

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

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ABSTRACT

Autoimmune pancreatitis (AIP) is characterized by lymphoplasmacytic 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.

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1. Introduction

Autoimmune pancreatitis (AIP) is characterized by irregular narrowing of the main pancreatic duct, swelling of the pancreas, histologic evidence of lymphoplasmacytic 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 lymphoplasmacytic 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/ μ l 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.gov/SNP/>), Applied Biosystems (<http://www.appliedbiosystems.com>), and HapMap (<http://www.hapmap.org/>) databases, 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.

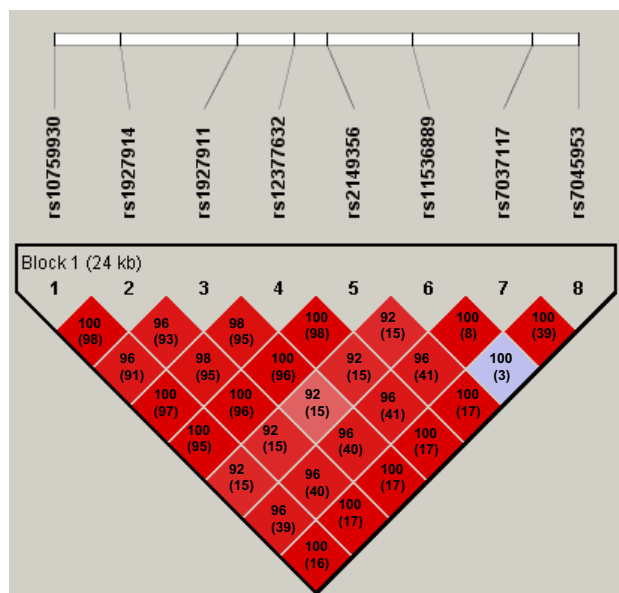


Fig. 1. Structure of linkage disequilibrium (LD) plot of 8 SNPs of the *TLR4* gene in the controls. The D' value and r^2 value (in parentheses) corresponding to each SNP pair are expressed as a percentage and shown within the respective square. Higher D' is indicated by a brighter red. The 8 SNPs constitute a haplotype block spanning 24 kb of the *TLR4* gene.

The haplotype frequency of the eight SNPs was estimated with the expectation-maximization algorithm. Nine unique SNP haplotypes were found altogether, and five had frequencies greater than 5% (Table 3). Association analysis using haplotypes calculated by expectation-maximization algorithms showed none of haplotypes were associated with either susceptibility or resistance to AIP.

We previously reported that the *HLA DRB1*0405-DQB1*0401* haplotype was associated with AIP [11,12]; thus, we further investigated the genetic association between *HLA* haplotype and *TLR4* SNPs in patients with AIP. Analysis of allelic frequencies revealed no significant difference in SNP5 ($\chi^2 = 0.52$, $p = 0.77$) between patients with and without the *HLA DRB1*0405-DQB1*0401* haplotype.

3.2. Associations among *TLR4* SNPs, patient characteristics, and AIP relapse

Next, we examined associations between the *TLR4* SNPs and clinical parameters. We found no associations between any of the eight *TLR4* SNPs and age, gender, or serum IgG4 concentrations (data not shown). In particular, the median serum IgG4 concentration was not significantly different between patients with and without the SNP5G allele (730 vs 728 mg/dl; $p = 0.94$).

Previous studies found that AIP was associated with the autoimmune diseases, sclerosing cholangitis (34/44; 77%) and hypothyroidism (11/50; 22%) [13]. Thus, we evaluated whether sclerosing cholangitis or hypothyroidism were associated with any of the eight *TLR4* SNPs. We found no significant association between the *TLR4* SNPs and the two diseases (data not shown). Other studies found an association between relapse of AIP and genetics [13,19]; thus, we further analyzed the relationship between *TLR4* SNPs and risk of AIP relapse. In our cohort, 16 of 55 patients (29%) experienced relapse during the follow-up period. However, we found no significant associations between the SNPs or the haplotypes and the relapse of AIP.

4. Discussion

In previous studies, we determined that the *HLA DRB1*0405-DQB1*0401* haplotype correlated with an increased prevalence of AIP in the Japanese population [11,12]. However, none of the pre-

viously identified genetic markers were sufficient to fully explain the disease etiology. Therefore, we suspected that a number of genes outside the major histocompatibility complex region might play a role in AIP susceptibility. For instance, we previously identified polymorphisms of the *FCRL3* and *CTLA4* genes that correlated with an increased prevalence of AIP in the Japanese population [13,14]. However, these findings have not been examined and confirmed in other ethnicities. *TLR4* is an interesting candidate for a gene related to AIP susceptibility because it has previously been implicated in other autoimmune diseases and allergic diseases, including rheumatoid arthritis, Behçet's disease, bronchial asthma, and atopic dermatitis [16–18,31,32].

Of the two co-segregating missense mutations in the gene encoding *TLR4*, A896G and C1196T (which result in Asp299Gly and Thr399Ile amino acid changes, respectively), only A896G interrupted *TLR-4* signaling [10]. Most studies that reported disease associations with *TLR4* SNPs have shown significantly higher frequencies of SNPs related to the A896G and C1196T mutations. However, we did not detect polymorphisms on these two SNPs in 100 Japanese healthy controls, consistent with other reports including HapMap data. In the present study, we examined eight SNPs. Only rs2149356 SNP was statistically associated with AIP before correction of the p value. However, this SNP, among all SNPs

Table 2
TLR4 polymorphisms in 59 patients with AIP and 126 healthy subjects

dBSNP	Frequency (%)		p	OR	95% CI
	AIP ($n = 59$)	Controls ($n = 126$)			
rs10759930					
Allele frequency					
C allele	38.1	33.3	0.37	1.24	0.78–1.94
T allele	61.9	66.7			
rs1927914					
Allele frequency					
G allele	38.1	32.9	0.33	1.26	0.80–1.98
A allele	61.9	67.1			
rs1927911					
Allele frequency					
A allele	35.6	32.9	0.61	1.13	0.71–1.78
G allele	64.4	67.1			
rs12377632					
Allele frequency					
C allele	64.4	67.5	0.57	1.15	0.72–1.81
T allele	35.6	32.5			
rs2149356					
Genotype frequency					
G/G	39.0	47.6	0.014*		
G/T	59.3	40.5			
T/T	1.7	11.9			
Allele carrier frequency					
G (G/G+G/T)	98.3	88.1	0.021**	7.84	1.01–60.82
T (G/T+T/T)	61.0	52.4	0.27	1.43	0.76–2.67
Allele frequency					
G allele	68.6	67.9	0.98	1.04	0.65–1.66
T allele	31.4	32.1			
rs11536889					
Allele frequency					
A allele	26.3	26.2	0.91	1.00	0.61–1.65
G allele	73.7	73.8			
rs7037117					
Allele frequency					
A allele	79.7	82.5	0.60	1.21	0.69–2.10
G allele	20.3	17.5			
rs7045953					
Allele frequency					
G allele	89.8	92.5	0.52	1.39	0.65–2.96
T allele	10.2	7.5			

AIP, autoimmune pancreatitis; OR, odds ratio; 95% CI, 95% confidence interval. p Value was calculated by χ^2 test 2×2 contingency table ($df = 1$), or test 3×2 contingency table ($df = 2$).

* p_c (corrected p value) = 0.042, ** p_c = 0.17.

Table 3
TLR4 haplotypes in patients with AIP and healthy subjects

Haplotype	8 SNPs within a haplotype block spanning 24 Kb								Proportion of indicated haplotype (%)		p value
	1	2	3	4	5	6	7	8	AIP	Controls	
	rs10759930	rs1927914	rs1927911	rs12377632	rs2149356	rs11536889	rs7037117	rs7045953	(n = 118)	(n = 252)	
HP1	T	A	G	C	G	G	A	A	33.9	41.4	0.21
HP2	T	A	G	C	G	C	A	A	28.0	24.0	0.43
HP3	C	G	A	T	T	G	A	A	11.0	15.1	0.32
HP4	C	G	A	T	T	G	G	A	10.2	9.5	0.85
HP5	C	G	A	T	T	G	G	G	10.2	6.9	0.26

AIP, autoimmune pancreatitis.

Values for n indicate two times the number of individuals since each person carries two haplotypes.

tested in the patients group, significantly deviated from HWE in the patient group. The deviation might be explained by collecting samples of affected individuals selectively, because HWE failure is generally caused by migration, mutation, gene flow, genetic drift, nonrandom mating, and natural selection. This phenomenon is thought to be the cause of decreasing homozygous TT and GG carriers in contrast to increasing heterozygous TG carriers in the patients. The functional effects of increased TG heterozygotes in patients are still uncertain. However, as there were only a small number of patients in our cohort, it will be necessary to confirm this association in future studies with larger cohorts. Although the statistical power was enough as 0.9349 in this study, type II error was also a possible explanation for the lack of association between TLR4 SNPs and AIP.

Although previous work has shown that serum IgG4 concentrations were closely associated with AIP, we found no significant correlations between serum IgG4 concentrations and TLR4 SNPs or haplotypes in this study. In addition, our analysis of patients with AIP that also had sclerosing cholangitis and hypothyroidism indicated that there were no associations between extrapancreatic complications and TLR4 SNPs or haplotypes.

We checked whether the HLA DRB1*0405-DQB1*0401 haplotype and rs2149356 SNP was independently associated with AIP. However, we found no confounding association between the HLA DRB1*0405-DQB1*0401 haplotype and rs2149356 SNP. This result was similar to that shown in a previous study that found no association between the HLA DRB1*0405-DQB1*0401 haplotype and FCRL3-110 alleles, although each was independently associated with AIP [14].

Long-term follow-up studies have shown that a 30–40% rate of AIP relapse can occur after remission with corticosteroids [13,19,23,33]. However, no characteristic risk factors or predictive markers have been identified that might be associated with a relapse of AIP. We recently found that patients that experienced relapse had high associations with CTLA4 +49A/A or +6230A/A genotypes [13]. Furthermore, substitution of aspartate to a non-aspartate residue at HLA DQβ1 57 was associated with a relapse of AIP in a Korean cohort [19]. In the present study, we examined whether any of the TLR4 SNPs were associated with an AIP relapse but found no associations. Further studies are required to identify predictive markers of AIP relapse.

In conclusion, we found that TLR4 gene polymorphisms were not significantly associated with susceptibility to AIP in Japan. However, the connection between genetic variations and susceptibility to AIP and AIP relapse remains to be addressed in future investigations.

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