CD24 Polymorphisms Affect Risk and Progression of Chronic Hepatitis B Virus Infection

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T-cell immunity to hepatitis B virus (HBV) is involved in both viral clearance and the pathogenesis of cirrhosis and hepatocellular carcinoma following chronic HBV infection. It is therefore of great interest to analyze whether genetic polymorphism of genes involved in the immune response may determine the outcomes of chronic HBV infection. Here we report that CD24 polymorphisms affect the risk and progression of chronic HBV infection. Thus the CD24 P170T allele, which is expressed at a higher level, is associated with an increased risk of chronic HBV infection. Among the chronic HBV patients this allele shows recessive association with more rapid progression to liver cirrhosis and hepatocellular carcinoma in comparison to the P170C allele. In contrast, a dinucleotide deletion at position 1527-1528 (P1527del), which reduces CD24 expression, is associated with a significantly reduced risk of chronic HBV infection. To confirm the role for CD24 in liver carcinogenesis, we compared the size of liver tumor developed in CD24+/−/− and CD24+/+ mice. Our data demonstrate that targeted mutation of CD24 drastically reduced the sizes of spontaneous liver cancer in the HBV transgenic mice. Conclusion: These data demonstrate that genetic variation of CD24 may be an important determinant for the outcome of chronic HBV infection. (HEPATOLOGY 2009;50:735-742.)
CD24 is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein with expression in a variety of cell types, including activated T cells, B cells, macrophages, and dendritic cells. Using mice with a targeted mutation of CD24, we reported that in lymphoid organs of an immune-competent host, CD24 on antigen-presenting cell (APC) mediates a costimulatory pathway for both CD4 and CD8 T-cell response that is essential, if and only if, the CD28 gene is also absent. However, CD24 expression in the target organ, such as the central nervous system, is necessary for the chronic inflammation induced by autoreactive T cells. Because HBV is a non-cytopathic and hepatotropic virus, genes that regulate the local immune response may be of particular significance in the pathogenesis. It is therefore of great interest to determine whether the costimulatory molecule that is essential in the nonlymphoid organs, such as CD24, affect the risk and progression of chronic HBV infection. More recently, we demonstrated that the CD24-Siglec 10 pathway selectively inhibits innate host immunity to tissue injury, but not to molecular patterns associated with pathogens.

Human CD24 messenger RNA (mRNA) has a 0.24-kb open-reading frame (ORF) and a 1.8 kb 3′-untranslated region (UTR). A C>T single-nucleotide polymorphism (SNP) at position 170 from the CD24 translation start site (P170) in the CD24 putative cleavage site for the GPI anchor (−1 position) results in a nonconservative replacement of an amino acid from alanine (A) to valine (V). The P170C/T genotype expressed higher cell-surface CD24 than the P170TT genotype, which had an increased risk and more rapid progression of multiple sclerosis (MS). Another SNP, a dinucleotide deletion in 3′UTR, strongly associates with protection of both MS and systemic lupus erythematosus (SLE) because the deletion drastically reduced the stability of the CD24 mRNA. These data raised the possibility that genetic variations in CD24 may affect the risk and outcome of autoimmune diseases. Meanwhile, accumulating evidence suggests that the level of CD24 expression may serve as an important prognosis marker for cancer.

Given the importance of CD24 in the development and progression of autoimmune diseases and cancer, and given the essential role of chronic inflammation in HBV-associated LC or HCC development, we tested the hypothesis that CD24 polymorphisms may affect the risk and progression of chronic HBV infection. Our studies involving 609 HBV patients with chronic HBV infection supports this notion. To directly demonstrate a role for CD24 in the development of liver cancer, we compared CD24+/− and CD24−/− HBV transgenic mice. Our data demonstrated that targeted mutation of the CD24 gene dramatically reduced the size of HCC in 14-month-old mice. Taken together, our data revealed a critical role for CD24 in the pathogenesis of HBV infection.

**Patients and Methods**

For description of patients and methods used, see Supporting Information online.

**Results**

**CD24 Polymorphisms Associated with Risk for Chronic HBV Infection.** Our previous studies have revealed two SNPs that significantly affect the risk and progression of autoimmune diseases (P170 C/T-rs8734; P1527 TG/del-rs3838646). To address whether these two SNPs influence the disease progression of the chronic HBV patients, we used the PCR-based RFLP polymerase chain reaction-restriction fragment length polymorphism) to genotype these two polymorphic sites in chronic HBV patients and healthy controls. As shown in Fig. 1, the CD24 genotypes can be distinguished by digesting the PCR products of CD24 with BstXI for P170, BsrI for P1527. The P170CC or P1527del/del products were completely resistant to the digestion, whereas the P170TT or P1527TG/TG products cleaved into two fragments of 275 basepairs (bp) and 129 bp for P170, and 645 bp and 202 bp for P1527. Partial digestion of 50% or less indicated the P170CT or P1527TG/del genotype. The validity of the PCR-RFLP analysis was confirmed by direct sequencing of several PCR samples with each genotype.

We analyzed 609 samples of chronic HBV patients and 383 normal controls for the distribution of CD24 genotypes. The P1527 genotyping was unsuccessful in 16 samples of chronic HBV patients. The frequencies of the CD24 alleles among our control population was similar to Caucasian, as we have reported previously (Supporting Table 1). Moreover, the genotype distributions of P170 and P1527 in the chronic HBV patients and controls did not deviate from Hardy-Weinberg equilibrium. These analyses confirmed the validity of genotyping. We therefore compared the distributions of the genotypes between normal controls and chronic HBV patients by the χ² test.

As shown in Table 1, the distribution of P170 genotypes among the chronic HBV patients significantly differed from that of the healthy controls, assuming a recessive model of genetic effect for P170T allele (sex-adjusted odds ratio [OR] = 1.65; 95% confidence interval [CI] = 1.05-2.62, P = 0.031). The overpresentation of the P170TT genotype suggests that more efficiently
expressed CD24 allele may increase the risk of chronic HBV infection.

Conversely, we observed a reduced frequency of the P1527del allele among the chronic HBV patients (Table 2). The P1527del allele has a significant gene dosage effect for the risk of chronic HBV infection (sex-adjusted OR, 0.67; 95% CI = 0.50–0.89, \( P = 0.006 \)).

**CD24 Polymorphism and Serum Viral Load.** We compared serum HBV-DNA load in the chronic HBV patients of different CD24 genotypes. As shown in Fig. 2A, the P170T/T patients had a higher viral load compared with the P170C/C or P170C/T patients (\( P = 0.036 \)). However, no significant difference was found in the viral load between patients of different P1527 genotypes (Fig. 2B).

**Associations of CD24 Polymorphism with Progression of Chronic HBV Infection.** Patients with chronic HBV infection often progress to LC and HCC. An important issue is whether the CD24 polymorphism affects the progression. Although the time of infection cannot be accurately defined, the overwhelming majority of the chronic HBV patients in China were infected at birth. Therefore, the age at which the patients developed either LC or HCC may provide a valuable approximation of the progression of chronic hepatitis B. We carried out Kaplan-Meier survival analysis to determine whether the CD24 polymorphism affects

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**Table 1. CD24 P170 Genotype Frequencies for All Subjects and OR Against Controls**

<table>
<thead>
<tr>
<th>Genotype or Allele</th>
<th>Controls</th>
<th>Cases</th>
<th>OR* (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype or Allele</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>180 (47.0)</td>
<td>227 (45.5)</td>
<td>1.00</td>
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<tr>
<td>CT</td>
<td>172 (44.9)</td>
<td>262 (53.0)</td>
<td>0.98 (0.75–1.30)</td>
<td>0.904</td>
</tr>
<tr>
<td>TT</td>
<td>31 (8.1)</td>
<td>70 (14.5)</td>
<td>1.64 (1.02–2.65)</td>
<td>0.042</td>
</tr>
<tr>
<td>CT+CC (against TT)</td>
<td>165 (4.3)</td>
<td>275 (5.5)</td>
<td>1.65 (1.05–2.62)</td>
<td>0.031</td>
</tr>
<tr>
<td>CC (against CT+TT)</td>
<td>0.93 (0.71–1.21)</td>
<td>0.573</td>
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<td></td>
</tr>
</tbody>
</table>

Gene dosage model (multiplicative per T allele)

1.16 (0.95–1.41) 0.155

*OR were adjusted by gender.

**Table 2. CD24 P1527 Genotype Frequencies for All Subjects and OR Against Controls**

<table>
<thead>
<tr>
<th>Genotype or Allele</th>
<th>Controls</th>
<th>Cases</th>
<th>OR* (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype or Allele</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG/TG</td>
<td>286 (74.7)</td>
<td>477 (80.4)</td>
<td>1.00</td>
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<tr>
<td>TG/del</td>
<td>88 (23.0)</td>
<td>111 (18.7)</td>
<td>0.89 (0.50–0.96)</td>
<td>0.028</td>
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<tr>
<td>del/del</td>
<td>0.06 (2.3)</td>
<td>5 (8.0)</td>
<td>0.36 (0.11–1.10)</td>
<td>0.073</td>
</tr>
<tr>
<td>TG/del+TG/TG (against del/del)</td>
<td>0.38 (0.12–1.19)</td>
<td>0.096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG/TG (against TG/ del+del/del)</td>
<td>1.50 (1.10–2.06)</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Gene dosage model (multiplicative per del allele)

0.67 (0.50–0.89) 0.006

*OR were adjusted by gender.
the progression of chronic HBV infection using LC and HCC as endpoints. As shown in Fig. 3A, comparison of the survival curve reveals that the P170 genotypes had a significant impact on the progression ($P/\text{H}^{0.017}$). Pairwise comparisons further show that P170T/T patients progressed more rapidly toward LC or HCC than both P170C/T patients ($P/\text{H}^{0.007}$) and P170C/C patients ($P/\text{H}^{0.060}$). When the CT and CC genotypes were compared with the TT genotypes, it is clear that the impact of CD24 P170 affected the disease progression by a T-recessive or C-dominant fashion ($P/\text{H}^{0.016}$). In all, 50% of the P170T/T patients developed LC or HCC in 41-year-old (95% CI = 39-43), whereas those with P170C/C and P170C/T patients developed LC or HCC in 47-year-old (95% CI = 45-49) and 49-year-old (95% CI = 46-52) patients. In addition, patients of P1527TG/del and P1527del/del genotypes were combined to compare with the P1527TG/TG genotype because of a low number of cases of LC and HCC patients with the P1527del/del. However, as shown in Fig. 3B, no significant difference was found in the patients having different genotypes in the SNP of P1527 ($P = 0.8369$).

**Expression of CD24 on Inflammatory Cells, But Not on HCC Cells or Hepatocytes of LC Patients.** To determine expression of CD24 in LC and HCC patients, frozen sections were first stained with anti-CD24 mAb SN3 by immunohistochemistry. As shown in Fig. 4A, expression of CD24 can be found in a high proportion of inflammatory cells, but not in the hepatocytes. We further used two color immunofluorescence microscopy to identify the types of CD24-expressing inflammatory cells in frozen sections of the HCC samples. As show in Fig. 4B, CD3+ T cell blasts expressed high levels of CD24,
although a significant proportion of T cells are CD24− (data not shown). Likewise, the majority of CD11c+ dendritic cells and CD19+ B cells are CD24+ (Fig. 4C,D). Therefore, multiple inflammatory cell types expressed CD24 in HCC tissues.

Targeted Mutation of CD24 Reduces the Size of HCC in HBV Transgenic Mice. We have previously reported that CD24 polymorphisms were associated with the expression levels of CD24. Because the CD24 P170T allele is expressed at a higher level than the P170C allele and conferred more rapid progression to LC or HCC, it is intriguing that higher CD24 levels may be a major risk factor for HBV-infected liver. To confirm a role for CD24 in the pathogenesis of HBV-expressing liver cells, we bred the CD24-null allele into a line of transgenic mice expressing S, pre-S, and X genes of HBV under the control of albumin promoter and spontaneously developing HCC.

We compared the size of liver tumors of the CD24+/−/HBV Tg mice and CD24−/−/HBV Tg littermates at 14 months. Male mice were used for the study as they developed tumors at a higher rate. As shown in Fig. 5A, the tumor nodules in CD24+/−/HBV Tg mice were strikingly larger than those found in the livers of CD24−/−/HBV Tg littermates. The mean largest volume of HCC per mouse was 5.5-fold greater in CD24+/−/HBV Tg mice than in CD24−/−/HBV Tg littermates (P = 0.045, Fig. 5B). When the volume of all tumors from one mouse were combined, CD24+/−/HBV Tg mice also showed significantly larger tumor volumes than CD24−/−/HBV Tg littermates, and the total volume was 4.2-fold larger in CD24+/−/HBV Tg mice (P = 0.036, Fig. 5C). Histology analysis (Fig. 5D) indicates that the tumors in both CD24+/− and CD24−/− mice are malignant HCC, characterized by large and heterogeneous nuclei, cancer cells with double nuclei (white arrow), as well as mitotic cells (yellow arrow). These data indicate that CD24 gene correlates with rapid cancer progression in the liver.

Discussion

Chronic HBV infection has been considered a multifactorial and polygenic disorder with viral, immunological, and genetic components. In the present study we examined the association between CD24 polymorphisms and the risk and progression of chronic HBV infection. Analysis of the distribution of the CD24 genotypes among 609 HBV patients and 383 normal controls indicated that the frequencies of the P1527T del or P1527del/del
genotypes were significantly lower in the chronic HBV infection group than in the normal controls. Thus, the CD24 P1527del allele may be a protective genetic susceptibility factor for HBV infection. Moreover, an increased risk of chronic HBV infection was found in subjects with the P170T/T genotype compared with P170C/T and P170C/C genotypes using a logistic regression model after sex adjustment. In contrast, the frequency of the P170T/T is significantly higher among the HBV patients in comparison to normal control. Given the fact that CD24 regulates local immune response, it is tempting to suggest that the different risks reflect a consequence of host response, although the possibility that the CD24 genotype affects the risk of infection during the perinatal period or early childhood cannot be ruled out.

In addition to an increased risk of chronic HBV infection, HBV patients with the P170T/T genotype developed LC and/or HCC at considerably younger age. Thus, among the patients that have reached LC or HCC, 50% of the CD24T/T patients reached that milestone in 41 years, and CD24C/C and CD24T/T patients did so in 47 and 49 years, respectively. Because chronic infection occurs in ~90% of infants infected at birth, in 25%-50% of children infected between the ages of 1 and 5 years, and in less than 5% of those infected during adult life,29 there is a high likelihood that the earlier onsets reflect faster progression from chronic HBV infection. More rapid progression in the CD24T/T patients suggests that more aggressive treatment may be warranted in this group of patients. However, we found no association between P1527 and the progression of chronic HBV infection. Although the underlying reason for the differential impact of the two SNPs remains unclear, it is of interest to note that P170 but not P1527 is associated with serum viral load.

An important issue is how the CD24 SNPs affect the risk and progression of chronic HBV infection. Our previous data have suggested that the protein encoded by the P170T allele, which exacerbates the progression of chronic HBV infection, is expressed at a higher level than P170C, presumably by enhancing the efficiency of posttranslational GPI cleavage.23 The protective allele, P1527del, on the other hand, reduces CD24 mRNA stability.24 These data are consistent with the notion that reduced CD24 function is protective against establishment of chronic HBV infection and its progression to severe liver diseases, including LC and HCC.

In order to demonstrate a role for CD24 for outcomes in liver cells chronically expressing HBV genes, we compared the tumor sizes of old male CD24+/−/HBV Tg mice and CD24−/−/HBV Tg littermates. This model recapitulates part of the pathogenesis of HBV-associated HCC as it is analogous to the stage of HBV infection when replication has ceased and viral DNA has integrated into the host cells.30 Our data clearly demonstrate that targeted mutation of CD24 greatly reduces the size of liver cancer caused by transgenic expression of HBV. A critical role for CD24 in liver cancer development raised an interesting possibility of targeting this gene for cancer therapy.

Although CD24 is expressed predominantly in the hematopoietic and neuronal cells, many tumor cells have been shown to overexpress CD24.25-27 Furthermore, Huang and Hsu31 showed that CD24 mRNA is overexpressed in liver tumors by differential display. Su et al.32 suggested that CD24 expression is a prognostic marker for intrahepatic cholangiocarcinoma. Potential expres-
sion of CD24 outside the hematopoietic cells raised an interesting issue as to whether CD24 modulates risk and progression of chronic HBV infection by affecting the immune/inflammatory response or malignant growth of hepatocytes. However, CD24 protein expression in hepatocyte or HCC has not been reported. In this regard, our immunohistochemical and immunofluorescence staining for CD24 on liver tissues from LC and HCC patients indicated that, whereas high levels of CD24 are observed on infiltrating leukocytes, including T cell blasts, B cells, and dendritic cells, no expression of CD24 in hepatocytes or tumor cells was found. These data suggest that the CD24 gene may modulate the function of leukocytes rather than HBV-infected hepatocytes, although the latter cannot be ruled out at this stage.

In theory, CD24 may affect the inflammatory response in the liver by two different mechanisms. First, as in the case of inflammation in the central nervous system, CD24 may be required for local T-cell activation. Because T-cell-mediated inflammation appears necessary for development of HCC in the HBV-transgenic mice, targeted mutation of CD24 may delay tumor growth by reducing the antigen-specific T-cell response locally. In contrast, we recently demonstrated that the CD24-Siglec 10 pathway negatively regulates production of inflammatory cytokines triggered by necrotic liver cells. Among the cytokines, the tumor necrosis factor has been demonstrated to suppress viral gene expression and therefore may delay the development of HCC in this model. Interestingly, the CD24^vprotein, which is encoded by the CD24^ allele, appears more efficiently associated with Siglec 10 (Supporting Fig. 1). As such, patients expressing this allele are expected to produce less tumor necrosis factor. A reduction in inflammatory cytokine may explain the higher HBV titer (Fig. 2A) and more rapid disease progression. It is unclear how these two factors are integrated to determine the consequence of HBV infection. In order to determine whether CD24 is required for local immune response in the liver, we compared the number and phenotype of inflammatory cells in the livers of CD24^/^- and CD24^+/+ HBV transgenic mice. As shown in Supporting Table S2, the number and subsets of T cells are comparable. Moreover, the levels of PD-1, a negative regulator for T-cell function in chronic infection, were also comparable in liver-infiltrating T cells. Therefore, the delay in growth of hepatocellular carcinoma cannot be merely attributed to a lack of T-cell infiltration to liver. Nevertheless, the types of local immune response may be qualitatively different. Clearly, further studies are needed to elucidate the molecular and immunological mechanisms by which CD24 gene dosage and/or polymorphism affects tumor sizes.

A recent study demonstrated that CD24 is expressed in liver progenitor cells during liver damage. Given the importance of both liver damage and the progenitor cells in the carcinogenesis of HCC, it would be of great interest to test whether CD24 expression in these cells facilitates cancer development in HBV transgenic mice.

In summary, our data indicated that human CD24 gene polymorphisms may affect both the risk and the progression of chronic HBV infection. Furthermore, in a murine HBV transgenic model the CD24 gene promoted the development of liver cancer. Our data provide important insights into the pathogenesis and immunotherapy of chronic HBV infection, LC, and HCC.

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References


