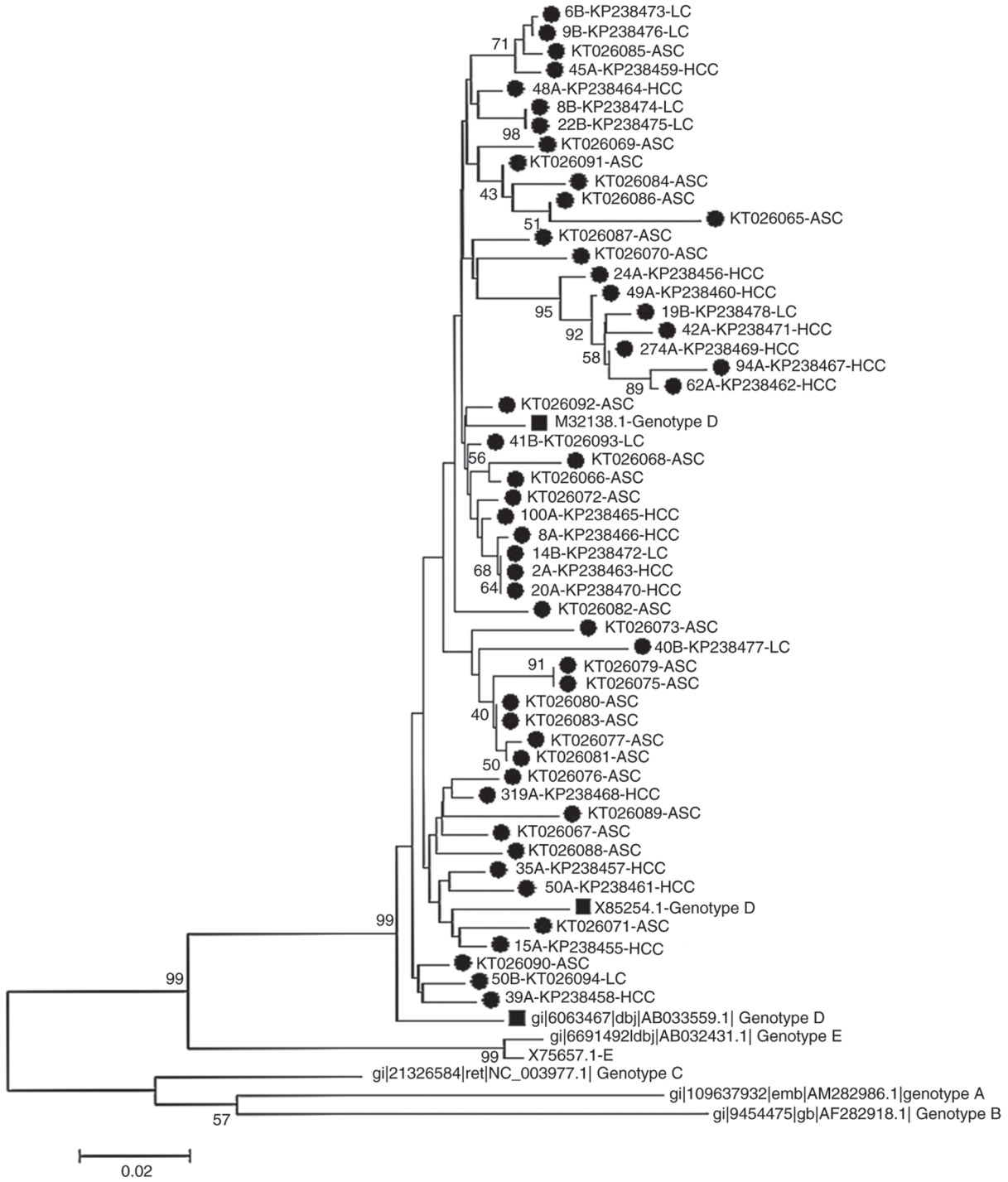


Fig. 3. Neighbor-joining phylogenetic tree based on pre-S1/S2 regions from 52 HBV-infected patients. The new isolated sequences are marked by black circles and HBV genotype D references are marked by black squares. The numbers at the nodes represent the bootstrap values (% obtained for 500 replicates)

variations in ASCs. As mentioned above, nt 2,879 A>T and nt 2,960 A>G variations that were located in this region were found significantly more frequently in ASCs individuals. Mutation in the regulatory region may change the binding ability of trans-regulating nuclear factors (such as Sp1 and hepatocyte nuclear factor 4) [22]. Although the exact role of the CCAAT-box in HBV infection progression is unclear [11], it was previously reported that a disrupted regulatory element may lead to occult hepatitis B [17], or in the present case, it may induce an asymptomatic state.

An L11Q point mutation was also located in the hepatocyte binding site (p10-36 in pre-S1). Schulze *et al.* showed that codon 11 of the pre-S1 protein is

responsible for viral infectivity [23]. Therefore, L11Q substitution may interfere with the HBV life cycle and lead to an ASC state in infected individuals.

The pre-S1 region (amino acids 21 to 47) encompasses the binding site for HepG2 cells. Also, the pre-S2 region encompasses the binding site for human serum albumin, which is believed to be involved in the attachment of HBV. Therefore, mutations of the pre-S gene may significantly influence the sequence of HBV infection [7]. Moreover, in the transactivation domain (p10-88 in pre-S1), more substitutions were detected in the ASC subjects. As this domain induces transcription of transforming growth factor α, a cytokine that contributes to fibrosis development [24], its disruption may make disease progression slower.

The current study results also revealed that deletion mutations were only present in the ASC group, which was a statistically significant result when compared with the LC/HCC group. Mohebbi *et al.* [12] found that the frequency of deletion mutations in the pre-S1/S2 region was similar among ASC, chronic active and cirrhotic subjects infected with HBV genotype B; therefore, the possible association of variations with disease outcome was rejected.

Furthermore, a recent study showed that partial deletion of the pre-S1/S2 region was detected in a similar pattern for CHB and HCC cases, but in a lower percentage of cirrhotic cases [25]. By contrast, other studies, such as that of Qu *et al.* [10] and Yeung *et al.* [11], demonstrated an association of pre-S1 deletions with HCC in HBV genotype B and C in China and Taiwan. However, even among these genotypes, a significant genotype-dependent pattern for deletion was demonstrated in different states of infection [26].

Regarding the clinical relevance of pre-S1/S2 deletions with infection outcome, Wu *et al.* [27] demonstrated that large fragment deletions reduced the necro-inflammatory grade in relapsing liver failure, an indication of slower disease progression. As large-fragment deletions were also frequent in the ASC patients in the present study, we hypothesize that reducing the pathogenesis is involved in the establishment of an ASC state. The deletions that were recognized in the present study were located in T-cell and B-cell epitopes, which may cause evasion of the virus from immune recognition, as shown to happen naturally by another study [17]. Moreover, deletion of small sequences from a pre-S region is also associated with an occult HBV state in patients with genotype C [17, 26, 27].

Similarly, in another study on genotype D, Mohebbi *et al.* [12] reported that the frequency of deletions in the pre-S1 region was greater than that in the pre-S1 region, and there was no significant difference between the patient groups. Moreover, Biswas *et al.* [13] and Chen *et al.* [14] showed a genotype-dependent pattern of the pre-S variations for determining the outcome of the disease. Accordingly, a controversial pattern of mutations, particularly for pre-S1/S2 deletions, is imaginable for genotype D in comparison with other genotypes.

In summary, the present findings suggested a different mutation trend in the pre-S1/S2 region of different groups according to HBV genotype D compared with non-D genotype. These variations led to a reduction in HBs antigen and virus production, and were more prevalent in the ASC group. Brunetto *et al.* [28] demonstrated that the serum levels of HBsAg vary during chronic infection with genotype D and are significantly lower in ASCs, a possible explanation for frequent mutations of HBsAg in the ASC samples.

In the pioneering study about genotype distribution by Amini-Bavil-Olyaee *et al.* [15], genotype D was defined as the predominant genotype in Iran. In other reports also [12], an HBV genotyping survey demonstrated that genotype D was dominant among different patients in a different area of the country from a referral medical center in Tehran. The present analysis also showed that genotype D was the only determined genotype, with the absence of amino acids 2-13 in pre-S and phylogenetic analysis.

Although variations in the pre-S1/S2 regions may reduce virus production, alleviate immunopathogenicity and influence disease progression in genotype D, further investigation is required. The lack of functional methods, small sample size (particularly for LC/HCC) and a shortage in viral biomarkers, such as HBsAg level, are among the drawbacks that hinder a definitive conclusion. Although certain limitations, including small sample size and absence of a more precise sequencing method, are evident, the findings in pure genotype D patients could be beneficial.

In conclusion, according to the present results, the point mutations L11Q, N37S and K38R, as well as certain deletion mutations, may correlate with the development of a healthy carrier state. On the other hand, a higher frequency of A49V in LC/HCC patients implies that this substitution is possibly associated with HBV infection progression toward LC/HCC. Further studies are required to clarify the significance of these findings.

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Disclosure

Authors report no conflict of interest.

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