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# Identification of hepatitis B virus DNA reverse transcriptase variants associated with partial response to entecavir

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## KEY MESSAGES

1. Entecavir is a potent antiviral agent against hepatitis B virus (HBV). In a cohort of 370 entecavir-treated chronic hepatitis B patients, 76 (21%) had a partial virological response, defined by detectable HBV DNA at year 1.
2. In HBV reverse transcriptase sequence analysis, 20 amino acid variants were identified solely in the partial responders.
3. There were 4 reverse transcriptase (rt) amino acid variants that had significantly different distribution between the optimal and partial entecavir responders.
4. HBV quasispecies complexity and diversity were higher in the optimal responders than in the partial responders.
5. In multivariate analysis, high baseline HBV DNA, hepatitis Be antigen-positivity, and rt variant rt124N were significantly associated with partial entecavir response.

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## Introduction

Nucleos(t)ide analogues (NA) are potent antiviral agents against hepatitis B virus (HBV) infection, drastically reducing viral load and improving clinical outcome. Lamivudine, adefovir, entecavir, telbivudine, and tenofovir are licensed in Hong Kong for treatment of chronic hepatitis B. Some patients have high HBV DNA, even at the initial stage.<sup>1</sup> One explanation is that some intrinsic viral properties may confer non-responsiveness to a particular NA. As the main target of NAs is HBV reverse transcriptase (rt), some natural polymorphisms in the HBV rt sequences may confer primary non-responsiveness.

HBV in a mixed viral population is known as quasispecies. Viral complexity and diversity change dynamically during the natural history of chronic hepatitis B infection. Studies of their effect on response to antiviral agents are rare. This study aimed to investigate whether there are some pre-treatment HBV rt sequence variations that predict response to entecavir. In addition, the association between quasispecies complexity and diversity and entecavir response were evaluated.

## Methods

This study was conducted from May 2009 to November 2011. Between January 2002 and September 2009, 370 chronic hepatitis B patients at Queen Mary Hospital underwent daily entecavir (0.5 mg) therapy and were followed up for >1 year.

None had previously received NA or interferon/pegylated-interferon therapy. Patients with severe liver disease (chronic hepatitis C and autoimmune and alcoholic liver disease) were excluded.

Patient HBV DNA was measured at baseline and year 1 using the COBAS TaqMan HBV Test (Roche Diagnostics), with a lower limit and upper limit of detection of 60 copies/mL and  $6.4 \times 10^8$  copies/mL, respectively. Optimal virological response was defined as profound HBV DNA suppression to  $\leq 60$  copies/mL at the end of the first year. Partial virological response was defined as a HBV DNA level of  $>60$  copies/mL at year 1.

Baseline HBV rt sequence was amplified using a High Fidelity Taq Polymerase, followed by DNA sequencing. To identify sequence variants present at  $<20\%$  in the viral population, HBV amplicons of the partial responders and randomly selected optimal responders were subjected to clonal sequencing. Differences in the pretreatment DNA polymorphism and HBV quasispecies complexity and diversity between the optimal and partial responders were noted.

## Results

### Baseline characteristics

Among the 370 (129 HBeAg-positive and 241 HBeAg-negative) patients, 76 (21%) exhibited a partial virological response to entecavir at year 1. Of these partial responders, 41% (53/129) were HBeAg-positive and 9.5% (23/241) were HBeAg-negative

( $P < 0.0001$ ). The gender ratio, baseline alanine transaminase (ALT), albumin, and bilirubin levels were comparable between the optimal and partial responders (Table 1). The mean age was higher in the HBeAg-negative optimal responders than partial responders ( $P = 0.026$ ). The partial responders had a higher baseline HBV DNA level than the optimal responders for HBeAg-positive ( $P < 0.001$ ) and HBeAg-negative ( $P = 0.001$ ) patients.

### HBV rt sequence analysis

HBV rt DNA was successfully amplified by PCR in 305 patients (114 HBeAg-positive and 191 HBeAg-negative). Of these, 64 (21%) [47 HBeAg-positive and 17 HBeAg-negative] had a partial response to entecavir at year 1. For the partial responders, the

median HBV DNA level at year 1 was 639 copies/mL (range,  $72-2.98 \times 10^7$  copies/mL), and the median baseline HBV DNA level was higher than that of the optimal responders (HBeAg-positive patients:  $8.81$  vs  $7.83 \log_{10}$  copies/mL,  $P < 0.001$ ; HBeAg-negative patients:  $8.34$  vs  $6.62 \log_{10}$  copies/mL,  $P = 0.001$ ).

Of the 344 amino acid residues in the HBV rt region, 217 were conserved among these 305 patients. Major known drug resistance mutations (rtL80I/V, I169T, V173L, L180M, A181V/T, T184G, A194T, S202I, M204I/V, N236T, and M250V) were not detected, except in one patient in whom concomitant rtL80I and rtL180M mutations were detected. Twenty amino acid variations (rt7D, rt7N, rt12Y, rt18K, rt53I, rt80I, rt118H, rt127W, rt128I, rt131S, rt135F, rt139Q, rt153W, rt180M, rt217R,

TABLE 1. Baseline characteristics of the optimal and partial entecavir responders

Characteristic	HBeAg-positive patients (n=129)			HBeAg-negative patients (n=241)		
	Optimal responders (n=76)	Partial responders (n=53)	P value	Optimal responders (n=218)	Partial responders (n=23)	P value
Mean±SD age (years)	42.6±12.3	40.7±10.8	0.36	51.3±9.3	46.8±8.4	0.026
No. of males:females	55:21	33:20	0.225	147:71	19:4	0.161
Median (range) hepatitis B virus DNA ( $\log_{10}$ copies/mL)	7.76 (4.40-8.81)	8.81 (5.41-8.81)	<0.001	6.43 (3.10-8.81)	7.64 (4.94-8.81)	0.001
Median (range) alanine transaminase (IU/L)	92.5 (12-2019)	107 (20-2144)	0.271	71.5 (11-3000)	93 (38-961)	0.133
Median (range) albumin (g/L)	42 (16-50)	42 (25-48)	0.657	42 (23-51)	42 (30-48)	0.763
Median (range) bilirubin (mmol/L)	14 (6-52)	12 (2-67)	0.162	11 (4-261)	14 (5-216)	0.086

TABLE 2. Distribution of reverse transcriptase (rt) polymorphisms found exclusively in partial responders receiving entecavir treatment for hepatitis B virus (HBV)

Gender	Age at baseline (years)	Baseline HBeAg status	HBV genotype	Baseline HBV DNA (copies/mL)	Year 1 HBV DNA (copies/mL)	rt variations exclusively found in partial responders
M	62.9	Positive	A	$>6.4 \times 10^8$	$5.5 \times 10^5$	rt7D + 53I + 139Q + 153W + 217R
M	62.3	Negative	C	$2.3 \times 10^8$	$3.6 \times 10^2$	rt7N
F	53.8	Positive	C	$>6.4 \times 10^8$	$4.4 \times 10^3$	rt12Y
M	22.3	Positive	B	$1.0 \times 10^8$	$1.6 \times 10^2$	rt18K
M	34.6	Positive	C	$1.0 \times 10^8$	$2.4 \times 10^2$	rt18K
M	48.2	Positive	C	$1.1 \times 10^8$	$2.1 \times 10^2$	rt80I + 180M
M	37.1	Negative	B	$2.2 \times 10^8$	$8.2 \times 10^1$	rt118H
M	28.7	Positive	B	$>6.4 \times 10^8$	$1.3 \times 10^3$	rt127W
M	37.6	Negative	B	$>6.4 \times 10^8$	$2.7 \times 10^2$	rt128I
M	45.7	Positive	B	$>6.4 \times 10^8$	$2.3 \times 10^2$	rt131S
M	31.4	Positive	B	$1.0 \times 10^8$	$1.6 \times 10^2$	rt135F
M	51.8	Positive	C	$8.0 \times 10^6$	$2.1 \times 10^3$	rt153W
M	54.8	Negative	C	$1.5 \times 10^5$	$8.5 \times 10^1$	rt219T
F	52.5	Negative	B	$>6.4 \times 10^8$	$4.5 \times 10^2$	rt233L
F	35.1	Positive	C	$>6.4 \times 10^8$	$4.5 \times 10^2$	rt322I
M	22.0	Positive	C	$1.0 \times 10^8$	$5.1 \times 10^4$	rt329N
F	39.9	Positive	C	$>6.4 \times 10^8$	$1.8 \times 10^3$	rt336Q

rt219T, rt233L, rt322I, rt329N, and rt336Q) were found exclusively in partial responders. These variations were distributed among 17 partial responders (Table 2). Variants rt18K and rt153W were found in two cases, and all other variants were found only once. Due to the rarity, association between these polymorphisms and partial/slow response was not significantly different and needed to be determined by phenotypic assays.

The distribution of some rt amino acid variations differed significantly between optimal and partial responders. Specifically, 17 amino acid variants were present at a significantly higher frequency in 64 partial responders than in the 241 optimal responders (Table 3). After controlling for false discovery during multiple comparisons using Bonferroni correction, four rt variants, namely rt 53N, rt118N, rt124N, and rt332S, remained to have a significantly higher frequency in the partial responders than in the optimal responders (Table 3).

Multivariate analysis was performed to determine the independent factors associated with partial entecavir response at year 1. All factors with significantly different distribution among the partial and optimal responders were analysed, namely baseline HBeAg status, age, HBV genotype, baseline HBV DNA level, rt53N, rt118N, rt124N, and rt332S (the four rt variants with differential distribution) (Table 3). Multivariate logistic regression analysis revealed that high baseline HBV DNA levels ( $P < 0.0001$ , OR=2.30, 95% CI=1.60-3.34), HBeAg-

positivity ( $P < 0.001$ , OR=3.68, 95% CI=1.79-7.62), and rt variant rt124N ( $P = 0.001$ , OR=3.09, 95% CI=1.54-6.18) were independently associated with suboptimal entecavir response at year 1.

### Minor sequence variations identified by clonal sequencing

Clonal sequencing was performed on the 64 partial responders and 34 randomly selected optimal responders. Among the polymorphisms found exclusively in the partial responders (Table 2), the variant rt18K was detected as minor species in five additional cases, rt118H was detected in one additional case, rt128I in two additional cases, rt131S in four additional cases, rt135F in one additional case, and rt153W in one additional case.

### HBV Quasispecies complexity and diversity

Quasispecies complexity and diversity were compared between the optimal and partial responders. The optimal responders had a higher quasispecies complexity (higher normalised Shannon entropy) than the partial responders at the nucleotide level ( $P = 0.036$ ) and at the amino acid level ( $P = 0.087$ ) [Table 4]. Similarly, the optimal responders had a higher quasispecies diversity than the partial responders (all  $P < 0.05$ , Table 4).

## Discussion

Entecavir is a potent nucleoside analogue that

TABLE 3. Reverse transcriptase (rt) variants with significantly different distribution among partial responders and optimal responders

rt variant	% of variants found in partial responders	% of variants found in optimal responders	P value (unadjusted)	P value (after Bonferroni correction)
rt9H	68.8	53.0	0.025	1
rt16T	50.0	29.6	0.002	0.254
rt53N	51.6	27.4	0.00024	0.031
rt109S	54.7	35.3	0.005	0.635
rt118N	48.4	25.3	0.00034	0.043
rt121I	51.6	29.5	0.001	0.127
rt124N	50.0	23.7	0.000038	0.0048
rt127R	46.9	29.0	0.007	0.889
rt131N	50.0	28.2	0.001	0.127
rt134N	35.9	16.2	0.00048	0.061
rt151Y	51.6	30.3	0.001	0.127
rt221Y	53.1	37.8	0.026	1
rt222A	40.6	21.6	0.002	0.254
rt238H	50.0	31.1	0.005	0.635
rt271M	48.4	26.1	0.001	0.127
rt278V	70.3	54.8	0.025	1
rt332S	43.8	20.1	0.00010	0.013

TABLE 4. Quasispecies complexity and diversity of the entecavir optimal and partial responders

Quasispecies	Optimal responders (n=34)	Partial responders (n=63)	P value
Complexity: normalised Shannon entropy			
Mean (range) nucleotide level	0.9673 (0.6868-1)	0.9316 (0.3324-1)	0.036
Mean (range) amino acid level	0.8668 (0.4930-1)	0.7869 (0.2192-1)	0.087
Diversity			
Mean (range) genetic distance (nucleotide level) [10 <sup>-3</sup> substitutions]	8.7083 (3.2747-36.4403)	5.2904 (0.8906-56.1354)	0.019
Mean (range) genetic distance (amino acid level) [10 <sup>-3</sup> substitutions]	14.0147 (3.3561-66.2545)	8.6151 (0.8923-94.822)	0.032
Mean (range) No. of synonymous substitutions per site (10 <sup>-3</sup> )	12.4076 (3.8811-42.0139)	7.4706 (0.6533-78.0112)	0.015
Mean (range) No. of non-synonymous substitutions per site (10 <sup>-3</sup> )	6.6121 (1.6396-30.8776)	3.9649 (0.4040-43.8353)	0.039

suppresses HBV DNA replication. The present study showed that 21% of patients still had detectable HBV DNA after 1 year of therapy. HBV rt sequence analysis of 64 partial responders and 241 optimal responders showed that 20 HBV rt variants were present exclusively in partial responders. However, these variants occurred only in one or two cases, and the association was not significant. In addition to the 20 variants found solely in partial responders, four variants were present in a higher proportion in the partial responders than in the optimal responders. Multivariate analysis showed that high baseline HBV DNA, HBeAg-positivity, and the rt variant rt124N were independent factors associated with slow entecavir response. This is consistent with our previous study in which patients with positive HBeAg and HBV DNA of >8 log copies/mL had a lower rate of HBV DNA undetectability at year 1 to 3.<sup>1</sup> rt124N variant was associated with partial entecavir response, even though only 50% of the partial responders harboured this variant. Further long-term study is needed to demonstrate the possible role of rt124N and the variants identified solely in the partial responders in susceptibility to entecavir.

Two case reports have identified HBV rt variants S219A + Y245H and S246T in entecavir non-responders.<sup>2,3</sup> Phenotypic assays determined that the rt variant pattern S219A + Y245H (rather than S246T) are associated with a lower susceptibility to entecavir in vitro. The present study did not identify S219A + Y245H variants. Nevertheless, the findings of rt124N and the 20 'unique' variants may provide a preliminary identification of putative variant pattern of reduced susceptibility to entecavir.

In the clonal sequencing data, the optimal responders had a significantly higher quasispecies complexity and diversity than the partial responders. It is possible that in the optimal responders a higher complexity and diversity was present. There may be a 'less-fit' subpopulation of viral quasispecies more susceptible to entecavir. In contrast, in the partial responders, the viral population was less diverse

and more replication competent, causing a slower entecavir response. Further studies using technology with a greater sequencing depth (such as ultra-deep pyrosequencing or next generation sequencing) are needed to further elucidate the association between quasispecies complexity/diversity and treatment response.

## Conclusions

We identified 20 rt variants found exclusively in entecavir partial responders and four rt variants with significantly different distribution among the optimal and partial responders. High baseline HBV DNA levels, HBeAg-positivity, and rt124N were associated with partial entecavir response at year 1. The possible association between rt variants and entecavir response needs to be proved by in vitro phenotypic studies.

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