Table 2. Clinical characteristics of patients with systemic JIA with or without macrophage activation syndrome (MAS).

Characteristics	Systemic JIA with MAS (n = 33)	Systemic JIA without MAS (n = 48)	p
Male, n (%)	16 (48.5)	24 (50.0)	0.54
Age at systemic JIA onset, yrs, mean	4.8	4.7	0.92
Observation period, mo, mean	97.8	102.2	0.43
Time interval between JIA onset and MAS development, mo, mean	24.8	—	—

JIA: juvenile idiopathic arthritis.

study[18]. None of the gene polymorphisms under study was associated with susceptibility to systemic JIA (Table 3).

However, the rs2004640 SNP was found to be associated with MAS susceptibility (Table 4). Patients with the rs2004640 T allele had a high risk of developing MAS compared to those without this allele even after the Bonferroni correction (p_c = 0.003, OR 4.12, 95% CI 1.84, 9.16). Moreover, all the patients with the TT genotype at rs2004640 finally developed MAS (Table 4, Figure 1). Patients carrying the TT genotype at rs2004640 had an early onset of MAS (a mean of 12.1 mo after onset of JIA). Additionally, the ATT haplotype of the *IRF5* gene

(rs729302-rs2004640-rs2280714) showed a statistically significant association with susceptibility to MAS (p < 0.001, OR 4.61, 95% CI 1.73, 12.3; Table 5). A haplotype block showed the correlation between the SNP genotyped (Figure 2).

DISCUSSION

In the clinical setting, MAS apparently develops under the influence of systemic inflammatory responses of systemic JIA together with environmental factor(s), supposedly viral infection[5]. Susceptibility to these environmental factors may be subject to genetic influences. The combined pres-

Table 3. Association of polymorphisms in the *IRF5* gene with susceptibility to systemic juvenile idiopathic arthritis (JIA).

SNP, Subject Subset	No. (%) with Genotype			Total	Allelic Association OR	(95% CI)	p
rs729302	AA	AC	CC				
Systemic JIA	42 (51.9)	33 (40.7)	6 (7.4)	81	0.84	0.56–1.24	0.37
Control	116 (61.1)	57 (30.0)	17 (8.9)	190	1.0	—	—
rs2004640	GG	GT	TT				
Systemic JIA	36 (44.4)	36 (44.4)	9 (11.1)	81	1.05	0.71–1.55	0.80
Control	82 (43.2)	85 (44.7)	23 (12.1)	190	1.0	—	—
rs2280714	CC	CT	TT				
Systemic JIA	28 (34.6)	44 (54.3)	9 (11.1)	81	1.19	0.81–1.73	0.38
Control	58 (30.5)	94 (49.5)	38 (20.0)	190	1.0	—	—

IRF5: interferon regulatory factor 5; SNP: single-nucleotide polymorphism.

Table 4. Association of polymorphisms in the *IRF5* gene with susceptibility to macrophage activation syndrome (MAS) in patients with systemic juvenile idiopathic arthritis (JIA).

SNP, Subject Subset	No. (%) with Genotype			Total	Allelic Association OR	(95% CI)	p	p_c
rs729302	AA	AC	CC					
MAS	22 (66.7)	10 (30.3)	1 (3.0)	33	2.45	1.11–5.42	0.03	0.08
Non-MAS	20 (41.7)	23 (47.9)	5 (10.4)	48	1.0	—	—	—
rs2004640	GG	GT	TT					
MAS	9 (27.3)	15 (45.5)	9 (27.3)	33	0.24	0.11–0.54	0.001	0.003
Non-MAS	27 (56.3)	21 (43.8)	0 (0.0)	48	1.0	—	—	—
rs2280714	CC	CT	TT					
MAS	14 (42.4)	17 (51.5)	2 (6.1)	33	2.12	1.08–4.40	0.045	0.13
Non-MAS	14 (29.2)	27 (56.3)	7 (14.6)	48	1.0	—	—	—

p_c: corrected combined p value using the Bonferroni method. *IRF5*: interferon regulatory factor 5; SNP: single-nucleotide polymorphism.

is a clinical impression, however, that there are at least 2 subsets of patients with systemic JIA, one never experiencing MAS and the other with recurring MAS.

Interferon regulatory factor 5 (IRF5) is a member of the IRF family of transcription factors, and is known to have a crucial role in the Toll-like receptor (TLR) signaling pathway[11]. The activation of TLR is central to innate and adaptive immunity. IRF5 acts as a master transcription factor in the activation of proinflammatory cytokine genes. In *IRF5*-knockout mice, a severely impaired induction of IL-6, IL-12, and TNF-α was observed[11]. Recent investigations revealed associations of single-nucleotide polymorphism (SNP) in the *IRF5* gene with susceptibility to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)[12,13]. Thus, IRF5 has a regulatory potential for proinflammatory cytokines in certain inflammatory diseases that manifest with abnormal expression of proinflammatory cytokines.

We hypothesized that polymorphisms in the *IRF5* gene may constitute the genetic differences between the 2 tentative subsets of systemic JIA. We found a close relationship between *IRF5* gene polymorphism/haplotype and susceptibility to MAS in patients with systemic JIA.

MATERIALS AND METHODS

Study population. Patients were eligible if they met the International League of Associations for Rheumatology classification criteria for systemic JIA[14]. A total of 81 children, 40 boys and 41 girls, enrolled in this study were followed at the Yokohama City University Hospital between December 2007 and December 2009. The mean age of the patients was 4.7 years at onset of systemic JIA. The observation period of patients without MAS was at least 25 months, with a mean observation period of 102.2 months (range 25–284 mo).

Patients were diagnosed as having MAS based on the clinical symptoms and laboratory abnormalities as suggested in the preliminary diagnostic guidelines for MAS complicating systemic JIA[15], as follows: (1) clinical criteria including central nerve dysfunctions, hemorrhages, and hepatomegaly; and (2) laboratory criteria including decreased platelet counts ($< 26.2 \times 10^9$/l), elevated levels of aspartate aminotransferase (> 59 U/l), decreased white blood cell counts ($< 4.0 \times 10^9$/l), and hypofibrinogenemia (< 2.5 g/l). The diagnosis of MAS requires the presence of 2 or more criteria. Evidence of hemophagocytosis in bone marrow aspirates was sought only for confirmation of doubtful cases.

We conducted our study in accordance with the Declaration of Helsinki and with the approval of the Ethics Committee of the Yokohama City University School of Medicine. Written informed consent was obtained from each patient and/or their guardians.

Genotyping. Three SNP (rs729302, rs2004640, and rs2280714) in the *IRF5* gene were selected based on previous research associating them with SLE and RA[12,13]. The patients with systemic JIA (n = 81) and 190 healthy controls were genotyped. Genomic DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen K.K., Tokyo, Japan). Genotyping was performed using the TaqMan SNP Genotyping Assays (AB assay ID: C_2691216_10 for rs729302, C_9491614_10 for rs2004640, and C_2691243_1 for rs2280714). These SNP were analyzed by real-time polymerase chain reaction (PCR) using the AB7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) under the conditions recommended by the manufacturer. The TaqMan SNP Genotyping Assay for rs2004640 was performed by TaqMan gene expression master mix instead of by TaqMan genotyping master mix. Results of genotyping at rs2004640 by TaqMan gene expression master mix were consistent with results from

direct sequencing, while results by TaqMan genotyping master mix were not consistent with results from direct sequencing. The rs41298401 SNP, located 6 base pairs downstream of rs2004640, influenced these conflicting results, presumably because rs41298401 is in the base sequence annealing with TaqMan probe and causes the annealing to be insecure.

Allele discrimination was done using SDS software version 1.4 (Applied Biosystems). Confirmation of which bases were present for 5 cases of each genotype at each of these SNP sites of the genomic DNA sample was carried out using direct sequencing in the Applied Biosystems 3730xl and Sequence Scanner version 1.0 under the conditions recommended by the manufacturer.

Statistical analysis. The SNPassoc package using the R-language version 2.8 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) was used to evaluate associations between systemic JIA/MAS and these SNP, by logistic regression analysis[16]. Haplotype phases and haplotype frequencies were estimated using the Expectation-Maximization algorithm as implemented in the haplostat package (minimum haplotype frequency: > 0.05; www.docstoc.com)[17]. Haplotype blocks were assessed using Haploview (The Broad Institute, Cambridge, MA, USA; www.broadinstitute.org). Logistic regression analysis was also performed to evaluate the association between systemic JIA/MAS and the *IRF5* haplotypes. Association between MAS and *IRF5* gene polymorphism was analyzed by Kaplan-Meier curves with log-rank test.

RESULTS

Of the 81 patients with systemic JIA, 33 (13 boys and 20 girls) developed MAS during the followup period, according to the preliminary diagnosis guideline (Table 1)[15]. The mean lengths of followup were 97.8 months in patients with MAS and 102.2 months in patients without MAS (Table 2). MAS was recognized at a mean of 24.8 months (range 0–166 mo) after the onset of systemic JIA. However, the remaining 48 patients did not develop MAS during the followup. Age at onset of systemic JIA (p = 0.92, Welch's t test) and sex (p = 0.54, Fisher's exact test) were not associated with susceptibility to MAS in our study population (Table 2).

The genotype frequencies for the 3 SNP of the patients with systemic JIA and the healthy controls were both in Hardy-Weinberg equilibrium (p > 0.05). These results were consistent with the findings of a recent Japanese population

Table 1. The frequency of clinical, laboratory, and histopathological features of macrophage activation syndrome (MAS) in the preliminary diagnostic guideline[15]. Total number of patients was 81.

| Features	No. Patients (%)
Laboratory criteria	
Decreased platelet count ($< 26.2 \times 10^9$/l)	27 (81.8)
Elevated levels of aspartate aminotransferase (> 59 U/l)	25 (75.8)
Decreased white blood cell count ($< 4.0 \times 10^9$/l)	11 (33.3)
Hypofibrinogenemia (< 2.5 g/l)	9 (27.3)
Clinical criteria	
Central nervous system dysfunction (seizure)	1 (3.0)
Hemorrhages (purpura, mucosal bleeding)	1 (3.0)
Hepatomegaly (> 3 cm below the costal arch)	3 (9.1)
Histopathological criterion	
Evidence of macrophage hemophagocytosis in the bone marrow aspirate	3 (9.1)

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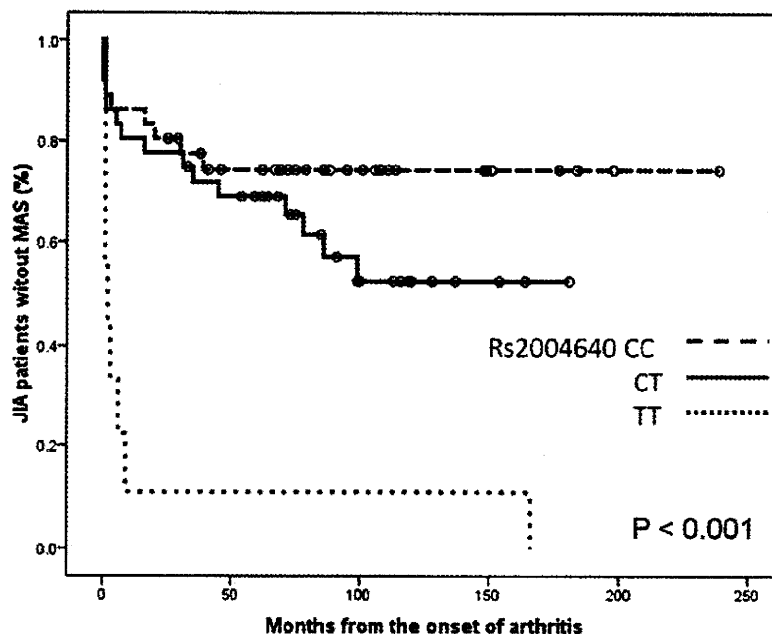
Figure 1. Kaplan-Meier analysis of survival without macrophage activation syndrome and rs2004640 genotypes. JIA: juvenile idiopathic arthritis.

Table 5. Comparison of IRF5 haplotypes in patients with systemic juvenile idiopathic arthritis (JIA) with or without MAS. The order of SNP (single-nucleotide polymorphisms) in the haplotype is rs729302-rs2004640-rs2280714.

Haplotype	Haplotype Frequencies in Patients without MAS	Haplotype Frequencies in Patients with MAS	p	OR	95% CI
A-G-C	0.438	0.303	0.06	1.0	—
C-G-T	0.330	0.182	0.04	0.92	0.35–2.39
A-T-C	0.017	0.015	0.93	1.19	0.08–17.6
A-T-T	0.201	0.485	< 0.001	4.61	1.73–12.3

IRF5: interferon regulatory factor 5; MAS: macrophage activation syndrome.

ence of fairly frequent polymorphisms in multiple genes involved in the regulation of innate and adaptive immunity may be one of the major determinants in the initiation of rheumatic diseases[19]. To develop innate and adaptive immune responses, the activation of a TLR signaling pathway is essential. The transcription factor IRF5 is generally involved downstream of the TLR signaling pathway for induction genes for proinflammatory cytokines such as IL-6, IL-12, and TNF-α[11,20]. As mentioned, proinflammatory cytokines such as IFN-γ and TNF-α are responsible for the clinical and laboratory abnormalities seen in MAS[4,5]. Thus, we examined the association of polymorphisms in the *IRF5* gene with susceptibility to MAS in patients with systemic JIA.

Our investigation revealed that the rs2004640 T allele and the ATT haplotype in the *IRF5* gene were associated with MAS developing in patients with systemic JIA. All the patients with the TT genotype at rs2004640 had MAS, and

they had an early onset of MAS compared to those with non-TT genotypes (Figure 1). While the ATT haplotype of the *IRF5* gene was associated with susceptibility to MAS in patients with systemic JIA in our study, it was also reported that this is the common haplotype conferring increased risk of SLE[12]. The T alleles of both rs2004640 and rs2280714 were associated with higher levels of IRF5 messenger RNA expression[12]. Further, the other IRF5 haplotype was associated with high serum IFN-α activity in patients with SLE[21]. Although we did not address the association between proinflammatory cytokine activity and genotype/haplotype in the *IRF5* gene, there may be the potential role of IRF5-associated immune response in the pathogenesis of MAS. Further research is needed to determine the influence of gene polymorphisms in the *IRF5* gene on proinflammatory cytokine activities.

Although several drugs, such as tolmetin and tocilizumab, and viral infections were considered the triggering caus-
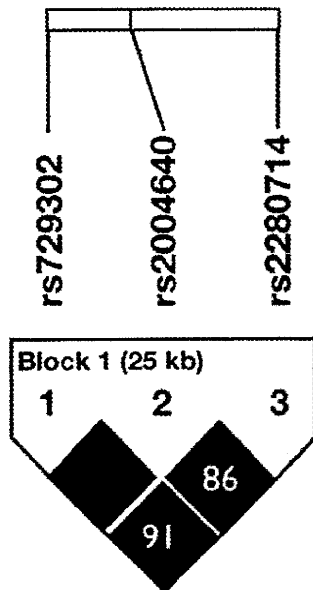
Figure 2. Haplotype blocks of interferon regulatory factor 5 gene polymorphisms.

es of MAS in our cohort, the triggers were not determined in most cases. All patients with MAS recovered from their severe complication. We could not find any association between IRF5 genotypes/haplotypes and the characteristics of clinical symptoms and severity of MAS in this study, presumably because of a small study population. We need a larger cohort to determine this association.

There are some limitations to our study. The incidence of MAS (40.7%) was significantly higher than generally seen (about 10%)[4,5]. Our hospital is one of the pediatric rheumatology centers in Japan and we have treated many patients with severe systemic JIA. The incidence of MAS is so high partly because of the characteristics of our hospital. In addition, there are ethnic differences in the incidence by the subtypes of JIA. Specifically, systemic JIA accounts for about 20% of JIA in Japan but for only about 10% in Europe and the United States[22]. Therefore there may also be ethnic differences in susceptibility to MAS.

A second issue is that we could not carry out a validation study. Although the genetic association study should be validated, the incidence of MAS complicating systemic JIA is too low to validate this association in a single institution. Therefore it is important for the association between the IRF5 genotype/haplotype and MAS susceptibility to be confirmed by other groups.

We found a strong association between polymorphisms in the *IRF5* gene and susceptibility to MAS in patients with systemic JIA. This finding suggests a potentially important role of the IRF5-associated immune response in the pathogenesis of MAS.

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ORIGINAL ARTICLE

Association of HLA-A*02:06 and HLA-DRB1*04:05 with clinical subtypes of juvenile idiopathic arthritis

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Juvenile idiopathic arthritis (JIA) is one of the most common forms of pediatric chronic arthritis. JIA is a clinically heterogeneous disease. Therefore, the genetic background of JIA may also be heterogeneous. The aim of this study was to investigate associations between human leukocyte antigen (HLA) and susceptibility to JIA and/or uveitis, which is one of the most devastating complications of JIA. A total of 106 Japanese articular JIA patients (67 with polyarthritis and 39 with oligoarthritis) and 678 healthy controls were genotyped for HLA-A, -B and -DRB1 by PCR-sequence-specific oligonucleotide probe methodology. HLA-A*02:06 was the risk factor for JIA accompanied by uveitis after adjustment for clinical factors (corrected P-value <0.001, odds ratio (OR) 11.7, 95% confidence interval (CI) 3.2–43.0). On the other hand, HLA-DRB1*04:05 was associated with polyarticular JIA (corrected P-value <0.001, OR 2.9, 95% CI 1.7–4.8). We found an association of HLA-A*02:06 with susceptibility to JIA accompanied by uveitis, which might be considered a separate clinical JIA entity. We also found an association between HLA-DRB1*04:05 and polyarticular JIA. Thus, clinical subtypes of JIA can be classified by the presence of the specific HLA alleles, HLA-A*02:06 and DRB1*04:05.
Journal of Human Genetics advance online publication, 23 December 2010; doi:10.1038/jhg.2010.159

Keywords: HLA-A*02:06; HLA-DRB1*04:05; juvenile idiopathic arthritis; uveitis

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is one of the most common forms of pediatric chronic arthritis, with an incidence of approximately about 10 per 100 000 children less than 16 years old.[1] JIA is clinically classified into subtypes, such as systemic arthritis, oligoarthritis and polyarthritis, according to the International League of Associations for Rheumatology (ILAR) classification criteria for JIA.[2]

JIA is the leading cause of chronic anterior uveitis in pediatric cases, accounting for 30–40% of incidence.[3,4] The prevalence of JIA-associated uveitis varies among different ethnic groups. Children of European ancestry have a higher relative risk for developing JIA-associated uveitis than those of non-European ancestry.[5] Risk factors for developing JIA-associated uveitis include antinuclear antibody positivity, early age at onset of JIA and female gender.[3,5] However, the contribution of each risk factor may also vary among ethnic groups, presumably because of variations in genetic background.[6]

Human leukocyte antigen (HLA) typing is considered useful for assisting in the diagnosis of autoimmune disease-associated uveitis such as HLA-B27-associated uveitis.[7] It has also been reported that HLA-DRB1*13 is associated with susceptibility to JIA-associated uveitis in a Caucasoid population.[8] Because the distribution of HLA

alleles vary in different ethnic groups, the HLA-linked genetic background for JIA is also likely to vary among different populations. Currently, there is no information in the literature on the association between JIA-associated uveitis and HLA in Japanese. The aim of this study was therefore to investigate the association between HLA genes and clinical subtypes of JIA, especially JIA accompanied by uveitis in Japanese children.

This is the first report on the association between HLA alleles and JIA accompanied by uveitis in the Asian population.

MATERIALS AND METHODS

Studied population

Patients were eligible, if they met the ILAR classification criteria for JIA oligoarthritis or polyarthritis (articular JIA).[2] All patients enrolled in this study were followed-up at the Yokohama City University Hospital between December 2006 and November 2009. A total of 106 Japanese articular JIA patients (86 female and 20 male; 67 with polyarthritis and 39 with oligoarthritis) were included in this study. Clinical data, including age at onset of arthritis and uveitis, gender, antinuclear antibody, rheumatoid factor and anti-cyclic citrullinated peptide status, were reviewed. Presence of uveitis was diagnosed by ophthalmologists at routine visits every 6 months. Written informed consent

was obtained from each patient and/or their guardians. Japanese healthy control subjects (n=678) were randomly selected, and an informed consent was obtained from each subject before blood sampling.

The study was performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the Yokohama City University School of Medicine Ethics Committee and the Ethics Committee of Medical Research Institute, Tokyo Medical and Dental University.

HLA genotyping

JIA patients were genotyped for HLA-A, -B and -DRB1 by the PCR-sequence-specific oligonucleotide probe (SSOP)–Luminex method using Genosearch HLA-A, -B and -DRB1 Ver. 2 (Medical & Biological Laboratories, Nagoya, Japan), as described previously.[9] HLA genotyping data from 678 healthy Japanese controls selected at random were obtained for HLA-A and -DRB1 by the PCR-SSOP-based typing method as described previously[10,11] and for HLA-B using PCR-reverse SSOP-based RELI SSOP HLA-B typing kits (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Frequencies of HLA allele carriers were calculated for each locus in patients and controls. The statistical significance of the differences in the frequencies between patients and controls was evaluated by Fisher's exact test. A corrected P-value (P_c) was calculated by multiplying the P-value by the number of alleles tested at each locus. A two-locus analysis was carried out to investigate interactions of the disease-associated HLA alleles for increasing disease susceptibility, according to the method of Svejgaard and Ryder.[12]

Associations between JIA-associated uveitis and clinical variables were analyzed by Student's t-test and Fisher's exact test. Multiple logistic regression analysis was performed to investigate associations with JIA-associated uveitis. All statistical analyses were carried out using SAS system version 9 (SAS Institute, Cary, NC, USA).

RESULTS

Association between HLA and JIA susceptibility

A total of 106 articular JIA patients and 678 healthy controls were genotyped for HLA-A, -B and -DRB1 to investigate associations of HLA with JIA susceptibility. It was found that frequencies of HLA-DRB1*04:05 and HLA-A*02:06 tended to increase in the patients

($P<0.05$, P_c=not significant) (Table 1). Because the marginal association between HLA and JIA overall might reflect a stronger association between HLA and a specific clinical subtype of JIA, patients were stratified into polyarticular and oligoarticular types. This approach revealed that HLA-DRB1*04:05 was indeed highly significantly associated with polyarticular JIA ($P_c<0.001$, odds ratio (OR) 2.9, 95% confidence interval (CI) 1.7–4.8), but tended to decrease in oligoarticular JIA (Table 1). In the latter, HLA-A*02:06 and HLA-DRB1*09:01 showed a tendency toward increased frequencies, but this did not reach significance after correction for the number of alleles tested ($P<0.05$, P_c=not significant) (Table 1). No other HLA-A, -B or -DRB1 alleles showed any significant associations with either form of articular JIA.

Clinical and genetic characteristics of JIA accompanied by uveitis

Because different HLA alleles showed associations with different subtypes of articular JIA, it is likely that the association of HLA with JIA would reflect the association with other specific clinical manifestations. Therefore, we investigated the clinical and genetic characteristics of JIA accompanied by uveitis.

Of 106 patients with articular JIA, 13 (12.2%) developed uveitis. All the patients with uveitis were female, but the gender difference was not statistically significant (P=0.055). Mean age at onset of JIA in the patients with uveitis was 3.2 years (range 0–14) (Table 2). The mean age for developing uveitis was 5.2 years (range 1–14), and the mean time interval between the onset of JIA and development of uveitis was 21.9 months (range −4 to 48), with one case developing uveitis 4 months before the onset of JIA. Oligoarticular onset, rheumatoid factor negativity ($<14\,IU\,ml^{-1}$) and low anti-cyclic citrullinated peptide ($<4.5\,U\,ml^{-1}$), as well as younger age at onset of JIA were all significantly associated with JIA accompanied by uveitis (Table 2). Nine patients with JIA accompanied by uveitis responded well to topical corticosteroids and could discontinue medications for uveitis, but the remaining four additionally required systemic glucocorticoids or anti-tumor necrosis factor-α therapy for uveitis in the follow-up period.[13]

Table 1 Association of HLA with susceptibility to JIA

	Articular JIA (N=106)				Polyarticular JIA (N=67)				Oligoarticular JIA (N=39)				Control (N=678)
	N (%)	OR (95% CI)	P-value	P_c	N (%)	OR (95% CI)	P-value	P_c	N (%)	OR (95% CI)	P-value	P_c	N (%)
HLA-A*02:06	24 (22.6)	1.9 (1.1–3.1)	0.012	NS	13 (19.4)	1.6 (0.8–3.0)	0.177	NS	11 (28.2)	2.5 (1.2–5.3)	0.010	NS	91 (13.4)
HLA-DRB1*04:05	36 (34.0)	1.6 (1.0–2.5)	0.032	NS	32 (47.8)	2.9 (1.7–4.8)	<0.001	<0.001	4 (10.3)	0.4 (0.1–1.0)	0.045	NS	164 (24.2)
HLA-DRB1*09:01	42 (39.6)	1.5 (0.97–2.3)	0.066	NS	24 (35.8)	1.3 (0.7–2.1)	0.385	NS	18 (46.2)	1.9 (1.0–3.7)	0.043	NS	208 (30.7)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; JIA, juvenile idiopathic arthritis; NS, not significant; OR, odds ratio; P_c, corrected P-value by multiplying a P-value by the number of alleles tested in each locus.

Table 2 Clinical variables in association with JIA accompanied by uveitis

	JIA patients with uveitis (N=13)	JIA patients without uveitis (N=93)	P-value	OR	95% CI
Age at JIA onset (years, mean)	3.2	7.6	0.002	0.7	0.6–0.9
Gender (female, %)	13 (100%)	73 (78.5%)	0.055	ND	ND
Subtype (oligoarticular onset, %)	10 (76.9%)	29 (31.2%)	0.004	7.4	1.9–28.7
ANA (≧1:160, %)	8 (61.5%)	30 (32.3%)	0.061	—	—
RF (≧14.0 (IU ml^{-1}), %)	1 (7.7%)	56 (60.2%)	0.001	0.1	0.07–0.4
Anti-CCP (≧4.5 (U ml^{-1}), %)	1 (7.7%)	47 (50.5%)	0.006	0.1	0.01–0.7

Abbreviations: ANA, antinuclear antibody; CCP, cyclic citrullinated peptide; CI, confidence interval; JIA, juvenile idiopathic arthritis; ND, not detectable; OR, odds ratio; RF, rheumatoid factor.

Table 3 Association between HLA and susceptibility to uveitis in patients with JIA

	With uveitis (N=13)	Without uveitis (N=93)	OR	95% CI	P-value	P_c
HLA-A*02:06	9 (69.2%)	15 (16.1%)	11.7	3.2–43.0	<0.001	<0.001
HLA-DRB1*04:05	0 (0%)	36 (38.7%)	0.6	0.5–0.7	0.006	NS
HLA-DRB1*09:01	9 (69.2%)	33 (35.5%)	4.1	1.2–14.3	0.020	NS

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; JIA, juvenile idiopathic arthritis; NS, not significant; OR, odds ratio; P_c, corrected P-value by multiplying a P-value by the number of alleles tested in each locus; with uveitis, JIA patients with uveitis; without uveitis, JIA patients without uveitis.

Table 4 Two-locus analysis of HLA-A*02:06 and HLA-DRB1*09:01

HLA-A*02:06	HLA-DRB1*09:01	JIA-associated uveitis (N=13) (%)	Control (N=678) (%)
(a) Basic data			
Positive (+)	Positive (+)	6 (46.2)	30 (4.4)
Positive (+)	Negative (−)	3 (23.1)	61 (9.0)
Negative (−)	Positive (+)	3 (23.1)	178 (26.3)
Negative (−)	Negative (−)	1 (7.7)	409 (60.3)

(b) Stratification analysis in JIA-associated uveitis

Comparison		Individual association		Independent association for A		Independent association for B		Difference between A and B association	Combinatory association
Factor A	Factor B	Test (1)	Test (2)	Test (3)	Test (4)	Test (5)	Test (6)	Test (7)	Test (8)
HLA-A*02:06	HLA-DRB1*09:01			++ vs −+	+− vs −−	++ vs +−	−+ vs −−	+− vs −+	++ vs −−
		OR_A	OR_B	$OR_{A, non-B}$		$OR_{B, non-A}$			$OR_{A, B}$
OR		14.5	5.1	20.1	6.9	11.9	4.1	2.9	81.8
95% CI		4.4–48.1	1.5–16.7	2.1–196.5	0.7–66.7	2.8–50.0	1.0–17.4	0.6–14.8	9.5–701.7
P-value		<0.001	0.024	0.002	0.428	<0.001	0.356	1.0	<0.001

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; JIA, juvenile idiopathic arthritis; OR, odds ratio.

To further investigate the association between HLA and JIA accompanied by uveitis, we compared the frequencies of HLA alleles among JIA patients with or without uveitis. As shown in Table 3, the frequency of HLA-A*02:06 carriers was significantly higher in the JIA patients with uveitis (P_c<0.001, OR 11.7, 95% CI 3.2–43.0). The frequency of HLA-DRB1*09:01 carriers was also higher in patients with uveitis, although this was not statistically significant after correction for multiple testing (P=0.020, P_c=not significant) (Table 3). No other HLA-A, -B or -DRB1 alleles, including HLA-B*27 (B27), DRB1*11 (DR11) and DRB1*13 (DR13), showed any significant associations with JIA accompanied by uveitis.[3,5,8]

To investigate the contribution of HLA-A*02:06 and HLA-DRB1*09:01 with susceptibility to JIA accompanied by uveitis, we performed a two-locus analysis according to Svejgaard and Ryder (Table 4).[12] This suggested a synergistic interaction of these two HLA alleles in susceptibility to this form of JIA. Thus, the odds risks conferred by A*02:06 in the presence or absence of DRB1*09:01 were 20.1 (test (3)) and 6.9 (test (4)), respectively; for DRB1*09:01 in the presence or absence of A*02:06, these values were 11.9 (test (5)) and 4.1 (test (6)), respectively (Table 4). In addition, the odds risk conferred by the presence of both HLA alleles was 81.8 (test (8)), which was much higher than A*02:06 alone (test (4)) or DRB1*09:01 alone (test (6)), further supporting a synergistic interaction.

Finally, we performed multiple logistic regression analysis to evaluate risk factors for JIA accompanied by uveitis, and demonstrated that HLA-A*02:06 was the most significant of these (P=0.014, OR 7.8, 95% CI 1.5–40.5).

DISCUSSION

It is well known that genetic factors influence susceptibility to autoimmune disorders, such as rheumatoid arthritis and JIA.[14–16] HLA is one of the most important genetic factors for these kinds of diseases; there are also ethnic differences in the contribution of HLA to disease susceptibility. In the present study, we evaluated associations between HLA and susceptibility to articular JIA and its clinical subtypes, polyarticular and oligoarticular JIA in Japanese children (Table 1). We found that HLA-DRB1*04:05 was significantly associated with polyarticular JIA, whereas HLA-A*02:06 and HLA-DRB1*09:01 tended to be associated with oligoarticular JIA. These findings imply the existence of genetic differences between the polyarticular and oligoarticular JIA.

HLA-DRB1*04:05 encodes an amino-acid sequence in the DRβ chain, which is well known as an epitope shared between the HLA-DR molecules associated with susceptibility to rheumatoid arthritis in many ethnic groups[17,18] including Japanese,[19] as well as polyarticular JIA in Taiwanese.[20] Although there is debate about whether rheumatoid arthritis and polyarticular JIA could be similar clinical conditions,[21] HLA-DRB1*04:05 may contribute to the pathogenesis of chronic arthritis at different ages and in different ethnic groups. Further studies are required to determine the molecular mechanisms by which HLA-DRB1*04:05 contributes to pathogenesis.

Herein, we found an association of both HLA-A*02:06 and DRB1*09:01 with susceptibility to JIA accompanied by uveitis (Tables 3 and 4). In the clinical setting, the frequency of occurrence

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of uveitis is one of the main differences between polyarticular and oligoarticular JIA (Table 2).[4] Uveitis is one of the most devastating complications of JIA, because it causes posterior synechia, band keratopathy and cataract, which can result in a poor visual prognosis.[22,23] Clinical risk factors for JIA-associated uveitis were also found to be associated with JIA-associated uveitis in Japanese (Table 2). In addition to these known risk factors, we found that HLA-A*02:06 was one of the risk factors. Using a two-locus analysis, HLA-A*02:06 and HLA-DRB1*09:01 were found to synergistically interact to increase susceptibility to JIA accompanied by uveitis. Because HLA-A*02:06 and HLA-DRB1*09:01 are not in linkage disequilibrium in Japanese,[24] two different genes in the HLA region might be responsible.

No patients with JIA accompanied by uveitis typed HLA-DRB1*13 positive in this study, although this allele has been reported to be significantly associated with such Caucasian patients.[8] There are several subtypes of HLA-DRB1*13; for example, DRB1*13:01 and DRB1*13:02, which vary in different ethnic groups. Because the majority of the DRB1*13 subtype in Japanese is DRB1*13:02, whereas it is DRB1*13:01 in Caucasians, the lack of association with DRB1*13 in our study might reflect these ethnic differences. In this regard, it should be noted that HLA-A*02:06 and HLA-DRB1*09:01 are relatively frequent in Japanese, but rare in European populations.

In this study, we demonstrated that JIA accompanied by uveitis was associated with HLA-A*02:06, which was not otherwise associated with JIA. This suggests that JIA accompanied by uveitis might be a specific clinical entity. It should be noted that HLA-A*02 (A2) is associated with susceptibility to anterior uveitis in adult patients[25] and JIA-associated uveitis in Caucasians.[14] In this regard, we should carefully follow-up the JIA patients carrying HLA-A*02:06 with respect to ocular complications.

We could not validate the association in this study. Although the genetic association should be validated in another cohort, the incidence of JIA and uveitis is too low to be validated in a single institution. Therefore, it is important to replicate the association between HLA-A*02:06/-DRB1*09:01 and JIA accompanied by uveitis in other cohorts including various ethnic groups.

In conclusion, we found an association of HLA-A*02:06 and possibly HLA-DRB1*09:01 with susceptibility to JIA accompanied by uveitis, which might be considered as a distinct clinical entity within JIA. Clinical subtypes of JIA may be classified by the presence of the specific HLA alleles, such as HLA-A*02:06 and DRB1*04:05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Category; Original Articles
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National Survey of Childhood Febrile Illness Cases with Fever of Unknown Origin in
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Japan
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Short title ; National Survey of Fever of Unknown Origin Cases on Childhood
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＊Number of text pages:, reference pages,figures:6
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Abstract   25

Background: In Japan, an actual condition survey on childhood febrile diseases with   26

fever of unknown origin has never been performed.We performed national survey on   27

childhood febrile illnesses in order to collect useful information for the differential   28

diagnosis. *Methods:* A nationwide survey using questionnaires was performed on febrile   29

illness cases with fever of unknown origin (FUO) experienced by 2,843 pediatrics   30

institutions with sick beds during a 5-year period before 2007. FUO was defined as a   31

febrile illness of at least 2 weeks' duration with a temperature $\geq 38℃$, and failure to   32

establish a diagnosis in spite of intensive evaluation during seven days' hospitalization.   33

Results: Two hundred fifty-five of 2,843 questionnaire-surveyed institutions had 960   34

FUO cases, of which 132 could not be diagnosed, and 828 could be diagnosed after   35

detailed medical examinations. The diagnoses they clarified included infectious diseases   36

in 190 cases (23%), rheumatic diseases in 448 cases (54%), neoplasms in 67 cases (8%),   37

and others in 123 cases (15%). *Conclusion:* Clarification of illnesses that ought to be   38

differentiated in the diagnostic approach to an FUO case is essential for arriving at its   39

definitive diagnosis by exclusion.   40

                                                                                          41

Keywords   42

Child , Febrile diseases , Fever of unknown origin , Final diagnosis , National survey

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Introduction

We often experience cases with fever of unknown origin (FUO) in a clinical setting, yet research on the actual state of childhood febrile illnesses has rarely been done in our country although such research will be useful for the differential diagnosis of FUO. It is unclear in many aspects what diagnoses are made for FUO cases and how their differential diagnosis is made. Making a definite diagnosis of an FUO case is considered important for determination of therapeutic indication for an FUO case that really needs treatment. Therefore, we made a nationwide survey on childhood febrile illnesses on this occasion in order to acquire information useful for the differential diagnosis of FUO.

Survey Institutions and Methods

Survey institutions were 2,843 nationwide children's institutions with sick beds. They were asked to answer primary and secondary retrospective inquiries about FUO cases they had experienced during a 5-year period before 2007. FUO was defined as a febrile illness of at least 2 weeks' duration with a temperature $\geqq 38\,^{\circ}\text{C}$ and failure to establish a diagnosis in spite of evaluation during seven days' hospitalization.

In the primary survey, number of FUO cases, final diagnosis, gender, and age were investigated. The secondary survey was made to investigate in detail symptoms and signs as well as differential diagnostic approaches taken in the cases that had been reported to have final diagnoses in the primary survey.

This study protocol was approved by the Ethics Committee of Yokohama City University Hospital (Approval No.042 , Approval date: July 27, 2007).

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Results

In the primary survey, of 2,843 institutions to which questionnaires were sent, 68

1,071 (37.7%) returned the questionnaire sheets. Valid answers were acquired from 69

1,045 institutions excluding 26 ones where pediatrics departments were closed. Two 70

hundred fifty-five institutions experienced 960 applicable cases (Fig.1), of which 132 71

could not be diagnosed, and 828 were diagnosed after detailed examinations: infectious 72

diseases in 190 (23%), rheumatic diseases in 448 (58%), neoplasms in 67 (8%), and 73

others in 123 (15%) (Fig. 3). 74

In the secondary survey, we made a more detailed investigation on 828 cases which 75

had been reported in the primary survey with their established diagnoses. We sent 76

questionnaire sheets to 230 institutions, 146 of which returned valid answered sheets. 77

Eighteen institutions replied but their data were invalid for analysis because their 78

pediatrics departments were closed in some and case records that had been returned in 79

the primary survey were incomplete in others. Of the above 146 institutions that 80

answered properly, 127 reported 328 applicable cases (Fig. 2). These 127 institutions 81

included 53 special hospitals, 60 municipal hospitals, and 14 non-specified facilities. 82

Among the above 328 cases, only 185 meet the above definitions. 83

Patients' background: 101 patients were boys, and a male/female ratio was 1.2. 84

Symptoms appeared at the age of 2 month to 18 years (mean, 7 years and 0 month) and 85

diagnoses were made at the age of 2 months to 22 years (mean, 7 years and 3 months). 86

Time from fever onset to diagnosis was 86.1 days in average. Diagnosis was established 87

after close examinations in 153 out of 185 cases. 88

Classification of illnesses: There were 29 cases (15.7%) of infectious diseases, 108 89

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(58.4%) of rheumatic diseases, 14 (7.6%) of neoplasms, and 34 (18.4%) of other diseases.

1. Infectious diseases (Fig. 4)   91

Cat scratch disease was most frequent in 10 cases, followed by 7 cases of infectious   92

diseases affected by viruses such as EB virus, coxsackie virus, adenovirus and others,   93

next came 2 cases of nontuberculous mycobacteriosis. Rare cases of Q fever,   94

tsutsugamushi fever, brucellosis, typhoid fever, and splenic abscess were included.   95

2. Rheumatic diseases (Fig. 5)   96

The most frequent illness was systemic-onset juvenile idiopathic arthritis, composing   97

about 60% (68 cases). Others were 9 cases of Takayasu's arteritis, 8 cases of   98

inflammatory bowel disease, and 4 cases of systemic lupus erythematosus.   99

3. Neoplasms   100

This category included 5 cases of Langerhans-cell histiocytosis, 4 cases of acute   101

lymphocytic leukemia, 2 cases of malignant lymphoma, 2 cases of neuroblastoma, and 1   102

myofibromatosis case.   103

4. Others (Fig. 6)   104

Thirty-four other cases included 14 cases of histiocytic necrotizing lymphadenitis that   105

was most frequent, 5 cases of hemophagocytic syndrome, and 3 cases of acute   106

disseminated encephalomyelitis.   107

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Discussion   109

We surveyed the reality of cases with fever of unknown origin (FUO) that developed   110

over years from 2003 to 2007 for the purpose to study what illnesses were differentiated 111

and to utilize it for diagnostic approach of new FUO cases.   112

FUO was defined by Petersdorf et al. for the first time in 1961 as a febrile illness of at 113

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least three weeks' duration, with a temperature $\geq$ 38.3°C and failure to establish a   115
diagnosis in spite of one-week intensive inpatient evaluation.[1] This body temperature   116
of 38.3°C was that measured in the oral cavity and could have been lower by 0.3 to 0.5   117
degrees if measured in the axilla. In 1968, Dechovitz et al. reported 155 cases of   118
childhood FUO defined as a febrile illness of at least two weeks' duration with failure to   119
identify a cause.[2]   119

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In this study, in accordance with these reports, we defined a group of illnesses as a   121
febrile illness with a temperature $\geq$ 38℃ lasting for two weeks or longer and failure   122
to establish a diagnosis in spite of evaluation in one-week hospitalization. After the   123
report of Petersdorf et al., several papers on FUO concerning pathogenetic classification   124
in particular were published. However, papers on children cases are very scarce, so that   125
the present nationwide study performed in Japan is considered significant in this   126
context.   127

Most of a large number of papers grouped roughly etiologies of FUO into infectious   128
diseases, rheumatic diseases, neoplasms, others, and unknown in descending order of   129
frequency. (Pizzo et al. reported 100 prolonged fever cases in children. Fifty-two were   130
infectious, 20 collagen-inflammatory, 6 malignancy, 10 miscellaneous, and 12   131
undiagnosed.→omit) Chantada et al. reported that 113 childhood FUO cases included   132
41 cases of infectious diseases, 15 of rheumatic diseases, 11 of neoplasms, and 22 of   133
unknown etiology.[3] As mentioned above, most of studies concerning childhood FUO   134
reported that infection was the most frequent cause of FUO.[4] In contrast, our present   135
survey revealed that rheumatic diseases comprised the etiologies in 54%, which   136
exceeded greatly 23% for infectious diseases. A similar trend was observed in a report   137

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concerning adults by Iikuni et al.[5]

In their report, among 79 adult FUO cases, 29.4% of them had rheumatic diseases, 140

28.8% infectious diseases, and 14.4% neoplasms, indicating a decrease in the rate of 141

infectious diseases or neoplasms and an increase in that of rheumatic diseases as 142

compared to previous reports. One of the reasons why rheumatic disease was the most 143

common cause of FUO in the present study as well like in the above report was that it 145

took a long time to make a diagnosis of illnesses associated with major conditions of 146

systemic inflammation or vasculitis which had no specific markers. Systemic-onset 147

juvenile idiopathic arthritis has no specific markers helpful for its diagnosis, so that 148

symptoms such as skin rash and arthritis are a determinant of reaching a diagnosis 149

after all. 150

However, its diagnosis can be hard to make in the initial phase because of lack of 151

pathognomonic symptoms or signs including arthritis. This situation allowed the 152

disease to fulfill the definition of FUO in many cases and the disease thus comprised the 153

rheumatic disease group in around 60% in the present study. 154

Similarly, in an investigation of adult FUO by Goto et al., adult-onset Still's disease, 155

which simulated systemic-onset juvenile idiopathic arthritis in clinical conditions, 156

comprised nearly 40% of the non-infectious inflammatory disease group including 157

rheumatic diseases.[6] Whereas it remains to be difficult to diagnose these rheumatic 158

diseases, the rate of the correct diagnosis of infectious diseases or neoplasms seems to 159

be better than before. The spread of rapid diagnostic methods, the progress of antibody 160

as well as culture examinations, and the expanded use of anti-bacterial agents may 161

have resulted in alleviation of symptoms and signs. This situation may have thus 162

reduced infectious disease cases that meet the FUO definition, while the development of 163

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imaging examinations may have improved the diagnosis rate of neoplasms.

When an affirmative diagnosis is difficult with the help of markers etc., exclusion of   165

other illnesses plays an important role for diagnosis. FDG-PET has been increasingly   166

reported to be useful for the diagnosis of FUO.[7] Although it is evident that the device is   167

a powerful tool for the establishment of inflammatory pathologic conditions, its   168

applicability is limited to special facilities as things are because of problems involving   169

equipment investment and indication for children.   170

When an 'FUO' case is presented, it is tempting to give priority to the establishment   171

of a diagnosis by way of differentiating illnesses listed in the present survey. However,   172

we consider it more important to evaluate the 'severity' of the case on the basis of   173

available information since the 'severity' suggests the 'morbid state,' which in turn   174

determines whether further appropriate examinations are required.   175

The present study has a limitation. Because this is the retrospective and multicenter   176

study, there is a possibility of a recall bias about whether all data in all patients were   177

included.   178

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