A new polymorphism in the GRP78 is not associated with HBV invasion

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INTRODUCTION

Hepatitis B virus (HBV) is one of the major infectious diseases worldwide and is responsible for significant morbidity and mortality, particularly in developing countries. Of patients with chronic HBV infection, a quarter develop chronic liver disease, which can be associated with hepatoma in some cases. Increasing evidence indicates that genetic factors influence the natural history of HBV infection. Recent studies have proposed that a number of polymorphisms influence the progression of patients with HBV infection.

The glucose-regulated protein 78 (GRP78), also called heat shock 70 ku protein, is a stress-inducible endoplasmic reticulum calcium-binding chaperone. The GRP78 pathway is one of the most important response pathways to disease-associated stress with HBV invasion. The GRP78 gene is not related to the clinical risk and acute exacerbation of HBV infection. Recent studies have proposed that a number of polymorphisms influence the progression of patients with HBV infection.

Abstract

AIM: To examine the association between -86 bp (T > A) in the glucose-regulated protein 78 gene (GRP78) and hepatitis B virus (HBV) invasion.

METHODS: DNA was genotyped for the single-nucleotide polymorphism by polymerase chain reaction followed by sequencing in a sample of 382 unrelated HBV carriers and a total of 350 sex- and age-matched healthy controls. Serological markers for HBV infection were determined by enzyme-linked immunosorbent assay kits or clinical chemistry testing.

RESULTS: The distributions of allelotype and genotype in cases were not significantly different from those in controls. In addition, our findings suggested that neither alanine aminotransferase/hepatitis B e antigen nor HBV-DNA were associated with the allele/genotype variation in HBV infected individuals.

CONCLUSION: -86 bp T > A polymorphism in GRP78 gene is not related to the clinical risk and acute exacerbation of HBV infection.

Key words: Acute exacerbation; Glucose-regulated protein 78; Hepatitis B virus; Single-nucleotide polymorphism

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The aim of the present study was to elucidate the potential association of -86 bp T > A, a new single-nucleotide polymorphism (SNP) from the estimated translation start site of GRP78, as a host genetic factor with the risk and acute exacerbation of HBV infection in a Chinese population.

MATERIALS AND METHODS

Subjects
A total of 382 unrelated HBV carriers (233 males and 149 females) between 24 and 39 years old (average age 30.5 ± 7.2 years) and a total of 350 sex- and age-matched healthy volunteers (203 males and 147 females) aged between 22 and 36 years old (average age 27.8 ± 7.0 years) who had no history of HBV infection or other conspicuous diseases from the Affiliated Tumor Hospital of Guangzhou Medical College were included in this study. The two groups had a similar frequency of distribution in age and gender (P > 0.05) (Table 1). The diagnosis of HBV infection was based on the presence of hepatitis B surface antigen and hepatitis B e antigen (HBeAg) or e antibodies, together with the absence of anti-HBs, for at least 36 mo prior to enrolment. The present study was approved by the Ethics Committee of Sun Yat-sen University and adhered to the tenets of the Declaration of Helsinki. Informed consent was also obtained from each participant.

PCR and resequencing
Blood samples were obtained and stored at -80°C until DNA extraction. Genomic DNA was extracted from peripheral blood leukocytes using the QIAGEN QIAamp DNA Mini Blood Kit (Hilden, Germany). The primers for PCR and resequencing were designed based on the published Homo sapiens GRP78 DNA sequence (GenBank access No. NT_008470.18). Oligonucleotide primers were synthesized on a oligonucleotide synthesizer (Applied Biosystem, ABI) by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

PCRs were performed in 50 μL reaction systems containing 200 ng sample DNA, 5 μL of 10 × Ex Taq buffer (Mg2+ free; Takara, Japan), 2 mmol/L MgCl2, 20 pmol of each primer (forward primer, AAGGGAGAACAACGAGTAG and reverse primer, TGTCCTGGAATTGTAAGC), 0.2 mmol/L of each deoxynucleoside triphosphate, and 5 U Ex Taq polymerase (Takara, Shiga, Japan). The amplification was performed using initial denaturation at 94°C for 10 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 90 s on a GeneAmp 9700 thermal cycler (PerkinElmer Applied Biosystems, Inc., Foster City, CA, USA).

Resequencing was performed with the primer ATC CGC AAC CCC ACT TAC C, Taq polymerase, ABI PRISM® BigDye™ terminators on an ABI 3730xl DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA).

Table 1 Characterization of the participants (mean ± SD) (n (%))

| Characteristics          | Cases    | Controls | P     |
|--------------------------|----------|----------|-------|
| Age (yr)                 | 30.5 ± 7.2 | 27.8 ± 7.0 | 0.426 |
| Gender                   |          |          |       |
| Females                  | 149 (39.01) | 147 (42.00) |       |
| Males                    | 233 (60.99) | 203 (58.00) | 0.410 |
| ALT (U/L)                | 125.5 ± 81.7 | 28.2 ± 10.9 |       |
| HBeAg (+)                | 382 (100)   | 0         |       |
| Anti-HBs                 | 0         | 0         |       |
| HBeAg (+)                | 134 (35.07) | 0         |       |
| Anti-HBe                 | 248 (64.93) | 0         |       |

Table 2 Allele and genotype frequencies of the new SNP (-86 bp T > A) in GRP78 among cases and controls, and risk of HBV n (%)

| Allele/genotype | Cases    | Controls | OR (95% CI) | P     |
|-----------------|----------|----------|-------------|-------|
| T               | 749 (98.1) | 689 (98.3) | Reference   |       |
| A               | 15 (1.9)   | 11 (1.5)  | 1.24 (0.56-2.78) | 0.534 |
| TT              | 367 (96.1) | 339 (96.8) | Reference   |       |
| AT              | 15 (3.9)   | 11 (3.2)  | 1.25 (0.57-2.80) | 0.529 |
| AA              | 0         | 0         |             |       |

1Mann-Whitney test; 2Pearson χ² test. ALT: Alanine aminotransferase.

Serological testing
Serological markers for HBV infection were determined by commercially available enzyme-linked immunosorbent assay kits (Sina-American Biotechnology Co., Ltd., China) or clinical chemistry testing. The normal range for serum alanine aminotransferase (ALT) is 0-40 IU/L. The extraction and quantification of serum HBV-DNA were carried out using a quantitative HBV PCR fluorescence diagnostic kit (Shenzhen PG Biotech., China) in the LightCycler Systems (Roche Diagnostic, Rotkreuz, Switzerland). HBV DNA levels were expressed as log copies/mL.

Statistical analysis
χ² test was used to determine whether there was a significant difference between cases and controls in terms of gender. Mann-Whitney U-test was used to test the difference among the age groups. Hardy-Weinberg equilibrium (HWE) of genotype distribution among cases and controls was carried out using the Pearson χ² test. A significant difference in polymorphism between cases and controls (or between different subgroup cases) was ascertained by unconditional logistic regression model adjusted for age and gender, in which the odds ratios (ORs) and 95% confidence intervals (CIs) were acquired synchronously. One way analysis of variance (ANOVA) and the Bonferroni test were used to evaluate the association between serum HBV DNA levels and alleles/genotypes in cases. All statistical tests were two-sided and P values less than 0.05 were considered statistically significant.
Table 3 ORs and 95% CIs calculated by logistic regression with adjustment for age and gender between different case subgroups according to the alleles and genotypes of the new SNP (-86 bp T > A) in all HBV carriers

| Alleles/genotypes | Serum HBV DNA levels (lg copies/mL) | Serum ALT > 40 IU/L (n) | Serum ALT ≤ 40 IU/L (n) | OR (95% CI) | P \(^2\) | Serum HBV DNA levels (lg copies/mL) | Serum HBeAg + (n) | Serum HBeAg - (n) | OR (95% CI) | P \(^2\) |
|-------------------|-----------------------------------|-------------------------|-------------------------|-------------|---------|-----------------------------------|-------------------|-------------------|-------------|---------|
| T                 | T                                 | 194                     | 555                     | Reference   |         | T                                 | 286               | 483               | Reference   |         |
| A                 | A                                 | 2                       | 13                      | 0.45 (0.10-1.99) | 0.262    | A                                 | 2                 | 13                | 0.28 (0.06-1.25) | 0.076    |
| TT                | TT                                | 96                      | 271                     | Reference   |         | TT                                | 132               | 235               | Reference   |         |
| AT                | AT                                | 2                       | 13                      | 0.44 (0.10-1.97) | 0.254    | AT                                | 2                 | 13                | 0.29 (0.06-1.23) | 0.070    |
| AA                | AA                                | 0                       | 0                       |             |         | AA                                | 0                 | 0                 |             |         |

\(^1\)P values for ALT > 40 IU/L cases vs ALT ≤ 40 IU/L cases; \(^2\)P values for HBeAg positive cases vs HBeAg negative cases. +: Positive; -: Negative.

**RESULTS**

The observed genotype frequencies conformed to the HWE in both cases and controls (data not shown). According to the logistic regression analysis with adjustment for age and gender, the distributions of allelotype and genotype in HBV cases were not significantly different from those in the controls (P > 0.05, respectively) (Table 2).

To assess the possible association between the polymorphism and acute exacerbation of HBV infection, the cases were divided into two groups based on a normal or abnormal ALT value, or the absence or presence of HBeAg. There were no significant differences in allele frequencies or genotype distributions of -86 bp (T > A) between ALT abnormal cases (ALT > 40 IU/L) and ALT normal cases (ALT ≤ 40 IU/L), or between HBeAg positive cases and HBeAg negative cases (P > 0.05, respectively) (Table 3). In addition, the viral load demonstrated no significant differences among different alleles (OR: 3.96, 95% CI: 3.34-4.61, P = 0.165 for allele A) or genotypes (OR: 3.95, 95% CI: 3.33-4.64, P = 0.175 for AT) compared with the respective reference groups (OR: 3.60, 95% CI: 3.50-3.68 for allele T; OR: 3.57, 95% CI: 3.46-3.72 for genotype TT) by ANOVA and Bonferroni testing (Figure 1).

**DISCUSSION**

The findings in this study indicate that the -86 bp variation is not susceptible to HBV invasion and acute exacerbation.

As some of the polymorphisms may reflect the risk of onset and the severity of disease, assessing the effect of variations on the risk and progression of HBV invasion is important. The role of GRP78 as a predisposing gene in the pathogenesis of viral diseases such as HBV has not been previously studied. Although no association was noted with GRP78 using the -86 bp (T > A) SNP marker, there are a number of other loss/gain-of-function SNPs and haplotypes in the GRP78 gene that have not been included in this study, which need further evaluation to conclusively exclude GRP78 as a susceptibility locus.

The data presented in this study also demonstrate that neither ALT/HBeAg nor HBV-DNA were associated with the allele/genotype variation in HBV infected individuals. Because ALT, HBeAg and HBV-DNA are markers for acute infection of HBV, we can infer that -86 bp (T > A) is not associated with acute exacerbation of HBV invasion.

Moreover, a prospective study on the influence of polymorphisms on disease risk and progression mainly explored a statistical association between alleles/genotypes and clinical events. Therefore, the present study suggests a lack of association between the -86 bp and clinical risk as well as acute exacerbation. These data, however, do not exclude a possible physiopathological role of the GRP78 in HBV progression.

To the best of our knowledge, this new found SNP in a Chinese Han population has not been deposited in a public database (http://www.ncbi.nlm.nih.gov/SNP). Although the functional effects of this polymorphism have not been elucidated, our data show that the specific
polymorphism evaluated in this study is not related to HBV susceptibility or acute exacerbation and suggest that, at least in this Chinese population, its role in HBV invasion could be less than expected. However, it must be underlined that this polymorphism should not be completely eliminated as studies with a larger sample size may demonstrate the small differences found in this study to be statistically significant.

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