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Evaluation of anti-HBV drug resistant mutations among patients with acute symptomatic hepatitis B in the United States

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Background & Aims: Reported HBV drug resistance mutations among previously untreated patients with chronic hepatitis B are variable. Whether resistant HBV strains are transmitted in the acute setting is uncertain. We sought to document the presence of antiviral resistance (AVR) mutations in patients with acute HBV (AHB) infection.

Methods: AHB infection was defined by HBsAg/IgM anti-HBc positivity, ALT>10X ULN and compatible clinical history. The TRUGENE HBV kit was used to perform genotyping and direct sequencing of the viral polymerase. INNO-LiPA HBV DRv2 and DRv3 were used to detect AVR mutations. Clonal sequencing was conducted on selected specimens.

Results: Twenty-three patients were evaluated (mean age, 43 years; 54% male; 39% African American, 39% Caucasian, 13% Hispanic and 4% Asian). The mean peak ALT was 1554.2 IU/L and mean peak total serum bilirubin was 12 mg/dl. The HBV DNA median viral load (N = 15) was 5.14 $\log_{10}$ IU/ml. Nineteen patients were genotype A, and 1 each were genotype C, D, E and G. HBV drug resistance mutations were not detected by direct sequencing or INNO-LiPA. Clonal sequencing was conducted on 192 clones isolated from three patients and showed rtA181T, rtM250V and rtS202G mutations at an overall frequency of 1.54%, 1.39%, and 1.67% respectively.

Conclusions: We detected adefovir/lamivudine and entecavir relevant mutations in a minor population (<2%) of viral clones by clonal sequencing only. The clinical significance of these mutations is uncertain and may represent small populations of quasi-species *vs.* transmission of drug resistant strains.

Keywords: Hepatitis B virus; Acute infection; Drug resistance mutations.
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Abbreviations: AVR, antiviral resistance; AHB, acute HBV infection; ALT, alanine aminotransferase; CHB, chronic HBV infection, DHHS, Department of Health and Human Services.

Introduction

There are approximately 2 billion people infected with HBV worldwide, with between 800,000 and 1.2 million of those being chronic hepatitis B (CHB) infections in the United States [1,2]. These numbers are expected to rise even in the face of current vaccination programs, due to factors such as travel and immigration, as there are 350–400 million CHB infections worldwide [3,4]. Directed antiviral therapy as a treatment option has been available in the last 10 years for CHB infection and is comprised of the L-nucleosides (lamivudine and telbivudine), the alkylphosphonates (adefovir and tenofovir) and the cyclopentane drug, entecavir [5]. Treatment with these antiviral agents requires lengthy application towards the goal of reduction of viral load to undetectable levels and sustained virologic response. Over time, therapy relevant mutations emerge that may confer loss of susceptibility to these agents. As it is demonstrated in those CHB infections treated with lamivudine, the percentage of observed resistance is reported to be 24% if treated for over 1 year and 70% if treated for more than 4 years [6].

The dynamics of HBV infection is a major factor in the generation of drug resistance. It is estimated that there are 10^{13} virions produced per day during active infection, with a mutation rate of 6.28×10^{-4} for the 3.2 kb genome [7,8]. Due to this high rate, it is hypothesized that the generation of therapy resistant mutations is inevitable, as every possible polymorphism would be created. The establishment of a resistant virus is due to the selective pressure of the therapeutic regimen, although other factors such as host immune response and therapy adherence play a role. Mutations that confer a fitness advantage over the susceptible wild type phenotype in the presence of antiviral drugs result in the outgrowth of these particular variants. There are now reports of antiviral resistance mutations detected in both drug naïve CHB and acute HBV infection [9–11]. The significance of the presence of these mutations as minor variants is unknown but there is a threat for the lack of durability of antiviral agents in patients that harbor these viral species.

In an attempt to detect the presence of therapy-related mutations in drug naïve acutely infected HBV patients seen at our institution, 25 individuals were investigated for the presence of

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circulating therapy relevant HBV polymorphisms by direct sequencing, line probe assay and clonal analysis.

Patients and methods

Patients

HBV positive patients were enrolled in this IRB approved cohort study from either the Emergency Department or Hepatology Clinic setting from January 2008 through August 2010. The case definition for HBV acute infection was based on positive hepatitis B surface antigen (HBsAg), IgM anti-HBc positivity and ALT levels greater than 10 times the upper limit of normal. Patients also had a compatible clinical history for acute HBV infection.

Laboratory data

Demographical data, including patient age, race, gender, peak ALT U/L and peak bilirubin levels, were collected from medical records in conjunction with sample collection. HBV viral load $\log_{10}$ IU/ml was also documented when available.

Most patients submitted whole EDTA blood specimens upon enrollment into the study or returned for molecular testing within 1 week of enrollment. Plasma was collected by centrifugation of whole blood for 10 min at 400g. Plasma was removed and stored at −80 °C until assayed. Viral DNA was extracted using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

Direct sequencing

Direct sequencing was conducted on a 1.2 Kb amplicon that overlaps the surface antigen and domains B through E of the reverse transcriptase region of HBV, using the TRUGENE HBV Genotyping Kit (Siemens, Deerfield, IL). The assay was conducted according to manufacturer's instructions, with 17 μl of DNA extract used as template. This assay identified HBV genotype and detected polymorphisms. Assay controls included a commercial panel of HBV DNA consisting of genotypes A, B, C, D, E and G (BBI Diagnostics, West Bridgewater, MA).

INNO-LiPA detection

Assessment of specific therapy-related mutations was accomplished using the Inno-Line Probe Assay (Innogenetics, Gent, Belgium) versions DRv2 and DRv3 for drug resistance detection. An 867-bp amplicon that spans the domains A through F of the polymerase gene was analyzed using overlapping probes that identified amino acid changes in reported therapy relevant positions. The assay was conducted according to manufacturer's instructions using the autoblot hybridization system. Colorimetric identification of polymorphisms was done in manual fashion by comparison to the kit standard. Controls included known HBV negative patient specimens and the commercial HBV DNA panel previously mentioned.

Clonal sequencing

A clonal analysis of HBV domains A through E of the reverse transcriptase gene was completed. The pol/rt gene, amino acids 75–255, was amplified by nested PCR using primers OS1 5′-GCCTCATTTTGTGGGTCACCATA-3′ and P1R 5′-TCAGGGTGGTTTCCATGCGA-3′ for the first round amplification of 35 cycles. The second round amplification was conducted using primers 313F 5′-GCAGTCCCCAACCTCCAATCACTCACCAACC-3′ and 1131R 5′-TTGTTGACACAGAAAGGCCTTGTAAGTTGGCG-3′. The amplification reaction consisted of HotStarTaq Master Mix (Qiagen, Valencia, CA) and High Fidelity Platinum Taq (Life Technologies, Grand Island, NY). Twenty microliters of HBV DNA template was used in the first round amplification. Five microliters of first round amplicon was used for the second round PCR. The reaction parameters were as follows: 95 °C for 15 min and then 35 cycles of 94 °C, 1 min; 58 °C, 30 s; 72 °C, 1.5 min and final extension of 72 °C for 7 min. Cloning of PCR amplicons was conducted using the Stratagene TopoTA kit according to manufacturer's instructions. Approximately 60 clones from each patient assayed were selected and sequenced using dideoxy sequencing. Sequencing was conducted using the second round amplification primers with the reaction conditions as follows: 25 cycles of 96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min. The frequency of each mutation was calculated as a percentage of clones harboring that particular mutation for each patient surveyed.

Clonal analysis was also conducted on the stem-loop structure of the *cis*-encapsidation signal from patients 2 and 3 [12]. This region was amplified using a nested PCR with forward primer (HBV2) 5′-GACCTTGAGGCATATTTCAAAGAC-3′ and reverse primer (HBV1) 5′-CTGAGTGCTGTATGGTGAGGTGA-3′ for round one and forward primer (HBV3) 5′-AGGAGATTAGGTTAATGGTCTTTGT-3′ and the same reverse primer for round two. Cloning was conducted as described above. Approximately 40 clones were submitted for bidirectional sequencing using primers HBV1 and HBV3 as described previously.

Results

Twenty-five patients met the criteria for HBV acute infection and were enrolled in this cohort study from January 2008 to August 2010. One patient subsequently developed acute liver failure and underwent successful liver transplantation. Among these 25 patients, 23 were genotyped and sequenced using the direct sequencing assay. The demographical data for these patients are described in Table 1. There was no suspected risk of antiviral resistance for any of these patients reported at the time of enrollment.

The laboratory results for all 23 patients are presented in Table 2. Viral load results were only available for 15 of the patients since viral load is not routinely ordered on presentation at the Emergency Department, where most patients were identified. Of the 23 patients, only 8 had follow-up data for HBV resolution. Seven were observed to resolve their acute infection and one did not. Two of the patients that resolved infection did receive tenofovir as therapy. The patient identified by direct sequencing as genotype G was subsequently identified as co-infected with HIV. While the direct sequencing method did detect several polymorphisms, none of these were at positions previously reported to be associated with antiviral drug resistance.

To determine whether mutations were present in the patient samples below the limit of our detection, we used the INNO-LiPA methodology on 15 of the patients for which we had residual plasma. Sensitivity for this assay is reported to be as low as 5% of the viral population. According to the assay results, no mutations were identified in our patient cohort for the probes represented on the assay blots.

For further verification, it was decided that a small number of specimens would be sent for clonal analysis. Three blinded specimens corresponding to three separate patients were sent to Gilead Laboratories (Durham, NC) where clonal selection and dideoxy sequencing were performed on 192 clones from three patients. The results of the detected polymorphisms in therapy-relevant positions are reported in Table 3. The A181T mutation that has an impact on lamivudine and adefovir susceptibility was detected in two of the patients, as were S202G mutations relevant to entecavir therapy. None of the clones harbored all three mutations. The overall frequencies of these mutations were 1.54% and 1.67%, respectively. Another patient also possessed a clone that had the M250V mutation relevant to entecavir resistance. No other therapy relevant mutations were detected. However, polymorphisms were detected at the therapy relevant locations of I169M, M204T, M250T and N236S [13]. Interestingly, polymorphisms were detected at positions immediately next to relevant resistance sites in clones isolated from all three patients (Table 4). There was only one relevant polymorphism detected at or near known resistance sites per clone with the exception of the two

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Table 1. Characteristics of the patient population with acute HBV infection.

	n = 23
Gender	
Male	48%
Female	52%
Mean age (yr)	43
Race	
Caucasian	39%
African American	39%
Hispanic	13%
Asian	4%
Reported risk	
Sexual	30%
Intravenous drug use	9%
Unknown	61%

Table 2. Laboratory results of patients infected with acute HBV.

	n = 23
Mean peak ALT (IU/ml) range (224-4136)	1588.1
Mean peak bilirubin (mg/dl) range (1.4-29.3)	11
Mean viral load (log_{10} IU/ml) range (3.14-7.93) n = 15	5.14
Genotype	
A	83%
C	4%
D	4%
E	4%
G	4%
Infection resolved	
Yes	30%
No	4%
Lost to follow-up	61%
Received therapy	
Yes (tenofovir)*	17%
No	83%

*Four patients received tenofovir treatment, two of which resolved their acute infection.

alterations observed in clone 31 from patient 3. The remaining polymorphisms detected from the three patients analyzed are listed in Supplementary Fig. 1. These are provided to illustrate the variety of polymorphisms detected.

As a control to rule out the introduction of polymorphisms from our PCR and cloning methodology, samples from patients 2 and 3 were utilized for cloning of the conserved stem-loop region of HBV. As few substitutions are allowable in the region from nt 1847 to nt 1909, we surveyed 89 clones from two patients in this region (Supplementary Fig. 2). Patient 2 did not contain any substitutions in this conserved region. Patient 3 had three substitutions, T to C at position 1853, G to A at position 1876 and T to C at position 1889 from three clones respectively.

Discussion

HBV antiviral therapy options have been available in the last 10 years. Lamivudine was approved for use in 1998. Adefovir, telbivudine, entecavir and tenofovir followed in 2003, 2005, 2006 and 2008, respectively. Increased use of antiviral drugs for CHB has led to increased antiviral resistance. Because of this potential, transmission of resistant HBV is a growing concern. This scenario has already been witnessed with acute HIV infection [14,15]. As a result, the DHHS guidelines for HIV treatment recommend obtaining a baseline resistance genotype in therapy naive patients [16]. Since the number of HBV antiviral drugs is limited, transmission of mutant virus is of particular importance for HBV infection as mutations that confer crossreactivity can leave patients with few therapy options. Indeed, there are recent reports of lamivudine- and adefovir-related mutations in acute HBV infection in both China and Japan [9,11]. Such mutations are usually detected at low frequencies. Alternatively, in a retrospective sequencing survey conducted on banked serum specimens from an acute HBV infected cohort collected between 1997 and 2001 in England, no therapy relevant mutations were detected [17]. To our knowledge, our report is the first prospective study done in the United States evaluating the presence of therapy-relevant mutations in acute HBV symptomatic patients. The aforementioned studies underscore both the need for surveillance and the importance of geographic distribution of resistance to HBV-directed therapy.

There are two possibilities for the presence of these mutations in the acutely infected population. They may be due to transmission of mutant viral species or *de novo* generation of quasi-species. If resistance were transmitted, one would expect that with time, in the absence of drug-induced selective pressure, the predominant species would revert to wild type. This transition is expected due to the observation that most resistant subtypes are less fit than the wild type species and are outcompeted for the space. If this was the case, it would also be expected that the resistant species would be archived in the form of covalently closed circular DNA present in infected hepatocytes [18]. Sequencing of viral nucleic acid from hepatocyte biopsy would help in this determination.

If the resistance was generated *de novo*, one might expect that compensatory mutations would be present that would allow for near wild type expression. In this case, such mutations could be detected by clonal whole genome sequencing. In either event, whether resistance is established in a latent reservoir or newly generated, the potential for future therapy failure is present.

Detection of potential resistant quasi-species in our cohort was not possible with the most common clinical testing methodology. The direct sequencing methodology utilized by the TRUGENE assay is believed to be able to detect minor mutation variants when the mutation is present in greater than 20% of the viral population [19]. The line probe assay has a much greater sensitivity for detecting minor variants down to approximately 5% of the viral population with some still greater sensitivity depending on the specific mutation [20]. One potential limitation of this methodology is that mutations adjacent to a resistance site

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Table 3. Mutations detected at drug resistance sites from individual clones isolated from each of three different patients.

Patient ID	HBV pol/RT position	Calculated frequency¥	Clone harboring substitution*
2	A181T	1.72%	59
6	A181T	1.39%	32
2	I169M	1.72%	56
2	M204T	3.45%	8 and 30
3	M250T	1.61%	60
6	M250V	1.39%	42
3	N236S	1.61%	43
6	N236S	1.39%	74
2	S202G	1.72%	78
3	S202G	1.61%	31

¥Frequency was calculated as the number of clones harboring the polymorphism divided by the total number of clones sequenced.
*Fifty-eight clones from patient 2, 62 clones from patient 3, and 72 clones from patient 6 were evaluated.

may reduce the selectivity of the probes present on the blot. Multiple overlapping probes are located on the blot, designed to minimize this occurrence. As evidenced by the polymorphisms listed in Table 4, there were several alterations detected adjacent to resistance sites in samples taken from three patients in our cohort. It is unclear whether the presence of these mutations prevented the detection of resistance by the INNO-LiPA albeit these mutations were detected at a frequency between 1% and 2%.

Clonal sequencing at present is not a viable option for monitoring genotypic resistance in the clinical setting, being labor intensive and expensive. For the other assays to be of utility as tools for monitoring resistance, greater numbers of mutations at higher viral loads will be necessary. For this reason, monitoring of viral breakthrough via HBV viral load determination for those individuals on therapy will be a critical indicator for resistance testing.

The existence of antiviral resistant mutations in therapy naïve HBV-infected individuals has been reported for patients with chronic HBV infection. Whether resistance mutations are transmitted upon infection or are selected by pressures other than antiviral therapy is not certain. Further, the clinical significance of the presence of these mutations is yet to be fully appreciated.

Though only a limited number of therapy-relevant mutations were found in three of our patient specimens, this phenomenon may still be significant, as the accrual of mutations builds towards an eventual loss of susceptibility. The A181T mutation is relevant to lamivudine with cross resistance to adefovir. Having this mutation present in the population would make sense if this was a remnant of a transmission strain, as lamivudine was the first reverse transcriptase inhibitor used as HBV therapy. Alternatively, the A181T mutation may have been selected by immunologic pressure.

The S202G mutations have an impact on entecavir susceptibility only after the L180M and/or M204V/I mutations are acquired. It is hypothesized that entecavir resistance is a two-step phenomenon and first requires lamivudine mutations to be present [21]. Since these mutations were not present in our clonal analysis, it can be argued that the S202G mutation was spontaneously generated and not a result of a transmission event. Further evidence in our cohort for spontaneous generation of mutation is that polymorphisms at/or near resistance sites were detected predominantly alone in single clones and not as multiples in a large number of clones. As other polymorphisms occurred in resistance sites (M204T, M250T, N236S), it would appear that the virus was exploring enhanced fitness opportunities.

To rule out the introduction of substitutions by our PCR and cloning methodology, we cloned and sequenced the conserved region of the stem loop structure of the *cis*-encapsidation signal. As expected, this is a highly conserved region and few substitutions were observed. A single clone possessed a T to C substitution at position 1853. This position was reported previously to be included in a 20-bp deletion from position 1853 to 1772 of the precore region that resulted in the loss of surface antigen positivity but not viral replication [22]. A second clone possessed a G

Table 4. Polymorphisms detected adjacent to reported drug resistance sites from individual clones isolated from each of three different patients.

Patient ID	HBV pol/RT position	Calculated frequency¥	Clone harboring the substitution*	Therapy relevant mutation
6	F183F/S	1.39%	10	T184I
6	F183S	1.39%	33	T184I
2	F201L	1.72%	31	S202I
3	G172E	1.61%	73	V173I
3	G174S	1.61%	67	V173I
3	G251D	1.61%	41	M250V
3	S185G	1.61%	62	T184I
6	S185G	1.39%	62	T184I
6	S81L	1.39%	65	L80I/V
2	S81P	1.72%	12	L80I/V
3	S81S/T	1.61%	13	L80I/V
3	Y203D	1.61%	31	S202I/M204I
2	L179H	1.72%	48	L180M

¥Frequency was calculated as the number of clones harboring the polymorphism divided by the total number of clones sequenced.
*Fifty-eight clones from patient 2, 62 clones from patient 3 and 72 clones from patient 6 were evaluated.

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to A substitution at position 1876. There are two separate reports of HBV chronically infected patients possessing the same substitutions at this site [23,24]. A third clone harbored a T to C substitution at position 1889. Homs *et al.* recently reported, in a survey done by ultra deep sequencing, that this nucleotide bulge had 0.2% variability at this position [25]. Given the low number of substitutions detected by clonal sequencing at this conserved region, it would appear that the previously detected polymorphisms in the therapy relevant regions were not a result of the methodology employed.

It is now appreciated that ultra-low deep sequencing can detect the presence of mutations that are missed by direct sequencing [26,27]. The clinical significance of these mutations at such low frequencies has yet to be determined. Given the number of polymorphisms detected throughout the clones generated from the three patients analyzed (Supplementary Fig. 1), it would appear that these are *de novo* mutations. One would expect that these mutations would impact viral fitness and therefore would be detected at a lower frequency. Mutations that would benefit viral fitness would be expected to be present at a much higher frequency. As the viral half-life is reported to be 4.8–284 days, it is possible that transmitted mutations were captured at the time point collected [7]. As this is a cross-sectional view of mutation populations, future longitudinal studies will be needed to ascertain the importance of these observations.

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Conflict of interest

The authors have no conflict of interest related to this project. Dr. Gordon is an advisor and speaker for Gilead.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.09.014.

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