

RESEARCH ARTICLE

Prevalence of Common YMDD Motif Mutations in Long Term Treated Chronic HBV Infections in a Turkish Population

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Abstract

In the current study we aimed to show the common YMDD motif mutations in viral polymerase gene in chronic hepatitis B patients during lamivudine and adefovir therapy. Forty-one serum samples obtained from chronic hepatitis B patients (24 male, 17 female; age range: 34-68 years) were included in the study. HBV-DNA was extracted from the peripheral blood of the patients using an extraction kit (Invisorb, Instant Spin DNA/RNA Virus Mini Kit, Germany). A line probe assay and direct sequencing analyses (INNO-LIPA HBV DR v2; INNOGENETICS N.V, Ghent, Belgium) were applied to determine target mutations of the viral polymerase gene in positive HBV-DNA samples. A total of 41 mutations located in 21 different codons were detected in the current results. In 17 (41.5%) patients various point mutations were detected leading to lamivudine, adefovir and/or combined drug resistance. Wild polymerase gene profiles were detected in 24 (58.5%) HBV positive patients of the current cohort. Eight of the 17 samples (47.1%) having rtM204V/I/A missense transition and/or transversion point mutations and resistance to lamivudine. Six of the the mutated samples (35.3%) having rtL180M missense transversion mutation and resistance to combined adefovir and lamivudine. Three of the mutated samples (17.6%) having rtG215H by the double base substitution and resistance to adefovir. Three of the mutated samples (17.6%) having codon rtL181W due to the missense transversion point mutations and showed resistance to combined adefovir and lamivudine. Unreported novel point mutations were detected in the different codons of polymerase gene region in the current HBV positive cohort from Turkish population. The current results provide evidence that rtL180M and rtM204V/I/A mutations of HBV-DNA may be associated with a poor antiviral response and HBV chronicity during conventional therapy in Turkish patients.

Keywords: YMDD - HBV - polymerase gene mutation - long term treatment - HCC risk

Asian Pac J Cancer Prev, 14 (9), 5489-5494

Introduction

Hepatitis B virus (HBV) infection is a global health problem and major cause of morbidity and mortality worldwide. The spectrum of clinical manifestations of HBV infection varies both in acute and chronic phase. Recently, due to the viral gene recombinations and increased point mutations the CHB therapy is not a black and white picture but a highly complicated challenge requiring systematic diagnostic procedures. More than 300 million people worldwide are estimated to have chronic HBV infection (Lee et al., 2006). The HBV infection is major cause of cirrhosis, liver failure as well as hepatocellular carcinoma (HCC) worldwide (Ganem and Prince 2004; Liu et al., 2005; Lin and Kao 2008). Treatment of chronic hepatitis B (CHB) infection aims to clear HBV DNA and prevent the development of complications. There are currently seven types of drugs available for the treatment of CHB: five nucleos(t)ide analogues and two for interferon-based therapies (Aspinall

et al., 2011). Lamivudine (2', 3'-dideoxy-3'-thiacytidine) is an analogue of cytidine base and commonly called as 3TC. A great number of studies have shown that administration of lamivudine for at least 4 weeks before orthotopic liver transplantation resulted in eradication of serum HBV DNA in 62.5% to 100% of treated patients (Markowitz et al., 1998; Fontana et al., 2002). Unfortunately long term use of the lamivudine is resulted in drug resistance due to mutations in YMDD (tyrosine, methionine, aspartate, aspartate) motif and in the proximal FLLAQ motif (phenylalanine, leucine, leucine, alanine, glutamine) of the viral polymerase of the viral DNA polymerase gene in 10-25% of patients (Lai et al., 1998; Fontana et al., 2002). The YMDD motif is a highly conserved amino acid sequence involved in deoxynucleoside triphosphate (dNTP) binding in the catalytic site of a number of RNA-dependent DNA polymerases, including HBV DNA polymerase (Poch et al., 1989; Kim et al., 2012). Wide range of point mutations in different populations were reported in HBV DNA polymerase gene (Sato et al., 1995; Carman et al., 1996;

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Baumert et al., 1996; Gaillard et al., 2002; Kim et al., 2012). Gene consists of four distinct regions; a primer involved in priming of reverse transcription, a spacer with no known function, a reverse transcriptase which is responsible for the reverse transcription of pregenomic RNA into the first negative strand HBV DNA and for the synthesis of the second positive strand HBV DNA and a RNase H, which removes RNA template (Poch et al., 1989). The reverse transcriptase gene has five conserved regions A, B, C, D and E. Domains A, C and D are involved in nucleoside triphosphate binding and catalysis, while domains B and E participate in the positioning of RNA template and the primer, relative to catalytic site (Johnson et al., 1986; Kohlstaedt et al., 1992; Allen et al., 1998). Lots of literature findings have clarified that persistent inflammation together with genetic/epigenetic alterations is strongly associated with chronic HBV infection-related hepatocarcinogenesis (Chen et al., 2013; Gao et al., 2013; Lavarone and Colombo, 2013; Singh et al., 2013). Patients of chronic HBV infection followed by chronic HCV infection were at higher risk of developing HCC in India (Sarma et al., 2012) and Iranian populations (Geramizadeh et al., 2013).

The putative catalytic domain is believed to reside in the YMDD locus in domain C. In the current case control study we aimed to show the percentage of common YMDD motif mutations of viral polymerase gene in chronic hepatitis B patients during mono and/or combined lamivudine-adefovir therapy in Turkish population.

Materials and Methods

Patient group

In the current case-control study it was aimed to investigate the prevalence of whether pre-existing YMDD motif mutants were selected during therapy. Forty- one serum samples obtained from chronic hepatitis B patients; [24 male (58.5%), 17 female (41.5%) and mean age-min-max; 49.9±7.41(34-68)] were included in the current retrospective case-control study. The chronic hepatitis B patients who were taking alone or combined LVD and ADV therapy in Cumhuriyet University training and research hospital by the collaboration of department of medical genetics and gastroenterology between March 2006 and January 2010 were included in the presented retrospective study. The patients with resistant motifs for longer than at least 9 months, (mean therapy period was 6 months) were selected and evaluated in the current study. HBV-DNA was extracted from the peripheral blood samples of the patients by using extraction kit (Invisorb, Instant Spin DNA/RNA Virus Mini Kit, Germany). A line probe assay and direct sequencing analyses (INNO-LIPA HBV DR v2; INNOGENETICS N.V, Ghent, Belgium) were used to determine target mutations at viral polymerase gene fragment in HBV-DNA samples.

Viral polymerase sequencing

YMDD mutations in the HBV DNA polymerase gene were determined using nested PCR and direct sequencing. Viral DNA was isolated from peripheral blood serum of patients with CHB using Invitex RTP DNA/RNA Virus

Mini Kit according to manufacturer's description. Viral polymerase region was amplified with nested PCR technique. The first set of primers as follows; Forward (POL1): 5'-CACCTGCAGCCTCATTTTGTGGGTCACCATA-3' and Reverse (POL2): 5'-CATAAGCTTCACAATTCGTT-GACATACTTT CCA AT -3'.

PCR reactions were carried out in a final volume of 50µl, in a mixture of 25pM of each primer, 10µl of genomic DNA, 6 mM dNTPs, 3µl of MgCl (Fermentas), 5µl of 10× PCR buffer (Fermentas), 1 U Taq DNA polymerase (Fermentas) and 26.5µl of DNase RNase free water. The following PCR conditions were used: at 95°C for 5 min 35 cycles with 95°C for 1 min, 45°C for 2 min and 72°C for 2 min were performed. Resulting PCR products was used as template for second PCR. The second set of primers for nested PCR as follows; Forward (372):5'-TCG CTG GAT GTG TCT GCG GCG TTTTAT-3' and Reverse (840): 5'-ACC CCA TCT TTT TGT TTT GTT AGG- 3'.

Nested PCR reactions were carried out in a final volume of 50µl, in a mixture of 25pM of each primer, 3µl of first PCR product, 6mM dNTPs, 2µl of MgCl (Fermentas), 5µl of 10× PCR buffer (Fermentas), 1U Taq DNA polymerase (Fermentas) and 31.5µl of DNase RNase free water. The following PCR conditions were used for second PCR: at 95°C for 5 min 25 cycles with 95°C for 1 min, 50°C for 2 min and 72°C for 2 min were performed.

All obtained PCR fragments were purified with a PCR cycle kit (Invisorb Spin PCRapid kit, Germany) according to the manufacturer's instructions. The sequencing reaction was performed with 3-9µl of the purified PCR product with 2µl BigDye Terminator Cycle Sequencing extract (Perkin-Elmer, Foster City, CA, USA). The PCR products were purified and sequenced on both strands with the same primers used for the second PCR. The DNA sequences were analysed on an ABI Prism Genetic Analyser 310 (Applied Biosystems, Foster City, USA). The software SPSS for Windows version 12.0 was used to perform statistical analysis and estimate the prevalence of the mutated alleles of common YMDD codons in HBV positive patients.

Results

Common mutations (Table 1) that located in 21 different codons were detected in the current results. YMDD motif mutations were detected in all, 100% (41/41) of current studied CHB patients. Silent mutations were detected in 24.4% (10/41) and missens mutations were detected in 75.6% (31/41) of the patients (Table 1). In 17 (41.5%) patients various point mutations were detected leading to lamivudin, adefovir and/or combine drug resistance. Wild polymerase gene profiles were detected in 24 (58.5%) HBV positive patients of current cohort. Eight of the 17 samples (19.5%) having rtM204V/I/A missense transition and/or transversion point mutations and showed resistance to lamivudin. Six of the the mutated samples (14.6%) having rtL180M missense transversion mutation and showed resistance to combined adefovir and lamivudin (Table 1). Three of the mutated samples (7.5%) having rtG215H by the double base substitution and showed resistance to adefovir (Table 1). Three of the

Table 1. The Prevalence of Some Clinical Characteristics, Mutation Type, Wild - Mutated Codons, Aminoacid and ADV and LDV Resistance Types of Current HBV Positive Patients

| Case No | Codon No | Triple Codon | | Aminoacid | | Point Mutation Type | Drug Resistance |
|---------|----------|--------------|---------|-----------|---------|--------------------------|------------------------------|
| | | Wild | Mutated | Wild | Mutated | | |
| 1 | 181 | TTG | TGG | L | W | Missense transversion | Lamivudine/Adefovir combined |
| 10 | 181 | TTG | TGG | L | W | Missense transversion | Lamivudine/Adefovir combined |
| 17 | 180 | CTG | ATG | L | M | Missense transversion | Lamivudine/Adefovir combined |
| | 181 | TTG | TGG | L | W | Missense transversion | Lamivudine/Adefovir combined |
| | 204 | ATG | GCG | M | A | Missense transition | Lamivudine |
| 20 | 80 | CTG | GTG | L | V | Missense transversion | Ttolerable |
| | 204 | ATG | ATA | M | I | Missense transition | Lamivudine |
| 22 | 215 | GGT | CAT | G | H | Double base substitution | Adefovir |
| 24 | 215 | GGT | CAT | G | H | Double base substitution | Adefovir |
| | 216 | CAT | CAC | H | H | Silent transition | Normal |
| | 217 | CTT | CGT | L | R | Double base substitution | Unknown |
| | 219 | TCC | GCC | S | A | Missense transversion | Adefovir |
| 25 | 231 | CTG | TTG | L | L | Silent transition | Normal |
| | 232 | TGT | GGT | C | C | Silent transversion | Normal |
| 27 | 215 | GGT | CAT | G | H | Double base substitution | Adefovir |
| 30 | 207 | GTG | ATG | V | M | Missense transition | Unknown |
| | 208 | GTA | CAA | V | Q | Double base substitution | Unknown |
| | 229 | TTG | TTT | L | F | Missense transversion | Unknown |
| 32 | 173 | GTG | GGG | V | G | Missense transversion | Ttolerable |
| | 176 | AGC | AGT | S | S | Silent transition | Normal |
| | 180 | CTG | ATG | L | M | Missense transversion | Lamivudine/Adefovir combined |
| | 184 | ACT | AGT | T | S | Missense transversion | Unknown |
| | 204 | ATG | ATA | M | I | Missense transition | Lamivudine |
| 33 | 216 | CAT | CAC | H | H | Silent transition | Normal |
| | 219 | TCC | GCC | S | A | Missense transversion | Unknown |
| | 220 | TTT | TGG | F | W | Double base substitution | Unknown |
| 36 | 180 | CTG | ATG | L | M | Missense transversion | Lamivudine/Adefovir combined |
| | 204 | ATG | GTG | M | V | Missense transition | Lamivudine |
| 37 | 172 | GGA | GGC | G | G | Silent transition | Normal |
| | 197 | CAC | CAT | H | H | Silent transition | Normal |
| | 204 | ATG | ATT | M | I | Missense transversion | Lamivudine |
| 38 | 180 | CTG | ATG | L | M | Missense transversion | Lamivudine/Adefovir combined |
| | 201 | TTC | TTT | F | F | Silent transition | Normal |
| | 204 | ATG | ATT | M | I | Missense transversion | Lamivudine |
| | 212 | AAG | AAA | K | K | Silent transition | Normal |
| 39 | 232 | GGT | GGC | G | G | Silent transition | Normal |
| 40 | 180 | CTG | ATG | L | M | Missense transversion | Lamivudine/Adefovir combined |
| | 204 | ATG | GTG | M | V | Missense transition | Lamivudine |
| | 229 | TTG | GTG | L | V | Missense transversion | Unknown |
| 41 | 180 | CTG | ATG | L | M | Missense transversion | Lamivudine/Adefovir combined |
| | 204 | ATG | GTG | M | V | Missense transition | Lamivudine |

mutated samples (7.5%) having codon rtL181W due to the missense transversion point mutations and resistance to combined adefovir and lamivudin (Table 1). The rest of twenty one mutated samples (50.9%) having various mutations types in different codons (Figure 1).

Point mutation types have showed; 19 (46.4%) missense transversion, 10 (24.4) silent, 6 (14.6) missense transition and 6(14.6) double base substitution in the presented cohort respectively. Silent mutations were detected in codons; rtG172G, rtS176S, rtH197H, rtF201F, rtL212K, rtH216H, rtL231L, rtC232C and rtG232G with no drug resistance (Table 1). Eight (19.5%) case were resistance to lamivudine, four (9.8%) adefovir, ten (24.4%) were showed no resistance (normal) due to silent mutations, two (4.9%) were tolerable, nine (21.9%) were combined adefovir and lamivudine resistance and eight (19.5%) were unknown respectively.

Different denovo point mutations were detected

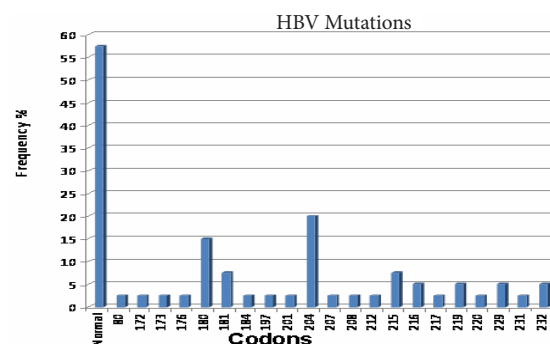


Figure 1. The Prevalence of Normal and Mutated Codons in the Studied Cohort

in nine codons such as; rtT184S, rtV201M, rtV208Q, rtL217R, rtS219A, rtF220W, rtL229F and rtL229W that have unknown drug resistance profiles were detected in the long time treated patients (Table 1).

Discussion

The Lamivudine (LVD) therapy has been commonly used in the treatment of chronic HBV infections as a first line antiviral agent. In the prolonged administration of LVD usually is necessary to obtain significant clinical benefits, but at the same time, the risk for developing drug-resistant mutations increases with duration of therapy (Villamil, 2002; Kim et al., 2012). Longterm LVD therapy can usually suppress HBV replication, however prolonged mono therapy leads to the lamivudine resistant HBV (Balzarini et al., 2013). The lamivudine resistant HBV viruses have a characteristic amino acid substitution over tyrosine-methionine-aspartate-aspartate (YMDD)-motif of the RNA-dependent DNA polymerase and mainly associated with substitution of methionine by either isoleucine (rtM204I) or valine (rtM204V) in YMDD motif of the HBV polymerase C domain (Balzarini et al., 2013). Novel types of mutations, including rtL180M, rtL80V/I, rtV173L and rtL80V/I, have been associated with resistance to LVD (Ogata et al., 1999; Bartholomeusz and Locarnini, 2006; Hadziyannis et al., 2006; Lee et al., 2006).

Adefovir (ADV) is a potent nucleotide analogue against both the wild-type and lamivudine-resistant HBV (Fung et al., 2006). However ADV-resistant strains have been reported after either switching or adding ADV in patients with LVD resistance, and several recent clinical studies have found that combined LVD with ADV is associated with improvements in virological response and lower rates of ADV resistance than sequential ADV monotherapy (Vassiliadis et al., 2005). Vassiliadis et al. (2005) have also strongly claimed that the combination of rtL80V/I and rtM204I is frequently observed in resistance to the LVD therapy. Resistance to ADV is prevalent due to rtA181V/T and rtN236T mutations and is associated with significant rebound viremia and at times hepatic decompensation (Gwak et al., 2011).

Kim et al. (2011) have investigated the influence of YMDD mutation patterns on clinical outcomes in patients with adefovir add-on lamivudine combination treatment in seventy-eight CHB patients with confirmed genotypic resistance to LVD. They found that biochemical response at 12 months from baseline was better in patients with a rtM204I mutation than rtM204V+rtM204I/V mutations. Gwak et al. (2011) have investigated the clinical impact of the development of YMDD mutants in hepatitis B virus-associated glomerulonephritis. They found aggravation of proteinuria and/or progressive renal deterioration in three patients after development of YMDD mutations and suggested that YMDD mutant-related viral breakthrough after prolonged LVD therapy might lead to the aggravation of renal disease in HBV-associated GN patients. Matsuda et al. (2004) have claimed that HBV DNA level may not have a positive correlation with YMDD mutations and LVD is clinically effective for CHB patients with YMDD mutations.

The current retrospective cohort analysis revealed and proved that the presence of several mutations, including the rtM204V and rtL180M substitutions, which augments the combined adefovir and lamivudine

resistant in conjunction with rtM204V/I/A in the untreated patients in Turkish population. Forty one mutations that located in 21 different codons were detected in the current cohort; 8 (19.5%) having rtM204V/I/A, 6 (14.6%) 3 (7.5%) having rtG215H by the double base substitution and 3 (7.5%) having codon rtL181W due to the missense transversion point mutations. Wild polymerase gene profiles were detected in 24 (58.5%) HBV positive patients of current cohort. The point mutation types have showed; 19 (46.4%) missense transversion, 10 (24.4%) silent, 6 (14.6%) missense transition and 6 (14.6%) double base substitution in the presented cohort respectively.

Consequently we assume that the silent mutations that identified in in codons; rtG172G, rtS176S, rtH197H, rtF201F, rtLK212K, rtH216H, rtL231L, rtC232C and rtG232G and unexpected point mutations such as; rtT184S, rtV201M, rtV208Q, rtL217R, rtS219A, rtF220W, rtL229F and rtL229W that reported in the current needs to be tested in further studies for combined drug resistance (Table 1). The nature and mechanisms of resistance for each therapy are important to develop treatment strategies, including for patients already harboring resistant viruses. Selection pressure caused by adefovir can lead to upraise of the mutations rtA181V and rtN236T (Angus et al., 2003) or rtI233V (Wang et al., 2012). The second two substitutions were not found in the current cohort. The current hypothesis is that interactions between residues at position 204 and those at 184, 202 or 250 are important for a functional HBV RT enzyme, or perhaps that the LVD_r, ADV_r and/or both substitutions further decrease the replication capacity of the more impaired M204I virus to below levels required to survive. Recently, lots of reports have demonstrated that YMDD motif mutations can naturally occur in chronic HBV patients without antiviral therapy (Ağca et al., 2012; Lee et al., 2012; Mello et al., 2012; Tan et al., 2012; Wu et al., 2012). Li et al. (2013) have claimed that HBV-related HCC patients with YMDD motif mutations have no response to lamivudine therapy. In Western China, the YMDD motif mutations were reported at a rate of 15.56% in chronic HBV infected patients (Zhao et al., 2012). All studied chronic HBV patients (100%) have showed various YMDD motif mutations in the current results. The current results were not included the clinical following-up of the presented cohort for hepatocellular carcinoma (HCC) but as claimed by Schildgen et al. (2010) the chronic HBV infection should be re-consider as a possible major cause of HCC risk in that infected patients. As claimed by some researchers the chronic infection with HBV is responsible for 60% of HCCs in Asia and Africa and at least 20% of the tumors in Europe, Japan, and the United States (Chen et al., 2013; Gao et al., 2013; Lavarone and Colombo, 2013). Chen et al. (2013) have also claimed that HBV subgenotypes and mutations in enhancer II, basal core promoter, and precore regions of HBV in relation to risks of liver cirrhosis (LC) and HCC in Southeast China. As claimed by Guerrieri et al. (2013) and Yang et al. (2013) the mutated YMDD motifs of HBV infection may contributes HCC by inducing both genomic instability and direct insertional mutagenesis of cancer related genes at the early steps of clonal tumor expansion and promote

its tumor development by eliciting epigenetic changes.

In conclusion, the current results show evidence that rtL180M and rtM204V/I/A mutations of HBV-DNA may be associated with a poor antiviral response and HBV chronicity in Turkish population. It is very important to aim to identify the mutation profile of HBV polymerase in such a cases for sufficient treatment, avoid or reduce chronicity of HBV and possibility of HCC development.

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