RESEARCH ARTICLE

Mutations in the *S* gene region of hepatitis B virus genotype *D* in Turkish patients

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Abstract

The *S* gene region of the hepatitis B virus (HBV) is responsible for the expression of surface antigens and includes the 'a'-determinant region. Thus, mutation(s) in this region would afford HBV variants a distinct survival advantage, permitting the mutant virus to escape from the immune system. The aim of this study was to search for mutations of the *S* gene region in different patient groups infected with genotype *D* variants of HBV, and to analyse the biological significance of these mutations. Moreover, we investigated *S* gene mutation inductance among family members. Forty HBV-DNA-positive patients were determined among 132 hepatitis B surface antigen (HbsAg) carriers by the first stage of seminested 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 identified. All variants were designated as genotype *D* and subtype *ayw3*. Ten kinds of point mutations were identified within the *S* region. The highest rates of mutation were found in chronic hepatitis patients and their family members. The amino acid mutations 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 in the S gene region known to be stable and observed that some of these mutations affected *S* gene expression.

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Introduction

Hepatitis B virus(HBV) infection is a major health problem throughout the world, and more than 350 million people are chronic carriers of this virus that can cause chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (McMahon 2005). The reported prevalence in Turkish population is 4%–10%, with the highest prevalence rates seen in southeastern Turkey (Aydin 2002; Kao 2002), an area with high intrafamilial transmission of HBV (Ustacelebi 2004).

Mutations in the HbsAg, also called *S* mutations, have now been documented in many areas of the world, but are most common in Asian infants (2% to 3% of vaccine recipients) (Hunt *et al*. 2002). Intrafamilial transmission of HBV has been studied in the eastern Anatolian region of Turkey (Serpil *et al*. 2003). 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 (Carman 1997). The same mutations also occur in liver transplant recipients receiving HBIg. Less frequently, they develop spontaneously during the course of a chronic HBV infection (Yamamoto *et al*. 1994).

HBV is classified into four serotypes (*adr, adw, ayr* and *ayw*) based on antigenic determinants of the HbsAg (Le Bouvier *et al*. 1972). These serotypes can be further classified into nine serotypes (*ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+* and *adrq−*) (Courouce-Pauty *et al*. 1978). 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 (Norder *et al*. 2004). Naturally occurring HBV with *S* mutations has been reported in a variety of patient groups, with variable rates of occurrence. However, the biological significance of this mutation is still unclear,

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especially for the populations of southeastern Turkey.

Mutations have been described in all the four open reading frames of the HBV. 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 hepatocellular carcinoma. Transmission to other individuals may also occur. Despite encouraging results in vaccinated chimpanzees, HBIg and HBV vaccination do not protect humans from *S* mutant infections (Ogata *et al*. 1999). 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, is of clinical concern. Moreover, loss of antigenicity at the 'a'-determinant, leading to lack of detection of these mutants by some monoclonal HBsAg immunoassay kits, has also been reported (Ogata *et al*. 1997; Carman *et al*. 1995).

We aimed to investigate the HBV *S* region mutations in patients with chronic liver disease, patients on haemodialysis, hospital personnel and healthy blood donors. Moreover, we studied these mutations for their effects on expression of HBsAg and 'a'-determinant epitopes, and also evaluated these mutations in family members of affected individuals. A specific region of the *S* gene was selected for screening mutations. This specific region was between nucleic-acid residues 250–715, a region that provides common regions for three types of surface antigens, and the first loop of 'a'-determinant responsible for antigenicity.

Materials and methods

Sample selection

Serum samples were taken from 132 HBsAg positive patients from different groups, including chronic HBV patients, their family members, hospital personnels, patients on haemodialysis, and healthy blood donors from various hospitals in southeastern Turkey. In addition to these samples, HBV-DNA-positive and HBV-DNA-negative control groups were also used. These serums were selected from patients who are not been vaccinated and were not under drug therapy for HBV, thus permitting the identification of *S* gene mutations with real biological effects.

Primer selection

To achieve maximal sensitivity, a nested PCR protocol was used. We used specific primers suitable for seminested PCR for amplifying the *S* gene region providing common regions for three types of surface antigens and first loop of 'a'-determinant. *S* gene region and determinant mutations have not previously been reported from Turkey.

At the first stage of PCR, 770-bp fragments corresponding to nucleotide positions from 250 – 1019 were amplified using the following primers:

5′- CTAGACTCGTGGTGGACTTCTC -3′ (forward), and

5′- CTAGACTCGTGGTGGACTTCTC -3′ (reverse).

The second stage of seminested PCR protocol utilized the primers

5′- CTAGACTCGTGGTGGACTTCTC -3′ (forward), and

5′- AAGCCANACARTGGGGGAAAGC -3′ (reverse), and yielded a 482-bp amplification product corresponding to nucleotide positions 250 – 732.

The sequencing primer was 5′- AAAGCCCTACGACCACTGA -3′ (reverse) (nucleotides 250 – 715; sequence product: 466 bp). Primers were synthesized by Metis Biotechnology (Ankara, Turkey).

DNA isolation

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 Proteinase K) and incubated at 37°C for 2 h. The mixture was centrifuged at 11,000 rpm at 4°C for 6 min, with the addition of 700 μl phenol buffer. Supernatant was extracted by 700 μl chloroform : isoamyl alcohol 5 (24 : 1) and centrifuged at 11,000 rpm for 6 min. Sodium acetate (3 M, pH 4.9 – 5) and 96% ethanol were added to the supernatant at −20°C. DNA was precipitated at −20°C for a minimum of 1 h, or overnight. Pellet was washed with 70% ethanol at 12,000 rpm for 2 min at −20°C. Then, alcohol was poured out, and DNA was dried at 65°C for 15 min. Finally, DNA was dissolved in 20 μl of TE (Tris + EDTA) (pH 8.0) and stored at −20°C for PCR.

DNA amplification

HBV-DNA was amplified by seminested PCR. For the first stage PCR, 50 μl reaction mixture contained PCR buffer, dNTP (10mM) (Fermentas, Burlington, Canada), $MgCl_2$ (25mM), primer (sense + antisense) and *Taq* DNA polymerase (5 U/ml) (Fermentas, Burlington, Canada). Amplification was carried out in thermal cycler (Techne Genius FGENO5TD, Staffordshire, UK) 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 s, primer annealing at 55°C for 40 s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 s. For the second stage, the conditions of each cycle were 94°C for 20 s, 60°C for 30 s, 72°C for 40 s. The first stage PCR products were analysed by gel electrophoresis on 2% agarose gel stained with ethidium bromide to determine HBV-DNA-positive samples and HBV-DNA-negative samples. The positive samples were reamplified by the second stage of seminested PCR and second stage products were run on 1% agarose gel.

DNA sequencing

The sequencing analysis of the surface antigen region (nucleotides 250 – 732) obtained from the second stage PCR was done with a DNA sequence analyzer (Visible Genetic Inc.,

Toronto, Canada). Tagged reverse primer that starting at nucleotide position 715, and was specific for *TNF-α* gene region was used, resulting in the sequencing of fragment of ~466 bp. Reaction mixture was prepared by Cy5/5.5 Dye Primer Cycle sequencing Kit (Visible Genetic Inc., Toronto, Canada).

Genotyping and mutation analysis

The resulting sequences were available in the GeneBank database with the corresponding AJ131956 accession number among various HBV genotype and serotype sequences. Thus, two genotypes and serotypes were determined. Variants were compared with original sequence of this serotype for identifying mutations.

Results

From the 132 HBsAg positive patients, HBV-DNA could be amplified in the serum of 40 patients (30.3%). The seminested PCR products of HBV-DNA, which corresponded to 482 bp, were run with 50-bp digested molecular weight marker (figure 1). Sequence results corresponded with AJ131956 accession number in the GeneBank. According to this result, genotype *D* and serotype *ayw3* were determined in all the HBV-DNA-positive serum samples. Genotype *D* includes subtypes *ayw2* and *ayw3* (Kidd-Ljunggren *et al*. 2002). Bozdayi *et al*. (2001) and Sentürker *et al*. (2004) have reported *ayw3* to be frequent in Turkey and our finding of *ayw3* subtype in all clones from southeastern Turkey corroborates those earlier findings with data from an hitherto unstudied region of Turkey.

Ten kinds of point mutations were observed. The region of nucleotides 250 – 715 was selected as sequencing region, however mutation density was determined in nucleotides 490 – 550 region. Point mutations of *C498A, A531G* and *T536C* were observed in all of the isolates. In addition, *C501A* point mutation was described in 82.5% of the isolates. Other mutations observed were *T496C, C517T, G523A, C479T, T320C, G296G.* Mutations *T320C* and *G296G* were observed in only two patients. As a result, mutations generally were observed in the nucleotides 460–490 region (figures 2, 3).

In one of the chronic patients (P4), two nucleotides exchanged their locations on A531/532A 5′ position. The point mutation rates were found to be highest in chronic HBV patients. The rates were also high in their family members. The prevalence of mutations in this region was the least in dialysis patients (figure 4).

In addition to the common mutation points *(C498A, A531G, T536C)*, the *C501A* point mutation was observed most frequently in patients. According to patient groups, this mutation was detected mainly in family members. *G296G* and *T320C* mutations were also found only in family members of HBV patients, whereas *C479T* was observed only in dialysis patients (figure 5).

Intrafamilial S gene mutation inductance occurring from parents to offspring

We also investigated whether the occurrence of *S* gene mutants in mothers increased the risk of *S* mutation occurrence in their offspring. Two members of a family were followed for two years. Other than common mutations, two mutations *G296G* and *T320C* occurred in one individual (figure 6: M1-2004), and these mutations led to amino acid exchanges 47 (V → A) and 55 (S → F). Some amino acid substitutions were observed in transcripts of immunologically important subregions within the studied region. The region of nucleotides 450–490 where most nucleotide changes occurred corresponded to amino acid positions 112–131, according to GeneBank. Within the region of nucleotides 284 – 350, corresponding to amino acids 43–61 amino acid position, few changes occurred. Amino acid substitutions of 125 (M → T) and 127 (T → P), in the region containing the surface antigen, were found in all samples. These mutations occurred on the first loop of 'a'-determinant (amino acids 124 – 137)

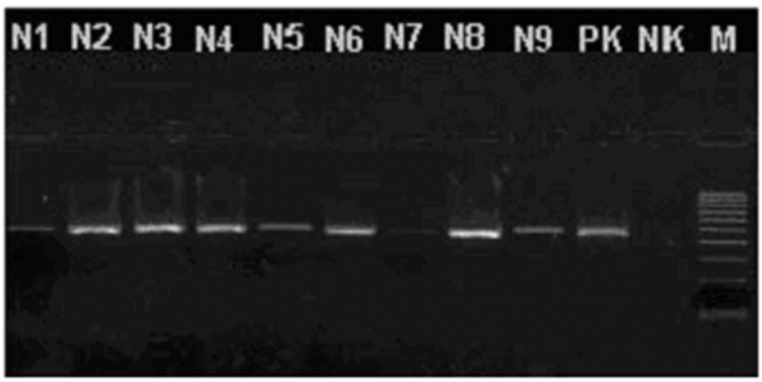

Figure 1. Seminested PCR product on a 1% agarose gel. PK, positive control; NK, negative control; M, DNA ladder; N, samples.

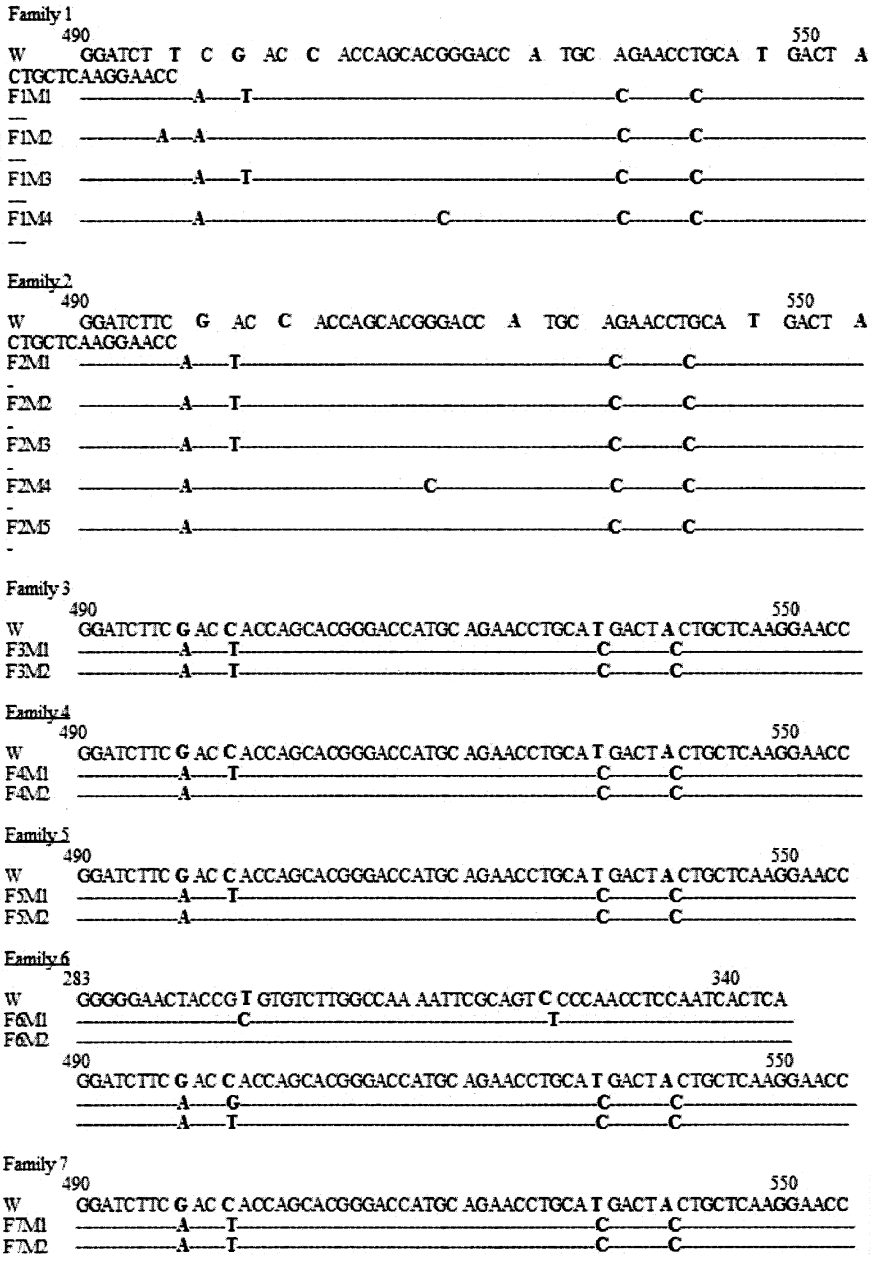

Figure 2. Nucleotide changes in various tested family members of patients. F, family; M, member; W, sequence of genotype *D* serotype from GenBank, accession number AJ131956.

of HBsAg. In one patient, two point mutations led to amino acid substitutions affecting protein synthesis by altering the mutation 125 (M → T) to 125 (M → S). The 114 (S → T) alteration was rarely found. The amino acid substitutions 47 (V → A) and 55 (S → F) were found in the member of one family. As seen in the data, amino acid changes exhibited similarity in all the patients (figure 7). The point mutation at nucleotide 531 led to a thymidine to cytosine substitution, with a corresponding amino acid change 125 (M → T) in the S protein, and the mutation at nucleotide 536 to an adenine to cytosine substitution, resulting in amino acid change from 127 (T → P) in all clones. Both of these mutations were located in first loop of the 'a'-determinant.

Discussion

In earlier studies, Huang *et al.* (2004) found changes at amino acid positions 126, 129, 135, 136 and 145 of 'a'-determinant in conjunction with the absence of detectable anti HBsAg in samples from Qidong, China. Carman *et al.* (1990) found changes at nucleotide position 587 of the 'a'-determinant region. Both these studies used vaccinated

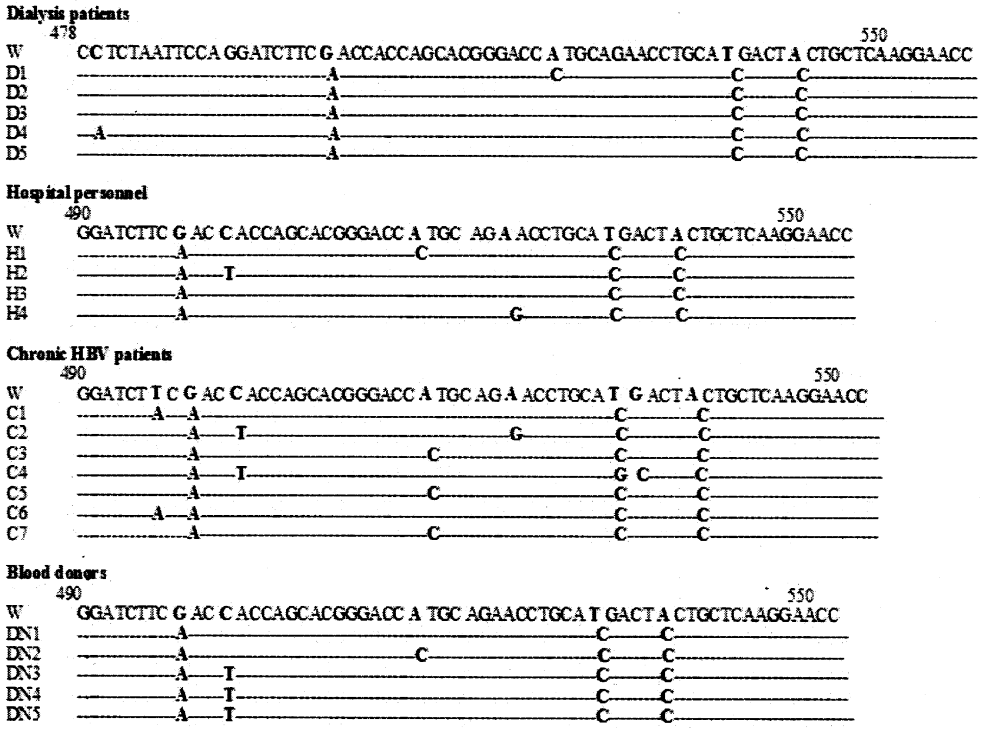

Figure 3. Nucleotide changes in various tested groups other than family members of patients. W, sequence of genotype *D* serotype from GenBank, accession number AJ131956.

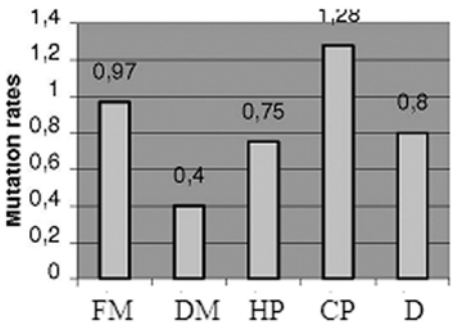

Figure 4. Mutation rates in various tested groups. FM, family members; DM, dialysis patients; HP, hospital personnels; CP, chronic patients; D, blood donors.

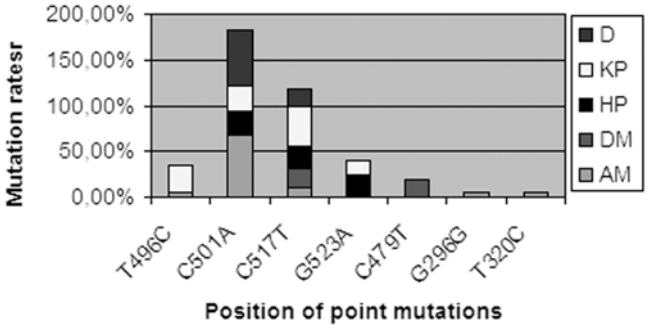

Figure 5. Mutation rates and positions observed in all clones. AM, family members; DM, dialysis patients; HP, hospital personnels; KP, chronic patients; D, blood donors.

patients. In contrast, our samples were unvaccinated anti HBs-negative patients and unlike the earlier studies on pre core-region mutations (Bozdayi *et al*. 1999) and *S* gene genotypes and serotypes (Sentürker *et al*. 2004) in Turkey, we focused on *S* gene mutations in the 'a'-determinant region, and their effects. This is also the first clear report of intrafamilial mutation inductance for HBV S mutations from Turkey, although Serpil *et al*. (2003) earlier found the prevalence of HBsAg and HBV infection among family members to be significantly higher than in the control group.

Recent studies have revealed significant diversity in sequences of HBV isolates, accounting for the allelic differences 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 seven genotypes A–G. However, genotyping can also 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. We found samples to be predominantly genotype

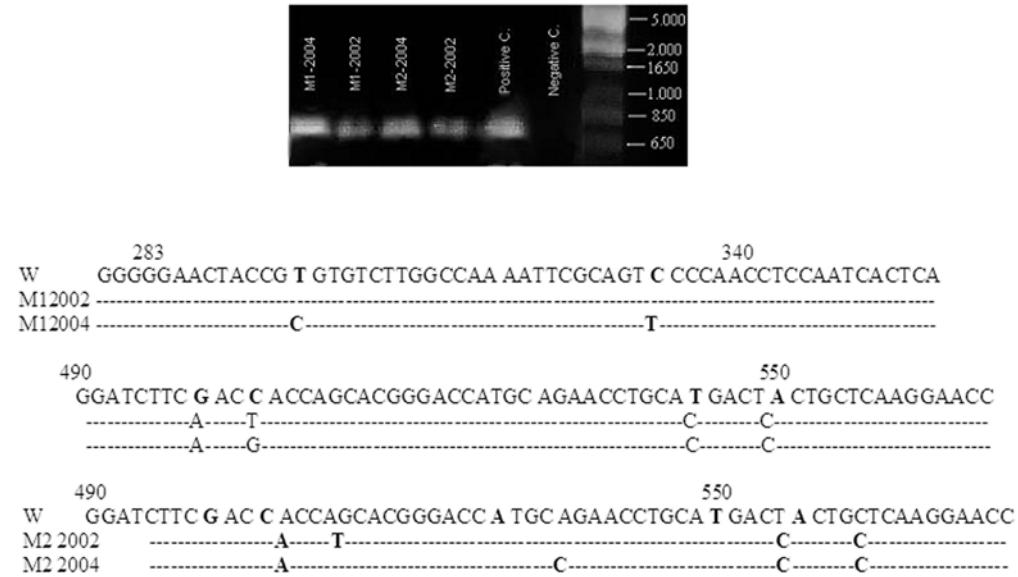

Figure 6. Mutation occurrences in two members of a family in two years, and their DNA fragments on agarose gel.

Nucleotide region 490 – 550 112 131

C	G S S T T S T G P C 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----------

Figure 7. Amino acid sequence of a part of HBV *S* gene in some samples. C, consensus sequence of genotype *D*, serotype *ayw3*; CP, chronic HBV patients.

D and serotype *ayw3*, which is similar to other reports for areas neighbouring Turkey (Ogata *et al*. 1999). The surface proteins of HBV 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 coexist in the infected host (Carman *et al*. 1990, 1995). We found 10 mutations in this region, although it was considered to be stable, and investigated these mutations in separate group of patients. In principle, observed differences in the nucleic acid sequence may be PCR artifacts, or reflect heterogeneity of the viral population, or be caused by point mutations. The rate of point mutations caused by the *Taq* polymerase itself ranges between 1×10^{-4} and 6×10^{-5}, depending on the *Taq* polymerase used (Tindall and Kunkel 1988; Gunther *et al*. 1998). Thus, PCR error is an unlikely explanation of our results, especially since many clones contained the same mutations in parallel.

In our study, *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 stable, determination of these mutations in our study that are collected on a part of *S* region, leads to the conclusion that these variants may trigger a new serotype in future. Maximum mutation rates were determined in chronic HBV patients. This was expected because of the long carrier phase of virion until chronic phase. Moreover, mutations might be directly triggered by the immune system due to nonvaccination and not undergoing drug therapy. High secondary mutation rates were also found in family members of HBV patients. Existence of mutant virions in parents may stimulate mutations in infants, resulting in high mutation rates in family members of patients. Lower mutation rates occurred in hospital personnels and blood donors, and the lowest mutation rate was seen in the group of haemodialysis patients.

The largest number of mutations were found on a part of *S* gene (nucleotides 460–550) including the first loop of the 'a'-determinant, which 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 to 147 is within the major hydrophilic region (Yamamoto *et al*. 1994; Wallace and Carman 1994). The conformation of 'a'-determinant residues between Cys-124 and Cys-137 (first loop) and Cys-139 and Cys-147 (second loop) (Fiordalisi *et al*. 1994; Brown *et al*. 1984) tends to be highly conserved (Yamamoto *et al*. 1995). If this conformation is altered, previously produced antibodies against the native 'a'-determinants cannot be protective (Carman *et al*. 1990). This area is highly immunogenic and forms the basis of HBsAg vaccines.

It is likely that a mutant 'a'-determinant will not be neutralized by vaccine-induced antibodies, because these muta-

tions affect the conformation of the 'a'-determinant epitope, and thus alter the antigenicity of HBsAg, leading to potential escape from detection by standard HBsAg assays. Further, the altered conformation of the 'a'-determinant epitope may lead to a considerable decrease of properly folded surface antigen, which may render the virus granules less immunogenic in inducing an effective neutralizing antibody to clear the virus. The 125 (M → T) and 127 (T → P) amino acid substitutions observed in our study occur in the first loop of the 'a'-determinant region in all the isolates, and may result in an increased likelihood of HBV escaping identification by the immune system.

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