Mutations on S Gene Region of Hepatitis B Virus Genotype D and Biological Significant Analyses of These Mutations

Mehmet Ozaslan, Canan Can and Arzu Barsgan

S gene region of HBV-DNA is responsible for expression of surface antigens and includes a-determinant region. Thus, mutation(s) in this region would afford HBV variants a distinct survival advantage. The aim of this study was to search the mutations of the S gene region in different patient groups infected with Genotype D variants and was to analyze biological significance of these mutations. Beside this, we investigated S gene mutation inductance among family members. Forty HBV-DNA positive patients were determined among 132 HBsAg carriers by first stage of Semi-Nested PCR. Genotypes and subtypes were established by sequencing of the amplified S gene regions. Variants were compared with original sequences of these serotypes and mutations were fixed. All variants were designated as Genotype D and subtype ayw3. Ten kinds of point mutations were identified within the S region. The rates of mutations were determined in chronic patients and family members as being maximum among the other groups. The amino acid mutations of 125 (M-T) and 127 (T-P) were found on the first loop of a-determinant. The other consequence was mutation inductance in a family member. We found some mutations on S gene region known to be stable and observed that some of these mutations were effective on S gene expressions. Identified mutations on a-determinant may provide advantage to mutant about escaping from immune system.

Key words: HBV, S gene region, mutation, semi-nested PCR, genotype D
INTRODUCTION

Hepatitis B virus is an agent causing the Hepatitis B infection, the major health problem and more than 350 million of the world population are chronic carriers of the virus[6]. Infected persons rate in Turkey population is 4-10% and this rate is 4-7% in only the Southeast of Turkey comunidad that various regions for distribution of HBV. The virus infection can cause chronic hepatitis, liver cirrhosis and hepatocellular carcinoma[23,24].

Intra-familial transmission of Hepatitis B virus denotes higher prevalence than the other groups especially in Southeast of Turkey[9].

It is observed, in some children whose mother is positive can be chronic HBV patient but in some of them don’t be. Mutants are parallel with chronic road[9].

HBsAg or S mutations have been now documented in many areas of the world but are most common in Asian infants (2-3% of vaccine recipients)[9]. Erol et al.[11] investigated intrafamilial transmission of hepatitis B virus in Eastern Anatolian region of Turkey. High maternal viral loads and mutations elsewhere in the mother’s HBV S gene appear to increase the risk of S mutations occurring in the offspring[9]. The same mutations also occur in liver transplant recipients receiving HBIG. Less frequently, they develop spontaneously during the course of a chronic HBV infection[9].

Hepatitis B virus is classified into four serotypes (adr, adw, ayr and ayw) based on antigenic determinants of the hepatitis B surface antigen[9]. These serotypes can be further classified into nine subtypes (ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrg1 and adrg2). Allelic differences exist among the four major HBV serotypes. Based on an intergroup divergence of 8% or more in the complete nucleotide sequence, HBV can be classified into eight genotypes A-H[10].

Naturally occurring HBV with the S mutation has been reported in a lot of patient groups. It is known that the rate of occurrence has varied in the literature. However, the biological significance of this mutation is still unclear especially for Southeast of Turkey.

Mutations have been described in all four ORFs of the hepatitis B virus. From a clinical perspective, the S escape mutant is the most worrisome, because in the absence of surveillance systems and/or a high index of suspicion the diagnosis can be difficult to establish. Undiagnosed cases can progress to liver failure and HCC. Transmission to others, including transmission via the blood transfusion route, might also occur. Despite encouraging results in vaccinated chimpanzees, HBIG and HBV vaccination do not protect humans from S mutant infections[24]. Of the clinical significance is the fact that the variation does not alter the ability of the virus to be attached to the hepatocyte and replicate, leading to chronic liver disease. A loss of antigenicity at the a-determinant, leading to lack of detection of these mutants by some monoclonal HBsAg immunoassay kits, has been reported[11,14].

HBV S region mutations were therefore, investigated in chronic liver diseases, hemodialize patients, family members, hospital personalists, donors as well as the association with HBV genotypes in Southeast of Turkey that various region for distribution of HBV (4-7%). And were also searched these mutation effects on expression of HBsAg and a-determinant epitopes. And was evaluated these mutations in family members.

A specific region on S gene was selected for determination of these mutations. This specific region was between nucleic acid residues 250-715 that provide common sequence response expression of surface antigens’ three types. In addition region contained first loop of a determinant.

For detection of HBV-DNA in serum, high sensitive semi-nested PCR technique was used. Then we analyzed the sequences of the region. Sequence datums were compared with Gen Bank datums for genotyping and mutation analyzing. Genotype D and serotype ayw3 was established in all samples. Mutation rates were observed as maximum in chronic liver diseases, family members. Some of point mutations were effective on region expressions. All these mutation points and kinds were determined and discussed their biological importance.

MATERIALS AND METHODS

Serum samples were taken from 132 HBsAg positive patients from different groups including chronic HBV patients, family members, hospital personalists, dialize patients and donors from several health centers (medical center, municipal hospitals, private hospitals, etc.) in Southeast of Turkey over two years from May 2002 to June 2004. Together with these samples, HBV-DNA positive and negative control groups were used. These sera were selected from the patients that included the non-vaccinated and not under the drug therapy. Thus, real variants could be determined so that the human immune system would be responsible from S gene mutations.

To achieve maximal sensitivity, a nested PCR protocol was used. We used specific primers that suitable for semi-nested PCR for amplifying S gene region. This region provides common regions for three types of surface antigens and first loop of a-determinant responsible of antigenity. S gene region and a-determinant mutations
have not previously been reported from Turkey. At the first stage of PCR, 770 bp fragment corresponding to nucleotide position from 250 to 1019 was amplified using following primers;

5’-CTAGACTCCTAGGGAGCTTCCTC-3’ (forward) and 5’-CTAGACTCCTAGGGAGCTTCCTC-3’ (reverse). Second stage of Semi-nested PCR protocol was realized using 5’-CTAGACTCCTAGGGAGCTTCCTC-3’ (forward) and 5’-AAAGCCCTAGGGAGAAAC-3’ (reverse) primers constituted 482 bp amplification product (nucleotide position: 250-732).

Sequencing primer was 5’-AAAGCCCTAGGGAGAAAC-3’ (reverse) (nt: 250-715; Sequence product: 466 bp). Primers were synthesized by Metis Biotechnology (Turkey).

HBV-DNA was isolated from 200 μL serum for each sample and serum was treated with 500 μL K Buffer (20 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.2% SDS, 20 μL mL-1 Proteinase K) and incubated at 37°C for 2 h. It was centrifuged at 11000 rpm for 6 min, by adding 700 μL phenol buffer (4°C). Supernatant was extracted by 700 μL chloroform: isooxyl alcohol (24:1) and centrifuged at 11000 rpm for 6 min. Sodium acetate (3 M, pH 4.9-5) and 96% ethanol (-20°C) were added to supernatant. DNA was precipitated at -20°C for minimum 1 h or overnight. Pellet was washed with 70% ethanol (-20°C) at 12000 rpm for 2 min. Then, alcohol was poured and DNA was dried at 65°C for 15 min. Lastly, DNA was dissolved in 20 μL of TE buffer (Tris + EDTA), pH 8.0 and stored at -20°C for PCR.

HBV-DNA was amplified by semi-nested PCR. For the first stage PCR, 50 μL reaction mixture contained PCR buffer, dNTP (10 mM) (Fermentas), MgCl2 (2.5 mM), primer (sense+antisense) and Taq DNA polymerase (5U mL-1) (Fermentas). Amplification was carried out in thermal cycler (Techne Genius FGEO STD, England) for 30 cycles. After the first amplification, 1 μL of the PCR products were reamplified with second stage primers for 30-cycles. For the first stage, each cycle entailed denaturation at 94°C for 20 sec, primer annealing at 55°C for 40 sec and extension at 72°C for 60 sec with a final extension at 72°C for 5 sec. For the second stage, the conditions of each cycle performed as 94°C for 20 sec, 60°C for 30 sec, 72°C for 40 sec. The first stage PCR products were analyzed by gel electrophoresis on a 2% agarose gel stained with ethidium bromide to determine HBV-DNA positive and negative samples. The positive samples were reamplified by the second stage of semi-nested PCR and second stage products were run on a 1% agarose gel.

The sequencing analysis of the surface antigen region (250-732) provided from the second stage PCR was done with a DNA sequence analyzer (Visible Genetic Inc, Canada). Tagged reverse primer that starting at nucleotide position of 715 and specific for TNF-α gene region was used. Therefore the fragment about 466 bp was sequenced. Reaction mixture was prepared by Cy5/5.5 Dye. Primer Cycle sequencing Kit (Visible Genetic Inc, Canada).

The resulting sequences were available in the Gene Bank database with the corresponding AJ131956 accession number among various HBV genotype and serotype sequences. Thus, genotypes and serotypes were determined. Variants were compared with original sequence of this serotype for fixing mutations. For comparison of sequences and determination of mutations, computer alignment programme was used.

RESULTS

From the 132 HBsAg positive patients, HBV-DNA could be amplified in the serum of 40 patients (30.30%). The semi-nested PCR products of HBV-DNA which corresponded to 482 bp were run with 50 bp digested molecular weight marker (Fig. 1).

Sequence results corresponded with AJ131956 accession number in the Gene Bank. According to this result, genotype D and serotype ayw3 were determined in all of the HBV-DNA positive serum samples.

Genotype D includes of the ayw2, ayw3 subtypes. Bozdayi et al.[10] and Şentürker et al.[11] have reported ayw3 dominance in Turkey. We determined ayw3 subtype in all clones for the Southeast of Turkey that have not previously reported.

Bozdayi et al.[10] investigated pre-core region mutations and Şentürker et al.[11] studied the S gene region for determining genotype and serotype of HBV. There was no report on a-determinant region, S gene mutations and their effects from Turkey.

![Fig. 1: Semi-nested PCR product on a 1% agarose gel.](image-url)

PK: Positive control, NK: Negative control, M: 50 bp. DNA ladder, N: samples
**Family members**

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<th>Genotype</th>
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<td>Family 1</td>
<td>W, M1, M2, M3</td>
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<td>Family 5</td>
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<td>Family 6</td>
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<td>Family 7</td>
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**Diabetes members**

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**Hospital Personnel**

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**Chronic HBV patients**

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**Donors**

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<td>DN1, DN2, DN3, DN4, DN5</td>
<td>GGATCTTG ACCACGCAACGGGAAACCA TGC AGAACCCTGACTA CTGCTCAAAGGAACC</td>
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Fig. 2: Nucleotide Changes positions in only Patient Groups (F = Family, M = Member, D = Dialize member, H = Hospital personnel, C = Chronic HBV patient, DN = Donor, W = sequence of Genotype D serotype from GenBank, accession number; AJ131956)
Ten kinds of point mutations were observed. The region of 250-715 bp was selected as sequencing region, however mutation density was determined in 490-550 bp region. Point mutations of C498A, A531G and T536C were observed in all of the isolates. Also C501A point mutation was described in 82.5% of the isolates. T496C, C517T, G523A, C479T, T320C and G296G were the other mutations. T320C and G296G mutations were observed in only two patients. As a result mutations generally were observed on 460-490 bp region (Fig. 2-4).

In one of the chronic patient (C4), two nucleotide exchanged their locations on A531/532A position (Fig. 2).

The point mutations rates were found as maximum in Chronic HBV patients. Secondary high rates were observed in family members. Beside these mutation prevalence of dialize members were minimum (Fig. 3).

Except common mutation points (C498A, A531G and T536C), C501A point mutation was observed most frequently in patients. According to patient groups, this mutation was determined showing high prevalence in family members. G296G and T320C mutations were found in only family members and C479T was in only dialize members (Fig. 4).

Intra-familial S gene mutation inductance occurring from parents to offspring. We investigated the S gene mutants, occurred in mother affection, on increasing the risk of S mutation occurrence in the offspring. But any inductance was observed because of some mutation points in family members corresponding with

Fig. 3: Mutation rates of patient groups. This figure was occurred calculating data of Figure 1. FM: Family members, DM: Dialize members, HP: Hospital Personals, CP: Chronic Patients, D: Donors

Fig. 4: Mutation kinds except observed in all clones

Fig. 5: A: Their DNA fragments on agarose gel. B: Mutation occurrence in two members of a family in two years for testing mutation inductance in family members
Nucleotide region 490 – 550

112 - 131
C G S S T T S T G P R T C M T T A Q G T
CP1 --T------------------------T----P----
CP2 --------------------------T----P----
CP3 --------------------------T----P----
CP1 --------------------------------S--P--
CP2 --------------------------------T--P--
CP3 ----T------------------------T----P----

Fig. 6  Amino acid sequence of a part of HBV-DNA S gene in some samples. C: Consensus sequence of genotype D and ayw3 serotype. CP: Chronic HBV patients

Analysis of the base substitutions and their effects on the amino acid sequence were revealed. Some amino acid substitutions were observed in transcripts of immunologically important region among synthesis region. Nevertheless, 490-550 bp region that nucleotide changes mostly occurred, corresponded to 112-131 amino acid position according to Gen Bank (Fig. 2 and 6). Within 284-350 bp region little changes occurred, in which corresponded to 43-61 amino acid position.

Amino acid substitutions of 125. (M→T) and 127. (T→P), on the region containing the surface antigen synthesis, were found in all samples. These mutations occurred on the first loop of α-determinant (124-137 a.a.) of HBsAg. In only one patient two point mutations led to amino acid substitution affecting the amino acid synthesis by altering to 125. (M→S) instead of 125. (M→T). The 114. (S→T) alteration was rarely found. The amino acid substitutions 47. (V→A) and 55. (S→P) were found in the member of a family. As seen in the data, amino acid changes exhibited similarity in all the patients (Fig. 6).

The point mutation at 531. nucleotide led to a thymidine to cytosine substitution with an amino acid exchange from Methionine (M) to Thiamine (T) in the S protein (125.a.a), and the mutation at nt 536 to an adenine to cytosine substitution with amino acid exchange from Thiamine (T) to Proline (P) (127. a.a) in all clones. Both of these mutations were located in first loop of the α epitope (Fig. 7).

Huang et al. found the changes at the position of 126, 129, 135,136 and 145 amino acid residues on α-determinant and correlated with the absence of detectable anti-HBsAg from Qidong area, China. Carman et al. found the changes at 587. nucleotide position on α-determinant region. Both of them studied with vaccinated patients. But our samples were not vaccinated and they were anti-HBs negative patients.

**DISCUSSION**

Prevalence of real virion is possible by only fixation of HBV-DNA. HBsAg serological marker gave positive results in 132 patients but only 40 HBV-DNA positive serum samples were found in 132 HbsAg positive sera. For this study highly sensitive semi-nested PCR technique was used.

Advances in molecular biology techniques revealed significant diversities in sequences of HBV isolates, accounting for the allelic differences among four major HBV serotypes. Based on an intergroup divergence of 8% or more in the complete nucleotide sequence, HBV can be

other groups (Fig. 5B). Two members of a family were followed for two years. G296G and T320C mutations except common mutation point occurred in one member (Fig. 5B: M1-2004) and mutations led to amino acid exchanges (47. from Valine to Alanine, 55. from Serine to Phenylalanine).

Intra-familial mutation inductance was not reported in Turkey. Erol et al. investigated intrafamilial transmission of Hepatitis B virus in the Easternanotolian of Turkey and assigned the prevalence of HBsAg and HBV infection among the family members to be significantly higher than in the control group.
classified into seven genotypes A-G. However, genotyping can be accomplished based on a partial sequence of the HBV genome such as the S gene. First of all, we determined the HBV serotype for our region due to the absence of previous genotype findings of the region. Thus, genotype D and serotype ayw3 dominance was put forward in all isolates. Genotype D dominance was seen in the Mediterranean region, Turkey’s neighbors on world genotypic distribution map. As a result of this study, HBV genotype of Turkish region was similar to the peripheral regions.

The surface proteins have many functions, including attachment and penetration of the virus into hepatocytes at the beginning of the infection process. Since HBV infections depend on persistence of viremia for transmission from person to person, both the surface proteins and the intact virus co-exist in the infected host. We established ten kinds of mutations on this region although this region was known as stable. Presentation of these mutations in patient groups were investigated separately.

Differences in the nucleic acid sequence may be PCR artifacts or reflect heterogeneity of the viral population or because of point mutations. According to literature the rate of point mutations caused by the DNA Taq polymerase itself ranges between $1 \times 10^{-4}$ and $6 \times 10^{-5}$ bp depending on the Taq polymerase used.

Ten kinds of mutations were determined. The C498A, A531G and T536C point mutations were found in all the isolates tested. The C501A point mutation was found in 82.5% of all isolates. Although S gene region is known to be a stable, determination of these mutations in this study that are collected on a part of S region, leads to the conclusion that these variants may tend to trigger a new serotype in future.

Maximum mutation rates were determined in chronic HBV patients. This was an expected result because of long carriehup of the virus until chronic phase. Besides this, mutations might be directly triggered by immune system due to non-vaccination and not undergoing on a drug therapy.

Secondary high mutation rates were found in family members. Existence of mutant virions in parents may stimulate occurrence of mutations in infants therefore, determining high mutation rates in family members could be expected because of inductance.

Third degree of mutation rates occurred in hospital personals and donors. The least mutation was found in the group of hemodialysis patients. The reason of this may be to use of same non-sterile machines.

Most number of mutations were found to be collected on a part of S gene (460-550 nt). This region included first loop of a determinant. The a-determinant is a common constituent of each pair of mutually exclusive determinants, d or y and w or r, resulting in four major antigenic subtypes, namely, adw, ayw, adr and ayr. The group-specific a-determinant encompassing codon 124-147 is within the major hydrophilic region. The conformation of a-determinant residues between Cys-124 and Cys-137 (first loop) and Cys-139 and Cys-147 (second loop). The 4 Cys residues at the basis of the two loops were found to be highly conserved. If this conformation is altered, previously produced antibodies against the native a-determinants can't be protective. This area is highly immunogenic and forms the basis of HBsAg vaccines.

We considered that the mutant a epitope could not be neutralized by vaccine-induced antibody, because these mutations affected the conformation of the a epitope and altered the antigenicity of HBsAg, leading to mutant HBsAg escape from the detection by standard HBsAg assays. Furthermore, the altered conformation of the a epitope may lead to a considerable decrease of properly folded surface antigen which may render the virus granules less immunogenic in producing an effective neutralizing anti-a to clear the virus.

Amino acid mutations at 125 (M→T) and 127 (T→P) obtained in study was found at the first loop of a-determinant region, in all the isolates. Amino acid changes like those of the nucleotide changes were not related to patient groups which studied in this paper. But nucleotide mutations led to two important amino acid changes that were fix in all clones. This result was consistent with other studies on reducing capacity of a-determinant binding to antibody and escaping advantage from immune system.

When present study correspond with the reference studies it may conclude that mutations on a-determinant could be occurred independently from vaccine or anti-HBs.

REFERENCES

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