Association of CD40 -1C/T Polymorphism in the 5′-Untranslated Region with Chronic HBV Infection

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Key Words
CD40-1C/T polymorphism • Asymptomatic HBV carriers • CD40 expression • Soluble CD40 • Immune tolerance

Abstract
Background: CD40 is an important costimulatory molecule in both cellular and humoral immune responses, involved in the pathogenic processes of chronic inflammatory diseases. Few studies were performed on the association of CD40 single nucleotide polymorphism (SNP) with chronic hepatitis B virus (HBV) infection. In this study, we studied whether the CD40-1C/T polymorphism had any effect on the progression of chronic HBV infection in Chinese population. Methods: CD40 -1C/T polymorphism in the 5′-untranslated region was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 453 chronic HBV carriers, who were divided into asymptomatic HBV carriers (ASC), moderate chronic hepatitis B group (MCHB) and severe chronic hepatitis B group (SCHB). 202 healthy individuals in the same region were enrolled in this study as the controls. The CD40 expression on B lymphocytes was detected by flow cytometry. The concentrations of soluble CD40 (sCD40) in sera were assayed by a commercial ELISA kit. Results: Our results showed the frequencies of TT genotype and T allele of CD40-1C/T polymorphism were higher significantly in ASC than those in controls (P < 0.05), while this result was not found in either MCHB or SCHB. On the surface of B lymphocytes, the CD40 expression levels in the individuals with TT genotype were significantly lower than those with CC and CT genotypes in either ASC group or healthy controls (P<0.001). The sCD40 levels in the sera of ASC, MCHB and SCHB groups were significantly higher than the controls (P<0.001). Conclusions: The CD40 -1C/T polymorphism may contribute to the susceptibility of asymptomatic HBV carriers through its effect on cell-surface CD40 expression, which indicated CD40 signaling was involved in immune tolerance of chronic HBV infection.

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Introduction

Hepatitis B virus (HBV) infection is the most common cause of liver disease worldwide. More than 350 million persons worldwide have chronic HBV infections. Patients with chronic HBV infection are not only at high risk of progression to liver cirrhosis and hepatocellular carcinoma, but also act as virus reservoir of HBV. The clinical presentations of chronic HBV infection vary from asymptomatic state to fulminant hepatitis [1-3]. Hepatitis B clearance occurs more often in individuals who develop a broad and strong immune response than in those with weak and narrowly focused responses [4]. Many studies indicated that the genetic variants of immune molecules were associated with the body immune status, which is correlated with different clinical outcomes of HBV infections [5-7].

CD40 is a 50-kD cell-surface glycoprotein of the tumor necrosis factor receptor superfamily. It is widely expressed by both immune and non-immune cells, such as B cells, macrophages, dendritic cells (DCs), epithelial, endothelial, hepatocytes, platelets, tumor cells, etc. CD40 interaction with its ligand CD40L promotes DCs to mature and triggers the T-cell activation and differentiation effectively. CD40 ligation of B cells also induces germinal center formation, immunoglobulin isotype switching, somatic hypermutation, which are the key steps for the production of antibodies. Besides, CD40 stimulation also induces the production of different cytokines, chemokines, etc. Therefore, CD40 signaling is crucial in regulating both adaptive immunity (T-cell and B-cell immune responses) and innate immunity [8-10]. Blockade of CD40 signaling results in compromised immune response [11, 12]. Several studies reported CD40 had been highly expressed in the livers of patients with viral hepatitis as well as hepatocytes infected with HBV virus [13-15], which was correlated with intrahepatic inflammation, necrosis and hepatocyte apoptosis [16, 17]. A recent report demonstrated that CD40 signaling was able to inhibit replication of hepatitis C virus in primary human hepatocytes by c-Jun N terminal kinase activation [18]. Besides, the concentration of sCD40 was also showed to be higher in the sera of patients with liver diseases (including hepatitis B) than those of the controls [19-21]. All these results suggested that CD40/CD40L system play an important role in the pathogenesis of viral hepatitis.

CD40-1 C/T polymorphism located in Kozak sequence can influence the initiation of CD40 translation [22], which was reported to be associated with the susceptibility of many immune-related, chronic inflammatory diseases such as autoimmune thyroid disease, multiple sclerosis, coronary syndrome, etc [23-26]. But few studies have been found to focus on this polymorphism association with chronic HBV infections. In this paper, we evaluated the association of CD40-1 C/T polymorphism with the susceptibility to disease progression of chronic HBV infection. Moreover, the relationships of this genetic variant with CD40 expression on B lymphocytes and the serum sCD40 level, have been also analyzed in the patients with chronic HBV infection.

Subject and Methods

Patients and control subjects

A total of 453 patients with chronic HBV infection from the First Affiliated Hospital of Zhejiang University (Hangzhou, China) were recruited for the study. The diagnosis of chronic HBV infection was confirmed by seropositivity for HBsAg over 6 months (commercially available enzyme-linked immunosorbent assay kit, Abbott Laboratories, Chicago, IL). Among the subjects, 174 cases were asymptomatic HBV carriers (ASC) with normal serum levels of alanine aminotransferase (ALT) / aspartate aminotransferase (AST) and negative for HBeAg and HBV DNA, without previous history of hepatitis B or any other clinical symptom within 1 year during the study. 165 cases were moderate chronic hepatitis B (MCHB) with previous history of hepatitis B and continuously elevated levels of ALT/AST, but serum total bilirubin (TBil) was less than 10 times of normal upper limit (171 μM) and International Normalized Ratio (INR) less than 1.5, while 114 chronic hepatitis B with TBil more than 10 times of normal upper limit and/or INR exceeds 1.5 were diagnosed as severe chronic hepatitis B (SCHB). Patients with other immune-related diseases or coinfected with other
type hepatitis virus or alcoholic liver disease were excluded. Meanwhile, 202 ethnically matched healthy individuals, who were negative for HBsAg, HBeAg, HBeAb, HBcAg, HBcAb from the same geographical area were recruited as controls. All subjects were informed consent and the study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang University.

**Genotyping of CD40 polymorphisms**

Genomic DNA from samples was extracted with the whole blood genomic DNA extraction mini-kit according to the manufacturer’s instructions (Hangzhou SIMGEN Biotechnology Co., Ltd. Hangzhou, China). CD40-1C/T polymorphism was determined by polymerase chain reaction (PCR) and the analysis of restriction fragment lengths polymorphism (RFLP) as previously reported [25]. PCR was performed to amplified the 5’-untranslated region of CD40 using the following primers: 5'-GAAACTCCTGCGCGGTGAAT-3' and 5'-CCTCTTCCC-GGAAGTCTTCC-3' (NCBI Reference Sequence: NC_000020.11). 100ng genomic DNA was amplified in 25ul reaction containing 0.25uL of rTaq (Takara Biotechnology CO. LTD., Dalian, China). The PCR procedure is initiated at 95℃ for 5 min, followed by 30 cycles of 94℃ for 30 s, 60℃ for 30s, and 72℃for 45s, and extended at 72℃ for 5min. Then, 5ul PCR product was digested with 5U of NcoI restriction enzyme (Takara Biotech., Dalian, China) at 37℃ for 6h. The digestion products were analyzed on 2.5% agarose gel. In order to determine the accuracy of the PCR-RFLP method, the results were verified again with direct sequencing in about 10% randomly selected samples and found no errors.

**Flow cytometry**

The whole blood from subjects was incubated for an hour at room temperature with PE-conjugated anti-human CD19 (BD Biosciences) and FITC-conjugated anti-human CD40 (invitrogen). FITC-conjugated mouse IgG1 was used as isotype control. Then the blood was hemolyzed and fixed. The cells were analyzed in a Coulter, Epics XL flow cytometer (Beckman Coulter, Germany).

**Detection for human sCD40**

Human sCD40 in sera from the subjects were dectected by a commercial ELISA kit (Bender MedSystems Diagnostics GmbH, Vienna, Austria). The procedure was performed according to the kit protocol.

**Statistical analysis**

Hardy–Weinberg equilibrium was tested for each polymorphism included in the study with Chi-square test. The frequencies of genotypes and alleles were determined by direct gene counting method and compared by Chi-square test. The associations of the genotype and allele frequencies with chronic HBV infection, were analyzed by unconditional logistic regression after adjusting for the age and gender. Continuous variables among the groups were analyzed by one-way ANOVA. P-value less than 0.05 was regarded to be statistically significant. All the statistical analysis was performed by SPSS software version 19.0 (SPSS, Inc., Chicago, IL).

**Results**

**Clinical and demographic characteristics of population with HBV infection**

The baseline and laboratory characteristics in normal controls, ASC, MCHB, and SCHB are summarized in Table 1. The age was significantly different among the four groups (F=2.894, P=0.035, one-way ANOVA). Patients with SCHB were younger than the control and ASC groups (Table 1, P<0.001). As for the gender, more male patients were presented in patients with MCHB and SCHB than those in normal controls (controls vs MCHB, P <0.05; controls vs SCHB, P <0.001), as well as in ASC (ASC vs MCHB, P <0.05; ASC vs SCHB, P <0.001). Also, the percentage of men in SCHB group was higher than that of MCHB (P <0.05). However, there was no significant differences in both the age and gender between the control and ASC groups (P >0.05). The data showed that the age and gender were in good matches between control and ASC groups except MCHB and SCHB groups, indicating the gender and age are sensitive factors related with the different outcomes of HBV infection, which is in accordance with the previous reports [27, 28].
Distribution of CD40 -1C/T genotype and allele frequencies

The genotype distributions for CD40-1C/T polymorphism were in Hardy–Weinberg equilibrium in each group (P>0.05). The genotype and allele frequencies of this CD40 SNP in patients and control groups are summarized in Table 2 and 3. There were significant differences in the distributions of the genotypes and alleles among the four groups (Table 2, for genotypes, χ² = 13.968, P=0.03; for alleles, χ² = 11.043, P=0.011). As shown in Table 3, the frequency of the CD40 TT genotype was significantly higher in ASC group than the control and SCHB groups (ASC vs controls, P=0.005; ASC vs SCHB, P=0.023), and the T allele frequency of ASC group was also significantly higher than the control, MCHB and SCHB groups (ASC vs controls, P=0.002; ASC vs MCHB, P=0.045; ASC vs SCHB, P=0.010). However, neither genotype nor allele frequency showed significant differences among the control, MCHB and SCHB groups. Moreover, we analyzed the data by nonconditional logistic regression after adjustment with age and sex. The results showed that the subjects with TT genotype had an
increased susceptibility to asymptomatic HBV carriers compared to those with at least 1C allele (for recessive model, OR 2.278; 95% CI, 1.354–3.833; \( P = 0.002 \), and for additive model, OR 2.212; 95% CI, 1.267–3.861; \( P = 0.005 \), shown in Table 4). Thus, genetic variation of CD40 -1 C/T polymorphism was strongly associated with susceptibility to asymptomatic carriers after HBV infection.

**Effect of the CD40-1C/T polymorphism on CD40 expression on B lymphocytes**

B lymphocytes are the main cells that express CD40 on their surface. They are also the antibody-produced cells in adaptive immune response. The CD40 -1 C/T SNP is located in the Kozak consensus sequence of CD40 gene, which plays a key role for CD40 translation [22]. In order to study whether the CD40-1C/T polymorphism has any effect on the CD40 expression in these subjects and whether there are any differences of CD40 expression between control and ASC groups, we detected the membrane CD40 expression on B lymphocytes by flow cytometry in 94 asymptomatic HBV-carriers and 77 healthy controls. Our data showed the membrane CD40 expressions on B lymphocytes were not different significantly between control and ASC groups [mean fluorescence intensity (MFI), 7.12 ± 1.18 vs 7.09 ± 1.73, \( P > 0.05 \)]. However, there are significant differences in CD40 membrane expressions of B lymphocyte among CC, CT and TT genotypes in both control and ASC groups (\( P < 0.001 \), one-way ANOVA.). As shown in Fig. 1, the highest expression of membrane CD40 on B cells existed in the individuals with CC genotype when compared to those with CT or TT genotype (CC vs CT, \( P < 0.05 \); CC vs TT, \( P < 0.001 \)) in both groups, and the individuals with CT genotype also showed higher levels of CD40 expression than those with TT genotype (CT vs TT, \( P < 0.05 \)). Thus, the CD40 -1C/T polymorphism has regulatory effects on CD40 membrane expression, which might affect the strength of CD40 signaling in both cellular and humoral immune responses.

### Table 4. The association test for CD40-1C/T genotypes between asymptomatic HBV carriers and controls.

The data were performed by logistic regression analysis adjusted with age and sex. \( ^a \), \( ^b \), \( ^c \)Comparison between control and ASC groups. \( ^a \)Comparison CC with CT+TT. \( ^b \)Comparison CC+CT with TT. \( ^c \)Comparison CC with CT. OR: odds rate, CI: confidence intervals.

| Genotypes | ASC \( n=174 \) | Controls \( n=202 \) | Dominant model\(^b\) | Recessive model\(^b\) | Additive model\(^b\) |
|-----------|----------------|-----------------|-----------------|-----------------|-----------------|
| CC        | 51 (29.3%)     | 77 (38.1%)     | 0.169 1.338     | 0.002 2.278     | 0.005 2.212     |
| CT        | 77 (44.3%)     | 98 (48.5%)     | (0.883-2.033)   | (1.354-3.833)   | (1.267-3.861)   |
| TT        | 46 (26.3%)     | 27 (13.4%)     |                |                 |                 |

**Fig.1.** CD40 expressions on B cells in the control and ASC groups with different genotypes of CD40 -1 C/T polymorphism. CD40 membrane expression on B cells were detected by flow cytometry and the obtained data were analyzed by one-way ANOVA. Compared with CC genotype in corresponding group, \( ^* P < 0.05 \). \( ^{**} P < 0.001 \). Compared with CT genotype in corresponding group, \( \Delta P < 0.001 \).
It was reported that serum sCD40 levels was significantly higher in patients with liver diseases including chronic viral hepatitis [19-21], and the renal function is a key factor in determining the sCD40 level [29]. Thus, in this study we have detected serum sCD40 concentrations in 76 controls and 214 cases with chronic HBV infection (96 ASC, 80 MCHB and 38 SCHB), all of whom have normal renal function. Similar results were also obtained in our study to confirm that serum sCD40 levels in chronic hepatitis B (including MCHB and SCHB) are significantly higher than those of the controls. Besides, we also found higher levels of serum sCD40 presented in asymptomatic HBV carriers (Fig. 2, \( P < 0.001 \)). However, there are no significant differences among ASC, MCHB and SCHB groups. Then, we are wondering whether there is any association between the serum levels of sCD40 and CD40-1C/T polymorphism. The results showed the serum levels of sCD40 are not associated with CD40 -1C/T genotypes in either chronic HBV infections or the healthy controls (data not shown). Since sCD40 is an important negative molecule in CD40 signaling, the above results suggested CD40 pathway might be involved in immune tolerance of chronic HBV infections, and CD40 -1C/T polymorphism have no effect on the expressions of the soluble form of CD40 in sera.

**Discussion**

It was estimated that about 70% of chronic HBV-infected individuals were asymptomatic inactive HBV carriers because of immune tolerance. Previous data showed about 10% of asymptomatic HBV carriers occurred liver cirrhosis, and/or even hepatocellular carcinoma after about 20 years. Persons with asymptomatic chronic HBV infection should be paid high attention and followed up continuously [3]. Therefore, researches on the mechanism about how the asymptomatic HBV carriers occur should be of great significance from both healthy and economic aspects. It is well-known that the clinical outcome of HBV infection depends on the interaction between viral replication and host immunity, and the genetic background was one of the most key factors that influence the host immunity [4-6]. But limited researches were reported on the genetic factors which influence the genesis of asymptomatic chronic HBV infections. Previous studies have demonstrated that activation of CD40 signaling is indispensable for the induction of effective virus-specific CD8+ T-cell responses, which is required for the virus clearance [30, 31]. Using a transgenic mouse model, Chisari et al. showed that the HBV antigen-specific CD8+ T cell exhaustion was able to be rescued by CD40-mediated mDC activation [32]. Thus, CD40 signaling plays critical roles in antiviral immune responses. The diversity of CD40 expression on cell surface is one of the major factors that
regulate the intensity of CD40 signaling. Among all the SNPs of CD40, −1C/T polymorphism, located at the KOZAK region of CD40 gene, is the only one SNP which is known to influence the CD40 expression. In the present study, we found the CD40 -1C/T polymorphism was associated with increased susceptibility to asymptomatic HBV carriers. The T allele and TT genotype frequencies were found significantly higher in asymptomatic HBV carriers. The CD40 -1C/T polymorphism was able to regulate the expression levels of membrane CD40 on B cells, since our results showed there were significant differences of CD40 membrane expressions among the individuals with CC, CT and TT genotypes. Besides, compared with the healthy controls, the serum sCD40 increased in chronic HBV infections, including ASC, MCHB and SCHB patients, but its levels were not correlated with CD40 -1C/T polymorphism. To our knowledge, this is the first report that the genetic variants of CD40 gene are correlated with an individual’s susceptibility to asymptomatic chronic HBV infection.

It is demonstrated that the SNP located in the Kozak sequence is able to influence the initiation rate of a gene translation [22]. In this study, we showed that the CD40 -1 C/T polymorphism can cause the alteration of CD40 translation on human B cells. The expression of CD40 in the individuals with TT genotype was significantly lower than those with CC and CT genotypes in both ASC and control groups, which is agreement with other studies [25, 33]. Our findings that the frequency of TT genotype was significantly higher in ASC groups might be understood as follows: TT genotype of CD40-1C/T polymorphism decreases CD40 expression on cell surface as we and others confirmed. Then the decreased CD40 expression down-regulates both viral-specific and innate immune responses via CD40 signaling, which in turn results in the host immune tolerance, the main immune characteristic of asymptomatic chronic HBV carriers.

Soluble form of CD40 was demonstrated to act as an agonist of CD40/CD40L interaction. Since its interaction with CD40 was able to reduce immunoglobulin production and T cell activation, this soluble molecule was supposed to have an immunosuppressive effect [34]. In HBV infections, this soluble form of CD40 was found significantly higher in patients with acute and chronic HBV hepatitis, which was correlated with ALT levels, indicating there should be association between sCD40 levels and liver inflammations [20, 21]. However, no data of the sCD40 concentrations were reported in the asymptomatic chronic HBV carriers. Here we showed that sCD40 was also significantly higher than those of healthy controls although the serum aminotransferases were normal in their bodies. The mechanism of increased sCD40 level in ASC is still elusive. It was reported that sCD40 was produced by either alternative splicing or the proteolytic cleavage of membrane-anchored CD40 by a tumor necrosis factor-converting enzyme (TACE) [35-37]. In liver diseases, Schmilovitz-Weiss et al. suggested that sCD40 probably derive from the liver [19]. Other studies in chronic HCV infection also found sCD40 was higher significantly than healthy controls, suggested that the virus might be able to stimulate the sCD40 formation resulting in the increased levels of sCD40 [38]. In our study, we found the lowest expression of membrane CD40 on B cells existed in the individuals with TT genotype compared with CC or CT genotype in both control and ASC groups (shown in Fig. 1) and the frequency of the CD40 TT genotype was significantly higher in ASC group. So, it seems unlikely that the elevated circulating sCD40 in ASC group is the proteolytic cleavage of membrane CD40 by TACE. Thus, in our opinion in ASC group, HBV might enhance the synthesis of sCD40 probably by either alternative splicing or an unknown signaling, which needs further study. However, it is not difficult for us to understand this result. Since sCD40 binds with membrane CD40 to counteract CD40/CD40L interaction, the increased sCD40 levels in ASC subjects are supposed to inhibit CD40 signaling, inducing the immunosuppressive status of ASC. Based on the previous finding that CD40 −1C/T polymorphism was able to influence the levels of sCD40L by feedback mechanism via its regulation on the CD40 expression [39], we also want to know if there is any effect of this CD40 SNP on the regulation of sCD40 levels and the results showed there was no relationship between the sCD40 concentrations and CD40 -1C/T genotypes, indicating sCD40 expression might not be influenced by this CD40 SNP. Because of the relative small number of samples assayed in this study, more data are needed to confirm the result.
In conclusion, our study first revealed the association of CD40-1C/T polymorphism with susceptibility to asymptomatic chronic HBV carriers, and the increased serum levels of sCD40 in these carriers, which suggested that the strength of CD40 signaling should be one of the most important pathways involved in host immune tolerance after HBV infection. This naturally occurring genetic variant of CD40 may have effects on the pathogenesis of chronic HBV infection by its regulation of CD40 expression, which might be useful in predicting the clinical outcomes of HBV infection.

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