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Brief Communications

Association Analysis of Toll-like Receptor 4 Polymorphisms in Japanese Primary Biliary Cirrhosis

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Abbreviated title: TLR4 and PBC in Japan

Abbreviations: PBC, primary biliary cirrhosis; TLR, toll-like receptor; LPS, lipopolysaccharide; SNPs, single nucleotide polymorphisms

Abstract

Primary biliary cirrhosis (PBC) is characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often result in liver failure. Toll-like receptor (TLR) 4 recognizes lipopolysaccharides of Gram-negative bacteria. Infectious agents have been suspected to play a crucial role in PBC pathogenesis since TLR4 expression was found in bile duct epithelial cells and periportal hepatocytes in liver tissues of PBC. To assess the potential contribution of *TLR4* SNPs to the development of this disease, we genotyped five SNPs in *TLR4* in 261 PBC patients and 359 controls using a TaqMan assay. No significant positive associations with either PBC susceptibility or progression were uncovered. These results indicate that *TLR4* polymorphisms do not play a prominent role in the development of PBC in Japanese patients.

Keywords: HLA; PBC; SNPs; susceptibility; TLR4

1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often result in cirrhosis and liver failure [1]. The cause of PBC remains poorly understood [2], although population and family studies suggest that genetic factors contribute to disease susceptibility and severity [3]. Significant associations of genetic factors, including HLA alleles [4-6], cytotoxic T-lymphocyte antigen 4 [7-10], and other loci [11] have been reported for PBC. Only HLA has consistently been associated with PBC among these susceptibility genes. Specifically, the *DRB1*08* family of alleles has been the most frequently described determinant for this disease [4-6].

Toll-like receptors (TLRs) are a class of evolutionarily conserved pathogen recognition receptors that play an important role in innate identification of foreign material [12]. Activation of TLRs induces both innate and adaptive immune reactions against invading pathogens. TLR4 is a receptor for bacterial lipopolysaccharide (LPS) which selectively binds the lipid A portion of LPS. It was also found to be expressed in bile duct epithelial cells and periportal hepatocytes in PBC patient liver tissues [13, 14]. Since several bacterial products were detected in sera or liver tissues of PBC patients [15-17], infectious agents might play a crucial role in disease pathogenesis [18]. *TLR4* single nucleotide polymorphisms (SNPs) have been reported to be associated with genetic susceptibility to autoimmune diseases [19-22], but these genes have not been examined with respect to PBC. As such, we hypothesized that *TLR4* SNPs may be associated with PBC in the Japanese population and examined eight SNPs for associations with susceptibility and progression in Japanese patients.

2. Subjects and Methods

2.1. Study subjects

Between January 2005 and December 2011, a total of 261 patients with PBC (234 women, median age: 58 years, range: 27-86 years) and 359 healthy subjects (319 women) participated in this study. All control subjects had indicated the absence of major illness on a standard questionnaire. Racial backgrounds were all Japanese. The diagnosis of PBC in all patients was based on criteria from the American Association for the Study of Liver Diseases [23]. Serum anti-mitochondrial antibody-M2 was determined by ELISA, where a >7.0 index was considered to be positive, as previously reported [24]. All patients were negative for hepatitis B surface antigen and antibodies to hepatitis B core antigen, hepatitis C virus, and human immunodeficiency virus. Patients were grouped into two stages of PBC based on their most recent follow-up: early stage patients were histologically classified as Scheuer stage I or II [25] or of unknown histological stage without liver cirrhosis, and late stage patients were histologically Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure [10]. Liver cirrhosis was diagnosed by histological examination and/or characteristic clinical signs of advanced liver disease [26]. Patients with late stage disease or cirrhosis were 53 (20%) and 44 (17%), respectively. All subjects and controls provided written informed consent for testing of DNA samples. This study was approved by the institutional ethics committee.

2.2. Genotypoing of TLR4 SNPs

Genomic DNA was isolated from whole blood extracts for all patients and controls using QuickGene-800 (FUJIFILM, Tokyo, Japan) and adjusted to 10-15 ng/μl. TLR4 is composed of four exons and has four transcript isoforms. We evaluated five SNPs (rs10759930, rs2149356, rs11536889, rs7037117, and rs7045953) which were localized within the exons and introns of the *TLR4* gene. SNPs were selected from among previous reports [27, 28] and had minor allele frequencies of >5%. SNP spans were approximately 1 kb to 5 kb and included 5 kb of the predicted 5'-untranslated region and 6 kb of the predicted 3'-untranslated region of the *TLR4* gene. Genotyping of all SNPs was performed with a TaqMan 5' exonuclease assay using primers supplied by ABI (Applied Biosystems, Foster City, CA, USA). The probe fluorescence signals were detected with a TaqMan Assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

HLA typing was carried out using a Luminex multi-analysis profiling system with a LAB type® SSO OneLambda typing kit One (Lamda, Ganoga Park, CA), which is based on polymerase chain reaction sequence-specific oligonucleotide probes. HLA genotypes were determined by sequence-based typing [6].

2.3. Statistical analysis

The Hardy-Weinberg equilibrium test was performed for each SNP between control and patient groups. Pairwise linkage disequilibrium pattern, haplotype block structure, and haplotype frequency analysis were assessed for all SNPs by the block definition by Gabriel *et al*. [29], and were based on a 95% confidence interval (CI) of D' with Haploview version 4.2 software [30]. We plotted r^2 values. The significance of allele

distribution between patients with PBC and healthy subjects was evaluated using the χ^2 test for 2×2 comparisons. A *P* value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 18.0J; SPSS, Chicago, IL).

3. Results

A total of five SNPs in the *TLR4* gene were genotyped in 261 patients with PBC and 359 healthy subjects. The observed genotype frequencies for patients and controls were all in Hardy-Weinberg equilibrium, and the minor allele frequencies of all SNPs were >5%. All five SNPs were located in one haplotype block, and the magnitude of linkage disequilibrium between each SNP was high (Figure 1). Analysis of allelic frequencies revealed no significant differences between PBC and controls for *TLR4* SNPs (Table 1).

The haplotype frequency of the five SNPs was estimated with the expectation-maximization algorithm. Six unique SNP haplotypes were identified, and five of them had frequencies of >5% (Table 2). Association analysis using haplotypes calculated by expectation-maximization algorithms showed that none of them were associated with either susceptibility or resistance to PBC.

Since we previously reported that the HLA *DRB1*08:03-DQ*06:01* haplotype was associated with PBC in Japan, we further investigated the genetic association between this haplotype and the *TLR4* SNPs. Analysis of allelic frequencies revealed no significant differences between the presence and absence of the HLA *DRB1*08:03-DQ*06:01* haplotype and these SNPs (data not shown).

Next, we examined associations between the five *TLR4* SNPs and disease progression. There were neither significant allelic associations nor significant haplotype associations found in comparisons of early and late stage groups with regard to liver cirrhosis or non-cirrhosis (data not shown).

4. Discussion

In the present study, we investigated the possibility of an association between *TLR4* SNPs and PBC in Japan. We found no associations for any of the SNPs analyzed. Several infectious organisms have been proposed as potential causes of PBC [15-17], and TLR4, a specific receptor for LPS, was found in bile duct epithelial cells and periportal hepatocytes in liver tissues of PBC patients [13, 14]. Ballot *et al.* [31] reported that 64% of PBC sera was positive for IgM antibodies against lipid A, an immunogenic and toxic component of LPS. This finding was specific for the disease and correlated with more florid histological lesions. Moreover, Mao *et al.* reported that PBC patients were hyper-responsive to LPS stimulation, and suggested that aberrant signaling through TLR4 may precipitate disease onset [32]. Therefore, it has been hypothesized that TLR4 and its ligands would be implicated in the development of PBC, but the results of our SNP analysis indicated otherwise. Until now, no reports have been published regarding an association between PBC and *TLR4* SNPs in other ethnicities. Furthermore, genome-wide association studies have shown no significant associations between *TLR4* SNPs and PBC in Caucasians, so our negative association of *TLR4* SNPs with Japanese PBC may be valid.

Of the two co-segregating missense mutations in the gene encoding *TLR4* rs4986790 (Asp299Gly) and rs4986791 (Thr399Ile), only rs4986790 interrupts TLR4 signaling. Most studies that reported disease associations with *TLR4* SNPs have shown significantly higher frequencies of SNPs related to Asp299Gly and Thr399Ile [33], but none have detected these nonsynonymous mutations in Asian populations. Moreover, they were monomorphic in our Japanese healthy controls, which was consistent with other reports, including HapMap data [28].

The HLA *DRB1*08:03-DQB1*06:01* haplotype has been associated with susceptibility to PBC in a Japanese population [6]. Therefore, we investigated whether the HLA *DRB1*08:03-DQB1*06:01* haplotype and *TLR4* SNPs or haplotypes were independently associated with PBC, but found no confounding associations. Although our prior study showed that the HLA *DRB1*09:01-DQ*03:03* haplotype was associated with disease progression [6], we observed no significant associations between *TLR4* SNPs or haplotypes with late stage PBC or cirrhosis in this study.

In conclusion, it appears that *TLR4* SNPs and haplotypes are not associated with susceptibility to PBC in Japan. Genetic variations associated with PBC vulnerability remain open for further investigation, indicating the need for a genome-wide association study of PBC in Japan.

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Figure Legend

Figure 1

Linkage disequilibrium plot of five SNPs of the *TLR4* gene in 261 patients with primary biliary cirrhosis and 359 healthy controls. Values of r^2 corresponding to each SNP pair are expressed as a percentage and shown within the respective squares. Higher *D′* values are indicated by a brighter red color. The five SNPs constitute a haplotype block spanning 24 kb of the *TLR4* gene.

Table 1. Allele frequencies of SNPs in the *TLR4* gene of PBC patients and healthy subjects

SNP No.	dbSNP	Position (bp)	Minor allele	MAF in PBC	MAF in controls	*P* value	OR	95%CI
1	rs10759930	119,501,442	C	0.366	0.350	0.55	1.07	0.85-1.36
2	rs2149356	119,514,020	T	0.337	0.345	0.76	0.96	0.76-1.22
3	rs11536889	119,517,952	C	0.226	0.253	0.27	0.86	0.66-1.12
4	rs7037117	119,523,484	G	0.190	0.187	0.89	1.02	0.76-1.36
5	rs7045953	119,525,616	G	0.090	0.078	0.45	1.17	0.78-1.75

CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; PBC, primary biliary cirrhosis;

TLR4; toll-like receptor 4; SNP, single nucleotide polymorphism

P values were calculated with a χ^2-test 2 x 2 contingency table (df=1).

Table 2. TLR4 haplotypes in PBC patients and healthy subjects

Haplotype	SNPs					Haplotype frequencies		*P* value
	1	2	3	4	5	PBC	Controls	
1	T	G	G	A	A	0.409	0.402	0.78
2	T	G	C	A	A	0.225	0.243	0.46
3	C	T	G	A	A	0.144	0.151	0.73
4	C	T	G	G	A	0.098	0.106	0.65
5	C	T	G	G	G	0.090	0.078	0.44

PBC, primary biliary cirrhosis; TLR4, toll-like receptor 4