Prevalence of hepatitis B virus precore stop codon mutations in chronically infected children

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Received: 2005-09-22 Accepted: 2005-11-18

Abstract

AIM: To find out whether there is a significant difference in the prevalence of the precore stop codon mutation between HBeAg positive and anti-HBe positive children.

METHODS: We investigated a large pediatric population of 155 European children (mean age 10.9 years) with chronic hepatitis B by PCR and direct sequencing. Ninety were HBeAg positive and 65 had seroconversion to anti-HBe. Additionally genotyping was performed.

RESULTS: Seventy-four (48%) of the sequenced HBV strains were attributed to genotype D and 81 (52%) to genotype A. In the group of 90 HBeAg positive patients, 2 (2.2%) 1896-G-to-A transitions leading to precore stop codon mutation were found, and in the group of 65 anti-HBe positive children, 5 (7.7%) were identified harbouring HBeAg-minus mutants. The difference was not statistically significant (P=0.13).

CONCLUSIONS: HBeAg minus variants as predominant viral HB strains play a minor role in the course of chronic hepatitis B in European children.

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Key words: Hepatitis B; Precore mutants; Children; HBeAg minus variants

Wintermeyer P, Gerner P, Gehring S, Karimi A, Wirth S. Prevalence of hepatitis B virus precore stop codon mutations in chronically infected children. World J Gastroenterol 2006; 12(14): 2235-2238

http://www.wjgnet.com/1007-9327/12/2235.asp

INTRODUCTION

Chronic hepatitis B (HB)-infection is a serious health problem worldwide and the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC). In Germany 0.3%-0.5% of the population are HBsAg carriers and it is estimated that approximately 5% of them are children. Mutations preventing the expression of HBeAg, so-called precore stop codon mutations, have been reported to aggravate liver disease and to cause fulminant hepatitis in children as well as in adults[1-3]. Currently it is not yet clear whether these mutations influence the response to antiviral treatment. However, a recent study has shown a better outcome after interferon-alpha treatment for chronic hepatitis in individuals without precore mutant strains before treatment[4]. The most common stop-mutation is the G1896A-substitution in the precore region of HB-virus. A guanine (G) to adenine (A) mutation of the HBV precore gene at nucleotide 1896 (numbered from the EcoRI site) leads to a conversion of codon 28 from TGG (tryptophan) to TAG, which is a stop codon, thereby rendering HBV incapable of producing HBeAg[5]. In adults the prevalence of this mutation ranges between 20%-95%, whereas it is more common in HBeAg-negative than in HBeAg-positive patients[6]. Results of a cross-sectional multicenter study of adults with chronic HBV infection in the United States show an association of the presence of precore variants with higher serum HBV DNA levels in HBeAg-negative but not in HBeAg-positive patients[7]. The selection of precore variants is dependent on HBV genotype. It is most common in patients with genotype D and rare in patients with genotype A[8]. This phenomenon is related to base pairing in the stem-loop structure of the pregenome encapsidation sequence[9,10].

Fukuda et al[11] have postulated that hepatitis B virus exists mainly as a quasi species. On account of this, a correlation of nucleotide sequences with clinical and serological findings has to be made with caution. Available data about prevalence and significance of precore mutations in childhood are limited. A study of children with chronic HBV infection (n=60) has shown the 1896-G-A-transition in 93% as mixed infection, with a similar prevalence of mixed viral populations in responders and non-responders to interferon treatment[12]. In the course of chronic HBV infection in children the frequency of precore mutant is increased after seroconversion from HBeAg to anti-HBe. It has been postulated that the higher
the aminotransferase levels are, the higher and the earlier the mutant emerges\textsuperscript{[13]}. The aim of this study was to evaluate the prevalence of precore mutations in a large cohort of European children with chronic hepatitis B.

MATERIALS AND METHODS

Patients
A cross sectional testing of sera from 155 randomly assigned HBsAg positive chronic hepatitis B virus carriers was performed. The median age of the children at the time of blood sampling was 10.9 years. Ninety were HBeAg positive and 65 had seroconversion to anti-HBe 1 - 3 years before blood sampling. Fifty-four (35\%) were female, 101 (65\%) male; 108 (70\%) of Caucasian, 42 (27\%) of Mediterranean and 5 (3\%) of Southeast Asian origin. ALT was elevated in 66 (73\%) of HBeAg positive individuals (mean: 1100 nkat/L) and in 18 (28\%) of anti-HBe positive individuals (mean: 367 nkat/L; normal <417 nkat/L).

HBV molecular analyses
DNA was extracted from sera with the QiaAmp blood kit (Qiagen, Chatsworth, CA, USA) and eluted with 50 \(\mu\)L distilled water according to the manufacturer’s recommendations. The precore region of the HBV genome was amplified and re-amplified with a proof reading of expand-polymerase (Expand High Fidelity PCR System) by nested PCR. The following primers were used for amplification (nucleotide positions are according to the unique EcoRI site\textsuperscript{[14]}): Sense P1: 5’-TGTCAGACGCCGACCTTGGAG-3’ (nt 1683-1702), anti-sense P2: 5’-CAATGCCTCAGGAAGCTCTAAGGC-3’ (nt 2045-2023); nested PCR: sense P3: 5’-GAGGTAGGGGATTCAAGAGGTCCAC-3’ (nt 1736-1756), anti-sense P4: 5’-GTTCCTGAACCTGGAGCCACCA-3’ (nt 2356-2380), sense P5: 5’-CGACTACCGTTTGGGACCGAC-3’ (nt 82-63). The PCR products were incubated with restriction enzymes Ava II and Dpn II (New England Biolabs, Inc., USA) according to the manufacturer’s recommendations. The products were visualized by 2 g/L agarose electrophoresis and ethidium bromide staining.

Statistical analysis
Fisher’s exact test was used for statistical analysis \(P<0.005\) was taken as significant.

RESULTS
A total of 155 patients were enrolled. Seventy-four (48\%) of the sequenced HBV strains were attributed to genotype D and 81 (52\%) to genotype A. In the group of 90 HBeAg positive patients, 2 (2.2\%) 1896-G-to-A transitions leading to precore stop codon were found, and in the group of 65 anti-HBe positive children, 5 (7.7\%) were identified harboring HBeAg-minus mutants. The difference was not statistically significant \(P=0.13\).

First, all sera were randomly selected for analysis without association with patients’ identities. We were then able to additionally investigate four HBeAg positive sera of the five patients who showed precore-mutations in anti-HBe positive status of the disease in order to find out if mutations already emerged in the HBeAg positive status. One of them was also positive for the G1896A-substitution in the HBeAg positive phase. All mutations identified were found in HBV DNA sequences of genotype D (Figures 1, 2).

The ALT levels in the two HBeAg positive sera with precore-mutation were 883 and 2983 nkat/L, reflecting a considerable elevation in one patient compared to the wild type sera of HBeAg positive status (mean: 1100 nkat/L). In the five anti-HBe positive individuals with the G1896A-substitution, the ALT levels were mildly elevated (mean: 467 nkat/L) compared to the group of 60 patients with wild type virus infection (mean: 367 nkat/L).

DISCUSSION
In contrast to previous studies with adults in particular, which reported HBeAg stop codon mutants in 20-95\% of investigated patients (6, 16), we found a very low prevalence in children, both in the HBeAg (2.2\%) and anti-HBe positive (8\%) phase of the disease. However, our results demonstrated consistently that precore variants were more common in anti-HBe positive children, but the difference did not reach statistical significance. We were
not able to show that mutations in anti-HBe positive sera have necessarily emerged already in the HBeAg positive phase of the disease. Thus, our data suggest that there is at least no strong evidence for the hypothesis that the mutant is selected by host immune pressure[13].

Due to the fact that the occurrence of the G1896A mutation is restricted to HBV genotypes with T at nucleotide position 1858 (genotype D)[17], it was not surprising that no mutations were identified in the group of genotype A virus strains. It has to be conceded that the existence of viral quasi-species including small amounts of precore stop mutations could not be excluded in our survey due to the method of direct sequencing. However, considering the high proportion of genotype D in our survey (48%), the postulation of a high prevalence of the G1896A precore mutation in these HBV strains[16] was not confirmed in chroniclly infected children.

It was reported that the precore mutation is common in Mediterranean and Asian populations, and two studies have shown a high prevalence of 16%-40% of the HBeAg minus variants in German adult populations as well[18,19]. It seems obvious that the age of the patients is more important than their ethnic origin.

Due to the elevated levels of transaminases in HBeAg positive sera, precore mutations may influence the inflammatory activity of the disease. But indeed, evidence is limited due to the small number of patients. In contrast to former studies, wherein a difference in clinical courses between genotypes B and C was described[26], this seems to be different for the most prevalent genotypes A and D in Europe, because they were equally distributed in our patients.

In summary, HBeAg minus variants as predominant viral HB strains play a minor role in the course of chronic disease in European children. Our results confirm the recent study of Söderström and colleagues who postulated that the most influential factors of HB-infection in childhood are epidemiologic parameters and the route of transmission[27].

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