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Viral minority variants in the core promoter and precore region identified by deep sequencing are associated with response to peginterferon and adefovir in HBeAg negative chronic hepatitis B patients

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ABSTRACT

Background and aim: Precore (PC) and basal core promoter (BCP) mutations are associated with responses to interferon-based treatment in HBeAg-positive chronic hepatitis B (CHB) patients. Here, we identify viral minority variants in these regions and assess association with response to peginterferon-alfa (Peg-IFN) and adefovir combination therapy.

Patients and methods: Ultra-deep pyrosequencing analysis of the BCP and PC region was performed for 89 CHB patients (42 HBeAg-positive; 47 HBeAg-negative), at baseline and during treatment. Specifically, associations of individual positions with the HBeAg-negative phenotype were studied, as well as the association of the most prevalent mutations with combined response in HBeAg-positive and −negative patients at week 72 (HBeAg negativity, HBV-DNA <2000 IU/mL and ALT normalization at 24 weeks of treatment-free follow-up).

Results: The mutations most strongly correlated with the HBeAg-negative phenotype were at positions 1762/1764 and 1896/1899 in the BCP and PC region, respectively. No major changes in nucleotide composition of these positions were observed during treatment. In HBeAg-negative patients, a combined presence of 1764A and 1896A was correlated with lower ALT levels ($p = 0.004$), whereas the presence of 1899A was correlated with higher age ($p = 0.030$), lower HBV-DNA level ($p = 0.036$), and previous IFN therapy ($p = 0.032$). The presence of 1764A/1896A or the absence of 1899A at baseline, was associated with lower response rates, after adjustment for HBV genotype ($p = 0.031$ and $p = 0.017$) and HBsAg level ($p = 0.035$ and $p = 0.022$).

Conclusion: We identified novel correlations between common BCP and PC variants with response to Peg-IFN and adefovir in HBeAg-negative patients. Ultimately, this may guide the selection of those patients most likely to benefit from Peg-IFN-based treatment.

Abbreviations: 454 DS, 454 deep sequencing; ALT, alanine aminotransferase; BCP, basal core promoter; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CR, combined response; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B *e* antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NUCs, nucleot(s)ide analogues; PC, precore; Peg-IFN, pegylated interferon alfa 2a; RMSD, root-mean-squared deviation; T1, time-point 1 (baseline); T2, time-point 2 (on-treatment); UDPS, ultra-deep pyrosequencing; ULN, upper limit of normal; Week 72, 24 weeks of treatment free follow-up; Week 144, two years of treatment free follow-up.

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1. Introduction

Prolonged hepatitis B virus (HBV) infection increases the risk of liver-related morbidity and mortality, including liver cirrhosis and hepatocellular carcinoma (Amin et al., 2006). Chronic hepatitis B (CHB) patients with a high viral load and active liver inflammation can currently be treated with either nucleos(t)ide analogues (NUCs) or pegylated-interferon alfa-2a (Peg-IFN) (European Association for the Study of the L, 2012; Lok and McMahon, 2009).

While NUCs potently reduce HBV-DNA and its associated complications (Marcellin et al., 2013), the HBV covalently closed circular DNA (cccDNA) within hepatocytes remains transcriptionally active, and discontinuation of NUCs usually results in recurrence of disease activity. In contrast, treatment with Peg-IFN can lead to a sustained off-treatment viral suppression in a subgroup of patients, but is associated with significant side-effects. Attempts to achieve improved outcomes with Peg-IFN and NUC combination therapy have been disappointing when lamivudine was included (Janssen et al., 2005; Lau et al., 2005; Marcellin et al., 2004). Despite this, the use of Peg-IFN with more potent NUCs remains of interest because of their proven differential effects on the innate and adaptive immune responses (Thimme and Dandri, 2013). Indeed, more recent studies show a benefit of Peg-IFN + NUC combination therapy, compared to both monotherapies (Brouwer et al., 2015; Marcellin et al., 2014). In addition, in our previously conducted study with a combination of peginterferon and adefovir a relatively high rate of HBsAg loss (11–17% at year 2) was observed, most specifically in HBeAg-negative patients (Takkenberg et al., 2013).

Nevertheless, the majority of patients are still treated without achieving a satisfactory outcome. It is therefore crucial to identify those patients who will benefit from Peg-IFN based treatment before the start of therapy, avoiding unnecessary use of this medication in patients with low chance of response. Previous research suggested several potential virus and host characteristics that were associated with response to treatment, including specific mutations in the viral genome.

The most extensively studied mutations in the HBV genome are those located in the precore (PC) and basal core promoter (BCP) region. Mutations in these regions have been implicated in reducing Hepatitis B *e* antigen (HBeAg) production and hence strongly associate with the HBeAg-negative status.

The most well-described PC mutation comprises a G-A substitution at nucleotide position 1896 of the HBV genome, creating a premature stop codon in the precore open reading frame which abrogates HBeAg synthesis. The proposed function of this mutation is related to its location in the encapsidation (ε) signal, which forms a secondary stem-loop structure of the RNA pregenome that is essential for the initiation of encapsidation of the pregenome (Lok et al., 1994). In the stem-loop, G1896A restores base pairing with T1858 leading to enhanced stability of this secondary structure (Lok et al., 1994). Similarly, the G1899A mutation further enhances the stability by base pairing with T1855.

In contrast, a cytosine (C) is present at position 1858 in HBV genotype A, F and some genotype C strains, making G1896 changes rare. In these patients mutations in the BCP region are preferably selected, whereas in other patients BCP mutations can be observed in conjunction with PC mutations (Chan et al., 1999). The most common BCP mutations, A1762T and G1764A, result in reduced HBeAg synthesis by influencing precore RNA transcription (Parekh et al., 2003; Buckwold et al., 1996).

Mutations in the PC and BCP region have previously been shown to influence IFN-based therapy response. Various studies, both with conventional IFN and Peg-IFN, associated an increased frequency of PC or BCP mutations with treatment-induced HBeAg seroconversion (Tseng et al., 2011; Chen et al., 2011; Tangkijvanich et al., 2009; Marrone et al., 2003; Erhardt et al., 2000). In contrast, a more recent study stated that the presence of BCP or PC mutations actually limits the probability of a sustained off-treatment (combined serological and virological) response to Peg-IFN (Sonneveld et al., 2012).

Importantly, data on the effect of PC and BCP mutations on IFN-response is limited to HBeAg-positive patients. Furthermore, HBV variants that coexist as minorities with wild-type strains may have been missed in previous studies since they were mostly performed with less sensitive direct sequencing methods. Next-generation sequencing techniques, based on ultra-deep pyrosequencing (UDPS), generate vast quantities of data and allow the quantitative analysis of mutant and wild-type species <1% (Eriksson et al., 2008). So far, UDPS data in HBV is limited to studies on HBeAg seroconversion in the natural history or resistance in nucleos(t)ide analogue treated patients (Homs et al., 2014; Rodriguez et al., 2013; Nishijima et al., 2012; Margeridon-Thermet et al., 2009).

Here, we studied mutations in the PC and BCP region in a well-characterized cohort of 89 chronic hepatitis B patients (42 HBeAg-positive; 47 HBeAg-negative) treated with a combination of Peg-IFN and adefovir. We aimed to shed new light on this well-studied region by analyzing mutational patterns quantified by UDPS both before and during treatment, and associate these with therapy response.

2. Patients and methods

2.1. Subjects

In total, 92 CHB patients with HBV-DNA levels above 100,000 copies/mL (17,182 IU/mL) participated in a prospective investigator-initiated study, of which detailed study characteristics have been described elsewhere (Takkenberg et al., 2013). In summary, patients were treated for 48 weeks with peginterferon alfa-2a 180 μg subcutaneously once a week, and adefovir dipivoxil 10 mg daily. After 48 weeks, treatment was discontinued and a treatment-free follow-up period started. The study was conducted according to the guidelines of the Declaration of Helsinki, with the principles of Good Clinical Practice and was approved by local ethics committees (*controlled-trials.com*; ISRCTN 77073364). All patients gave written informed consent.

Of the 92 patients treated in the initial study, 89 had baseline plasma samples available for sequencing analysis (Fig. 1). In addition, 84/89 patients completed 48 weeks of treatment and 2 years of follow-up, and comprised the per-protocol population to study associations with treatment response.

Achievement of a combined response (CR) and/or HBsAg loss (HBsAg<0.05 IU/mL) was determined after 24 weeks (week 72) and 2 years (week 144) of treatment-free follow-up. Combined response was defined as HBV-DNA levels ≤2000 IU/mL and persistent normal alanine aminotransferase (ALT) levels in both HBeAg-positive and -negative patients, including HBeAg loss in HBeAg-positive patients (European Association for the Study of the L, 2012).

2.2. Sample selection

For each patient a pre-treatment (baseline) plasma sample was subjected to both Sanger sequencing and 454 deep sequencing. In addition, an on-treatment sample was selected at Week 12 or at the latest time-point where the viral load was >10,000 IU/mL. Median sampling time of this second time-point was 12 weeks (iqr 6.1–24.0) for HBeAg-positive patients, and 1.6 weeks (iqr 0.6–4.0) for HBeAg-negative patients (Supplementary Fig. S1). Mean HBV-DNA levels at the second time-point were 3.94 (±0.61) and 3.52