Association of HLA-DRB1*13 and TNF-α gene polymorphisms with clearance of chronic hepatitis B infection and risk of hepatocellular carcinoma in Thai population

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SUMMARY. Considerable evidence suggests that host genetic factor play an important role in the pathogenesis and clinical outcome of chronic hepatitis B virus (HBV) infection in several ethnic groups. Association study was performed included 150 chronic HBV patients, 100 resolved hepatitis B and 150 healthy individuals with similar ethnic background. Interestingly, human leucocyte antigen (HLA)-DR13 show a strong association with the clearance of HBV [odds ratio (OR) = 0.04, 95% confidence interval (CI) = 0.00–0.26, corrected P-value (Pc) = 0.0008] similar to reports from several ethnic groups. TNF-α promoter polymorphisms (~863, ~308 and ~238) were also analysed. Only ~863C allele was found to be significantly decreased in chronic HBV patients compared with healthy control (Pc = 0.03, OR = 0.54, 95% CI = 0.35–0.84 respectively). This ~863C allele was not in linkage disequilibrium with HLA-DR13 suggesting that other genetic markers linked with ~863C might influence clearance of chronic HBV infection in Thai. When stratified chronic HBV patients into patients without hepatocellular carcinoma (HCC) and with HCC, the ~863 A allele was significantly increased in the HCC group compared to healthy control (Pc = 0.003, OR = 2.61, 95% CI = 1.49–4.60). Haplotype analysis (~863/~308/~238) revealed that the homozygosity of the haplotype (CGG/CGG) was a protective marker for HCC (OR = 0.37, 95% CI = 0.17–0.79, Pc = 0.02). One can propose that carriers of ~863A genotype are associated with increased levels of TNF-α in the liver in response to HBV infection and induce hepatocyte damage that may finally lead to HCC development. Additional study is needed to validate these finding and to further explore the genetic pathogenesis of HBV infection.

Keywords: hepatitis B, hepatocellular carcinoma, HLA–DRB1, polymorphisms, TNF alpha.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a global public health problem with an estimated 350 million people chronically infected worldwide. The evidence in support of genetic factors arises from twin studies conducted in Taiwan, in these studies, it was demonstrated that the degree of concordance for hepatitis B surface antigen (HBsAg) status was significantly higher in monzygotic twins than in dizygotic twins [1]. As HBV is generally believed not to have a direct cytopathic effect on hepatocyte, most available experimental evidence suggested that liver damage in hepatitis B is produces by the cellular immune response against viral antigens present in infected hepatocytes. Therefore, the genetic component of chronic HBV infection have been speculated in immune response genes and many studies have reported of the association between human leucocyte antigen (HLA) class II genes and cytokine genes with susceptibility of chronic HBV infection.

Interestingly, the clearance of HBV infection was associated specifically with HLA-DR13 in several ethnic groups. In Gambia, the HLA allele DRB1*1302 was associated with protection against persistent HBV infection in both children and adults [2]. A European study has confirmed that DRB1*1302 and DRB*1301 alleles also conferred resistance...
to chronic infection in Caucasians [3,4]. An association between HLA-DR13 alleles and clearance of HBV infection was also reported in Korean population [5]. However, the frequency of HLA-DR13 phenotype was quite low in Asian population (~3% in Thai population) [6]. Another more common HLA allele in Asian, HLA-DR12 was reported to be associated with protection against chronic hepatitis B in south China [7] which is related with Thai. Thus, the association of the specific HLA-DR: HLA-DR13 and HLA-DR12 and susceptibility to chronic HBV infection in Thai population were analysed in this study.

Several cytokines have been identified to participate in the process of viral clearance via host immune response to HBV [8]. In particular, TNF-α is an important cytokine in the immune pathogenesis of HBV infection and also located 850 kb telomeric of the class II HLA-DR locus of the short arm of chromosome 6 [9]. Biermer et al. reported that TNF-α inhibit HBV replication by noncytopathic suppression mediated by NF-κB pathway [10]. Considerable evidence suggests that TNF-α gene polymorphisms associated with clearance or susceptibility of chronic HBV infection (Table 1) [11–23]. However, association studies have many limitations and often gave inconsistent results. Independent studies from various ethnic groups are needed to confirm or disprove previous finding. Furthermore, TNF-α gene plays a critical role not only in persistent HBV infection but also associated with the development of hepatocellular carcinoma (HCC) [24]. A genetic polymorphism of TNF-α at promoter region has been found to be associated with susceptibility to various cancer [25–27]. Therefore, the association between the three commonly studied single nucleotide polymorphism (SNP) (~863, ~308, ~238) within TNF-α gene with susceptibility to chronic HBV infection and risk of HCC in Thai population were analysed in this study.

MATERIALS AND METHODS

Subjects
Thai patients from outpatients and inpatients service of King Chulalongkorn Memorial hospital were included in the study. Subjects were categorized into two different groups: chronic HBV infection group and resolved hepatitis B group: (i) chronic HBV infection group contained 150 subjects who had been HBsAg-positive for at least 6 months and did not have any type of liver disease such as chronic hepatitis C or alcoholic liver disease. Moreover, all patients had elevated serum ALT and AST level. Patients with chronic HBV infection were further divided into two groups: without (n = 100) (n = 100; male/female = 68/32; mean age = 50.8 ± 13.9; HBeAg positive 66%) and with HCC (n = 50; male/female = 38/12; mean age = 57.5 ± 14.2; HBeAg positive 40%) according to the absence or presence of concurrent HCC. Diagnosis of HCC was based on histopathology and/or a combination of mass lesion in the liver from hepatic imaging and serum alpha fetoprotein level >400 ng/mL; and (ii) resolved hepatitis B group to serve as control for the population-based case–control study contained 100 subjects (male/female = 48/52; mean age = 51.0 ± 12.3), who tested HBsAg negative and both HBV core antibody (anti-HBc) and HBV surface antibody (anti-HBs) positive, with normal liver function tests, and no history of HBV vaccination. Moreover, 150 ethnically and geographically matched controls (male/female = 80/70; mean age = 30.9 ± 10.6) from healthy blood donor of the Thai Red Cross Society were recruited as control group. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent.

DNA extraction
DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as anticoagulant, using a salting out method [28].

Analysis of HLA-DRB1 (HLA-DR12, HLA-DR13) polymorphism
Polymerase chain reaction-sequence specific primer (PCR-SSP) analysis of HLA-DR12 and HLA-DR13 were performed as previously described [29]. Internal control primers were used to check for successful PCR amplification. These primers amplify a human growth hormone sequence [30]. Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The DRB1 full typing of the eight patients that carry DR13 was performed using SSP UniTray (Pel-Freeze, Brown Deer, WI, USA).

Analysis of TNF-α polymorphism
Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to analyse TNF-α gene at promoter position ~308 (A/G), ~863 (A/C) and ~238 (G/A) as previously described [17,31,32]. Negative controls without DNA template were included in each experiment.

Statistical analysis
The genotype frequencies were checked by consistency among normal controls with those expected from Hardy–Weinberg equilibrium. Allele and genotype frequencies were compare between groups using the chi-squared (χ²) test or Fisher exact probability test, where appropriate. Gene frequencies were determined by gene counting. A P-value of <0.05 was considered significant. Odds ratio (OR) with 95% confidence interval (CI) were calculated using the statistical
<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Population</th>
<th>Controls</th>
<th>Polymorphism</th>
<th>Haplotype</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hohler (1998) [22]</td>
<td>Germany</td>
<td>Germany</td>
<td>NS</td>
<td>G/G (0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xu (2005) [21]</td>
<td>China</td>
<td>56</td>
<td>90 healthy control</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li (2005) [14]</td>
<td>China</td>
<td>443</td>
<td>244</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xiao (2005) [18]</td>
<td>India</td>
<td>184</td>
<td>214</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suneetha PV (2006) [20]</td>
<td>India</td>
<td>100</td>
<td>91</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li (2006) [15]</td>
<td>China</td>
<td>62</td>
<td>62</td>
<td>G/A (&lt;0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li (2006) [16]</td>
<td>China</td>
<td>62</td>
<td>246</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Du (2006) [23]</td>
<td>China</td>
<td>196</td>
<td>143</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS, not significance.

Table 1: TNF-α promoter polymorphisms associated with chronic hepatitis B virus (HBV) infection.
disequilibrium was considered statistically significant. The pairwise linkage determined using LDPlotter Tool.

HLA, human leucocyte antigen; HCC, hepatocellular carcinoma.

Haplotype analysis

The program PHASE (UW TechTransfer Digital Ventures, Seattle, WA, USA) was used to reconstructing haplotypes from population genotype data [34].

RESULTS

Protective effect of DR13 allele against chronic hepatitis B infection

The distribution of HLA-DR12 and HLA-DR13 alleles in chronic HBV patients and resolved hepatitis B patients were shown in Table 2. DR13 allele was not found in any chronic HBV patients. In contrast, DR13 allele was identified with high frequency of 8% in the resolved hepatitis B patients which was significantly higher than chronic HBV patients (0%) \( (P_c = 0.0008, \ OR = 0.04, \ 95\% \ CI = 0.00–0.26) \). HLA-DR12 allele was increased in chronic HBV patients (32.7%) compared with resolved hepatitis B patients (27%), but did not reach statistical significance. Additional DRB1 typing was performed to identify another allele for eight individuals that carried DR13 and revealed that all eight patients are heterozygous for DR13. There was no significance in distribution of HLA-DR12 in chronic HBV patients with HCC compared with chronic HBV patients without HCC.

Table 2 Distribution of specific HLA-DRB1 (DR12 and DR13) alleles in patients with chronic hepatitis B (with HCC and without HCC), resolved hepatitis B patients and healthy control

<table>
<thead>
<tr>
<th>HLA-DRB1 allele</th>
<th>Chronic hepatitis B</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With HCC, n = 50 (%)</td>
<td>Without HCC, n = 100 (%)</td>
<td>Total, n = 150 (%)</td>
<td>Resolved hepatitis B, n = 100 (%)</td>
<td>Healthy control, n = 150 (%)</td>
<td></td>
</tr>
<tr>
<td>DR13</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>8 (8)</td>
<td>7 (4.7)</td>
<td></td>
</tr>
<tr>
<td>DR12</td>
<td>18 (36)</td>
<td>31 (31)</td>
<td>49 (32.7)</td>
<td>27 (27)</td>
<td>42 (28)</td>
<td></td>
</tr>
</tbody>
</table>

HLA, human leucocyte antigen; HCC, hepatocellular carcinoma.

\( ^*_P_c = 0.0008 \) vs resolved hepatitis B. OR (95% CI) = 0.04 (0.00–0.26).

TNF-\( \alpha \) gene polymorphisms associated with clearance of chronic HBV infection

The distribution of genotypes of TNF-\( \alpha \) gene polymorphism at all three position \(-\)863(C/A), \(-\)308 (G/A), \(-\)238 (A/G) among healthy controls were in agreement with the prediction under the condition of Hardy–Weinberg equilibrium. The \(-\)863 C/C genotype and \( \alpha \) allele was found to be significantly decreased in chronic HBV patients compared with healthy control \((P_c = 0.01, \ OR = 0.46, \ 95\% \ CI = 0.27–0.77 \) and \( P_c = 0.03, \ OR = 0.54, \ 95\% \ CI = 0.35–0.84 \) respectively) (Tables 3 and 4). There were no significant differences in allele frequency of \(-\)308A/G and \(-\)238A/G polymorphism in the promoter of TNF-\( \alpha \) gene between chronic HBV patients and resolved hepatitis B patients. The haplotype frequencies of the TNF-\( \alpha \) promoter polymorphism were determined by PHASE program. In this study, CGG haplotype were the most common haplotype in Thai population. No significant differences in haplotype frequencies could be demonstrated between chronic HBV patients and resolved hepatitis B patients/healthy control.

Association of TNF-\( \alpha \) gene polymorphisms with decreased risk of hepatocellular carcinoma in chronic hepatitis B patients

When stratified chronic HBV patients into patients without HCC and with HCC, the \(-\)863 A allele was found to be significantly increased in the HCC group compared to healthy control \((P_c = 0.003, \ OR = 2.61, \ 95\% \ CI = 1.49–4.60) \). By analysis of mode of inheritance, \(-\)863 A/A, A/C genotypic frequencies were significantly increased in the HCC group compared to healthy control \((P_c = 0.001, \ OR = 3.62, \ 95\% \ CI = 1.77–7.46) \). The CGG haplotype and CGG/CGG genotype of \(-\)863/\(-\)308/\(-\)238 haplotype were found to be significantly decreased in chronic HBV patients with HCC, as compared with healthy control \((P_c = 0.03, \ OR = 0.39, \ 95\% \ CI = 0.18–0.80 \) and \( P_c = 0.02, \ OR = 0.50, \ 95\% \ CI = 0.30–0.83 \) respectively) (Tables 3 and 4). The homozygous CGG haplotype (CGG/CGG) also significantly decreased in chronic HBV patients with HCC compared to
DISCUSSION

The result of the present study show strong association between HLA-DR13 and clearance of HBV which support other studies in several ethnic groups. This similarity in various independent studied help indicate the important role of HLA-DR13 in chronic HBV infection. It has been suggested that the beneficial effect of HLA-DR13 phenotype on the outcome of HBV infection may be due to the induction of a vigorous HBc-specific CD4+ T-cell response, which might be either a more proficient antigen presentation by HLA-DR13 molecules themselves or due to a linked polymorphism in a neighboring immunoregulartory gene [35].
The identification of the specific peptide epitopes derived from the virus presented by these HLA molecules may provide suitable vaccine candidates both for prophylactic and therapeutic use. Cao et al. [36] reported that Hbc-specific CD4⁺ T-cell clone and T-cell lines derived from subjects carry DR13 who spontaneously recovered from acute HBV infection show a dominant recognition of HBCAg peptide spanning aa 1–20 (P1), 11–30 (P2), 41–60 (P5), 111–131 (P12) and 141–160 (P15). Most T cell generated from these subjects recognized a single epitope within HBCAg at aa 147–156 (147TVVRRRGRSP156). Diepolder showed that patients with acute hepatitis B who carrying HLA-DR13 mount a more vigorous HBC-specific CD4⁺ T cell than patients without HLA-DR13. However, peptide epitopes aa 50–69, aa 61–85, and 81–105 were recognized most frequently by both groups [35]. Additional study is needed to validate these finding and to further explore the role of HLA-DR13 phenotype in antigen presentation of HBV core epitopes to HBC-specific CD4⁺ T cell responses in patients with acute, self-limited HBV infection. Our preliminary result screening T cells from one DR13 individual that recovered from HBV infection with overlapping peptides reveal positive response to peptide from polymerase, not from precore or core protein (data not shown).

It was proposed that a genetically increased capacity to produce TNF-α would result in more effective inhibition of HBV; thus the resolution of HBV infection is associated with high TNF-α promoter allele. However, an extensive review of all previous analysis of TNF-α promoter polymorphism association with HBV infection summarized in Table 1 showed that although there were clearly some associations, different markers were involved in each study. This fact suggests that these associations might be due to linkage disequilibrium with other genes. There was report that −308A allele was in strong linkage disequilibrium with DR13 [13]. The present study demonstrated that the −863C allele and −863C/C genotype of TNF-α gene was increased in healthy control compared to chronic HBV patients. There are trend for association when compared to resolved hepatitis B patients although it is not statistically significant when corrected for multiple comparison (uncorrected P-value = 0.04). This −863C allele was not in linkage disequilibrium with HLA-DR13 suggesting that other genetic markers linked with −863C independent of DR13 might influence clearance of chronic HBV infection in Thai.

Besides role of TNF-α polymorphism in the pathogenesis of chronic HBV infection, its association with the development of HCC was reported [24]. That study in Taiwan showed that carriers of −308A allele, either homozygous or heterozygous subjects (associated with high TNF-α production), had a higher risk of developing HCC. Thus, the association analysis between TNF-α polymorphisms and chronic HBV infection risk was performed with the stratified chronic HBV patients according to progression to HCC and non-HCC compared with healthy control group. Although no significant association at −308 position was found, another high TNF-α production genetic marker, −863A allele was shown to associate with an increased risk of HCC with autosomal mode of inheritance in this study. The haplotype (−863A/−308A/−238) analysis revealed that the homozygosity of the low-production haplotype (CGG/CGG) was a protective marker for HCC in this study. However, various factors might influence the carcinogenesis process in HBV patients including the duration of infection. Given that the majority of Thai patients acquired HBV infection vertically from their mothers at birth or horizontally during childhood from carrier family members, their age would probably serve as a reasonable surrogate for the duration of HBV infection. The mean age of HCC patients was significantly older than non-HCC patients (57.5 ± 14.2 vs 50.8 ± 13.9, P ≤ 0.05). Therefore, it is likely that a subset of the non-HCC group will develop cancer later in life. In fact the main deviation of TNF-α allele frequency was observed between HCC group and healthy control rather than when compared with non-HCC group. However, the proposed role of TNF-α gene in HCC should be validated in further study that can control for all other risks for carcinogenesis.

Interestingly, the association of the TNF-α variant with chronic hepatitis B infection in our study is correlated with a pathophysiological mechanism of TNF-α in carcinogenesis. TNF-α has been found in high concentration in patients with cancer [37,38]. The existing evidence implicates the role of TNF-α inflammatory pathway that increased tumorigenesis [39]. More convincing evidence suggests that the TNF-α, which is a key player in inflammation can also activate signaling pathways, in both cancer cells and tumour-associated inflammatory cells, that promote malignancy [40]. However, it is also possible that this association is not due to the TNF-α gene per se, but to another gene in linkage disequilibrium in a neighboring immunoregulatory gene. Especially, haplotype analysis showed a strong protective haplotype. It has been suggested that self-elimination of HBV infection may be due to the influence from another protective allele.

In conclusion, our study showed that HLA-DR13 allele is a strong protective genetic marker in chronic hepatitis B infection. The functional significance of this particular HLA molecule in HBV immune response may provide insight to develop novel prophylactic and therapeutic tools. Furthermore, the −863A allele of TNF-α gene was identified as a genetic marker for HCC development in patient with chronic HBV infection. The hypothesis regarding TNF-α genetic polymorphism and hepatocarcinogenesis are based on the assumption that carriers of these genotypes are associated with increased levels of TNF-α in the liver in response to HBV infection and induce hepatocyte damage that may finally lead to HCC development. Additional study is needed to validate these finding and to further explore the genetic pathogenesis of HBV infection.

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ACKNOWLEDGEMENTS

The study was supported by the Thailand Research Fund, RSA4680021. We greatly appreciate the participants and all the staffs who participated in chronic HBV study at King Chulalongkorn Memorial Hospital. Also, we would like to thank the National Blood Center for the recruitment of healthy controls and collection of research materials.

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