Undetectable HBV DNA at month 12 of entecavir treatment predicts maintained viral suppression and HBeAg-seroconversion in chronic hepatitis B patients at 3 years

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SUMMARY

Background
On-treatment monitoring of serum hepatitis B virus (HBV) DNA to guide treatment strategy for patients on entecavir has received little attention.

Aim
To investigate the predictive value of on-treatment HBV DNA levels for responses to entecavir.

Methods
This was a retrospective cohort study among nucleos(t)ide analogue-naïve HBV-infected patients on entecavir with a minimum follow-up of 2 years. Maintained virological suppression was defined as undetectable HBV DNA (<20 IU/mL) until the last visit. Genotypic drug resistance was screened by using the INNO-LiPA DR assay.

Results
A total of 440 chronic hepatitis B patients (160 HBeAg-positive) followed for 34 ± 9 months were included. The cumulative probability of maintained virological suppression at year 1, 2 and 3 were 76.5%, 83.0% and 88.3% respectively. On multivariate analysis, lower baseline HBV DNA, undetectable HBV DNA at month 12 and negative HBeAg were the independent predictors of maintained virological suppression. M12 responders (who had undetectable HBV DNA at month 12) had higher probability of maintained virological suppression at 3 years (99.1%) as compared to non responders (57.5%; P < 0.001). The cumulative probability of HBeAg-seroconversion at year 1, 2 and 3 were 19.0%, 27.2% and 33.5% respectively. M12 responders had higher probability of HBeAg-seroconversion at 3 years (43.2%) than the non responders (19.0%; P = 0.003). M12 responders had lower probability of drug resistance at 3 years (0%) than the non responders (2.6%; P = 0.004).

Conclusion
Month 12 HBV DNA responses could predict the probability of maintained virological suppression, HBeAg-seroconversion and risk of drug resistance among patients on entecavir treatment at 3 years.

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INTRODUCTION
Chronic hepatitis B is the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) in Asia. Large prospective Asian studies have demonstrated that high level of hepatitis B virus (HBV) DNA is an independent risk factor for the development of HCC. In a recent meta-analysis, suppression of HBV DNA by antiviral therapy can reduce the risk of HCC by approximately 80%. However, the clinical benefit may be negated by viral relapse after cessation of antiviral treatment. Hence maintained viral suppression is a widely accepted surrogate marker to predict long-term outcome in nucleos(t)ide analogue-treated patients.

The importance of early viral suppression to reduce the risk of drug resistance to nucleos(t)ide analogues has been addressed in several studies. Greater HBV DNA reduction at 12 or 24 weeks was associated with reduced risk of subsequent lamivudine resistance. In the GLOBE study comparing the efficacy of telbivudine with lamivudine in both hepatitis B e antigen (HBeAg)-positive and HBeAg-negative chronic hepatitis B, complete viral suppression at 24 weeks of therapy was associated with reduced risk of drug resistance in patients in both treatment arms at the end of 2 years. Adefovir dipivoxil has a slower HBV DNA suppression as compared to lamivudine and telbivudine, and week 48 is a more appropriate time point to predict its effectiveness. On-treatment HBV DNA monitoring has formed the backbone of the ‘roadmap’ concept to modify antiviral treatment regimen, particularly for drugs with a lower genetic barrier of resistance. This is particularly important in the Asia Pacific region where drug cost is a major concern influencing the choice of antiviral therapy.

Entecavir is a potent antiviral agent superior to lamivudine and adefovir for virological suppression. Among treatment-naïve patients, extended use of entecavir has been associated with a very low risk of drug resistance up to 5 years. Data on the use of on-treatment HBV DNA suppression to predict treatment response and drug resistance to entecavir are lacking. The European guideline defined complete virological response to entecavir as HBV DNA undetectable at week 48, and partial virological response as more than 1 log decline in HBV DNA from baseline but a detectable viral load at week 48. However, the role of on-treatment virological response to guide subsequent treatment strategy is still controversial. This study included a real-life cohort of entecavir-treated chronic hepatitis B patients in a tertiary centre aiming to investigate the predictive value of on-treatment serum HBV DNA levels for long-term virological response.

MATERIALS AND METHODS

Study population
This was a retrospective study among all nucleos(t)ide analogue-naïve chronic hepatitis B patients who were started on entecavir on or before September 2009 in the Hepatitis Clinic, Prince of Wales Hospital. These patients were identified by case note review and from the database of HBV DNA monitoring in the laboratory. We included patients who had positive hepatitis B surface antigen (HBsAg) for 6 months or more, had been put on entecavir 0.5 mg daily for the treatment of chronic hepatitis B, had baseline and month 12 serum HBV DNA level results available; and had received at least 24 months of entecavir treatment with an HBV DNA checked at month 12 or beyond. We excluded patients whose serum HBV DNA levels were not available at baseline and month 12, those with concomitant liver diseases such as chronic hepatitis C, autoimmune or metabolic liver diseases (not including fatty liver), previous or concurrent use of oral nucleos(t)ide analogues (lamivudine, adefovir dipivoxil, entecavir, telbivudine and/or tenofovir) before entecavir treatment, use of interferon or peginterferon within 6 months of entecavir treatment and moribund state.

Clinical evaluation
All patients fulfilling the inclusion criteria were identified and the case notes were retrieved and studied. Patients taking entecavir were regularly followed up every 3–6 months, with serum HBV DNA monitoring at least every 6 months. Information including drug compliance, serial blood results including liver biochemistry, alpha fetoprotein, HBeAg, antibody against HBe (anti-HBe) and HBV DNA levels were collected from the case notes and the hospital computer database. Any change in antiviral therapy was also recorded. In our laboratory, HBV DNA was measured by Taqman real-time polymerase chain reaction (PCR) assay validated against the EUROPHEP standard with a linear range of detection from 20 to $2 \times 10^8$ IU/mL.

Clinical endpoints
The primary endpoint was the cumulative probability of maintained viral suppression, which was defined as undetectable serum HBV DNA maintained until last visit, and the time of reaching this primary endpoint was defined as the time at which serum HBV DNA first turned undetectable. The secondary endpoints were the cumulative probabilities of virological breakthrough, entecavir resistance,
HBeAg-seroconversion (in HBeAg-positive patients), and HBSAg-seroclearance. Virological breakthrough was defined as an increase of serum HBV DNA level by at least 1 log from the nadir during treatment.21

Detection of HBV variants conferring resistance to entecavir
For patients with detectable on-treatment serum HBV DNA at the last visit, a prototype line probe assay (LiPA), the INNO-LiPA DR (version 2 & 3) assay (LiPA Innogenetics, Ghent, Belgium), was used to detect and characterise the HBV polymerase mutations associated with entecavir resistance (supplementary Table S1).22 A practical feature of this assay is that the single-stage PCR product can be used for combined hybridisation of strips from the INNO-LiPA DR assay (version 3) and INNO-LiPA DR assay (version 2), the latter of which is used to detect the main variants resistant to lamivudine and adefovir dipivoxil.23 The INNO-LiPA DR assay was performed according to the manufacturer’s instructions. The INNO-LiPA DR assay can detect a variant in a mixed population when the variant accounts for more than 5% of the total population with a sensitivity of approximately 200 IU/mL.22

Statistical analyses
Statistical analysis was performed by using Statistical Package for Social Science (SPSS version 15.0, Chicago, IL, USA). Continuous variables were expressed in mean ± standard deviation or median (range) as appropriate. Qualitative and quantitative differences between subgroups were analysed using χ2 test or Fisher’s exact test for categorical parameters, and Student’s t-test or Mann–Whitney test for continuous parameters as appropriate. The Kaplan–Meier method was used to estimate the cumulative probability of different virological responses. The log-rank test was used to compare time-to-event curves between the patient cohorts with detectable or undetectable HBV DNA at month 12. Cox proportional hazard model was used to identify possible covariates as significant predictors of long-term virological responses. All statistical tests were two-sided. Statistical significance was taken as P < 0.05.

RESULTS

Baseline characteristics and on-treatment virological responses
From December 2005 to October 2011, 680 patients have taken entecavir for at least 24 months in the hepatitis clinics of Prince of Wales Hospital, Hong Kong. After excluding 217 patients who had previous treatment with other oral nucleos(t)ide analogues and 23 patients with unavailable results for baseline and/or month 12 serum HBV DNA, 440 patients were included in the analysis. None of the patients not included in the analysis as they received less than 24 months of entecavir were switched to other antiviral regimens because of suboptimal response. The clinical characteristics of these patients are shown in Table 1. These patients were predominantly men at mean age of 51 ± 11 years. Sixteen (3.6%) patients received (peg)interferon for a median of 11 (8–15) months prior to the use of entecavir. A total of 324 (73.6%) patients achieved undetectable HBV DNA at month 12, i.e. complete virological response as defined by the European guideline; whereas 114 (25.9%) achieved partial virological response and 2 (0.5%) patients were primary non-responders (i.e. <1 log reduction of HBV DNA).19

Prediction of virological responses
The mean follow-up duration of this patient cohort was 34 ± 9 months, and all patients had at least 24 months follow-up. The overall cumulative probability of maintained viral suppression at year 1, 2 and 3 were 76.5%,

| Table 1 | Baseline clinical characteristics of patients with respect to month 12 HBV DNA response |
|-----------------|-----------------|-----------------|------|
| Virological response at month 12 | HBV DNA undetectable (N = 324) | HBV DNA detectable (N = 116) | P values |
| Follow-up (months) | 35 ± 9 | 33 ± 9 | 0.07 |
| Male gender | 209 (65%) | 83 (72%) | 0.19 |
| Age (years) | 52 ± 11 | 49 ± 12 | 0.02 |
| Previous (peg)interferon therapy | 12 (4%) | 4 (4%) | 0.91 |
| Platelet (×10^9/L) | 173 ± 111 | 178 ± 65 | 0.24 |
| Creatinine (mmol/L) | 86 ± 57 | 98 ± 115 | 0.22 |
| Albumin (g/L) | 41 ± 8 | 41 ± 7 | 0.47 |
| Total bilirubin (µmol/L) | 33 ± 112 | 26 ± 49 | 0.18 |
| Alanine aminotransferase (IU/L) | 181 ± 338 | 212 ± 425 | 0.85 |
| Hepatitis B e antigen (HBeAg) | | | |
| Positive | 96 (30%) | 64 (55%) | <0.001 |
| Negative | 228 (70%) | 52 (45%) | |
| Baseline HBV DNA (log IU/mL) | 5.6 ± 1.4 | 6.6 ± 1.3 | <0.001 |
| Data are presented in mean ± s.d. | | | |
83.0% and 88.3% respectively. On survival analysis, more patients achieved and maintained viral suppression if HBV DNA was undetectable at month 12 (Figure 1). The 3-year cumulative probability of maintained viral suppression in patients with undetectable HBV DNA at month 12 was 99.1% [95% confidence interval (CI) 98.6%–99.6%], which was higher than that in patients with detectable serum HBV DNA at month 12 (57.5%; 95% CI 52.7%–62.3%; \( P < 0.001 \)). Undetectable month 12 HBV DNA had high sensitivity (81.2%), specificity (89.4%) and positive predictive value (98.5%) to predict maintained viral suppression at last visit, while its negative predictive value was 36.2%. The current sample size had statistical power of 99% to detect this 41.6% difference in maintained virological suppression between two cohorts at 5% significance level.

In the Cox proportional hazards model, undetectable HBV DNA at month 12, lower baseline serum HBV DNA level and negative HBeAg status, were independent factors predicting maintained viral suppression (Table 2). Age, gender ratio and ALT levels at baseline were not associated with maintained virological suppression.

**Prediction of virological breakthrough**

Overall, nine patients have virological breakthrough. The cumulative probability of virological breakthrough at year 1, 2 and 3 were 0.2%, 0.7% and 3.1% respectively. There was no association between on-treatment HBV DNA suppression at month 12 with the cumulative probability of virological breakthrough (Figure 2). The 3-year cumulative probability of virological breakthrough was comparable in patients with undetectable HBV DNA at month 12 (2.4%; 95% CI 1.3%–3.5%) vs. patients with detectable serum HBV DNA at month 12 (5.1%; 95% CI 2.5%–7.7%; \( P = 0.11 \)). Detectable month 12 HBV DNA had high specificity (74.0%) and negative predictive value (98.5%) to predict virological breakthrough at last visit, while its sensitivity and positive predictive value was 44.4% and 3.4% respectively.

Among the nine patients having virological breakthrough, four patients were found to have poor adherence to entecavir (taking entecavir in less than 80% of time); one of them had serum HBV DNA become undetectable 18 months after improving his drug adherence. Among those patients with apparent good drug adherence, one patient had entecavir stepped up from 0.5 mg daily to 1.0 mg daily empirically at 18 months, and his serum HBV DNA became undetectable 20 months after stepping up the entecavir dosage. Another patient had his serum HBV DNA become undetectable 12 months after the virological breakthrough without changing the entecavir dosage. Two patients had serum HBV DNA that remained detectable until last visit, but no drug resistance was detected by the INNO-LiPA DR assay. The remaining patient was subsequently found to have entecavir resistance (see below).

**Virological responses in HBeAg-positive patients**

The clinical characteristics of the 160 HBeAg-positive patients were shown in Table 3. Ninety-six (60.0%) HBeAg-positive patients achieved undetectable HBV DNA at month 12. The baseline serum HBV DNA levels
were lower in those with undetectable HBV DNA at month 12 (6.4 ± 1.4 log IU/mL vs. 6.8 ± 1.4 log IU/mL, P = 0.04), otherwise patients with detectable or undetectable HBV DNA at month 12 had comparable baseline characteristics (Table 3).

On survival analysis, more HBeAg-positive patients achieved maintained viral suppression if HBV DNA was undetectable at 12 (supplementary Figure S1A). The 3-year cumulative probability of maintained viral suppression in patients with undetectable HBV DNA at month 12 was 97.9% (95% CI 96.4%–99.4%), which was higher when compared to patients with detectable serum HBV DNA at month 12 (6.4 ± 1.4 log IU/mL vs. 6.8 ± 1.4 log IU/mL, P = 0.04). On the other hand, the 3-year cumulative probability of virological breakthrough was comparable in HBeAg-positive patients with undetectable HBV DNA at month 12 (3.0%; 95% CI 0.9%–5.1%) vs. patients with detectable serum HBV DNA at month 12 (8.9%; 95% CI 4.4%–13.4%) (P = 0.17; supplementary Figure S2A).

The cumulative probability of HBeAg-seroconversion at year 1, 2 and 3 were 19.0%, 27.2% and 33.5% respectively. Patients who had undetectable HBV DNA at month 12 had a higher probability of HBeAg-seroconversion at the last follow-up visit (Figure 3). HBeAg-positive patients with undetectable HBV DNA at month 12 had higher 3-year cumulative probability of HBeAg-seroconversion (43.2%; 95% CI 38.1%–48.3%) as compared to those with detectable serum HBV DNA at month 12 (19.0%; 95% CI 14.1%–23.9%; P = 0.003). Undetectable month 12 HBV DNA had modest sensitivity (75.9%) and negative predictive value (79.4%) to predict HBeAg-seroconversion at last visit, while its specificity and positive predictive value was 48.1% and 43.2% respectively. No patient developed HBsAg-seroclearance during the follow-up period.

### Virological responses in HBeAg-negative patients

The clinical characteristics of the 280 HBeAg-negative patients are shown in Table 3. A total of 228 (81.4%) HBeAg-negative patients had undetectable HBV DNA at month 12. The baseline serum HBV DNA levels were again lower in those with undetectable HBV DNA at month 12 (5.5 ± 1.3 log IU/mL vs. 6.1 ± 1.3 log IU/mL, 207 139 83 43

**Figure 2 | Cumulative probability of virological breakthrough in relation to serum HBV DNA at month 12.**
P = 0.04), otherwise patients with detectable or undetectable HBV DNA at month 12 had comparable baseline characteristics (Table 3).

On survival analysis, more HBeAg-negative patients achieved and maintained viral suppression if HBV DNA was undetectable at month 12 (supplementary Figure S1B). The 3-year cumulative probability of maintained viral suppression in patients with undetectable HBV DNA at month 12 was 99.5% (95% CI 99.0%–100%), which was higher when compared to patients with detectable serum HBV DNA at month 12 (71.7%; 95% CI 65.2%–78.2%; P < 0.001). On the other hand, the 3-year cumulative probability of virological breakthrough was again comparable in HBeAg-negative patients with undetectable HBOV DNA at month 12 (2.2%; 95% CI 0.9%–3.5%) vs. patients with detectable serum HBV DNA at month 12 (0%; 95% CI 0%–0%) (P = 0.43; supplementary Figure S2B).

**HBV variants conferring resistance to entecavir therapy**

All the 73 PCR-positive serum samples at the last visit were tested for drug resistance testing by INNO-LiPA assay. However, only 18 of these 73 on-treatment serum samples were amplifiable by the assay. The median (range) HBV DNA level of the 55 PCR-negative serum samples was 62 (20–182) IU/mL. Supplementary Table S2
shows the details of the LiPA results of these 18 serum samples. Three patients (Patient 1, 3 and 4) were found to have polymerase gene mutation that could confer resistance to entecavir (supplementary Table S2). Patient 1 only had rtS202C substitution without changes in rt180 or rt204; Patient 3 had the triple amino acid substitutions and Patient 4 had double substitutions conferring resistance to entecavir. The overall cumulative probability of entecavir resistant mutant development was 0.5% (95% CI 0.2–0.8%) at year 1, 0.7% (95% CI 0.3–1.1%) at year 2 and 0.7% (95% CI 0.3–1.1%) at year 3. Patients with detectable serum HBV DNA at month 12 had significantly higher cumulative probability of entecavir resistant mutant development (1.7%, 2.6% and 2.6% at year 1, 2 and 3 respectively) as compared to those with undetectable HBV DNA at month 12 (0% at year 1–3; \(P = 0.004\)).

To determine the timing of emergence of the entecavir resistant mutants, LiPA assay was performed in the previous stored serum samples of these three patients. Patient 1 had virological breakthrough and entecavir resistant mutant detected at the last visit at year 2 when patient’s drug adherence to entecavir was found to be poor, while no entecavir resistant mutant was detected at the baseline and year 1 samples (supplementary Table S3). Unfortunately this patient died of heart disease shortly after drug compliance was reinforced, hence, no information was available on the subsequent viral control. Patient 3 had detectable HBV DNA during the entire course of entecavir treatment. A mixed wild type mutant and entecavir resistant mutants were found at baseline as well as at year 1, but no exposure to lamivudine was found on detailed examination of the case history (supplementary Table S3). This patient received add-on adefovir dipivoxil at month 24, and the dose of entecavir was increased to 1 mg daily at month 42 as the viral suppression was still suboptimal. At month 45, while the serum HBV DNA level was 76 000 IU/mL, the drug regime was changed to tenofovir monotherapy. The serum HBV DNA level decreased to 2400 IU/mL 6 months after tenofovir monotherapy. Patient 4 had suboptimal viral response as serum HBV DNA remained detectable at 870 IU/mL after receiving 18 months of entecavir 0.5 mg daily despite good drug adherence all along. The dosage of entecavir was stepped up to 1.0 mg daily since 18 months but the serum HBV DNA remained detectable at 1726 IU/mL 12 months later. A mixed wild type mutant and entecavir resistant mutants were found at baseline as well as at year 1, but again no exposure to lamivudine was found on detailed examination of the case history.

Virological response to stepped-up dosage of entecavir

Five patients who had detectable HBV DNA at month 12 had entecavir dosage stepped up from 0.5 mg daily to 1.0 mg daily at 24 (18–30) months of therapy. All of them still had detectable serum HBV DNA levels (29–1726 IU) after a median of 11 (6–18) months of entecavir 1.0 mg daily treatment. The median reduction of serum HBV DNA was 0.3 (−0.3 to 0.9) log.

Virological response and clinical outcomes

During the follow-up period, four patients died and one patient developed HCC. Among patients with undetectable HBV DNA at month 12 (\(n = 324\)), one patient had HCC (0.3%), whereas three patients died (0.9%) of HCC, liver failure and colorectal carcinoma respectively. Among patients with detectable HBV DNA at month 12 (\(n = 116\)), one patient died (0.8%) of heart failure. The difference in death and HCC development was not statistically significant among two groups (\(P = 0.72\) and 0.74 respectively).

DISCUSSION

In this large-scaled real-life cohort study, we showed that chronic hepatitis B patients taking entecavir 0.5 mg daily had increasing probability of undetectable HBV DNA over the treatment. Although complete viral suppression to undetectable HBV DNA at month 12 could predict the probability of maintained viral suppression, HBeAg-seroconversion (in HBeAg-positive patients) and the development of entecavir resistant mutants in a median follow-up of 3 years, it did not influence the risk of virological breakthrough. This is in contrast to the observation on the use of nucleos(t)ide analogues with relative low genetic barrier of resistance such as lamivudine, adefovir dipivoxil and telbivudine.\(^6\)\(^–\)\(^10\) The difference was probably due to the overall low incidence of virological breakthrough among entecavir-treated patients, and virological breakthrough was often secondary to poor drug adherence instead of genotypic drug resistance to entecavir. Undetectable HBV DNA at month 12, on top of lower baseline HBV DNA and negative HBeAg status, was the most independent predictor (hazard ratio 7.9) of maintained viral suppression at 3 years.

Long-term follow-up of patients recruited into the pivotal entecavir trials demonstrated low incidence of resistance in nucleoside-naïve patients during 5 years of entecavir therapy.\(^18\) According to the study protocol, only 149 (41%) of the initially recruited 663 patients had been followed up for 2 years or more, and all patients
were shifted to entecavir 1.0 mg daily from 0.5 mg daily at 96 weeks. Therefore, the long-term virological responses of entecavir 0.5 mg daily, which is the recommended dose in clinical practice, cannot be evaluated. In a Japanese study among 167 patients with continuous entecavir 0.5 mg daily for up to 2.5 years, 140 (84%) patients were evaluable at the last follow-up and the probability of undetectable HBV DNA was approximately 88%. In another recent study in Hong Kong, 222 patients on entecavir 0.5 mg daily were studied and 188 (85%) and 101 (45%) patients were evaluable at years 2 and 3; the rate of undetectable HBV DNA at year 2 and 3 were 90.4% and 92.1% respectively. In the current study, to minimise the potential bias due to loss of patient follow-up, all 440 patients received entecavir 0.5 mg daily had followed up for at least 2 years. In line with previous reports, we also found a high and incremental cumulative probability of undetectable HBV DNA (76.5%, 83.0% and 88.3% at year 1, 2 and 3 respectively). We also confirmed a very low incidence of entecavir resistance (0.7% in 3 years) even vast majority of our patients were kept on entecavir 0.5 mg daily without increased dosage to 1.0 mg daily after month 24. Patients who developed entecavir resistance might either have poor drug adherence or pre-existing drug resistance before commencement of therapy.

On antiviral therapy, one clinical importance of HBeAg-seroconversion among HBeAg-positive patients is the possibility of stopping treatment. Among our 160 HBeAg-positive patients, the cumulative probability of HBeAg-seroconversion at year 1, 2 and 3 were 19.0%, 27.2% and 33.5% respectively. This was comparable to the registration trial of entecavir (21% at year 1 and 32% at year 2) and another report in Hong Kong (22.2% at year 1, 40.8% at year 2 and 43.9% at year 3). As we have no control group in this study, we could not comment on the comparative probability of HBeAg-seroconversion with other antiviral drugs. One interesting finding in our study was the association of on-treatment HBV DNA responses at month 12 with a higher chance of HBeAg-seroconversion. Rapid HBV DNA suppression might stimulate the T-cell response and enhance the probability of clearing the HBeAg. As the vast majority of patients have not stopped entecavir treatment at the time of data analysis, sustainability of HBeAg-seroconversion after stopping entecavir was not evaluated in this study. A recent study showed that early decline in HBeAg levels had a higher predictive value than that of HBV DNA for HBeAg-seroconversion in entecavir-treated HBeAg-positive patients. Nonetheless, quantitative HBeAg was not available in our laboratory hence this marker could not be evaluated in the current study.

Five of our patients received an increased dose of entecavir to 1.0 mg daily due to incomplete viral suppression by entecavir 0.5 mg daily. This resulted in only a further reduction of 0.3 (–0.3 to 0.9) log in serum HBV DNA and all patients still had detectable HBV DNA after a median of 11 (6–18) months. In other words, stepping up the dose of entecavir to 1 mg daily has only modest benefit among patients with incomplete viral suppression by entecavir 0.5 mg daily. On the other hand, switching to add-on tenofovir seems to have better virological suppression among suboptimal responders to entecavir. In a retrospective analysis of 42 Asian-American HBV-infected patients who had detectable HBV DNA after 6–12 months of entecavir monotherapy, 67% and 90% of them achieved complete viral suppression after switching to tenofovir monotherapy and add-on tenofovir to entecavir therapy respectively. Our results have potential important practical implications. Although undetectable HBV DNA at month 12 was predictive of undetectable HBV DNA and HBeAg-seroconversion at 3 years of entecavir treatment, it was not a risk factor of virological breakthrough and the overall risk of drug resistance remained low. There was only one patient who had genuine entecavir resistance, whereas the other two patients who had those amino acid substitutions were mainly secondary to non-adherence to the therapy. The cumulative probability of undetectable HBV DNA was also increasing with time on keeping entecavir at 0.5 mg daily. Apparently, increasing entecavir to 1.0 mg daily can only provide modest additional viral suppression. Although HBV DNA should still be regularly monitored during entecavir treatment, stepping up the dose of entecavir among patients who had incomplete viral suppression at month 12 cannot be recommended. Instead it is important to check patient’s drug adherence as well as genotypic drug resistance to entecavir. On the other hand, we failed to demonstrate any difference in hard clinical outcomes among patients with different on-treatment virological responses. We believe a longer follow-up duration is warranted to look for these clinical outcomes.

Our study has a few limitations. The retrospective nature of this study might have introduced some bias and incomplete data collection. One of the differences in the baseline characteristics was more patients with detectable HBV DNA at month 12 were HBeAg-positive, which partly explained the higher baseline HBV DNA in this
cohort. All the 16 patients who received previous (peg) interferon therapy were HBeAg-positive but none of them cleared HBeAg, such that they subsequently switched to entecavir as antiviral therapy. Patient inclusion and identification through the database of HBV DNA testing might miss those not adherent to follow-up. Fortunately, the compliance of HBV DNA monitoring every 6 months was high in entecavir-treated patients (>90%), as the test was largely free of charge to these patients. Despite 23 patients being excluded due to the lack of HBV DNA results, they had similar baseline characteristics as the study group (result not shown). Although our study has a reasonably long follow-up duration (mean 34 months), there might be more patients who developed virological breakthrough or even entecavir resistance in the future, particularly for those with persistently detectable serum HBV DNA. The lack of standard treatment protocol also led to heterogeneous treatment regimes in response to incomplete on-treatment viral suppression. For example, only five patients with detectable HBV DNA at month 12 had entecavir dosage stepped up from 0.5 mg daily to 1.0 mg daily, which implied that most of our patients with detectable HBV DNA did not follow the roadmap protocol for those with an incomplete virological response while on nucleos(t)ide analogue. This could be partly explained by the financial constraints of our patients, as entecavir 1.0 mg daily is not reimbursable in this region. Nonetheless we believe this might be an advantage that the results were reflecting the virological responses to entecavir in a real-life clinical setting.

In conclusion, month 12 HBV DNA responses could predict the probability of complete viral suppression and HBeAg-seroconversion but not virological breakthrough among patients on entecavir 0.5 mg daily treatment. Failure of complete HBV DNA suppression was associated with entecavir resistance secondary to poor drug compliance or pre-existing drug resistance before commencement of therapy, but the overall risk was very low at 0.7% in 3 years. Future studies will be needed on optimization of drug regime among month 12 partial responders to improve the virological suppression and to increase the chance of HBeAg-seroconversion.

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article:
Table S1. Interpretation of the prototype line probe assay (LiPA), the INNO-LiPA DR (version 2 & 3) assay (LiPA Innogenetics), for the detection and characterisation of the HBV polymerase mutations associated with entecavir resistance.
Table S2. Results of the line probe assay of the 18 serum samples with amplifiable HBV DNA results at last visit.
Table S3. Serial line probe assay results of the 3 patients with entecavir resistant mutants detected at last visit.
Figure S1. Cumulative probability of maintained viral suppression in relation to serum HBV DNA at month 12 in (A) HBeAg-positive and (B) HBeAg-negative patients.
Figure S2. Cumulative probability of virologic breakthrough in relation to serum HBV DNA at month 12 in (A) HBeAg-positive and (B) HBeAg-negative patients.
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REFERENCES
1. Chan HLY, Sung JJY. Hepatocellular carcinoma and hepatitis B virus. Semin Liver Dis 2006; 26: 153–61.
2. Chen CJ, Yang HI, Su J, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006; 295: 65–73.
3. Chan HLY, Tse CH, Mo F, et al. High viral load and hepatitis B virus subgenotype Ce are associated with increased risk of hepatocellular carcinoma. J Clin Oncol 2008; 26: 177–82.
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4. Sung JJY, Tsoi KKF, Wong VWS, et al. Meta-analysis: treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. *Aliment Pharmacol Ther* 2008; 28: 1067–77.

5. Wong VWS, Wong GLH, Chim AML, et al. Surrogate endpoints and long-term outcome in patients with chronic hepatitis B. *Clin Gastroenterol Hepatol* 2009; 7: 1113–20.

6. Chan HLY, Wang H, Niu J, et al. Two-year lamivudine treatment for hepatitis B e antigen-negative chronic hepatitis B: a double-blind, placebo-controlled trial. *Antivir Ther* 2007; 12: 345–53.

7. Yuen MF, Fong DY, Wong DK, et al. Hepatitis B virus DNA levels at week 4 of lamivudine treatment predict the 5-year ideal response. *Hepatology* 2007; 46: 1695–703.

8. Lai CL, Gane E, Liaw YF, et al. Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; 357: 2576–88.

9. Lui YYN, Chan HLY. Treatment of chronic hepatitis B: focus on telbivudine. *Expert Rev Anti Infect Ther* 2009; 7: 259–68.

10. Chan HLY, Heathcote EJ, Marcellin P, et al. Treatment of hepatitis B e antigen positive chronic hepatitis B with telbivudine or adefovir: a randomized trial. *Ann Intern Med* 2007; 147: 745–54.

11. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, et al. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; 131: 1743–51.

12. Keeffe EB, Zeuzem S, Koff RS, et al. Report of an international workshop: roadmap for management of patients receiving oral therapy for chronic hepatitis B. *Clin Gastroenterol Hepatol* 2007; 5: 890–7.

13. Wong GLH, Chan HLY. Predictors of treatment response in chronic hepatitis B. *Drugs* 2009; 69: 2167–77.

14. Chan HLY, Jia J. Chronic hepatitis B in Asia – New insight from the past decade. *J Gastroenterol Hepatol* 2011; 26(Suppl 1): 131–7.

15. Chang TT, Gish RG, de Man RA, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; 354: 1001–10.

16. Lai CL, Shouval D, Lok AS, et al. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; 354: 1011–20.

17. Leung N, Peng CY, Hann HW, et al. Early hepatitis B virus DNA reduction in hepatitis B e antigen-positive patients with chronic hepatitis B: a randomized international study of entecavir versus adefovir. *Hepatology* 2009; 49: 72–9.

18. Tenney DJ, Rose RE, Baldick CJ, et al. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; 49: 1503–14.

19. European Association For The Study Of The L. EASL clinical practice guidelines: management of chronic hepatitis B. *J Hepatol* 2009; 50: 227–42.

20. Chan HLY, Chui AK, Lau WY, et al. Factors associated with viral breakthrough in lamivudine monoprophylaxis of hepatitis B virus recurrence after liver transplantation. *J Med Virol* 2002; 68: 182–7.

21. Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–39.

22. Jardi R, Rodriguez-Frias F, Tabernero D, et al. Use of the novel INNO-LiPA line probe assay for detection of hepatitis B virus variants that confer resistance to entecavir therapy. *J Clin Microbiol* 2009; 47: 485–8.

23. Osioyewu C, Villeneuve JP, Heathcote J, et al. Detection of the rtN236T and rtA181V/T mutations associated with resistance to adefovir dipivoxil in samples from patients with chronic hepatitis B virus infection by the INNO-LiPA HBV DR line probe assay (version 2). *J Clin Microbiol* 2006; 44: 1994–7.

24. Yokosuka O, Takaguchi K, Fujioka S, et al. Long-term use of entecavir in nucleoside-naïve Japanese patients with chronic hepatitis B infection. *J Hepatol* 2010; 5: 791–9.

25. Yuen MF, Seto WK, Fung J, et al. Three years of continuous entecavir therapy in treatment-naïve chronic hepatitis B patients: viral suppression, viral resistance, and clinical safety. *Am J Gastroenterol* 2011; 106: 1264–71.

26. Gish RG, Lok AS, Chang TT, et al. Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; 133: 1437–44.

27. Boni C, Bertoletti A, Penna A, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* 1998; 102: 968–75.

28. Zhang X, Lin SM, Ye F, et al. An early decrease in serum HBeAg titre is a strong predictor of virological response to entecavir in HBeAg-positive patients. *J Viral Hepatitis* 2011; 18: e184–90.

29. Yip B, Trinh NH, Nguyen HA, et al. Response to alternative therapies in patients with chronic hepatitis B and entecavir (ETV) partial response (PR). *Hepatology* 2010; 52(Suppl 4): 535A.