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Mutation and genotype analysis of hepatitis B virus on acute and chronic infected selected patients in Turkey

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Abstract

Chronic hepatitis B virus infection is important in Turkey because of the intermediate prevalence (8 %) of persistent HBV infection. The purpose of this study is to demonstrate X and core gene mutations in chronic and acute HBV infected patients in the Turkish population. HBV genotypes were analysed using restriction fragment length polymorphism (RFLP) of the S gene region. The genotypes of fifty HBV infected patients were determined. Three different genotypes were found, genotype D; 42 (84 %), genotype A; 6 (12 %), and genotype F; 2 (4 %). The X and overlapping core regions have been amplified by PCR and sequenced. Seven different mutations were detected in the core region. In the X region, 12 different point mutations and 4 different insertions were detected. All samples had C→T and T→C alterations and the others as follows; (G→A), (G→C), (A→T), (A→G), (C→A), (C→G), (A→C), (G→T), (T→G), (T→A). Isolates from five AntiHBe positive five patients contained specific mutations, which are reported to increase viral replication and result in a poor prognosis. Further investigations should be carried out using more diverse Turkish patients in order to understand the clinical outcome of these alterations.

Keywords: HBV, core region, X region, HBV mutations, RFLP.

Türkiye'de akut ve kronik infeksiyonlu hastalardaki hepatit B virüs mutasyon ve genotip analizi

Özet

Kronik hepatit B virus infeksiyonu Türkiye'de % 8 oranında bulunmaktadır. Bu çalışmada, akut ve kronik hepatit B infeksiyonlu hastalar seçilerek X ve kor genlerine ait baz değişikliklerinin saptanması amaçlanmıştır. Restriksiyon fragment uzunluk polimorfizm yöntemi ile 50 hastanın S gen bölgesine göre genotipleri belirlenmiştir. 42 olguda genotip D (% 84), genotip A 6 olguda (% 12), ve genotip F 2 hastada (% 4) olmak üzere üç farklı genotip saptanmıştır. X ve bu bölge ile üstüste gelen prekor-kor bölgeleri özgün primerler kullanılarak çoğaltılmıştır. Ardından yapılan dizi analizi reaksiyonları ile hastaların baz dizileri belirlenmiştir. HBV X gen bölgesinde 12 farklı nokta mutasyon ve 4 farklı insersiyon tanımlanmıştır. Tüm hastalarda C_T and T_C baz değişimleri gözlenmiş olup söz konusu diğer mutasyonlar sırasıyla, (G→A), (G→C), (A→T), (A→G), (C→A), (C→G), (A→C), (G→T), (T→G), (T→A)'dır. AntiHBe pozitif 5 olguda viral replikasyonu arttırarak prognozun kötüleşmesine neden olan spesifik mutasyonlar tesbit edilmiştir. HBV infeksiyonları ve mekanizmasının hastalığın seyrindeki önemin anlaşılması için farklı bölgelerden seçilmiş akut ve kronik HBV ile infekte hastalar üzerinde daha fazla çalışma yapılması gerekmektedir.

Anahtar Sözcükler: HBV, kor gen bölgesi, X gen bölgesi, HBV mutasyonları, RFLP.

Introduction

Hepatitis B virus (HBV), a member of the Hepadnaviridae, causes transient and chronic infections of the liver. Transient infections may produce serious illness with approximately 0.5% of infections resulting in fulminant hepatitis. Chronic infections also may have serious consequences: nearly 25% result in untreatable liver cancer (Baumert et al., 1998; Wai and Fontana, 2004; Marcellin et al., 2005). The number of carriers worldwide is estimated at 350 million. One of the reasons for chronic HBV infections is that the virus causes chronic, noncytocidal infections of hepatocytes, the principal cell type of the liver. Hepatocytes continuously shed virus into the bloodstream, ensuring that 100 % of the hepatocyte population is infected (Seeger and Mason, 2000).

HBVhas a circular genome of approximately 3200 base pair (bp), comprising partially double-stranded DNA. The genome comprises four open reading frames encoding the polymerase (P), surface antigen (S), nucleocapsid (C), and X proteins. Significantly, these reading frames overlap substantially, with approximately half the viral genome encoding more than one protein product (Uysal et al., 2001; Locarnini, 2004; Onganer, 2004).

HBV has been classified into eight genotypes (Ato H), which have been associated with different geographical areas: A with Europe and sub-Saharan Africa, B and C east Asia, D with the Mediterranean and Middle East region, E with western Africa, F with the Americas and G with France and United States. Recently, an additional genotype H, has been observed in the northern part of Latin America (Ruiz et al., 2002).

HBV has evolved a unique life cycle resulting in the production of enormous viral loads during active replication without directly killing the infected cell. Because the virus relies on error-prone reverse transcriptase during the replicative process, mutant viral genomes are found frequently. Particular selection pressures, both endogenous (host immune clearance) and exogenous (vaccines and antivirals), readily select for escape mutants (Lee et al., 1996). It is still not known which particular viral mutations or combination of mutations directly affect the clinical presentation of the liver disease, the nature of viral persistence, or the course and outcome of chronic infection. Sequence analysis of virus isolates from many individual patients has revealed the occurrence of certain mutational hot spots in the genome, some of which correlate with the patient's immunological and/or disease status; however, cause and effect are not always easily discernible (Su et al., 1998).

The aims of this study were to determine dominant HBV genotypes within the Turkish population and the frequency of mutations within the X and precore/core genes.

Materials and methods

Serum samples were provided by the Microbiology and Clinical Microbiology Laboratories of Haydarpasa Numune Hospital, and selected from HBV infected adults. All of the samples were HBsAg and anti HBcIgG positive. HBe antigen and Anti-HBe assays were performed for selective patients.

Extraction of viral DNA from serum

Fifty serum samples were obtained from Turkish patients presenting with chronic HBVinfection. A 100 µl aliquot of serum was incubated at 45°C for 2 h in a mixture of proteinase K (100 mg/µl), 0.5 % sodium dodecylsulfate, 5 mM EDTA, and 10 mM Tris HCI, pH 8.0. The solution was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol, followed by an ethanol precipitation. The precipitate was dissolved in 50 µl of distilled water.

Genotype analysis

PCR Amplification of HBVDNA S region

A 10 µl aliquot of serum DNA was subjected to polymerase chain reaction (PCR) with the following amplification mixture: 2.5 µl of 10x Taq polymerase buffer, 2.5 µl 2.5 mM deoxyribonucleotide triphosphate, 0.1 µl of Taq polymerase (Promega), 10 pmol of sense HBVF1(CCCTGCTGGT GGCTCCAAGTTC) and antisense primers HBVR2 (AAGCCAAACAGTGGGGGAAAGC) in a 25 µl reaction volume. PCR amplification was performed as follows: 2 min at 96°C, followed by 25 cycles of 94°C for 15 sec, 45 sec at 60°C and 45 sec at 72°C. A volume of 2.5 µl of the first-round PCR products was added to a second-round PCR mixture. Amplification

was performed as above but with an inner sense primer HBVF2 (GTCTAGACTCGTGGTGGACT TCTCTC) and antisense primer HBVR2 (AAGCC AAACAGTGGGGGAAAGC). The second-round PCR products, which were 485 bp long, were analyzed by electrophoresis in 3 % agarose gels in TBE buffer (89 mM Tris-HCI, 89 mM boric acid, 2 mM EDTA) stained with ethidium bromide, and then visualized under ultraviolet light.

RFLP Analysis for amplified S region of HBVDNA

HBVgenotyping was performed using RFLPanalysis. Restriction digestions were performed using 20 µl of second-round PCR products. Digestion proceeded for 3 hours and was performed according to the manufacturer's recommendations. Reactions were carried out with 10 units of Alw I, Nci I, Ear I, NIa IV or Hph I at 37°C. The digested PCR products were electrophoresed on a 3 % agarose gel in TBE buffer containing ethidium bromide. The RFLP pattern was then evaluated under ultraviolet light.

HBV precore/core region mutation analysis

PCR amplification of HBV DNA core region

PCR amplification mix was prepared as described above. First round PCR was carried out using primers B935(GCGCTGCAGAAGGTTTGTGGCTCCTCTG) and MDC1 (TTGATAAGATAGGGGCATTTG). Amplification consisted of 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 90 sec. Reaction mixtures comprised 10 µl DNA in a final reaction volume of 50 µl. Second round PCR was carried out on 5 µl of the first round product using primers CPRF1 (CAATGTCAACGACCGACC) and CPRR1 (GAG TAACTCCACAGTAGCTCC) and 30 cycles of the same reaction conditions. The second-round PCR products, which were 270 bp long, were analyzed by electrophoresis in 1 % agarose gels stained with ethidium bromide, and visualized under ultraviolet light.

Direct sequencing of PCR products for precore/core region

For the determination of nucleotide and amino acid sequence divergences, direct sequencing of PCR products was performed. PCR products were purified by the Wizard DNA Purification System (Promega), and direct sequencing was performed using primer CPRF1. Cycle Sequencing was performed with the ^{33}P Promega Cycle Sequencing kit. Sequencing reactions were run on polyacryamide urea gels. Patients' sequences were compared with the sequence of a HBV published in the Genbank.

Nucleotide sequence accession number: The GenBank accession numbers of the sequences reported in this paper are AF461043.1- AF461043 and AAL66350.

HBV X region mutation analysis

PCR amplification of HBV DNA X region

The PCR mix, except primers, was used as given at HBV DNA S region amplification section. PCR was carried out using primers Xbeg (GCCTGAA TTCCATGGCTGCTCGGTTGTGC) and Xend (CA TGAACTCGAGATGATTAGGCAGAG), using 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec. Reactions contained 5 µl DNA in a 50 µl final volume. PCR products, which were 518 bp long, were analyzed by electrophoresis in 1 % agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Direct sequencing of PCR products for X region

The products from PCR were purified with the Wizard DNA Purification System (Promega). In the course of our experiments, there have been unexpected regulations on radioactive material therefore we chose to change our procedure to use automatic sequencer. Sequencing was performed by primer walking in both directions with 20 pmol of template and 4 pmol of primer Xbeg. Cycle sequencing was performed with a Thermal Controller, using the Big Dye Terminator Cycle Sequencing kit (Genomics). Sequence electrophoresis was carried out with an automated DNA sequencer ABI 3100. Sequence results were compared with those in Genbank.

Nucleotide sequence accession number: The GenBank accession numbers of the sequences reported in this paper are AF461043.1- AF461043 and AAG17600.

Results

Genotype analysis

The HBV genotype specific regions were amplified and digested using the restriction enzymes HphI (Genotype F), Nci (Genotype E), AlwI (Genotype C), NIaIV (Genotype A and D) (Mizokami et al., 1999). Genotypes D, A and F were detected - genotype D (84 %) was found in 42 samples, genotype A (12 %) in 6 and genotype F (4 %) in only 2 of 50 samples examined.

HBV precore/core region mutation analysis

The 31 samples that were PCR positive for precore/core region were investigated by sequencing. Seven different mutations were detected within these samples. These mutations are as follows: nucleotide (nt) 1858 (C→T), 19 (61 %); nt 1899 (G→A), 17 (55 %); nt 1856 (C→T), 5 (16 %); nt 1862 (G→T), 2 (6.4 %); nt 1896 (G→A), 1 (% 3.2); nt 1762 (A→T) and nt 1764 (G→A), 1 (3.2 %). HBV precore/core region sequencing data is shown in Figure 1.

HBV X region mutation analysis

In the X region, 12 different point mutations and 4 different insertions were detected. All samples had a C→T and T→C alteration and the others were as follows; (G→A), (G→C), (A→T), (A→G), (C→A), (C→G), (A→C), (G→T), (T→G), (T→A). Sequencing results for the HBV X gene are shown in Figure 2.

Discussion

There is growing evidence that HBV genotypes may influence HBeAg seroconversion rates, mutational patterns in the precore and core promoter regions, and the severity of liver disease (Akahane et al., 1990; Lavine and Hirsch, 1989; Liang et al., 1994; Jazayeri et al., 2004). In addition, different HBV genotypes predominate in various parts of the world (Mizokami et al., 1999). Thus, the heterogeneity in disease manifestations and response to antiviral treatment among patients with chronic hepatitis B in different parts of the world may be attributed to differences in HBV genotypes (Aye et al., 1997; Tassopoulos et al., 1999; Tillmann et al., 1995). Hepatocytes are normally long-lived, with half-lives estimated at 6 to 12 months or longer. The combination of a long-lived, usually non-dividing host cell and a stable virus-host cell interaction virtually ensures the persistence of an infection in the absence of a robust host immune response (Robinson, 1994; Rodriguez-Frias et al., 1995). Liver disease in transient and chronic carriers is thought to be largely due to the host immune response to the infection. This response can induce a high level of hepatocyte destruction (Nowak et al., 1996), leading to scarring, disruption of blood flow, and obstruction of bile drainage without necessarily eliminating the infection. It is not yet known why this response is unable to clear the virus in many who become chronic carriers or, for that matter, why elimination sometimes occurs spontaneously after many years of virus production (Loriot et al., 1995; McMillan et al., 1996). Therefore, a major focus of HBV research is to understand the virus-host interactions, which determine whether an infection will persist or terminate (Lindh et al., 1996). The hope is that this knowledge will lead to better treatments and a reduced incidence of hepatocellular cancer.

Although a high prevalence HBV infection is present in Turkey, the genotype distribution is still unclear. The HBV is classified into eight genotypes based on intergroup divergence; however the genotype-related differences in the pathogenicity of HBV remain unknown.

This study showed a molecular heterogeneity of HBV in the Turkish population with 3 different genotypes observed in the 50 samples studied. Genotype D is by far the most common genotype in the Turkish population (Bozdayi et al., 2004) as is the case in all other Mediterranean countries. In view of Turkey's geographical setting which has served as a bridge in many migration events during history, the findings in chronic hepatitis B patients may not be as expected.

Although the frequency of the percentage of genotype A and F is rather low, the presence of such genotypes may result from the relationship of the Turkish people with Northern European (A) and South American (F) countries. It is not known whether there is a correlation between the HBV genotype and HBV

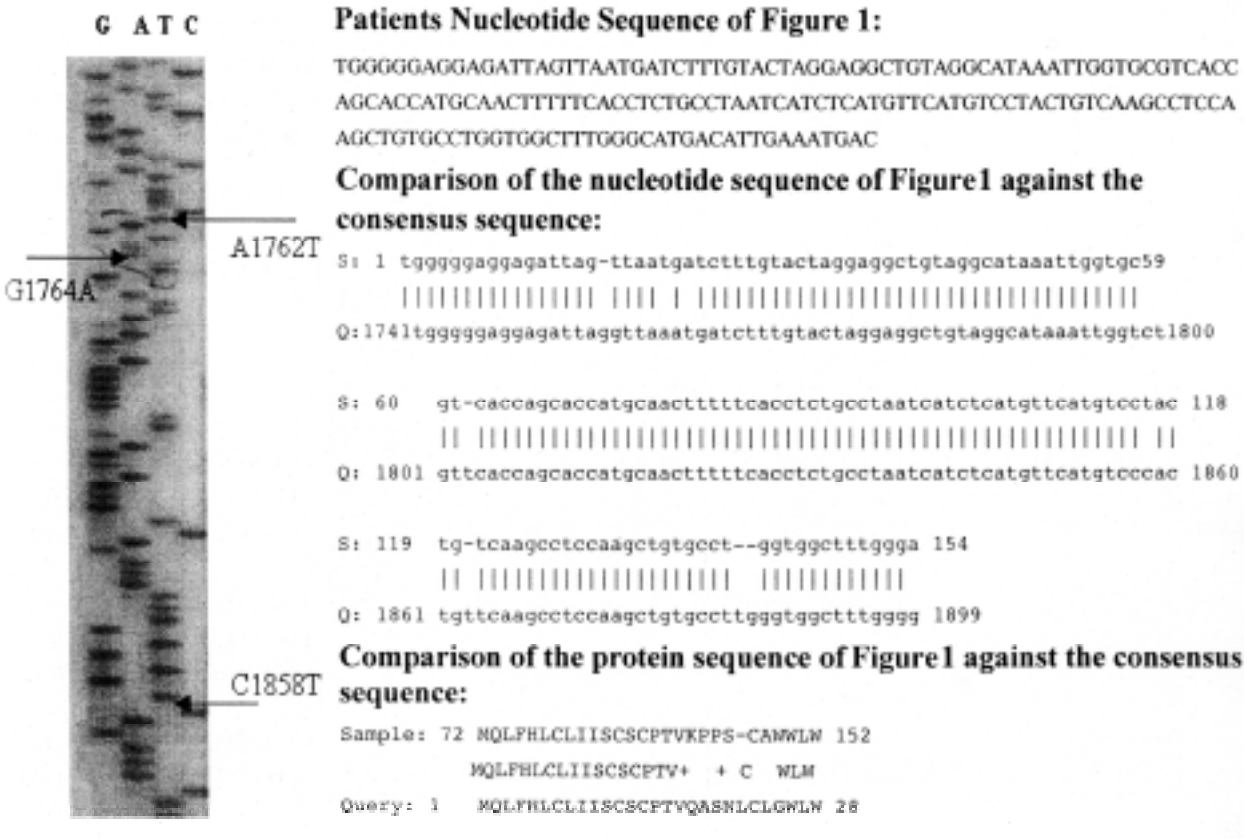
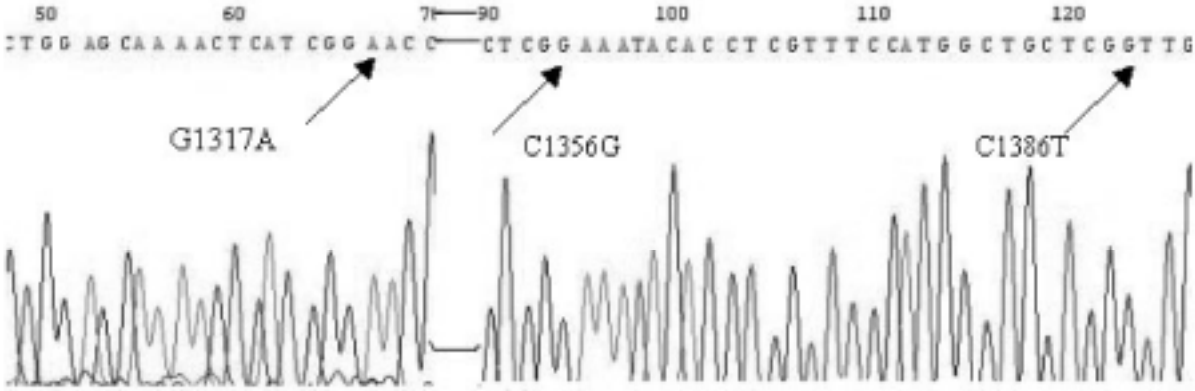


Figure 1. Sequencing result of a representative patient showing the precore/core region. Arrows represent the mutation regions and base changes. Sample's sequence was compared to Gen Bank sequence, S used for sample, Q used for Query.

replication, severity of liver disease or clinical outcome of treatment response. However, geographical distribution of HBV genotypes is related to the endemicity of HBV infection (Schaefer, 2005). The studies on the correlation of these genotypes and risk of progression to chronic infection are still ongoing. It is interesting that there are very few studies (Kao et al., 2001) investigating whether infection with one HBV genotype is protective against subsequent infection by other HBV genotypes.

In this study, we found that most (61 %) of the Turkish patients with infected Hepatitis B had the mutation at nt 1858 (cytosine to thymine change). On the other hand, low prevalence of precore stop codon mutation at nt 1896 (guanine to adenine change) was detected. Some reports suggest that, thymine at nt 1858 may create a more stable base pairing with adenine at nt 1896 instead of guanine in the encapsidation signal (Akahane et al., 1990; Li et al.,1993; Lok et al., 1994). Several studies have associated the precore stop mutation A1896 with evolves of the clinical symptoms of liver disease caused by HBV (Brunetto et al., 1991; Brunetto et al., 1993; Jardi et al., 2004). HBV strains showing this mutations cannot express the HBe antigen either or cell membrane or in the circulation (Brunetto et al., 1990; Carman et al., 1989; Okamoto et al., 1990). This mutation is found very often in the Far East and Mediterranean countries (Carman et al., 1989; Pollicino et al., 1995; Santantonio et al., 1991). HBV genotype A has cytosine at nt 1858 and as expected in this case G at nt 1896 (Brunetto et al., 1990; Carman et al., 1989). Also some studies shown that same pattern also observed at genotype F (De Castro et al.,



Patient Nucleotide Sequence of Figure 2:

GATCCATACTGCGGAACTCCTTGCAGCTTGTTTTGCTCGCAGCCGGTCTGGAGCAAAACTCATCGGAACCGACAACTCTGTTGTCC
TCTCTCGGAAATACACCTCGTTTCCATGGCTGCTCGGTTGTGCTGCCAACTGGATCCTGCGTGGGACGTCCTTTGTCTACGTCCCGT
CGGCGCTGAATCCCGCGGACGACCCGTCTCGGGGTCGCTTGGGGATCTATCGTCCCCTTCTCCGTCTGCCGTTCCGGCCGACCACG
GGGCGCACCTCTCTTTACGCGGTCTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGCACGTCACA
TGGAGACCACCGTGAACGCCCACCAGGTCTTGCCCAAGGTCTTACATAAGAGGACTCTTGGACTCTCAGCAATGTCAACGACCGA
CCTTGAGGCGTACTTCAAAGACTGTGTGTTTAAAGACTGGGAGGAGCNGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGA
GGCTGTAGGCATAAATTGGTCTGCGTACCAGCACCATGCAACTTTTTCACCTCTGCCCTAATCATCTCATGTTCATGTTCCACTGTT
CAA

Comparison of the nucleotide sequence of Figure2 against the consensus sequence

```
Sample:     1 gatccatactgcggaactccttgcagcttgttttgctcgcagccggtctggagcaaaact 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Query:   1263 gatccatactgcggaactccttgcagcttgttttgctcgcagccggtctggagcgaaact 1322


S: 61   catcggaaccgacaactctgttgtcctctctcggaaatacacctcgtttccatggctgct 120
        |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Q: 1323 catcggaaccgacaactctgttgtcctctctcgcaaatacacctcgtttccatggctgct 1382


S: 121  cggttgtgctgccaactggatcctgcgtgggacgtcctttgtctacgtcccgtcggcgct 180
        ||| |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Q: 1383 cggctgtgctg ccaactggatcctgcgcgggacgtcctttgtctacgtcccgtcggcgct 1442


S: 181  gaatcccgcggacgacccgtctcggggtcgcttggggatctatcgtcccctttctccgtct 240
        |||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||
Q: 1443 gaatcccgcggacgacccgtctcggggtcgcttggggctctaccgtcccctttctccgtct 1502


S: 241  gccgttccggccgaccacggggcgcacctctctttacgcggtctcccccgtctgtgccttc 300
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Q: 1503 gccgttccggccgaccacggggcgcacctctctttacgcggtctcccccgtctgtgccttc 1562


S: 301  tcatctgccggaccgtgtgcacttcgcttcacctctgcacgtcacatggagaccaccgtg 360
        |||||||||||| |||||||||||||||||||||||||||||| ||||||||||||||||
Q: 1563 tcatctgccggtccgtgtgcacttcgcttcacctctgcacgtcgcatggagaccaccgtg 1622

S: 361  aacgcccaccaggtcttgcccaaggtcttacataagaggactcttggactctcagcaatg 420
        |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Q: 1623 aacgccaaccaggtcttgcccaaggtcttacataagaggactcttggactctcagcactg 1682
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Comparison of the protein sequence of Figure2 against the consensus sequence:

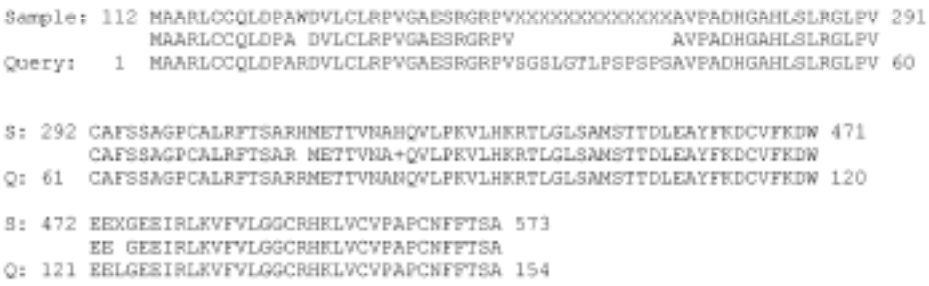

Figure 2. Sequencing result of a representative patient showing the X region. Arrows represent the mutation regions and base changes. Sample's sequence was compared to Gen Bank sequence, S used for sample, Q used for Query.

2001). These might explain that why we detected low frequent precore stop mutation in our studies. Besides the precore stop mutation, several point mutations can cause the initiation failure as well as deletions and insertions of nucleotides including frameshifts, have been shown in the precore regions (Okamoto et al., 1990; Chan et al., 1999; Hsu et al., 1995). Although in this study we found that most dominant genotype was D, A1896 were detected on only one patient. Another study from Sweden published that none of six genotype D isolates showed the precore stop codon mutation at nt 1896 during the follow-up of 17 months after anti HBe seroconversion (Blackberg et al., 2000).

In precore region an additional mutation at nt 1899 (guanine to adenine change) was found. This mutation has been described in association with the stop codon mutation at nt 1896 and may enhance the stability of the stem loop structure of the pregenome encapsidation signal (Akahane et al., 1990; Carman et al., 1989). High prevalence of (55 %) A1899 detection may also be another evidence for future stop codon mutation in these patients.

In vitro studies have shown that the T1762 and A1764 double mutation specifically suppress precore RNA transcription and enhances viral replication (Akarca and Lok 1994; Chen et al., 1995). Only one patient showed the double substitution-changing AGG to TGA at positions 1762-1764 in the core promoter region. Cytosine to thymine substitution at nt 1856 and guanine to thymine changes at nt 1862 also were

detected.

Five AntiHBe positive patients have mutations between nt 1823-1834 (direct repeat-DR1) and nt 1818-1921 (encapsidation regions). These mutations reportedly increase virus replication and result in a relatively poor prognosis (Loriot et al., 1995; Lindh et al., 1996; Li et al., 1993; Alexopoulou et al., 1997).

The core promoter region overlaps with the carboxyl half of the X open reading frame (Baumert et al., 1998). This could be a decreased need for those functions of the X protein that are associated with its C-terminal domain (Arii et al., 1992; Takada et al., 1994). However, deletions in this overlapping region could also be of advantage to the survival of HBV. The results of the sequence analysis in this study show that the observed nucleotide alterations within the X gene result in several amino acid changes in X protein.
Further investigations should be carried out on more diverse Turkish patients in order to understand clinical outcome of these alterations. Also to confirm that the change of procedure from radioactive to automatic sequence does not affect the results some crossed checked experiments should be performed.

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