Hepatitis B virus reverse transcriptase polymorphisms between treated and treatment-naïve chronically infected patients

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Abstract The aim of this study was investigation of variation(s) in the Hepatitis B virus (HBV) reverse transcriptase domain. 120 patients with chronic HBV infection recruited. 104 patients were received nucleos(t)ide analogs treatments. DNA extractions were done from plasma samples. Direct sequencing and alignment of Polymerase Chain Reaction products were applied for further analysis. HBV genotypes determined by NCBI's Genotyping Tool. Polymorphism(s) were detected by using DnaSP software. Of 120 samples, 98 were sequenced. All of products were HBV genotype D. 13/98 (13.27%) of patients had M539I/V substitutions corresponding to YMDD motif. FLFAQ to FLMAQ was observed among 22/98 (22.98) patients. Two substitutions N459Y and L515M were significantly correlated ($R^2 = 0.486$ and $R^2 = 0.941$ respectively) with FLFAQ motif variation. Mutation ratio among treatment-received patients to treatment-naïve patients was 0.2–0.6. Drug resistance conferring substitutions (DRCSs) were rtL180M (22/98), rtA194V (11/98), rtM204V (1/98), and rtM204I (11/98). Furthermore, six variants were observed among all patients. Appearance of DRCSs in HBV polymerase is a major obstacle to the virus treatments. In the present study, it was shown that DRCSs are more prevalent among treated patients. Therefore, replacement of current

anti-viral regimen with novel anti-HBV drugs is warranted in the future.

Keywords Chronic hepatitis B virus · Hepatitis B virus polymerase · HBV reverse transcriptase · Nucleotide analogues resistance mutation · Drug resistance conferring substitution

Introduction

The human hepatitis B virus (HBV) is an enveloped DNA virus that causes chronic hepatitis (CHB) and consequently cirrhosis and hepatocellular carcinoma (HCC) [2, 17, 31]. There are over 257 million chronically infected people worldwide and 600,000 death each year from HBV-related liver failure [18]. In order to reduce the risk of HCC and mortality, some nucleos(t)ide analogues (NAs) are recommended, including lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine, and tenofovir that modulate viral replication and inhibit the progression of liver disease [12].

Despite the inefficiency and toxicity, long-term usage of current anti-HBV drugs can cause drug resistance in patients [30]. The common target of NAs is the RT domain of polymerase gene containing the YMDD (Tyr- Met- Asp- Asp) motif and mutations in this domain would lead to drug resistance [1, 21]. The emergence of NAs resistance in HBV strains is associated with primary and secondary amino acid alterations in HBV reverse transcriptase. Primary mutation are resulted from amino acid substitutions that lead to decreased antiviral drugs susceptibility, while secondary mutations restore viral replication [30].

In a study by Gomes-Gouvea et al. [3] it has been shown that 39% of 702 treatment-naïve chronically infected

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patients had HBV mutation in at least one region of RT domain. They found a high genetic variability in their samples including 7 genotypes and 12 sub-genotypes [3]. In that study, the HBV NA resistance mutations were observed in 1.6% of patients and the compensatory mutations were found in only 0.4% of patients [3].

Mirandola et al. [15] have been studied 255 NA-naïve treatment patients with cHBV. Drug resistance mutations were observed in 13 (5%) patients including 3 cases in YMDD motif conferring LAM resistance, 3 cases with rtM250L/V mutation conferring ETV resistance, 4 isolates with rtI233V mutations that probably reduces sensitivity to ADV, and three patients with the rtL80V and rtV173L compensatory mutations [15].

In the continue of our previous works on HBV polymorphisms [19, 20], we analyzed substitutions of the viral polymerase in two groups of treated and treatment-naïve chronically infected patients. It was found that mutation rate in treatment-naïve patients was only little smaller than that in treated patients. However, the sample size of treatment-naïve CHB patients was not comparable to that of treatment-received patients.

Materials and methods

Sample collection and DNA extraction

In this cohort study, 120 patients with chronic HBV (cHBV) infection were included who have referred to specialist for HBV infection diagnosis. None of the patients had history of anti-HBV vaccination or immunoglobulin therapy; all patients were negative for antibodies against hepatitis C virus (HCV), hepatitis D virus (HDV) and human immunodeficiency virus (HIV). All patients gave their informed consent and participated voluntarily. Demographic data and blood samples were collected in anti-coagulant collection tube. HBV DNA was extracted from 200 µl plasma samples by using QIAamp DNA Mini Kit (QIAGEN, Hamburg, Germany) according to the manufacturer's protocol. Extracted DNA was stored at -20°C for polymerase chain reaction (PCR) assay.

DNA amplification

A forward primer (5'-GATGTGTCTGCGCGTTTTA-3') encompassing nucleotides 376–395, and a reverse primer (5'-CAGCAAAGCCCAAAAGACCCAC-3') encompassing nucleotides 1000–1021 were used for amplification of HBV reverse-transcriptase domain (rtPol) [9]. Mutations in the HBV DNA polymerase gene were determined by using polymerase chain reaction (PCR) and direct sequencing. Briefly, 5 µl of extracted DNA was added to an

amplification mixture containing 1 × PCR buffer, 2.5 mM MgCl_2 , 0.2 mM/l dNTP mixture (dATP, dGTP, dCTP, dTTP), 2.5U of Taq DNA polymerase (QIAGEN, Hamburg, Germany) and 1 pmol of each primers in a total volume of 50 µl. The amplification was performed in following thermal conditions: an initial incubation at 94°C for 3 min and 30 cycles comprised of denaturation for 30 s at 94°C , annealing for 30 s at 55.9°C and extension for 50 s at 72°C , with a final extension at 72°C for 2 min [31].

Sequencing and genotyping

The positive PCR products were subjected to automated sequencing (Macrogen Company). Desirable sequencing results were considered to have QV score higher than 20, which evaluated through DNA Baser v4.36.0. The genotypes of sequences were determined by alignment of data with all known HBV genotypes using NCBI Viral Genotyping Tool [24].

Multiple sequence alignment and genetic study

The sequences with known genotypes and enough quality were aligned to each other and a genotype-related reference sequence through the multiple sequence alignment (MSA) by using MAFFT server v7 [7]. The server options were kept as its default, but direction of nucleotide sequences has changed to “Adjust direction according to the first sequence” and output order was changed to “Same as input”. This allowed for comparison of nucleotide changes with the reference genotype sequence.

Phylip file format from MSA was used for genetic analysis through DNA Sequence Polymorphism (DnaSP) v5 [11]. Number of polymorphic sites, synonymous, non-synonymous, and insertions or deletions (InDels) was evaluated with DnaSP at nucleotide level. Translated sequences were used for substitution analysis at amino acid level with MEGA 6 [25].

Evaluation of drug-resistance conferring substitutions and HBV variants

Amino acid substitutions were analyzed for their potential to confer any anti-HBV drug resistance or functional impact on the structural configuration by Mutation Assessor server as described before [16].

Statistical analysis

The incidence rate of drug-resistance conferring mutations (substitutions) was evaluated by MS-Excel 2016. Further statistical analyses have also performed by R packages.

Mutation rate (MR) was calculated among different treated and treatment-naïve populations with following formula:

$$MR = \frac{\frac{T_i}{N-T_i}}{\frac{T_n}{N-T_n}}$$

where T_i is the number of drug-resistance conferring substitutions among patients under treatment with LAM or ADV within total population (N), and T_n is the same number of substitutions among treatment-naïve patients.

Results

Patient characteristics

120 cHBV infected patients with mean age of 36.417 ± 11.744 ranged from 17 to 72 were incorporated to the study. All patients were native residents of Golestan Province of Iran. 65% (78/120) were male and 35% (42/1420) were female.

After DNA extraction and PCR assay, sequencing was performed and analyzed. Only 98 sequences validated to have enough qualities for analysis. The sequences were submitted to GenBank database with accession numbers of MG652651-MG652748.

Of 98 samples provided for further study, 60 (61.22%) were male with age of 38.18 ± 12.01 and 38 (38.78%) were female with age of 35.68 ± 12.44 . Sixty-six (39 males and 27 females) patients have a history of ADV usage. In addition, 47 patients (34 males and 13 females) had a history of LAM administrations. 31 patients (31.63%; 21 males and 10 females) had a history both ADV and LAM treatments. Furthermore, 16 patients were treatment-naïve.

Mutation at nucleotide level

At nucleotide (nt) level, synonymous, non-synonymous, silent, and InDels were evaluated. Of synonymous point mutations, six single nucleotide polymorphisms (SNPs) have observed at positions T676C (1.02%), A681T (1.02%), T696C (1.02%), A702G (1.02%), A710G (1.02%), and A747T (1.02%). Also, two variants of C690T and A747C observed among all patients. Further results are provided in the Supplemented Materials. Non-synonymous mutations were also observed to be more prevalent than synonymous ones (Fig. 1). Accordingly, four variants of A677T, G728A, T752C, and A762G were occurred within all patients. Further polymorphisms were C662T (2.04%), T667A (3.06%), C671T (3.06%), T674G (1.02%), C680G (1.02%), C713T (1.02%), A721G (1.02%), T761C (4.08%), T766G (2.04%), A770T (1.02%), T771A

(12.24%), A775T (1.02%), G785A (1.02%), and A788T (1.02%). No InDels were observed in any patients. Further point mutations among CHB patients are provided in the Supplemented Materials.

Functional analysis of HBV polymerase substitutions

Functional impact of rtPol substitutions were analyzed using Mutation Assessor Server [23] (Fig. 2). Accordingly, 17 substitutions had low functional impact score (FIS) of 1.05 ± 0.852 . Two substitutions have medium functional impact (1.975). Additionally, 54 substitutions were at neutral FI state (-0.014 ± 0.55). As shown in Table 1, 51.16% (41/73) of substitutions were at Reverse Transcriptase (rt) position (amino acids 365–589). 39.73% (29/73) of substitutions were at the viral DNA polymerase C-terminal domain (amino acids 590–832) (Table 1).

Variations of YMDD and FLLAQ motifs

Of 98 samples, 47 patients were LAM treated. In addition, 66 patients were treated with ADV. Also, 31 patients were given both LAM and ADV. Substitution of YMDD motif have observed among 13.27% (13/98) of patients. Of which, 92.31% (12/13) of substitutions were at M539 residue. Variations at M539 were comprised of M539V (1/12) and M253I (11/12). Only one case had D541E substitution. Among female patients, only 10.53% (4/38) had YMDD substitutions. Among males, 15% (9/60) had YMDD variations. Pearson correlation has shown significant relations between YMDD variations and substitutions M539I ($R^2 = 0.909$; $p < 0.0001$), D541E ($R^2 = 0.260$; $p = 0.005$), and LAM treatment ($R^2 = 0.287$; $p = 0.002$). No YMDD correlations were observed with ADV or gender.

Further motif variation was only observed at FLLAQ. Among 98 patients, 22.45% (22/98) had FLLAQ to FLMAQ substitution. The variant has observed among 10.53% (4/38) of females and 23.3% (14/60) of males. FLLAQ to FLMAQ had highly positive correlation with N459Y ($R^2 = 0.486$; $p < 0.0001$) and L515M ($R^2 = 0.941$; $p < 0.0001$). Further substitutions and their partial correlations with either LAM or ADV treatments are shown in the Fig. 3. Further correlations of HBV polymerase point mutations are provided in the Supplemented Materials.

Mutation rates among treated and treatment-naïve patients

The rate of nucleotide mutation among patients under LAM, ADV, and LAM + ADV treatments in comparison with treatment-naïve patients were calculated as mentioned

Fig. 1 Bulk substitution among chronically HBV infected males and females treated either with LAM or ADV

Fig. 2 FI score of HBV polymerase substitution. Substitutions with low FI were Y592F (0.895), Y592H (0.895), E598N (1.15), H599R (1.67), Q602H (0.805), Q602P (1.15), K603R (1.845), R609I (1.525), L611H (1.67), P616T (1.845), C622G (1.845), I625M (1.67), V626G (1.525), L628F (1.67), G630A (1.67), F631L (1.845), and A632C (1.445). L629G/W had medium functional impact with FI score of 1.975. Further neutral substitutions with FI score of 0 were G442L,

S444P, L457F, N459H/Y, M464L, P465Q/H, D466K/N, Y470S/L, L481S, H491Q, F501L, R502Q, L515M, A521V, I522L, V526I, A529V, M539V, D541E, S548T/N, V549A/E, Q550H/S/P, L552H/R, S554A, F556Y, V559A, I568V, N573H/S, K574R, K576Q, Y580H, N583H, I589G, E598D, and I601R. Further neutral substitutions were C591S (− 1.1), I601K/M (0.69), I601V (− 0.625), Q602L (0.255), E606D/K (0.345), and I613V (− 1.355).

above. Accordingly, 9 mutations were observed among patients treated with only LAM. In comparison with 16 non-treated (non-LAM/ADV), the mutation rate was obtained as high as 0.6. In addition, mutation rate of ADV: treatment-naïve patients was 0.25. For LAM + ADV the

mutation rate was 0.405. Figure 4 shown diagram of point mutations among four population groups.

Table 1 The similarity search of the detected substitutions among a database containing dHBV variants at reverse transcriptase and DNA polymerase C-terminal domains of HBV polymerase

Count of Substitution	HBV Polymerase Domains
41	Reverse transcriptase (RT) amino acids 365: 589
30	RT 336:679
3	Variant 457:459 LNN → FNY (in strain: Latvia)
5	Variant 465:466 PD → QN (in strain: Latvia)
2	Variant 470:470 Y → S (in strain: Latvia)
1	Variant 583: 583 N → H (in strain: Latvia)
29	DNA polymerase C-terminal domain amino acids 590: 832
25	(By similarity ^a) 590:832
3	Variant 598:598 E → D (in strain: Latvia)
1	Variant 613:613 I → V (in strain: Latvia)
70	Grand total

^aIndicates that the substitution is found in a database containing HBV variants by similarity search **Fig. 3** Partial correlation of LAM or ADV treatments with investigated HBV polymerase substitutions. The circles are marked with R-square values. Most notable correlated substitutions with LAM were M539I ($R^2 = 0.306$; $p = 0.001$), F556Y ($R^2 = 0.185$; $p = 0.034$), Y592H ($R^2 = -0.245$; $p = 0.007$), and I601V ($R^2 = -0.195$; $p = 0.027$). Further substitutions correlated with ADV were also comprised of Q602L ($R^2 = 0.161$; $p = 0.056$) and L629G ($R^2 = 0.193$; $p = 0.028$)

Fig. 4 Diagram of mutations within treated and treatment-naïve population groups. Synonymous mutations are shown in Green and non-synonymous in Red. Synonymous A747C mutations were existed in all groups. Five other non-synonymous mutations, including A677T, G728A, T752C, A762G, and T771A were also identical in the population groups (color figure online)

Drug resistance-conferring substitutions at reverse transcriptase domain

The prevalence of known drug resistance-conferring substitutions (DRCSs) was 40.8% (40/98). These substitutions were rtL180M (22/98), rtA194V (11/98), rtM204V (1/98), and rtM204I (11/98). Of 60 male patients 28 (46.7%) had DRCSs. Of those, 57.14% (16/28) and 67.85% (19/28) were under LAM and ADV treatments, respectively. Among female patients, 12 (31.58%) had DRCSs. Of 13 females under LAM treatments, 7 (53.85%) had DRCSs. Moreover, incidence of DRCSs among female patients under ADV treatments (27/98) was 37.04% (10/27).

Distribution of drug efficiency-reducing substitutions (DERSSs) was 21.4% (21/98). The substitutions were comprised of rtV191I (11/98), rtV214A (1/98), rtV214E (1/98), rtQ215H (3/98), Q215S (2/98), rtQ215P (3/98), and rtI233V (1/98). Prevalence of these substitutions among males and females were 20% (12/60) and 23.68% (9/38). There was no significant difference between two groups.

Discussion

Emerging HBV resistance strains to the NAs in HBV reverse transcriptase is resulted from amino acid substitutions. These substitutions occur in A through E domains of the viral polymerase gene [8]. Drug resistance occurs in two forms, including primary and secondary drug resistance mutations [29].

Primary resistance mutations take place in 8 codons, including rtI169T, rtA181T/V, rtT184S/C/G/A, rtA194T,

rtS202G/C/I, rtM204V/I, rtN236T and rt250V; which codons 169, 180 and 184 are in B-domain, codons 202 and 204 are in C-domain, codon 236 is in D-domain and codon 250 is in E-domain. Substitutions of M204V and M204I conferring LAM resistance reduce RT activity [8]. Lamivudine acts as a competitive inhibitor of deoxycytidine triphosphate and prohibits from RT activation [8]. N236T mutation in D-domain reduces almost five to tenfolds sensitivity to ADV in vitro [8]. Adefovir restrains the joining of deoxyadenosine triphosphate to viral primer, therefore prevents the priming process [8]. In addition, secondary resistance substitutions take place in three codons rtL80I, rtV173L, and rt180M and restore the replicative capability of RT [8]. Since these escape mutations lead to resistance to common anti-HBV drugs, study of these mutations is warranted within different group of patients [1, 5, 22, 27, 28, 30].

In this cohort study, we investigate 120 patients with chronic HBV, of which 98 samples were sent for sequencing. Based on history of drug usage, the patients were categorized into four groups. Sixty-six (39 males and 27 females) patients with a history of ADV usage, in addition, 47 patients (34 males and 13 females) with a history of LAM administrations, 31 patients (31.63%; 21 males and 10 females) with both ADV and LAM treatments, and 16 drug naïve-treatment patients. Two variants of synonymous C690T and A747C point mutations were observed in all samples. Furthermore, there were four variants of non-synonymous mutations in all patients, including A677T, G728A, T752C and A762G. Non-synonymous mutations were more prevalent than synonymous point mutations.

Mostly, mutations that lead to NA drugs resistant in cHBV genome occurred in DNA polymerase domain of viral genome. In this study, out of 73 substitutions, 41 cases were observed in reverse transcriptase (rt) domain and 29 cases were in the viral DNA polymerase C-terminal domain.

Mutations that are related to tyrosine-methionine-aspartic acid- aspartic acid (YMDD) catalytic site motif, are the most significant cause of NA treatment failure [1]. In patients treated with LAM, the mutation in YMDD motif came up after 9–10 months [4]. The emergence of YMDD motif variations 3 months after initiation of LMV therapy is probably applicable for prediction of long-term outcome of 3TC therapy in HBe-Ag positive patients [4]. In consistence to [6], there was no significant association between age or gender and amino acid substitutions. In our study, out of 98 samples, there were 47 LAM-treated patients, 66 ADV-treated patients and 31 patients were treated with both drugs. 13.27% of patients had substitutions within the YMDD motif, of which 92.31% of them comprised of variations in M539 residue. Pearson correlation has shown

significant relations between YMDD variations (M539I, D541E) and LAM treatment. No YMDD correlations were observed with ADV or gender.

The results of this study showed that the mutation rate in treatment-naïve patients was nearly as high as (LAM/ADV) treated patients. There were 9/47 mutations among LAM-treated patients, 9/66 mutations in ADV-treated patients, and 9 mutations within LAM + ADV-treated patients. On the other hand, there were 7/16 mutations in treatments-naïve patients. According to the presented results, the prevalence of mutations was about 40.8%, of which 22.98% of them were related to rtL180m, 11.98% to rtA194V, 1.98% to rtM204V, and 1.98% to rtM204T. According to the results of this study, and with the knowledge that 204V/I substitution leads to primary resistance and mutation of 180M leads to secondary resistance, we found that among observed substitutions, 17.82% were related to primary and 22.98% to secondary mutations. The mutation at rt204 residue is known to related with resistance to LAM [14], the substitutions at this position was found in LAM treated patients. According to our study, it was shown that [13], pre-existence of drug-resistance substitutions was prevalent in treatment-naïve patients. We observed 43.75% of treatment-naïve cases had variations in the HBVpol. In a similar study, the authors have found 22% NA resistance mutations in treatment-naïve patients [26]. In addition, another study reported mutation rate of 5.2% in treatment-naïve CHB patients [15]. Furthermore, LAM resistance substitutions in HBeAg positive and negative patients were 39.5% and 25.9%, respectively [10]. Therefore, further studies is warranted on treatment-naïve CHB patients.

Our results indicates six variants of synonymous, C690T and A747C point mutations and four non-synonymous mutations, A677T, G728A, T752C and A762G in all patients in North of Iran. Almost half of the treatment-naïve patients had mutations in the polymerase gene and it was close to mutations in treated patients. However, the treatment-naïve group was small. With respect to the type of study, evaluation of HBeAg level was not possible. Therefore, we could not answer whether variations of HBV rt are associated with productive viral replication. Therefore, evaluation of HBeAg as well as viral load are essential factors for assessing drug resistance mutations.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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