DR. LILLY KA WAI YUEN (Orcid ID : 0000-0003-2934-0167)

DR. HENRY LY CHAN (Orcid ID : 0000-0002-7790-1611)

Article type : Original Paper

Original Article

TITLE:HBV variants are common in the “immune-tolerant” phase of chronic hepatitis B

Running Title:HBV variants in immune-tolerant phase

Lilly Yuen^{1}, *Peter A Revill^{1,7*}, *Gillian Rosenberg^{2*}, Josef Wagner¹, Margaret Littlejohn¹, Julianne Bayliss¹, Kathy Jackson¹, Susanna K. Tan³, Anuj Gaggar³, Kathryn Kitrinos³, Mani Subramanian³, Ed Gane⁴, Henry LY Chan⁵, Xin Li¹, Scott Bowden¹, Stephen Locarnini¹, Alexander Thompson⁶

*These authors contributed equally to this study.

1. Victorian Infectious Diseases Reference Laboratory, Royal Melbourne Hospital, at the Peter Doherty Institute for Infection and Immunity, Victoria, 3000, Australia

2. National Cancer Research Institute, London, England

3. Gilead Sciences, Inc., Foster City, CA USA

4. New Zealand Transplant Unit, Auckland, New Zealand

5. Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong SAR, Hong Kong

6. St Vincent's Hospital, Melbourne, Australia

7. Department of Microbiology and Immunology, University of Melbourne, Parkville, 3000, Victoria, Australia.

Corresponding author: Dr. Lilly Yuen. lilly.yuen@vidrl.org.au

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/jvh.13318](https://doi.org/10.1111/jvh.13318)

This article is protected by copyright. All rights reserved

27 **Conflict of Interest:**

- 28 • Peter Revill has received research funding from Gilead Sciences.
- 29 • Susanna Tan is an employees of Gilead Sciences
- 30 • Anuj Gaggar, Kathryn Kitrino and Mani Subramanian are employees and stockholders of Gilead Sciences
- 31 • Ed Gane is a member of the Scientific Advisory Board and Speakers Bureau for Gilead Sciences and on
- 32 Scientific Advisory Board for Janssen, VIR, Dicerna and Roche.
- 33 • Henry Chan is an advisor and speaker for Gilead Science
- 34 • Alexander Thompson is on the advisory board for Gilead Sciences, research grant from Gilead Sciences,
- 35 speaker fee for educational activity from Gilead Sciences
- 36 • All remaining co-authors do not have no conflict of interest

37 **Fundings:**

38 The study was funded in part by Gilead Sciences

39 **Ethics:**

40 All subjects in the study have signed an informed consent form prior to screening and in accordance with

41 local regulatory and ethics committee requirements. Experimental protocol in these trials was approved by

42 Gilead Sciences and all local regulatory agencies. The Clinical Trials Gov Identifier for Gilead trial GS-US-203-

43 0101 was NCT0050750.

44 ABSTRACT

45 Nucleos(t)ide analogues (NUC) treatment prevents progression of liver fibrosis in subjects with chronic
46 hepatitis B (CHB). However, risk for hepatocellular carcinoma (HCC) persists despite viral suppression.
47 Specific HBV variants have been associated with adverse outcomes, including HCC, however the frequency of
48 these variants during the seemingly benign immunotolerant (IT) phase is unknown. Next generation
49 sequencing and detailed virological characterization on a cohort of treatment-naïve IT subjects was
50 performed to determine the frequency of clinically relevant viral variants. Samples from 97 subjects
51 (genotype B/C 55%/45%, median HBV-DNA 8.5 log₁₀ IU/mL, median HBsAg 4.8 log₁₀ IU/mL, median HBeAg
52 3.6 log₁₀ PEIU/mL) were analysed. Despite subjects being in the IT phase, clinically relevant HBV variants
53 were common at baseline, particularly in the basal core promoter (BCP, overlaps the hepatitis B X (HBx)
54 gene), precore, and PreS regions. BCP/HBx variants were independently associated with lower baseline
55 HBeAg, HBsAg and HBV-DNA titres. Precore variants were independently associated with higher baseline
56 ALT. Increased viral diversity was associated with increased age and lower HBV DNA, HBsAg and HBeAg
57 levels. Low level (<5%) drug resistance associated amino acid substitutions in the HBV reverse transcriptase
58 were detected in 9 (9%) subjects at pre-treatment but were not associated with reduced antiviral activity.
59 Future studies should evaluate whether detection of HBV variant during IT CHB is predictive of progression
60 to immune clearance and poor prognosis, and whether early initiation of antiviral therapy during IT CHB to
61 prevent the selection of HBV variants is clinically beneficial.

62 **KEYWORDS:** hepatitis B virus; chronic hepatitis B; immune tolerance; HBV variants; viral diversity

63 INTRODUCTION

64 Persistence of hepatitis B virus (HBV) infection is most common in individuals who are exposed as neonates
65 or young children.(1)The earliestphaseof chronic hepatitis B (CHB)infection, termed the immunotolerant (IT)
66 or the “HBeAg-positive chronic HBV infection” phase of disease, in these subjects is characterized by the
67 presence ofserum HBeAg, very high levels of serum HBV DNA and persistently normal serum ALT levels.The
68 IT phase has traditionally been thought to represent a benign phase of disease asnecroinflammation is mild
69 and fibrosis progression is unusual.(2)

70 While current guidelines do not recommend initiation of antiviral therapy for IT subjects(3-5),there is
71 increasing scientific rationale to consider treating these subjects.Sustained viral suppression from a young
72 age may be associated with reducedhepatocellular carcinoma(HCC) risk.(6)IT subjects have recently been
73 reported to have ahigh level of HBV DNA integration and clonal hepatocyteexpansionsimilar to subjects in

the HBeAg positive immune clearance phase of CHB infection, suggesting that hepatocarcinogenesis may already be underway in this early phase of the infection.(7)Recent data also suggest that HBV specific T cell function is intact in childhood rather than being in a state of immune tolerance, consistent with an early initiation of an anti-HBV immune response.(8)Few studies evaluating for viral variants using next-generation sequencing (NGS) technology have been performed in CHB subjects, particularly in IT subjects. This is important because the selection of HBV variants, including HBeAg-defective variants, such as those with mutations in the basal core promoter (BCP, A1762T/G1764A) which overlaps the gene of the hepatitis B x protein(HBx) and/or in the precore (PC, G1896A) of the viral genome may contribute to disease progression including HCC.(9, 10)The frequency of HBV variants with these mutations or other mutations known to be associated with adverse disease outcomes, referred to as clinically relevant HBV variants, in the IT phase of chronic HBV infection is unknown. Identification of these variants and early initiation of antiviral therapy to prevent their selection may contribute to a reduction in progressive liver disease and HCC risk.

Study GS-US-203-0101 was a randomized, double blind study that evaluated the efficacy of antiviral therapy in IT subjects.(11)HBeAg positive, treatment-naïve individuals with high HBV DNA and normal ALT received either oral tenofovir disoproxil fumarate (TDF, 300 mg) and placebo (n=64) or a combination of TDF (300 mg) and emtricitabine (FTC, 200 mg, n=62) for 192 weeks. The primary end point of study was to determine the proportion of subjects who achieved viral suppression to <69 IU/mL at week 192. Study showed the treatment was safe and efficacious, and TDF/FTC provided better viral suppression than TDF alone(11), with 55% and 76% of subjects who received TDF and TDF/FTC respectively achieved HBV DNA levels of <69 IU/mL at end of treatment. Among subjects with on-going low level viraemia, no confirmed TDF resistance was identified by Sanger sequencing. However, the rate of HBeAg loss was low (5%) and no subjects achieved HBsAg seroclearance, which was considerably lower compared to observations in IC subjects.(12)No HCC was observed, although the study was not designed to evaluate HCC as an outcome.

To date there have been few detailed studies profiling HBV virology among IT subjects. The aim of the current study was to perform a detailed virological investigation of baseline and on-treatment samples from subjects enrolled in Study GS-US-203-0101. We have previously shown that the detection by NGS of HBV variants with BCP(A1762T/G1764A) and/or PC (G1896A) mutations at baseline was associated with reduced likelihood of HBsAg loss, even when present in serum at levels as low as 1% of the viral quasispecies pool(14). We hypothesized that these and other clinically relevant HBV variants that are associated with disease progression and HCC risk may begin to emerge during the IT phase. Whole HBV genome sequencing by NGS was performed to identify the presence and frequency of HBV variants with these genetic changes, which were tested for association with baseline and on-treatment levels of serum HBeAg, HBsAg and HBV DNA.

107 MATERIALS AND METHODS

108 Subjects

109 The inclusion criteria for GS-US-203-101 were: age 18-69yrs., serum HBV DNA level $>1.7 \times 10^7$ IU/mL, serum
110 ALT $\leq$ upper limit of normal (ULN) (men:43 U/L, women:34 U/L), positive serum HBeAg and HBsAg, and
111 creatinine clearance ≥ 70 mL/min. Subjects with decompensated liver disease, history of HCC, and
112 co-infection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus were excluded.(11)A
113 total of 126 subjects received treatment in GS-US-203-0101, 64 received TDF and 62 received TDF/FTC. This
114 study was restricted to subjects infected with HBV genotypes B and C (n = 114, 90% of the cohort).

115 Viral load and serological characterisation

116 HBV viral load and HBsAg quantification were determined previously(11)using the Roche COBAS TaqMan
117 (Roche Diagnostics, Mannheim, Germany) and Abbott Architect (Abbott Laboratories, Abbott Park, IL)
118 assays, respectively.The lower limit of quantification for HBV DNA was 29 IU/mL and HBsAg was 0.05
119 IU/mL.HBeAg levels were determined using the Roche Elecsys HBeAg assay (Roche Diagnostics, Basel,
120 Switzerland) at the Victorian Infectious Diseases Reference Laboratory (VIDRL) as previously
121 described.(13)The upper limit of quantification was >6000 PEIU/mL and the lower limit was 0.3 PEIU/mL.(13)

122 Next Generationsequencing (NGS)

123 Whole HBV genome amplification was performed with HBV DNA extracted from 200 μ L serum using the
124 QIAamp DNA Minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A genome
125 length PCR product and an overlapping PCR product covering the primer binding region were generated
126 usingthe Hi-Fidelity FastStart DNA polymerase (Roche Diagnostics, Basel, Switzerland). PCR products
127 generated for Sanger sequencing were pooled in a 1:1 molar ratio. Library preparation was performed using
128 the Nextera XT kit (Illumina, San Diego, CA, USA) and NGS was donewiththe MiSeq platform (Illumina)
129 according to manufacturer's protocolat Micromon Genomics (Melbourne, Australia).Analysis of NGS data
130 was performed using the in-house HBV-QuasiMiner software package, as previously described(14) with
131 minor modifications. Briefly, the package was composed of a number of modules:raw data processing,
132 quality assurance testing, HBV genotype determination, significant single/multiple nucleotide
133 variant(SNV/MNV) calling, and auto-deduction of nucleotide to amino acid changes of the respective HBV
134 genes.The threshold for detection of SNV/MNVusing this approach was established at 1%, compared to
135 Sanger sequencing (limit of detection $>20\%$).NGS was successfully performed on 97 baseline and 27 on-
136 treatment (last sample with sufficient viral load for amplification) samples. Sanger sequencing was
137 performed for validation purposes and mutation analysis across the whole genome was carried out against
138 genotype specific consensus sequences as described previously.(15-17)

Clinically relevant HBV variants

Each HBV infected subject harbours a unique collection of HBV variants some of which may be associated with clinically important outcomes. The clinically relevant HBV variants analysed in this study included those with BCP mutation/deletion at A1762T and G1764A (BCP/HBx variant), PC mutation at G1896A which created a stop codon at position 28 of the HBe protein sequence (PC variant), PreS1/2 amino acid substitution or deletion at M1 (PreS variant), S amino acid substitutions within the 'a'-determinant region of the surface protein sequence (S variant), S amino acid substitution at G145R (vaccine-escape variant), drug resistance associated amino acid substitutions (RAS) in the RT of HBV polymerase (RAS variants), and the NRE mutation G1613A (NRE variant).

Viral Diversity Analysis

HBV variants in individual subjects occur at different relative frequencies, and their diversity level can be assessed by haplotype reconstruction (refer to Supplementary Material and Methods section for detail). Each reconstructed haplotype was equated to the genome sequence of a specific HBV variant in this study. Briefly, the NGS short reads were processed using Trimmomatic v0.2.36(18), mapped to a reference genome using SMALT v0.7.4 (Wellcome Sanger Institute, Cambridge UK), and the HBV genome haplotypes were reconstructed using ClaqueSNV v1.4.8(19). All haplotypes with a minimum abundance of 1% were used for analysis.

Phylogenetic Analysis

A maximum likelihood (ML) tree was generated in MEGA7(20) to show the phylogenetic relationships between all the haplotypes determined from baseline samples with a minimum abundance of 1%.

Statistical Analysis

Statistical analysis between clinical factors and single nucleotide or amino acid variants (SNV or SAV) detected in baseline and on-treatment samples of subjects were performed with R Statistical Software (v2.3.1; R Foundation for Statistical Computing, Vienna, Austria). For bivariate analysis, categorical data were evaluated using the Fisher's exact test, and continuous data using the t-test for parametric data and Wilcoxon Rank Sum test (or Kruskal-Wallis if more than 2 groups were analysed) for non-parametric data. Normality of continuous data was assessed using the Shapiro-Wilk normality test. Multiple logistic regression analysis was performed to determine independent factors associated with treatment response using the R package MuMIn.(21) All reported P values were 2-sided.

Statistical tests on the reconstructed haplotypes were performed using GraphPad Prism version 7.0e (GraphPad Software, La Jolla California USA, www.graphpad.com) (refer to Supplementary for detail). The t-

test was used to evaluate parametric data and Mann Whitney Test for non-parametric data. Normality of continuous data was tested with both D'Agostino & Pearson and Shapiro-Wilk normality tests. Correlations between clinical factors and haplotype frequency was assessed by measuring the Pearson correlation coefficient for parametric data and Spearman correlation coefficient for nonparametric data.

Results

i) Subject characteristics

Baseline samples from 53 HBV genotype B and 44 HBV genotype C subjects from the GS-US-203-101 study were successfully analysed using NGS(97/114 (85%) subjects, Table1). Samples of the remaining 17subjects could not be amplified or had failed NGS quality control. Thesubjects at baselinewere predominantly young (median age 31 [IQR 26-40] yrs.), Asian (96%), had high HBV DNA (median viral load 8.4[IQR 8.2-8.6] $\log_{10}$ IU/mL) and normal ALT (median 26 [IQR 21-31] U/L), consistent with baseline characteristics described in the overall GS-US-203-101 cohort.(11)

ii) Clinically relevant HBV variants were commonat baseline

Whole HBV genome NGS data was generated from baseline samples of 97 subjects, and analysis revealed presence of clinically revelvanthHBV variants was frequent despite the subjects were in the IT phase of CHB (Table 2), with 87% of subjects had at least one of the described HBV variants that are associated with adverse disease outcomes. BCP/HBx (n=19, 20%) and NRE (n=72, 74%) variants weremore common in subjects infected with HBV genotype C than those with genotype B(BCP/HBx 41% vs. 2%, NRE 100% vs. 53%; $p<0.001$). Other HBV variants detected include those with genetic variations in the PC, PreS and S regions of the viral genome. Subjects harbouring HBV variants with BCP/HBx, PC or PreS1/2 M1 immune evasion associated changes or deletions were older (35yrs vs. 30yrs, $p=0.042$) and had higher ALT levels (27 U/L vs. 23 U/L, $p=0.006$). RAS variants(22) were identified at low frequency ($<5\%$) in 9 subjects.

iii) BCP/PC variants were associated with HBeAg, HBsAg, HBV DNA and ALT levels at baseline

The associations between the BCP/HBx and PC variants and serum HBsAg, HbeAg, HBV DNA and ALT levelsof subjects at baselinewere assessed. The frequency of BCP/HBx variants when present at $>1\%$ was negatively correlated with HBsAg ($r=-0.503$; $p=0.028$), HbeAg ($r=-0.651$; $p=0.003$), and HBV DNA levels ($r=-0.446$; $p=0.056$). Furthermore, subjects with BCP/HBx variants present at $>20\%$ of the quasispecies pool (n=9, detectable by Sanger sequencing) had significantly lower HbeAg ($p=0.033$), HBsAg ($p=0.001$) and HBV DNA ($p=0.016$) levelsthan those with only wildtype (WT) variants (n=75) (Figure 1). Notably, subjects with PC variants even at low levels ($>1\%$, n=11) had significantly higher ALT levels than those infected with WT

200 variants (n=43)(p=0.015).However, since all IT subjects by definition have low ALT levels, the biological
201 relevance of these small yet statically significant differences is unclear.

202 **iv) An increase in viral diversity may indicate transition towards immune clearance phase**

203 The median number of haplotypes determined from the IT cohort at baseline was 3 [IQR: 2 to 5].
204 The relationship between viral diversity level in subjects and clinical factors at baseline were
205 assessed. Haplotype reconstruction showed that the level of viral diversity, expressed as number of
206 haplotypes per sample, correlated with baseline serum ALT level (r=0.314 and p=0.002)(Figure 2). In addition,
207 the level of viral diversity correlated with age of subjects (r = 0.367 and p <0.001), negatively correlated
208 albeit weakly with HBV DNA (r = -0.264 and p = 0.012), HbeAg (r = -0.254 and p <0.001) and HBsAg (r = -0.418
209 and p <0.001) levels at baseline. Viral diversity level also differed significantly by HBV genotype (p
210 <0.001, Figure 3), being higher in subjects infected with HBV genotype C (median = 4; IQR 2 to 6) than HBV
211 genotype B (median = 2; IQR 2 to 3). No subjects were infected with mixed HBV genotypes by phylogenetic
212 analysis, and all haplotypes clustered correctly in the maximum likelihood tree by the expected HBV
213 genotype (data not shown). Interestingly, the majority of the BCP/HBx variant haplotypes were determined
214 from HBV genotype C subjects (data not shown).

215 **v) Baseline HBV variants were not associated with treatment response**

216 Finally, we assessed whether baseline HBV variants can inform response to antiviral therapy in IT subjects. Of
217 77 subjects with a complete set of serological data available at week 192, 66% achieved viral suppression of
218 serum HBV DNA to <29 IU/mL (59% among TDF treated and 76% among FTC/TDF treated), 15% had >1
219 log₁₀ IU/mL decline in serum HBsAg, and 29% had >1 log₁₀ IU/mL decline in serum HbeAg at end of
220 treatment. Female subjects in this cohort were more likely to achieve viral suppression to <29 IU/mL at
221 week 192 than male subjects (Table 3), consistent with the parent study.(11)

222 Baseline clinically relevant HBV variants were not associated with reductions in HBV viral load or HBsAg, but
223 presence of the BCP/HBx variant (p=0.006), and higher viral diversity (haplotype count, p=0.005) at baseline
224 was independently associated with >1 log₁₀ decline in serum HbeAg at week 192 by univariate analysis
225 (Table 3). The dynamics of HBsAg and HbeAg change were also explored, with both markers exhibiting a
226 biphasic decline over 192 weeks of treatment (Supplementary Figure 1).

227 **vi) Persistent viraemia was not associated with selection of drug resistance associated substitutions**

228 RAS variants were detected at low frequency in 9(9%) subjects at baseline (Tables 2 and 4). The lamivudine
229 (LMV) resistance associated rtM204I substitution(22) was detected in 5 of the 9 subjects, the LMV and adefovir
230 (ADV) resistance associated rtA181S/T substitution(22) was detected in 3 subjects, and a subject had viral
231 variants with both rtM204I and rtA181T substitutions. All RAS were detected at levels below 5% of the HBV

quasiespecies pool, well below the 20% Sanger sequencing detection threshold, and were not associated with viral suppression in response to TDF treatment.

The quadruple mutation (rtS106C, rtH126Y, rtD134E and rtL269I, collectively referred to as the CYEI mutation) that may be associated with reduced sensitivity to TDF(23), recently identified in two patients(23), was not detected in the samples of any of the subjects.

No subjects with RAS variants at baseline experienced virological breakthrough nor HBeAg loss by week 192. Moreover, on-treatment samples from 4 subjects were successfully analysed by NGS, and the RAS variants were no longer detected (Table 4).

DISCUSSION

This is the first detailed study of HBV sequence variability in the setting of IT CHB. The standard clinical definition of ITCHB includes HbeAg positivity, high HBV DNA, normal ALT and minimal hepatic necroinflammation(1), with current clinical guidelines not recommending treatment for this phase.(3-5) This study has identified prevalent clinically relevant HBV variants among a cohort of subjects at baseline in the IT phase of CHB. Increase HBV diversity was associated with clinical factors suggestive of transition to IC phase disease, despite persistently normal ALT. HBV variants previously associated with disease progression (cirrhosis and HCC) were common using NGS and were detected in 87% of the cohort.

Of note, the BCP/HBx mutations/deletions located at nucleotide positions 1762 and 1764 of the HBV genome (BCP/HBx variant) was detected at baseline by NGS in 20% of the IT subjects. Presence of the BCP/HBx variant was associated with lower baseline levels of HbeAg, HBsAg and HBV DNA. This association was independent of gender and HBV genotype. Similarly, mutations or amino acid substitutions in the PC, core Pre-S and NRE regions of HBV genomes were detected in IT subjects. Analysis of viral diversity by haplotype reconstruction of NGS data confirmed that increased diversity was associated with clinical features suggestive of progression towards the immune clearance phase of CHB, including lower HBV DNA levels, HBsAg and HbeAg levels. Sequence diversity also varied by HBV genotype, with higher levels of sequence diversity in HBV genotype C than genotype B in IT subjects. The data suggest that the viral population is diverse during the IT phase of CHB, which we speculate reflects host-virus interaction but this requires further experimental confirmation. Future studies should evaluate the association between selection of HBV variants during the IT phase and markers of anti-HBV immunity.

The presence of clinically relevant HBV variants has been associated with clinical outcomes. BCP/HBx variants have been associated with disease progression, cirrhosis, and HCC.(10, 24) Variants with mutations/deletions in the PC and Pre-S regions of viral genomes have also been associated with increased HCC risk(10, 25). Long-term viral suppression with NUC therapy reduces HCC risk when treating subjects with

cirrhosis (26). Given the excellent long-term efficacy, resistance and safety profiles, the European guidelines suggest that treatment be considered in IT subjects if they are older than 30 years, regardless of the severity of liver histological lesions (27)). Detection of HBV variants associated with HCC risk in IT subjects with a mean age of 32 yrs in our study supports this approach. Prospective studies are required to evaluate whether commencing immunotolerant subjects on NUC therapy before the selection of HBV variants will be associated with a risk reduction for progression to HCC.

Another important clinical question is whether commencing treatment in IT subjects with very high viral loads would be associated with an increase in the risk of antiviral resistance. Despite subjects in this study having very high baseline HBV DNA levels, variants with resistance to tenofovir were not identified, although very low levels of variants with resistance to other NUC's were detected.

In summary, our data shows that clinically relevant HBV variants associated with increased risk of liver morbidity and mortality were detected in IT subjects. We surmise that individuals who may be transitioning to IC phase have an increase in viral haplotype diversity, and suggest that the classification of "immunotolerant CHB" may need to be revisited to consider the role of detectable viral evolution within the clinical definition. Further studies of subjects who transition from IT to IC phases are required, as are studies to evaluate the clinical benefit of early antiviral therapy to prevent the selection of HBV variants and reduce the risk of HCC.

REFERENCES

- 1 McMahon BJ. Natural history of chronic hepatitis B. *Clin Liver Disease*. 2010; 14(3):381-96.
- 2 Tseng TC, Kao JH. Treating Immune-tolerant Hepatitis B. *J Viral Hepat*. 2015; 22(2):77-84.
- 3 EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol*. 2012; 57(1):167-85.
- 4 Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology*. 2009; 50(3):661-2.
- 5 Liaw Y-F, Kao J-H, Piratvisuth T, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2012 update. *Hepatol Int*. 2012; 6(3):531-61.
- 6 Zoulim F, Mason WS. Reasons to consider earlier treatment of chronic HBV infections. *Gut*. 2012; 61(3):333-6.
- 7 Mason WS, Gill US, Litwin S, et al. HBV DNA Integration and Clonal Hepatocyte Expansion in Chronic Hepatitis B Patients Considered Immune Tolerant. *Gastroenterology*. 2016; 151(5):986-98 e4.
- 8 Kennedy PTF, Sandalova E, Jo J, et al. Preserved T-cell function in children and young adults with immune-tolerant chronic hepatitis B. *Gastroenterology*. 2012; 143(3):637-45.

- 295 9 Liao G, Wang Y, Chang J, *et al.* Hepatitis B virus precore protein augments genetic immunizations of
296 the truncated hepatitis C virus core in BALB/c mice. *Hepatology*. 2008; 47(1):25-34.
- 297 10 Tseng TC, Liu CJ, Yang HC, *et al.* Higher proportion of viral basal core promoter mutant increases the
298 risk of liver cirrhosis in hepatitis B carriers. *Gut*. 2015; 64(2):292-302.
- 299 11 Chan HL, Chan CK, Hui AJ, *et al.* Effects of Tenofovir Disoproxil Fumarate in Hepatitis B e Antigen-
300 Positive Patients With Normal Levels of Alanine Aminotransferase and High Levels of Hepatitis B Virus
301 DNA. *Gastroenterology*. 2014; 146(5):1240-8.
- 302 12 Marcellin P, Gane E, Buti M, *et al.* Regression of cirrhosis during treatment with tenofovir disoproxil
303 fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet*. 2013; 381(9865):468-75.
- 304 13 Zacher BJ, Moriconi F, Bowden S, *et al.* Multicenter evaluation of the Elecsys hepatitis B surface
305 antigen quantitative assay. *Clin Vaccine Immunol*. 2011; 18(11):1943-50.
- 306 14 Bayliss J, Yuen L, Rosenberg G, *et al.* Deep sequencing shows that HBV basal core promoter and
307 precore variants reduce the likelihood of HBsAg loss following tenofovir disoproxil fumarate therapy in
308 HBeAg-positive chronic hepatitis B. *Gut*. 2017; 66(11):2013-23.
- 309 15 Gunther S, Li BC, Miska S, Kruger DH, Meisel H, Will H. A novel method for efficient amplification of
310 whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in
311 immunosuppressed patients. *J Virol*. 1995; 69(9):5437-44.
- 312 16 Ayres A, Locarnini S, Bartholomeusz A. HBV genotyping and analysis for unique mutations. *Methods*
313 *Mol Med*. 2004; 95:125-49.
- 314 17 Yuen LK, Ayres A, Littlejohn M, *et al.* SeqHepB: a sequence analysis program and relational database
315 system for chronic hepatitis B. *Antiviral Res*. 2007; 75(1):64-74.
- 316 18 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.
317 *Bioinformatics*. 2014; 30(15):2114-20.
- 318 19 Knyazev S, Tsyvina V, Melnyk A, *et al.* CliqueSNV: Scalable reconstruction of Intra-host viral polulations
319 from NGS reads. *BioRxiv*. 2018; 264242.
- 320 20 Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for
321 Bigger Datasets. *Mol Biol Evol*. 2016; 33(7):1870-4.
- 322 21 Barton K. Mu-MIn: Multi-model inference. R Package Version 0.12.2/r18. 2009; [http://R-Forge.R-](http://R-Forge.R-project.org/projects/mumin/)
323 [project.org/projects/mumin/](http://R-Forge.R-project.org/projects/mumin/).
- 324 22 Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology*. 2009;
325 137(5):1593-608 e1-2.

- 326 23 Park ES, Lee AR, Kim DH, *et al.* Identification of a quadruple mutation that confers tenofovir resistance
327 in chronic hepatitis B patients. *J Hepatol.* 2019; 70(6):1093-102.
- 328 24 Tong S, Kim KH, Chante C, Wands J, Li J. Hepatitis B Virus e Antigen Variants. *Int J Med Sci.* 2005;
329 2(1):2-7.
- 330 25 Liao Y, Hu X, Chen J, *et al.* Precore mutation of hepatitis B virus may contribute to hepatocellular
331 carcinoma risk: evidence from an updated meta-analysis. *PLoS One.* 2012; 7(6):e38394.
- 332 26 Papatheodoridis GV, Idilman R, Dalekos GN, *et al.* The risk of hepatocellular carcinoma decreases after
333 the first 5 years of entecavir or tenofovir in Caucasians with chronic hepatitis B. *Hepatology.* 2017;
334 66(5):1444-53.
- 335 27 EASL. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J*
336 *Hepatol.* 2017; 67(2):370-98.
- 337 28 Urban S, Bartenschlager R, Kubitz R, Zoulim F. Strategies to Inhibit Entry of HBV and HDV into
338 Hepatocytes. *Gastroenterology.* 2014; 147(1):48-64.
- 339 29 Mun HS, Lee SA, Jee Y, *et al.* The prevalence of hepatitis B virus preS deletions occurring naturally in
340 Korean patients infected chronically with genotype C. *J Med Virol.* 2008; 80(7):1189-94.
- 341 30 Pollicino T, Amaddeo G, Restuccia A, *et al.* Impact of hepatitis B virus (HBV) preS/S genomic variability
342 on HBV surface antigen and HBV DNA serum levels. *Hepatology.* 2012; 56(2):434-43.
- 343 31 Blackberg J, Kidd-Ljunggren K. Mutations within the hepatitis B virus genome among chronic hepatitis
344 B patients with hepatocellular carcinoma. *J Med Virol.* 2003; 71(1):18-23.
- 345 32 Zhang D, Dong P, Zhang K, *et al.* Whole genome HBV deletion profiles and the accumulation of preS
346 deletion mutant during antiviral treatment. *BMC Microbiol.* 2012; 12:307.
- 347 33 Chotiyaputta W, Lok AS. Hepatitis B virus variants. *Nat Rev Gastro Hepat.* 2009; 6(8):453-62.
- 348 34 Carman WF, Zanetti AR, Karayiannis P, *et al.* Vaccine-induced escape mutant of hepatitis B virus.
349 *Lancet.* 1990; 336(8711):325-9.
- 350 35 Li MS, Lau TC, Chan SK, *et al.* The G1613A mutation in the HBV genome affects HBeAg expression and
351 viral replication through altered core promoter activity. *PloS one.* 2011; 6(7):e21856.
- 352 36 Nie H, Evans AA, London WT, Block TM, Ren XD. Quantitative dynamics of hepatitis B basal core
353 promoter and precore mutants before and after HBeAg seroconversion. *J Hepatol.* 2012; 56(4):795-
354 802.

355 37 Locarnini S. Primary resistance, multidrug resistance, and cross-resistance pathways in HBV as a
 356 consequence of treatment failure. *Hepatol Int.* 2008; 2(2):147-51.

357 **Table 1:** Comparison between baseline characteristics of subjects who had samples successfully assessed by
 358 NGS and those of the original study. (11)

	NGS Cohort (N = 97)		Original Study (N = 114)		p-values
Age (yrs), median [IQR]	33	[26-42]	34	[26-40]	0.535
Male, n (%)	45	(46)	54	(47)	0.891
Asian, n (%)	92	(95)	109	(96)	1
TDF monotherapy, n (%)	53	(55)	56	(49)	0.490
ALT (U/m), median [IQR]	25	[20-31]	26	[21-31]	0.679
HBV DNA (log ₁₀ IU/mL), median [IQR]	8.5	[8.2-8.6]	8.4	[8.2-8.6]	0.962
HBsAg (log ₁₀ IU/mL), median [IQR]	4.8	[4.6-5.0]	4.7	[4.6-5.0]	0.571
HbeAg (log ₁₀ PE IU/mL), median [IQR]	3.6	[3.4-3.7]	3.5	[3.4-3.7]	0.872
HBV Genotype, n (%)	B	53 (55)	64	(56)	0.890
	C	44 (45)	50	(44)	0.899

359
 360 **Table 2:** Baseline HBV variants with specific mutations or amino acid substitutions in the viral genome that
 361 have previously been associated with clinical phenotype, present at >1% frequency, that were detected by
 362 NGS from 97 subjects.

Mutations/Amino Acid Substitutions (†)	Associated phenotype	N (%) (‡)	Median frequency [IQR] when detected by NGS
PreS1 M1	Loss of large HBsAg, associated with infectivity(28), disease progression(29) and decreased HBsAg(30)	33 (34%) (B=17; C=16)	2% [1-9] (B: 1% [1-2]) (C: 11% [4-25])
PreS2 M1	Loss of medium HBsAg, associated with viral secretion(28), disease progression(31) and decreased HBsAg(30)	10 (11%) (B=2; C=8)	4% [3-20] (B: 22% [12-32]) (C: 4% [3-13])
PreS deletions (excluding those that involved M1)	Truncated large/medium HBsAg associated with decreased HBsAg(30) and immune escape(32)	5 (5%) (C=5)	20% [6-27] -
HBsAg 'a' determinant (codons 120-150)	Major antigenic region of the protein(33) and variants can lead to vaccine, immune, and diagnostic escape	43 (61%) (B=26; C=17)	2% [1-3] (B: 1% [1-2]) (C: 2% [1-3])
HBsAg G145G/R	Known vaccine escape variant(34)	10 (10%) (C=10)	12% [4-20] -

RT L80, A169, V173, L180, A181*, T184, S202, M204*, N236*, M250	Known drug resistance and compensatory variants(22)	9 (9%) § (B=4; C=5)	1% [1-2] (B: 1% [1-2]) (C:2% [1-2])
NRE G1613A	Associated with an increased risk of HCC in genotype C and alongside the BCP mutation(35), increase viral expression(35) and decreased HBeAg secretion(35)	72 (74%) (B=28; C=44)	4% [1-19] (B: 4% [1-25]) (C:4% [2-17])
BCP (mutations/deletions involving sites A1762 and G1764)	Associated with lower HBeAg expression, higher viral loads, disease progression(33). Suggestive of upcoming HBeAg seroconversion(36).	19 (20%) (B=1; C=18)	28% [4-73] (B: 53%) (C:25% [4-80])
Precore (G1896G/A)	Associated with loss of HBeAg(33). Suggestive of upcoming HBeAg seroconversion(36).	13 (15%) (B=7; C=6)	9% [3-20] (B: 13% [3-29]) (C: 9% [7-18])

†RT = reverse transcriptase, NRE = negative regulatory region, BCP = basal core promoter

‡ Number (percentage) of subjects with the mutation/amino acid substitution

§10 HBVRT amino acid substitutions are associated with drug resistance(37) and were screened for in this study, but only viral variants with at least one of the 3 primary resistance substitutions flagged with asterisk are considered a drug resistant variant. The others are secondary resistance associated substitutions. The RAS profiles of the 9 subjects are shown in Table 4.

368 **Table 3:** Association of viral variants with clinically relevant nucleotide/amino acid changes detected by NGS at baseline and associated clinical markers with
369 antiviral therapy response at 192 weeks

Baseline characteristics	Viral suppression (†)			HBsAg >1 log ₁₀ decline			HBeAg >1 log ₁₀ decline		
	R (N = 54)‡	NR (N = 27)‡	p-value§	R (N = 12)‡	NR (N = 67)‡	p-value§	R (N = 24)‡	NR (N = 57)‡	p-value§
TDF monotherapy, n (% of N)	26 (48)	19 (70)	0.068	6 (50)	37 (55)	0.763	15 (63)	30 (53)	0.470
Age, median [IQR]	34 [26-45]	32 [27-39]	0.627	30 [23-44]	33 [27-42]	0.461	36 [26-46]	32 [27-41]	0.612
Female Gender, n (% of N)	32 (59)	9 (33)	0.035	4 (33)	36 (54)	0.224	11 (46)	30 (53)	0.632
ALT, median [IQR]	24 [21-30]	28 [23-35]	0.218	28 [24-35]	24 [20-31]	0.127	28 [24-35]	24 [20-30]	0.006
HBV DNA, median [IQR]	8.4 [8.2-8.6]	8.5 [8.3-8.8]	0.331	8.6 [8.4-8.6]	8.4 [8.2-8.6]	0.541	8.4 [8.0-8.6]	8.5 [8.3-8.7]	0.102
HBsAg, median [IQR]	4.8 [4.5-5.0]	4.9 [4.8-5.1]	0.070	4.8 [4.6-5.1]	4.8 [4.6-5.0]	0.778	4.5 [4.3-4.8]	4.9 [4.7-5.0]	0.005
HBeAg, median [IQR]	3.6 [3.3-3.7]	3.6 [3.4-3.6]	0.670	3.5 [3.3-3.6]	3.6 [3.4-3.7]	0.244	3.5 [3.3-3.7]	3.6 [3.4-3.7]	0.757
Genotypes C, n (% of N)	31 (57)	12 (44)	0.346	3 (25)	39 (58)	0.057	13 (54)	30 (53)	1.000
BCP/HBx (A1762T, G1764A), n (% of N)	14 (26)	3 (11)	0.155	1 (8)	15 (22)	0.442	10 (42)	7 (12)	0.006
Precore (G1896A), n (% of N)	7 (13)	4 (15)	1.000	2 (17)	9 (13)	0.671	4 (17)	7 (12)	0.724
PreS1/2 (M1, deletion), n (% of N) ¶	19 (35)	10 (37)	1.000	5 (42)	22 (33)	0.742	9 (38)	20 (35)	1.000
HBsAg ('a' determinant), n (% of N)	19 (35)	14 (52)	0.161	4 (33)	27 (40)	0.756	13 (54)	20 (35)	0.140
HBsAg (G145R), n (% of N)	7 (13)	2 (7)	0.710	1 (1)	8 (12)	0.674	6 (25)	3 (5)	0.017
Drug-resistant changes, n (% of N)	12 (22)	8 (30)	0.586	4 (33)	15 (22)	0.469	5 (21)	15 (26)	0.779
NRE (G1613A), n (% of N)	41 (76)	24 (89)	0.240	7 (58)	56 (84)	0.060	18 (75)	47 (82)	0.543
Haplotype Count, median [IQR]	3 [2-5]	2 [1-4]	0.089	3 [2-4]	3 [2-5]	0.989	4 [2-8]	2 [2-4]	0.005

370 †Number of subjects who achieved viral suppression of serum HBV DNA to <29 IU/mL

371 ‡ R = responders; NR = non-responders

372 § p-values in bold and italic font are statistically significant

¶ PreS: include the 3 amino acid changes/deletions categories listed in Table 2

Table 4: Summary of drug-resistance associated amino acid substitutions (RAS) identified in the reverse transcriptase domain of HBV polymerase and overlapping HBsAg, at baseline in 9/97 subjects by NGS.

Subject† (Genotype)	Treatment	RAS Profile at baseline (%)	Overlapping HBsAg changes at baseline	HBeAg at Baseline (log ₁₀ PEIU/mL)	VL at Baseline (log ₁₀ IU/mL)	VL at week 192 (IU/mL)	VL first week <29 IU/ml	ALT at Baseline (U/L)	HBeAg >1 log ₁₀ decline at week 192	HBsAg >1 log ₁₀ decline at week 192
1 (C)	TDF	A181T (1.1) + M204I (1.9) + M250I (1.1)	W172*+W196*	3.60	8.36	<29	96	31	N	N
2 (C)	TDF	M204I (1.3) + M250I (1.2)	W196*	3.25	7.65	<29	48	21	Y	Y
3 (B)	TDF	A181T (1.4) + M250I (3.6)	W172*	3.66	8.71	44	160	15	N	N
4 (B)	TDF + FTC	M204I (1.5) + M250I (3.6)	W196L	3.20	8.61	<29	96	24	N	N
5 (C)	TDF	A181S (1.7)	W172C	3.35	9.02	90	72	29	N	N
6 (B)	TDF	M204I (1.6) + M250I (2.7)	W196L	3.26	8.45	early EOT	-	29	N	N
7 (B)	TDF	M204I (1.2) + M250I (1.6)	W196L	3.29	8.52	32	160	17	N	N
8 (C)	TDF + FTC	A181S/T (3.6/2.7)	W172C/*	1.33	7.87	<29	48	26	N	N
9 (C)	TDF	M204I (2.0)	W196*	1.97	7.42	<29	48	23	Y	N

† Second samples of Subjects 3 (week 24), 5 (week 24), 6 (week 24), and 7 (week 8) were successfully analysed by NGS, and no RAS variants were detected.

378 **FIGURE LEGENDS**

379 **Figure 1:** Baseline levels of HBsAg, HBeAg, HBV DNA and ALT determined from subjects infected with
380 immune/drug susceptible viral variants (WT), and those with BCP/HBx (BCP), PC and BCP+PC variants present
381 at different frequencies (>20%, >10% and >1%). Subject groups with significantly different serological
382 biomarker levels are marked with a bar above the boxplots.

383 **Figure 2:**Correlation relationships between viral diversity level (number of haplotypes), serological markers,
384 and age of subjects at baseline. Number of haplotypes versus: (a) ALT level, (b) HBV DNA level, (c) HBsAg
385 level, (d) HBeAg level, and (e) subject age.

386 **Figure 3:** Distribution of haplotype frequency determined from subjects with HBV genotype B and C
387 infections.

jvh_13318_f1.tif

jvh_13318_f2.tif

jvh_13318_f3.tif

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Yuen, L;Revill, PA;Rosenberg, G;Wagner, J;Littlejohn, M;Bayliss, J;Jackson, K;Tan, SK;Gaggar, A;Kitrinos, K;Subramanian, M;Gane, E;Chan, HLY;Li, X;Bowden, S;Locarnini, S;Thompson, A

Title:

HBV variants are common in the 'immune-tolerant' phase of chronic hepatitis B

Date:

2020-06-17

Citation:

Yuen, L., Revill, P. A., Rosenberg, G., Wagner, J., Littlejohn, M., Bayliss, J., Jackson, K., Tan, S. K., Gaggar, A., Kitrinos, K., Subramanian, M., Gane, E., Chan, H. L. Y., Li, X., Bowden, S., Locarnini, S. & Thompson, A. (2020). HBV variants are common in the 'immune-tolerant' phase of chronic hepatitis B. JOURNAL OF VIRAL HEPATITIS, 27 (10), pp.1061-1070.
<https://doi.org/10.1111/jvh.13318>.

Persistent Link:

<http://hdl.handle.net/11343/275917>