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The association of HBV core promoter double mutations (A1762T and G1764A) with viral load differs between HBeAg positive and anti-HBe positive individuals: A longitudinal analysis☆

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***Background/Aims*: Although there have been a few reports regarding the effect of basal core promoter (BCP) double mutations (A1762T and G1764A) on hepatitis B viral loads, the association remains uncertain. We aim to determine the association after controlling for HBeAg – a strong confounding factor.**

***Methods*: We selected randomly 190 individuals from a Chinese cohort of 2258 subjects for cross-sectional analysis and 56 of the 190 for longitudinal analysis of viral loads.**

***Results*: In multivariable analysis of the cross-sectional data, BCP double mutations are significantly associated with lower viral loads in HBeAg positive subjects but no difference was found in anti-HBe positive subjects. Triple mutations at nucleotide (nt) 1753, 1762 and 1764 and mutations between nt 1809 and 1817, precore stop mutation (nt 1896) and genotype are not associated with viral loads in either HBeAg or anti-HBe positive subjects. Analysis of the longitudinal data yielded similar results to the cross-sectional data. Viral loads differ significantly between individuals infected with wild-type and BCP double mutations prior to HBeAg seroconversion but this difference is lost after seroconversion.**

***Conclusions*: BCP double mutations are associated with lower viral loads in HBeAg positive individuals but have no effect on the viral loads of anti-HBe positive individuals.**

***Keywords*: Hepatitis B virus; Basal core promoter double mutations; Viral loads; Hepatitis B e antigen (HBeAg); Anti-HBe**

1. Introduction

Hepatitis B virus (HBV) has a 3.2 kb circular DNA genome containing four partially overlapping open reading frames (ORFs): C, encoding the nucleocapsid (core) protein (HBcAg) and secreted e antigen (HBeAg); P, the polymerase protein (Pol); S, the envelope proteins; and X, a transcriptional *trans*-activator protein. The core promoter located at nucleotides (nt) 1591–1822 plays a central role in virus replication, directing the synthesis of the pregenomic RNA, which, as well

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Abbreviations: BCP, basal core promoter; ORF, open reading frame; AFP, alpha fetoprotein.

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as being the template for genome synthesis, encodes HBcAg and Pol, and the precore RNA, which encodes HBeAg [1]. The basal core promoter (BCP) has been mapped to nt 1744–1804 [2].

The lack of proof-reading during reverse transcription of the pregenomic RNA favours the development of sequence variants during long-term HBV replication [3]. One of the most critical changes is the appearance of double mutations at nt 1762 (A–T) and 1764 (G–A) in the BCP. The mutations were first reported by Okamoto et al. who suggested that they might arrest the transcription of the precore RNA but not seriously affect that of the pregenomic RNA [4]. However, others suggested that these mutations seem to be insufficient to generate a HBeAg-negative phenotype [5] but may suppress the expression of HBeAg [6]. Subsequently, studies involving transfection of human hepatoma cell lines and analysis of clinical samples showed that these double mutations suppress, but do not abolish, the synthesis of HBeAg and may also and increase HBV DNA replication [2,7,8].

However, other reports do not support the conclusion that BCP double mutations increase virus replication, in that the BCP double mutations seem to have no effect on viral load [9–12] or even may be associated with lower serum HBV DNA concentrations [13]. Furthermore, others found in transfection studies that core promoter mutations other than those at nt 1762/1764 seem to upregulate viral DNA replication [14,15]. Therefore, the effect of the BCP double mutations on viral loads remains uncertain.

HBeAg has been used as a marker of infectivity and active virus replication in HBsAg-positive individuals [16,17]. Seroconversion from HBeAg to anti-HBe, either spontaneous or after antiviral therapy, usually results in lower viral loads, this decline occurring usually up to 12 months before seroconversion [18]. Furthermore, because of the complexity of the host immune system, viral loads may fluctuate over time [19]. Therefore, a single measurement, or several measurements made around the time of clearance of HBeAg, may not give a clear view of the effect of BCP double mutations on viral loads. In this study, taking the advantage of our Chinese cohort [20], we have carried out a cross-sectional analysis to determine the association of BCP double mutations and viral loads and a 3 year-longitudinal analysis to test this association further.

2. Study subjects and methods

2.1. The Long An cohort

In order to determine the value of screening carriers of hepatitis B surface antigen (HBsAg) for virus with BCP double mutations as a marker of an extremely high risk of developing HCC, a cohort of 2258 hepatitis B surface antigen positive subjects aged 30–55 was recruited in Guangxi, China. Written informed consent was obtained from each individual. The study protocol conformed to the ethical guidelines of the 1975 declaration of Helsinki and has been approved by the Guangxi Institutional Review Board and the UCL Committee on the Ethics of Non-NHS Human Research (Project no. 0042/001) [20].

From 1st March, 2004, the Chinese study team travelled to 128 villages in 12 townships of Long An county to visit agricultural workers aged 30–55 for a 3 ml sample of blood by venepuncture for screening for HBsAg. All samples were tested for HBsAg and those positive were tested in China for HBV DNA using nested PCR. We also detected and excluded those positive for anti-HCV or alpha-fetoprotein (AFP) to eliminate the confounding effect of HCV infection on the incidence of HCC and pre-existing HCC. We started to follow up our study subjects from 1st July, 2004. Each study subject completed a one-page questionnaire at the first visit and provided a serum sample every six months for the assessment of virological parameters and AFP concentrations and was monitored for HCC by ultrasonography (US).

2.2. Study subjects

2.2.1. Cross-sectional analysis

We selected randomly approximately 25 subjects within each of eight sub-groups defined by the various combinations of sex (male or female), HBV status (HBeAg positive or anti-HBe positive) and BCP sequence (wild-type or 1762/1762 double mutations) (Table 1) [20].

2.2.2. Longitudinal analysis

From these eight groups, we selected 58 study subjects for longitudinal analysis. Eligible subjects were those who did not convert from HBeAg to anti-HBe or from anti-HBe to HBeAg, whose HBV sequences remained stable at nt 1762, 1764 and 1896 over the 3 years, and for whom additional samples were available at each of three time points (12, 24 and 36 months). We also selected subjects for the analysis of occurrences of seroconversion and sequence evolution, and the association of these events with viral loads, including 10 individuals with evolution from wild-type BCP to double mutations but without HBeAg seroconversion and 17 individuals with clearance of HBeAg without evolution of the BCP. No subject received antiviral or immunosuppressive therapy during the 3-year period.

2.3. Serological testing

Sera were tested for HBsAg, HBeAg/anti-HBe using enzyme immunoassays (Zhong Shan Biological Technology Company, Limited, Guangzhou, China). Alanine aminotransferase (ALT) levels were determined using a Reitman kit (Sichuan Mike Scientific Technology Company, Limited, Chengdu, China).

2.4. Nested PCR for HBV DNA and nucleotide sequencing

DNA was extracted from 85 μl serum by pronase digestion followed by phenol/chloroform extraction. For nested PCR of the core promoter, first round PCR was carried out in a 50 μl reaction using primers B935 (nt 1240–1260, 5′-GAAGGTTTGTGGCTCCTCTG-3′) and MDC1 (nt 2304–2324, 5′-TTGATAAGATAGGGGCATTTG-3′), with 5 min hot start followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. Second round PCR was carried out on 5 μl of the first round product in a 50 μl reaction using primers CPRF1 (nt 1678–1695, 5′-CAATGTCAACGACCGACC-3′) and CPRR1 (nt 1928–1948, 5′-GAGTAACTCCACAGTAGCTCC-3′), with 5 min hot start followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

For nested PCR of the surface region, first round PCR was carried out in a 50 μl reaction using primers LSOB1 (nt 2739–2762, 5′-GGCATTATTTGCATACCCTTTGG-3′) and P2 [21] with 5 min hot start followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. Second round PCR was carried out on 5 μl of the first round products in a 50 μl reaction using primers SSEQ5 (226–246,