Diagnosis Escape Variants of Hepatitis B Virus are Predominant in Inactive Carriers and Display Varied Therapeutic Response in Treatment Eligible Patients

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Abstract

Background: Diagnostic escape variants (DEVs) of hepatitis B virus (HBV) are a genomic subpopulation of HBV that escape detection by the commercial diagnostic real-time PCR assay. They have altered genomic sequence in the precore/core region of HBV genome irrespective of known mutations in the same region. We devised an indigenous nested PCR assay which can detect such variants along with the wild-type form of the virus. Present work aimed to study the distribution of DEVs in different HBV related disease categories and their treatment response.

Methods: HBsAg positive patients (N = 1723) who attended the Asian Institute of Gastroenterology (AIG), Hyderabad, India, during the period of 2009 - 2013 were screened. The study was approved by the Institutional Review Board of AIG. After taking informed consent from the patients, sera was collected and viral load was estimated. In-house PCR assay was performed to detect wild type and DEVs of HBV simultaneously.

Results: The assay detected DEVs in 25% of the study subjects along with the wild type virus. In contrast to other disease categories, DEVs appeared as majority genomic population in 65% of inactive carriers. In treated subjects, virological relapse was significantly high (92%) in patients harboring DEVs at baseline compared to those having only wild type (14%).

Conclusion: Predominance of DEVs in inactive carriers indicate this group as a reservoir of such variants and some of them might appear eligible for treatment if DEVs are included in the viral load estimation. Patients having DEVs at baseline are more prone to relapse after discontinuation of anti-viral therapy.

Keywords: Diagnosis Escape Variants; Hepatitis B Virus; Inactive Carriers

Introduction

Hepatitis B virus (HBV) infection is a serious global health problem [1]. It is held that about 50 million people contact this disease every year, resulting in 1 - 2 million deaths annually [2]. In India, over 40 million are HBV carriers and more than 1,00,000 Indians die annually due to HBV infection [3]. Infection with hepatitis B virus (HBV) leads to a wide spectrum of clinical manifestations ranging from acute to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma. The severity of liver damage and the outcome of disease are influenced not only by the intensity and competence of the host’s antiviral immune response, but also by the genetic variation of the virus [4]. Improved management of chronic hepatitis B (CHB) patients has been achieved by the inventions of sensitive diagnostic assays and accessibility of powerful antiviral agents [5]. Guidelines for management and treatment of CHB infection have been established by several international guidelines to assist physicians in recognition, diagnosis, and optimum management of patients with CHB [6-8]. These guidelines recommend favored approaches towards the initiation and end point therapy. Treatment end-points are complete viral suppression (undetectable levels of HBV DNA replication), HBeAg clearance and seroconversion in hepatitis B e antigen (HBeAg)-positive patients, and if possible, HBSAg clearance and development of anti-HBs antibody. Patients achieving these serological end-points may discontinue treatment, after an additional 6- to 12-month period of consolidation therapy, on which the present work was designed [7,9]. Recent guidelines have recommended to treat HBV patients for lifetime to prevent hepatitis flare-ups and inhibit hepatocarcinogenesis to

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806

a great extent, as presently available treatment options cannot completely eradicate HBV [10]. The emergence of drug resistant variants has been one of the handicaps for successful HBV treatment of patients, immunosuppressed patients or infected patients after vaccination [11]. Surface antigen mutants allow for escape from humoral immune responses and reduce the effectiveness of diagnostic tests and vaccination [12]. Most of the corresponding mutations accumulate during the course of infection and persist until the late phase and these mutants are clinically important [13]. It is learned that the presence or emergence of specific mutant is associated with particular stages of chronic infections. In general, the enhancer II/core promoter and precore stop codon mutants appear to be associated with disease severity and progression [14]. HBV core deletion mutants detected by nested PCR assay has been found to be associated with advanced cirrhosis of liver [15]. The real time polymerase chain reaction (PCR) assay found its role in monitoring viral loads during anti-viral therapy, in treatment of HBV infection and to assess severity of liver disease [16]. While a mutant has genetic sequence alteration often associated with a phenotypic change, a variant, on the other hand, though possess such alteration may not imply phenotypic changes but can persist with the wild type virus in a quasispecies setting. HBV having a quasispecies nature can exist as a heterogenous population in certain patients, where commercial real time PCR is able to detect only the wild type virus but not the variants. In recent past, we identified a group of genomic variant of HBV and coined the term, Diagnostic escape variants (DEVs) since, it evade detection by the commercial diagnostic real-time PCR assay [17]. DEVs are a group of viral population having altered genomic sequence(s) in the precore/core region of the virus irrespective of known mutations in such region. Using strategically designed inner primer mismatched to wild type sequence, we devised an indigenous nested PCR assay that is able to detect such variants in HBV infected subjects along with the wild type virus [17].

Aim of the Study

The aim of the present work was to (1) study the distribution of DEVs in different disease categories of HBV; (2) study the response of treatment naive patients harboring DEVs who were treated with nucleos(t)ide analogs in a one year follow up time. It is believed that such a study would be helpful not only in detection of variant HBV population along with the wild type but also their role in disease progression and therapeutic response for improved patient management.

Materials and Methods

Study Subjects

A total of 1723 HBsAg positive patients who were treatment naive, without any other coinfections and attended the Asian Institute of Gastroenterology (AIG), Hyderabad, India during the period of 2009 to 2013 were subjected to HBV DNA analysis by commercial diagnostic real-time PCR and In-house PCR assays to estimate the viral load and to detect the DEVs respectively. Patients were categorized into different disease categories like, acute, immune tolerant (IT), inactive carriers (IC), and chronic active (CHB) based on the Asian Pacific Association for the Study of the Liver (APASL) guidelines [8]. Blood samples (5 mL) were collected from each patient for biochemical analysis and determination of viral loads in sera of patients by standard biochemical and diagnostic real time PCR (Roche COBAS TaqMan) assay respectively. The study was approved by the Institutional Review Board of AIG and informed consent was obtained from all the study subjects.

Furthermore, a total of 100 treatment naïve patients who were eligible for the treatment were studied to assess the response of DEVs to the currently used Nucleos(t)ide analogues (NA’s). Out of these patients, 80 were treated with Entecavir (0.5 mg/day) and 20 were treated with Telbivudine (600 mg/day) for a duration of 48 weeks. The choice of treatment was based on clinician’s discretion, affordability of the medicine and patient’s compliance. The patients were followed-up from baseline to 48 weeks of treatment (end of therapy) and for a further period of one year after stopping the anti-viral therapy.

Biochemical and Serological Tests

Standard automated analyzers were used to perform biochemical tests. Third-generation enzyme-linked immunosorbent assay (ELISA) was used to measure HBsAg, HBeAg, and Anti-HBe status.

Quantification of Serum HBV DNA

Viral DNA was isolated from 500 µL serum of study subjects using High Pure Viral Nucleic Acid Kit (Roche Molecular Systems, Inc., NJ, USA) as per manufacturer’s instructions. Serum HBV DNA levels were estimated by COBAS TaqMan HBV Test, v2.0 kit (Roche Molecular Systems, Inc., NJ, USA) as per manufacturer’s instructions using Roche COBAS TaqMan 48 Analyzer which has the lower detection limit of 25 IU/mL of serum.

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Detection of Diagnosis Escape Variants (DEVs)

Detection of DEVs along with the wild type HBV was achieved by an in-house nested PCR assay \[8\]. In this assay, the first PCR targets the wild type HBV genome, while strategically employed mismatch primers in the second PCR targets the genome of the DEVs of HBV as mentioned below.

**First PCR**

Extracted DNA (10 µl) were amplified in a 50 µl reaction volume using the primers S1 (CATAAGAGGACTCTTGGGACT) and AS1 (CAG-GTACAGTAAAGATAAGGCC) 5 µl (40 pmol/µl) each, 5 µl 10x reaction buffer containing MgCl2 (25 mM), 1.0 µl dNTPs (10 mM), 0.5 µl Taq polymerase (5 U/µl) and 32.5 µl sterile dH2O. The reaction was performed in a programmable thermocycler (Mastercycler, epgradient S, Eppendorf, Germany) using following protocol; initial denaturation at 94°C for 3 minutes followed by 30 cycles each of which involve denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds respectively. After completion of 30 cycles, a final extension step was performed at 72°C for 5 minutes to obtain a 858 bp amplicon.

**Second PCR**

Using first PCR product amplicon (5 µl) as template, the second PCR reaction was performed using inner primer set S2 (GGGAGGAGATTAGTTAA) and AS2 (AGAAGCTCCAAATTGCTTTAT). The strategically employed AS2 primer, mismatched to the consensus/wild type sequence of HBV. All other conditions of the reaction were identical to the first PCR reaction except the duration of the final extension step at 72°C was reduced to 3 minutes to obtain a product of 192 bp.

**Gel electrophoresis**

Amplicons (10 µl) were resolved by 2% agarose gel electrophoresis (100 V, 45 minutes) along with a 100 bp DNA ladder and visualized under UV fluorescence upon staining with ethidium bromide, where 858 bp product refers to the wild type population while 192 bp product of second PCR refers to the DEV population of HBV. Densitometric estimation of the DNA bands was performed by Bio-Capt gel imager system (Vilber Lourmat, France) using the software Image J 1.42 (Broken Symmetry Software, USA).

**Sequence validation**

To validate the in-house PCR generated genomic fragments of HBV, direct sequencing analysis was performed. Sequencing was done through Ampli-Taq facilitated cycle sequencing reaction by the dideoxy terminator method (Applied Biosystems, USA) was employed using 373A automated DNA sequencer (Applied Biosystems, USA) as per manufacturer’s instructions.

**Statistical analysis**

Descriptive statistics (mean, median, standard deviations, inter quartile range (IQR), Student’s t-test and Fischer’s exact tests were performed as and where applicable. The statistical analysis was carried out using statistical package for social sciences (SPSS 20th version). A value of \( p < 0.05 \) was considered statistically significant.

**Results**

The scheme of study, patient categorization and modules are depicted in figure 1. Out of 1723, HBsAg positive patients, a total of 1039 were positive for HBV DNA by commercial real time PCR assay. Of these, 747 patients were detected positive by In-house PCR assay. The mean age of patients was 40 ± 13 years, of which 84.1% were male and 18.9% were females. Majoritiy of the patients (82.7%) were negative for HBeAg and as well as positive for anti HBe indicating seroconversion. Their median ALT level was 43 U/L with IQR of 27 - 75, while the median AST was 31 U/L with IQR 22 - 55. The Mean HBV DNA level was 5.16 ± 1.98 Log copies/mL.

These patients were broadly categorized into Acute (n = 24), Chronic (n = 700), and Hepatocellular carcinoma (HCC) (n = 23). Further, chronic form was divided into 3 categories Immune Tolerant (IT) (n = 32), Inactive Carriers (IC) (n = 192) and active Chronic hepatitis B (CHB) (n = 476) infection (Figure 1).

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Detection of DEV’s in different disease categories

Among all 747 subjects, positive by In-house PCR assay, 562 (75%) patients were detected only with the wild type (W) as evident by the presence of 858 bp amplicon while, 185 (25%) patients were detected positive for variant along with the wild type by showing both 192 bp and the 858 bp amplicon (Figure 2). Direct sequencing of HBV amplicons obtained from the In-house PCR assay, identified as specific HBV DNA sequence and not a PCR artifact, when aligned with the consensus HBV DNA sequence using CLUSTAL O (1.2.3) multiple sequence alignment software program.

Variant HBV population along with wild type was detected across all the disease categories studied. Variant HBV population was found more in IT disease category followed by CHB, IC and Acute as 31%, 25%, 24% and 17% respectively (Figure 3).

Patients’ having variant viral population, in each disease category were further analyzed to evaluate the ratio between wild and variant viral population, for indication of any possible shift in the distribution of viral population among these patients. Interestingly, in contrast to other disease categories, variant types appeared as majority genomic population in 65% of the Inactive carrier group as determined by percentage ratio calculation (Figure 4).
Figure 2: Representative gel picture showing the detection of heterogenous HBV population in patients using In-house PCR assay.

Figure 3: Variant viral population was detected in all disease categories.

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Baseline clinical profile of the treated patients were as follows. Out of one hundred patients, 85 (85%) were male, and 15 (15%) were female, with a mean age of 42 ± 14 years. Eighty four patients (84%) were HBeAg negative, and the mean viral load was 6.35 ± 1.63 log10 copies/mL. All the patients had a median ALT value of 64 IU/mL (25 - 75th IQR, 52 - 116 IU/mL), and 15% had ALT values > 5 times ULN.

Out of 100 patients, 12 (12%) were positive for variant viral population along with wild type (WV) before starting the treatment (baseline). The mean viral load of variant positive patients at baseline was 6.07 ± 1.27 (Log 10 copies/mL) and the median ALT was 57 IU/mL (IQR, 52 - 106 IU/mL). No significant difference in HBV DNA levels and ALT levels were found between the patients having only wild type (W) and patients having variant along with wild type (WV) viral population at baseline.

The virological responses at end of therapy (EOT) and at 48 weeks after discontinuation of the anti-viral therapy (cumulatively up to 104 weeks) of patients with and without variant viral population at baseline are shown in figure 5. Starting from the baseline to end of therapy, the mean HBV DNA levels of DEV positive patients has declined (from 6.07 to 1.89 (Log copies/mL)) similarly to the patients having wild type only (from 6.38 to 2.27 (Log copies/mL)). Surprisingly, the mean HBV DNA levels of DEV positive patients, significantly (P = 0.001) increased to 6.87 Log copies/mL when followed up to 104 weeks after stopping the therapy in comparison to those having wild type virus only where mean HBV DNA was 2.7 (Log copies/mL) (Figure 5).

The biochemical response of the patients towards the therapy from baseline to end of the therapy (48 weeks) and after cessation of therapy (104 weeks) is shown in figure 6. Patients were grouped based on the presence of variant population at baseline. The mean ALT levels in DEV positive patients declined (from 100 to 31 (U/L)), (P = 0.001) from baseline to 48 week of anti-viral therapy like the patients harboring only wild type virus (from 119 to 33 (U/L)), but significantly (P = 0.001) increased to 67 U/L after stopping the therapy, when followed up to 104 weeks in comparison to 46 U/L those having wild type virus only at baseline (Figure 6).
Interestingly, out of 12 patients, who were positive for variant at baseline along with wild type (WV), 5 patients remained to be variant (V) positive at the end of the treatment at 48 weeks. Moreover, six patients who were variant negative at baseline, appeared to be variant positive (V) at the end of the treatment for 48 weeks. All these patients were detected HBV DNA negative by standard diagnostic real time PCR assay at 48 weeks anti-viral therapy.
Relapse rate in patients having DEV’s

At the end of the 48 weeks, the treatment was discontinued for all the patients. Out of 100 patients, 22 (22%) experienced relapse of HBV infection at 96 weeks from baseline after stopping treatment. There are no significant differences observed between patients with or without virological relapse regarding age (41 ± 15 years vs. 42 ± 14 years, P = 0.669), gender (P = 0.385), mean HBV DNA levels (6.19 ± 1.54 log copies/mL vs. 6.38 ± 1.73 log copies/mL, P = 0.650) and mean ALT levels [113 IU/L (IQR, 53 - 130 IU/L) vs. 117 IU/L (IQR, 49 - 116 IU/L) P = 0.909].

Remaining 88 patients, who were having only wild type viral population before anti-viral therapy, eleven (14%) had virological relapse of HBV infection at 48 weeks after stopping the anti-viral therapy.

Twelve patients, who were positive for variant viral population before starting the anti-viral therapy, a total of 11 (92%) showed relapse after stopping the therapy within a period of one year (P = 0.001). 100 percent relapse rate was observed in the patients who were positive for variant viral population at baseline and 48 weeks (n = 5) of therapy (P = 0.001). Six patients who were negative for variant viral population at baseline appeared positive for the same at 48 weeks of treatment. There were 11 patients who were positive for variant population at 48 weeks, irrespective of their baseline status, of which 10 patients (90.1%) had a relapse (P = 0.001) after 48 weeks.

Discussion

The present study was conducted to detect and ascertain the association of DEV’s of HBV in different stages of the disease and their response to anti-viral therapy. Heterogeneity of HBV genome in response to selective pressures is identified to be strongly associated with the HBV infection, progression and treatment outcomes [18]. The quasispecies nature of HBV may be one of the main reasons for immune escape and drug resistance of these hyper-variable viruses [19,20]. Many studies have proven the presence of vaccine escape, immune escape, and drug escape variants of HBV but, there is no study to evaluate whether the presence of genomic variants of HBV viral population can affect the detection efficacy of presently available standard diagnostic PCR assays [21-23].

Identification of genomic variants/quasispecies requires whole genome sequencing of the HBV using techniques such as Next-generation sequencing (NGS) and ultra-deep sequencing which are cost intensive procedures that are not affordable in routine clinical practice [24,25]. In the present study, a cost effective In-house PCR assay was developed and used to detect the viral genomic heterogeneity in a localized region of the viral genome. The In-house PCR assay detect wild type viral population along with the genomic variants of HBV population which is being escaped from the most sensitive routine diagnostic PCR assays (COBAS®TaqMan®48 analyzer) might be due to the sequence alteration in the primer binding site of the target genome of HBV used in the commercial assay.

Interestingly, in the present study, 65% of inactive carriers (IC) were found to harbor DEVs as majority population followed by HCC, CHB and Immune tolerant as 40%, 31% and 20% respectively (p < 0.001). This finding might have implication in the viral load of ICs where presence of higher percentage of variants, which are getting escaped from detection, reflected the lower viral load in selected patients of inactive carrier stage. The differentiation of inactive carriers from HBeAg negative chronic hepatitis B patients is important as the inactive carriers have a low incidence of cirrhosis and hepatocellular carcinoma while the chronic patients are associated with a high risk of developing cirrhosis and hepatocellular carcinoma and therefore may benefit more from timely antiviral treatment [26]. In this respect, the present study indicate that, most of the inactive carriers (above 65%) need to be considered as chronic HBV patients if we include the variant viral load to the existing viral estimation at similar levels of ALT elevation.

Even though viral load is less in inactive carriers in comparison to other disease categories, our previous study showed that the level of pre-genomic RNA (pgRNA), a replication intermediate of HBV, in inactive carriers was comparable with the other groups [27,28]. Furthermore, our study also demonstrated the reduced expression of DNA damage repair genes i.e. high mobility group box1 (HMGB1) and Poly(ADP-ribose) Polymerase1 (PARP1) in inactive carriers than other groups is suggestive of the possibility of viral genomic integration in this group [29,30]. These findings indicate that, though the routinely done viral load of IC patients is low, the host virus interactions are more intense in inactive carriers than the other disease categories of HBV.

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Among the 100 HBV patients who were treatment eligible, 12% of them were detected to harbor DEVs at baseline along with wild type viral population. The present study demonstrated that, patients having variant viral population along with wild type at baseline have shown significant viral breakthrough \((p < 0.001)\) at 48 weeks of therapy. These results corroborate with earlier reports which indicated that, virological breakthrough in antiviral therapy for HBV treatment is preceded by the emergence of quasispecies variants [31]. Furthermore, insufficient viral suppression, such as that associated with sequential mono therapy with non-mutagenic inhibitors, can promote selection of drug-resistant variants [32].

Interestingly 92% of the patients, who were positive for DEVs at baseline have shown the relapse within 48 weeks after cessation of the therapy while only 14% of the patients showed the relapse who were harbor only wild type viral DNA at baseline \((p < 0.0001)\). Earlier investigations have reported that HBV quasispecies if treated insufficiently, may undergo evolution resulting in drug resistant variants, hence relapse. Changes in the HBV quasispecies between baseline and after discontinuation of Entecavir and Tenofovir discontinuation suggest continuous evolution of HBV [33]. Therefore, there is a need to include DEVs which are not detected by routine diagnostic real time PCR assay as diagnostic add-on while estimating viral loads. This might help to alter the treatment regimen and avoid viral breakthrough and relapse for better patient management.

Although measurement of serum HBV DNA levels is an essential tool to assess disease severity and monitoring of anti-viral treatment response, patients with low serum HBV DNA levels and bearing risk of progression to cirrhosis as well as hepatocellular carcinoma (HCC) has been reported [34]. In patients with HBV-related decompensated cirrhosis, no association was found between serum HBV DNA and respective histological activity in the liver [35]. Insufficiency of serum HBV DNA level alone to begin anti-viral treatment has also been reported [36]. In the light of these observations, the present study suggest that commercial standard diagnostic real time PCR assays might underestimate the serum HBV DNA levels, which play crucial role in disease progression and monitoring anti-viral treatment response. Some of these patients who were positive for DEVs and not eligible for the treatment, because of low viral load obtained by the commercial diagnostic PCR assays, may actually be eligible for the therapy if genomic variant (DEVs) count is added to the existing viral loads. Since the In-house PCR assay is less sensitive, its customization to real time PCR format is highly warranted for detection of these variant viruses more precisely.

**Conclusion**

Genomic variants of HBV which are not detected in routine clinical practice, remain as a potential source of transmission. The presence of variants (DEVs) detected by the In-house PCR assay indicate the limitation of current estimation of viral load in a patient to be considered eligible for treatment. Variant population was found as the majority genomic population of HBV in 65% of Inactive carriers in contrast to other disease categories indicating that this group may be a possible reservoir of such variants. The results obtained in the present study indicate that subjects harboring variants along with the wild type virus at baseline are more prone to relapse/viral breakthrough at the end of Nucleos(t)ide analogue therapy. Subjects, positive for HBV DNA without variants before treatment can develop variant population upon selective pressure of NAs indicating a population shift from wild to variant or vice versa. Thus, patients having such variants need more attention and better clinical management than those who do not possess variant population of HBV.

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**Conflict of Interest**

None.

**Bibliography**

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