RESEARCH ARTICLE

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

Hakan Alagozlu[1], Oztürk Ozdemir[2,4]*, Binnur Koksal[3], Abdulkerim Yilmaz[1], Mahmut Coskun[4]

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 lamivudin, 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 (19.5%) having rtM204V/I/A missense transition and/or transversion point mutations and resistance to lamivudin. Six of the the mutated samples (14.6%) having rtL180M missense transversion mutation and resistance to combined adefovir and lamivudin. Three of the mutated samples (7.5%) having rtG215H by the double base substituation and resistance to adefovir. Three of the mutated samples (7.5%) having codon rtL181W due to the missense transversion point mutations and showed resistance to combined adefovir and lamivudin. Unreported novel point mutations were detected in the different codons of polymerase gene region in the current HBV positive cohort fromTurkish 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

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;

[1]Department of Gastroenterology, [3]Department of Medical Genetics, Faculty of Medicine, Cumhuriyet University, Sivas, [2]Department of Medical Genetics, [4]Department of Medical Biology, Faculty of Medicine, Canakkale Onsekiz Mart University, Canakkel, Turkey
*For correspondence: ozdemir615@yahoo.com

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 participitate 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 clarifed 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. Fourty-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 theraphy in Cumhuriyet University training and research hospital by the colleboration 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 sellected and evaluated in the current study. HBV-DNA was extracted from the pheripheric 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 Invitek 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 1min, 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 1min, 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 prevelance 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 Prevelance of Some Clinical Characteristics, Mutation Type, Wild - Mutated Codons, Aminiacid and ADV and LDV Resistance Types of Current HBV Positive Patients

Case No	Codon No	Triple Codon Wild	Triple Codon Mutated	Aminoacid Wild	Aminoacid Mutated	Point Mutation Type	Drug Resistance
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	Missence 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 transvertion	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 transvertion, 10 (24.4) silent, 6 (14.6) missense transition and 6(14.6) double base subsitation in the presented cohort respectively. Silent mutations were detected in codons; rtG172G, rtS176S, rtH197H, rtF201F, rtLK212K, 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
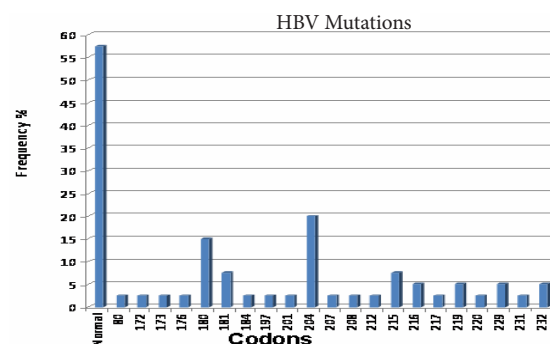
Figure 1. The Prevalence of Normal and Mutated Codons in the Studied Cohort

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 supress 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 proofed 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. Fourty 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 substation 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 transvertion, 10 (24.4%) silent, 6 (14.6%) missense transition and 6(14.6%) double base substituation 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 LVDr, ADVr 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 theraphy (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.

References

Ağca H, Sayıner AA, Sengönül A et al., (2011). A real-time PCR assay for the quantification of hepatitis B virus DNA and concurrent detection of YMDD motif mutations. *Mikrobiyol Bul*, **45**, 664-76.

Allen MI, Deslauriers M, Andrews CW, et al (1998). Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology*, **27**, 1670-7.

Angus P, Vaughan R, Xiong S, et al (2003). Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology*, **125**, 292-7.

Aspinall EJ, Hawkins G, Fraser A, et al (2011). Hepatitis B prevention, diagnosis, treatment and care: a review. *Occup Med (Lond)*, **61**, 531-40.

Balzarini J, Wedgwood O, Kruining J, et al (1996). Anti-HIV and anti-HBV activity and resistance profile of 3TC and its arylphosphoramidate derivative CF 1109. *Biochem Biophys Res Commun*, **2**, 363-9.

Bartholomeusz A, Locarnini SA (2006). Antiviral drug resistance: clinical consequences consequences and molecular aspects. *Semin Liver Dis*, **26**, 162-70.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ (1996). Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J Clin Invest*, **98**, 2268-76.

Carman WF, Trautwein C, van Deursen FJ, et al (1996). Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis. *Hepatology*, **24**, 489.

Chen Y, Wang L, Xu H, et al (2013). Exome capture sequencing reveals new insights into hepatitis B virus-induced hepatocellular carcinoma at the early stage of tumorigenesis. *Oncol Rep*, **30**, 1906-12.

Chen YM, Wu SH, Qiu CN, et al (2013). Hepatitis B virus subgenotype C2- and B2-associated mutation patterns may be responsible for liver cirrhosis and hepatocellular carcinoma, respectively. *Braz J Med Biol Res*, **46**, 614-22.

Fontana RJ, Keeffe EB, Carey W, et al (2002). Effect of lamivudine treatment on survival of 309 North American patients awaiting liver transplantation for chronic hepatitis B. *Liver Transpl*, **8**, 433-9.

Fung SK, Chae HB, Fontana RJ, et al (2006). Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J Hepatol*, **44**, 283-90

Gaillard RK, Barnard J, Lopez V, et al (2002). Kinetic analysis of wild-type and YMDD mutant hepatitis B virus polymerases and effects of deoxyribonucleotide concentrations on polymerase activity. *Antimicrob Agents Chemother*, **46**, 1005-13.

Ganem D, Prince AM (2004). Hepatitis B virus infection–natural history and clinical consequences. *N Engl J Med*, **350**, 1118-29.

Gao C, Fang L, Zhao HC, et al (2013). Potential role of diabetes mellitus in the progression of cirrhosis to hepatocellular carcinoma: a cross-sectional case-control study from Chinese patients with HBV infection. *Hepatobiliary Pancreat Dis Int*, **12**, 385-93.

Geramizadeh B, Nikeghbalian S, Kazemi K, et al (2013). Hepatocellular carcinoma in explanted livers of patients with genotype d HBV cirrhosis: report of the first experience from Iran. *Arch Iran Med*, **16**, 348-50.

Guerrieri F, Belloni L, Pediconi N, Levrero M (2013). Molecular mechanisms of HBV-associated hepatocarcinogenesis. *Semin Liver Dis*, **33**, 147-56.

Gwak GY, Lee CY, Lee DH, et al (2011). Clinical impact of the development of YMDD mutants in hepatitis B virus-associated glomerulonephritis. *Hepatogastroenterology*, **58**, 1291-5.

Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, et al (2006). Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis b for up to 5 years. *Gastroenterology*, **131**, 1743-51.

Johnson MS, McClure MA, Feng DF, et al (1986). Computer analysis of retroviral pol genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. *Proc Natl Acad Sci USA*, **83**, 7648-52.

Kim HJ, Park JH, Park DI, et al (2012). The influence of YMDD mutation patterns on clinical outcomes in patients with adefovir add-on lamivudine combination treatment. *Liver Int*, **32**, 303-10.

Kohlstaedt LA, Wang J, Friedman JM, et al (1992). Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science*, **256**, 1783-90.

Lai CL, Chien RN, Leung NW, et al (1998). A one year trial of lamivudine for chronic hepatitis B. Asia hepatitis lamivudine study group. *N Engl J Med*, **339**, 61-8.

Lavarone M, Colombo M (2013). HBV infection and hepatocellular carcinoma. *Clin Liver Dis*, **17**, 375-97.

Lee CZ, Lee HS, Huang GT, et al (2006). Detection of YMDD mutation using mutant-specific primers in chronic hepatitis B patients before and after lamivudine treatment. *World J Gastroenterol*, **12**, 5301-5.

Lee SH, Kim HS, Byun IS, et al (2012). Pre-existing YMDD mutants in treatment-naïve patients with chronic hepatitis B are not selected during lamivudine therapy. *J Med Virol*, **84**, 217-22.

Lee YS, Suh DJ, Lim YS, et al (2006). Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. *Hepatology*, **43**, 1385-91.

Li D, Cheng H, Gong W, et al (2013). Detection of primary YMDD mutations in HBV-related hepatocellular carcinoma using hybridization-fluorescence polarization. *J Virol Methods*, **187**, 259-63.

Lin CL, Kao JH (2008). Hepatitis B viral factors and clinical outcomes of chronic hepatitis B. *J Biomed Sci*, **15**, 137-45.

Liu K, Hou W, Zumbika E, Ni Q (2005). Clinical features of chronic hepatitis B patients with YMDD mutation after lamivudine therapy. *J Zhejiang Univ Science B*, **6**, 1182-8.

Markowitz JS, Martin P, Conrad AJ, et al (1998). Prophylaxis against hepatitis B recurrence following liver transplantation using combination lamivudine and hepatitis B immune globulin. *Hepatolog*, **28**, 585-9.

Matsuda M, Suzuki F, Suzuki Y, et al (2004). YMDD mutants in patients with chronic hepatitis B before treatment are not selected by lamivudine. *J Med Virol*, **74**, 361-6.

Mello FC, Lago BV, Lewis-Ximenez LL, et al (2012). Detection of mixed populations of wild-type and YMDD hepatitis B variants by pyrosequencing in acutely and chronically infected patients. *BMC Microbiol*, **12**, 96.

Ogata N, Fujii K, Takigawa S, et al (1999). Novel patterns of

amino acid mutations in the hepatitis B virus polymerase in association with resistance to lamivudine therapy in Japanese patients with chronic hepatitis B. *J Med Virol*, **59**, 270-6.

Poch O, Sauvaget I, Delarue M, Tordo N (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J*, **8**, 3867-74.

Sarma MP, Asim M, Medhi S, et al (2012). Viral genotypes and associated risk factors of hepatocellular carcinoma in India. *Cancer Biol Med*, **9**, 172-81.

Sato S, Suzuki K, Akahane Y, et al (1995). Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med*, **122**, 241-8.

Schildgen O, Sirma H, Funk A, et al (2006). Variant of hepatitis B virus with primary resistance to adefovir. *N Engl J Med*, **354**, 1807-12.

Schildgen V, Ziegler S, Tillmann RL, Schildgen O (2010). Novel mutation in YMDD motif and direct neighbourhood in a child with chronic HBV-infection and clinical lamivudine and adefovir resistance - a scholarly case. *Virol J*, **7**, 167.

Singh S, Singh PP, Roberts LR, Sanchez W (2013). Chemopreventive strategies in hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol*, [Epub ahead of print].

Tan YW, Ge GH, Zhao W, et al (2012). YMDD motif mutations in chronic hepatitis B antiviral treatment naïve patients: a multi-center study. *Braz J Infect Dis*, **16**, 250-5.

Vassiliadis T, Nikolaidis N, Giouleme O, et al (2005). Adefovir dipivoxil added to ongoing lamivudine therapy in patients with lamivudine-resistant hepatitis B e antigen-negative chronic hepatitis B. *Aliment Pharmacol Ther*, **21**, 531-7.

Villamil FG (2002). Hepatitis B progress in the last 15 years. *Liver Transpl*, **8**, 59-66.

Wang LL, Yu XJ, Dong QJ, et al (2012). HBV genotypes distribution and YMDD spontaneous mutation in Qingdao population. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, **26**, 250-2.

Wu F, Wu MJ, Zhuge XL et al (2012). Correlation of the occurrence of YMDD mutations with HBV genotypes, HBV-DNA levels, and HBeAg status in Chinese patients with chronic hepatitis B during lamivudine treatment. *Hepatobiliary Pancreat Dis Int*, **11**, 172-6.

Yang JH, Zhang H, Chen XB, et al (2013). Relationship between hepatocellular carcinoma and hepatitis B virus genotype with spontaneous YMDD mutations. *World J Gastroenterol*, **19**, 3861-5.

Zhao J, Guo Y, Yan Z, et al (2012). The natural YMDD mutations of hepatitis B virus in Western China. *Scand J Infect Dis*, **44**, 44-7.