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Association analysis of Toll-like receptor 4 polymorphisms with autoimmune pancreatitis

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ABSTRACT

Autoimmune pancreatitis (AIP) is characterized by lymphoplasmocytic inflammation, high serum IgG4 concentrations, and a favorable response to corticosteroid treatment. Although long-term follow-up studies have shown that a relapse rate of 30–40% can occur in AIP after remission with corticosteroids, there are few genetic characteristic predictors of relapse in AIP patients. Toll-like receptor (TLR) is an important mediator in both innate and adaptive immunity. Polymorphisms in *TLR4* gene have been linked with several autoimmune and allergic diseases. We therefore investigated the genetic association between *TLR4* polymorphisms and AIP susceptibility and relapse in a Japanese population. Eight SNPs in *TLR4* (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) were genotyped in 59 patients with AIP and 126 healthy controls using a TaqMan assay. Analysis of allelic frequencies revealed no statistical association with either susceptibility or relapse of AIP. These data indicate that *TLR4* polymorphisms do not play an important role in the development of AIP.

1. Introduction

Autoimmune pancreatitis (AIP) is characterized by irregular narrowing of the main pancreatic duct, swelling of the pancreas, histologic evidence of lymphoplasmocytic inflammation, and a favorable response to corticosteroid treatment [1–4]. We and others have previously reported that IgG4 concentrations are significantly and specifically higher in patients with AIP, suggesting that IgG4 plays a major role in AIP pathogenesis [5,6]. In addition, abundant IgG4-bearing plasma cells have been found infiltrating the pancreas in AIP [7,8]. This disease is also characterized by systemic complications involving various extra-pancreatic lymphoplasmocytic inflammation and IgG4-bearing plasma cell infiltration; thus AIP has been recognized as a systemic inflammatory condition [7–10]. Furthermore, we previously reported three susceptibility genetic markers [11–14]. However, because none of the genetic markers currently identified can sufficiently explain disease etiology, additional genes that influence immune tolerance are likely to be involved. Zen *et al.* recently reported that in patients with AIP, the Th2 and regulatory immune reactions were upregulated in the affected tissues [15]. These investigators indicated that the predominance of Th2 and regulatory immune reactions in AIP might reflect an allergic nature in the pathogenesis. According to recent studies on AIP, susceptibility and relapse of AIP are influenced by genetic factors, specific HLA alleles, amino acid sequences at the presentation site of the HLA molecule, and cytotoxic T-lymphocyte antigen 4 *(CTLA4)* SNPs [13,16].

Toll-like receptors (TLRs) are transmembrane proteins expressed by cells of the innate immune system, which recognize pathogen-associated molecular patterns and play important roles in immune and inflammatory responses to destroy the invaders. Among TLR family members, TLR4 (Toll-like receptor–4) has been the most thoroughly investigated. Apart from its involvement in the recognition of lipopolysaccharide, TLR4 also interacts with endogenous ligands, including heat-shock proteins. Some studies have reported that allergic diseases, including bronchial asthma and atopic dermatitis, are associated with single-nucleotide polymorphisms (SNPs) in the *TLR4* gene [17–19]. However, no study has comprehensively evaluated risk factors for AIP relapse and investigated the association between *TLR4* SNPs and AIP. Therefore, we examined the potential involvement of *TLR4* SNPs in the susceptibility and relapse of AIP.

2. Subjects and methods

2.1. Subjects

Between September 1994 and September 2007, we recruited 59 patients with AIP (49 men and 10 women), 38–76 years old (median, 63 years old), and 126 healthy control subjects. The diagnosis

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of AIP was based on criteria released by the Japan Pancreas Society using clinical data, imaging tests, and/or histopathologic findings, as reported previously [20]. Of the 59 patients with AIP, 37 (63%) had concurrent autoimmune diseases, including hypothyroidism (11 patients) and sclerosing cholangitis (34 patients); these diagnoses were described in prior studies [9,21]. All control subjects had indicated the absence of major illnesses on a standard questionnaire. This group was formed by enrolling volunteers from hospital staff. All racial/ethnic backgrounds were Japanese.

Serum levels of IgG4 were determined by single radial immunodiffusion kits (normal, <135 mg/dl) as reported previously [5]. High serum IgG4 concentrations (median, 730.0 mg/dl; interquartile range, 265.0–1037.5 mg/dl) were found in 55 of the 59 patients with AIP. Of the patients, 52 were treated with 40 mg prednisolone daily for 4 weeks; the dose was then reduced by 5 mg per week over a period of several weeks. All 52 patients responded favorably to corticosteroid therapy, resulting in improvements in clinical, laboratory, and imaging findings. We found no high concentrations of serum IgG4 in healthy subjects. All patients and controls were negative for the hepatitis B surface antigen and antibodies to hepatitis C in the serum [22].

In total, we followed the 55 patients with high IgG4 levels, including 52 patients who were treated with corticosteroids every month for a period of at least 12 months (median, 72 months; range, 12–178 months). Patients underwent regular follow-up visits with an interview every month; laboratory tests every 2–3 months, and imaging tests, including computed tomography or magnetic resonance imaging, every 6 months or, in the event of relapse, until September 2007. Of the 55 patients, 16 (29%) experienced relapse during follow-up. A relapse was defined as a recurrent attack of pancreatic swelling that resulted in irregular narrowing of the pancreatic duct or stenosis of the common bile duct, as reported previously [23].

All participants provided written informed consent for tests with DNA samples. After receiving permission, serum samples were obtained from patients and normal subjects. This study was approved by the institutional ethics committee.

2.2. TLR4 genotyping

Genomic DNA was isolated from whole blood of patients and healthy individuals using QuickGene-800 (Fujifilm, Tokyo, Japan). The concentration of genomic DNA was adjusted to 10–15 ng/μl for the TaqMan SNP genotyping assay. TLR4 is composed of four exons and has four transcript isoforms. We evaluated eight SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) that were localized within the exons and introns of the *TLR4* gene. These SNPs were selected from among previous reports [24–26] and public information sources, such as the NCBI dbSNP (http://www.ncbi.nlm.nih.bov/SNP/), Applied Biosystems (http://www.appliedbiosystems.com), and HapMap (http://www.hapmap.org/) databses, and had minor allele frequencies >5%. The SNP spans approximately 1–5 kb, and includes 5 kb of the predicted 5′-untranslated region (UTR) and 6 kb of the predicted 3′ UTR in the *TLR4* gene. Genotyping of all SNPs was performed by a TaqMan 5′ exonuclease assay using primers supplied by (Applied Biosystems, Foster City, CA). 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.

2.3. HLA typing

HLA class I and II alleles, and *DRB1* and *DQB1* alleles were identified, as reported previously [27,28]. These HLA typings had been done before, not for the purpose of this manuscript.

2.4. Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was done for each SNP among controls and patient groups. The pairwise linkage disequilibrium (LD) patterns, haplotype block structure, and haplotype frequency analysis for all SNPs were assessed by the block definition of Gabriel *et al.*, and was based on 95% CI of D′ with implementation of Haploview version 3.32 software [29,30] (http://www.broad.mit.edu/mpg/haploview/index.php). The significance of allele distribution between patients with AIP and healthy subjects was tested using the χ^2 test for 2 × 2 or 2 × 3 comparisons. When the number of subjects was less than 5, Fisher's exact test was used. A value of $p < 0.05$ was considered statistically significant. The corrected p value (p_c) was calculated by the Bonferroni's correction where the coefficient was the total number of the contingency tables tested.

3. Results

3.1. TLR4 genotyping in patients with AIP and healthy subjects

Eight SNPs in TLR4 were genotyped in 59 patients with AIP and in 126 healthy subjects (Table 1). In controls, the genotype distributions of all SNPs exhibited Hardy-Weinberg equilibrium, and the minor allele frequencies of all SNPs were more than 5%. However, in patients, the genotype distribution of one SNP (rs2149356) differed significantly from the expected Hardy-Weinberg values ($p < 0.05$) (Table 1). All eight SNPs were located in 1 haplotype block, and the magnitude of LD between each SNP was high (Fig. 1). Analysis of allelic frequencies (Table 2) revealed a significant difference between patients with AIP and healthy subjects for SNP rs2149356: Positivity for G was significantly higher in patients with AIP than in healthy subjects ($\chi^2 = 8.58$, $p = 0.014$). The G/T genotype was significantly increased in patients with AIP compared with healthy subjects. This SNP preliminary showing statistical significance was later confirmed as not significant after correction for multiple testing. No other SNPs were significantly associated with AIP. The statistical power of this study was 0.9349 and enough for analysis.

Table 1
Allele frequencies of SNPs of the TLR4 gene in AIP patients and controls

dbSNP	Alleles (1/2)	Position (bp)	Gene location	Patients ($n = 59$)		Controls ($n = 126$)	
				MAF (%)	HWE	MAF (%)	HWE
rs10759930	T/C	119,501,442	5′-UTR	38.1	0.276	33.3	0.801
rs1927914	A/G	119,504,546	5′-UTR	38.1	0.276	32.9	0.698
rs1927911	G/A	119,509,875	Intron	35.6	0.629	32.9	0.235
rs12377632	C/T	119,512,551	Intron	35.6	0.629	32.5	0.602
rs2149356	G/T	119,514,020	Intron	31.4	0.007	32.1	0.511
rs11536889	G/C	119,517,952	3′-UTR	26.3	1.000	26.2	1.000
rs7037117	A/G	119,523,484	3′-UTR	20.3	0.505	17.5	0.282
rs7045953	A/G	119,525,616	3′-UTR	10.2	1.000	7.5	0.961

1, major allele; 2, minor allele; bp, base pair.
Position is distance from short arm telomere.