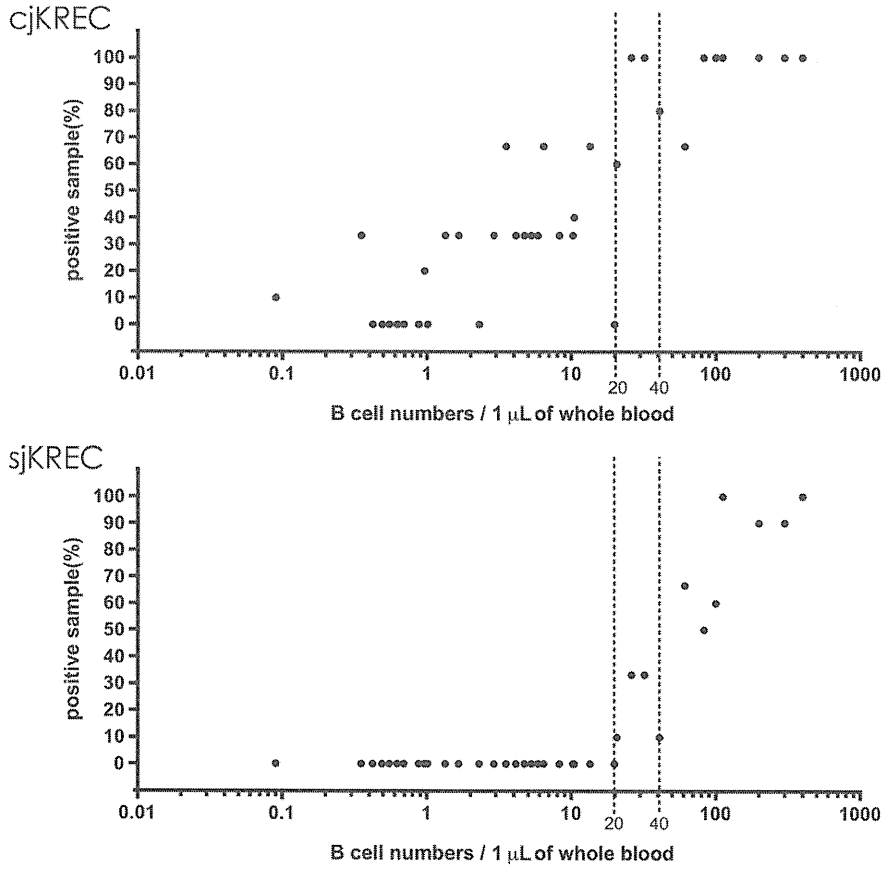

FIG 1. Sensitivity levels of cjKRECs and sjKRECs. Various numbers of purified normal B cells were serially added to whole PB from a patient with XLA (P20) to obtain B-cell–added XLA whole blood. cjKRECs and sjKRECs were measured in 3 to 10 samples of each concentration in triplicate. In all analyses, RNaseP (internal control) was positive ($2.3 \pm 0.2 \times 10^5$ copies/μg DNA). *X-axis,* B-cell numbers in 1 μL whole blood from a patient with XLA. *Y-axis,* Percentages of the KREC-positive results in the tests.

as the B-cell concentrations increased (Fig 1). None of the samples were positive for sjKRECs when the B-cell numbers were less than 20/μL, but cjKRECs were often positive. It has been reported that 90% of patients with XLA have less than 0.2% B cells in the PB at diagnosis.[1] Because peripheral lymphocyte numbers in neonates range from 1200 to 9800/μL,[8] the absolute B-cell numbers of 90% of patients with XLA are estimated to be 2.4 to 19.6/μL at the time of blood collection for Guthrie cards, although exact B-cell numbers of XLA in neonatal periods are not known at this moment. Because neonates are known to have fewer B cells than infants,[9] and we observed that B-cell numbers are constantly low in patients with XLA throughout infancy (Nakagawa, unpublished data, June 2010), which is consistent with the fact that BTK plays an essential role in B-cell maturation. It is likely that neonates with XLA also have severely decreased B cells. On the other hand, all samples obtained from 400 B cells/μL were positive for both cjKRECs and sjKRECs. We also observed that all healthy infants (1-11 months old; n = 15) were sjKREC-positive (Nakagawa, unpublished data, June 2010) and might have at least 600 B cells/μL whole blood.[9] From these data, it is assumed that at least 90% of patients with XLA are sjKREC-negative, and healthy neonates are positive for sjKRECs on neonatal Guthrie cards.

Next, we measured cjKRECs and sjKRECs in dried blood spots in filter papers or Guthrie cards from 30 patients with XLA and 5 patients with non-XLA and from 133 neonates born at the National Defense Medical College Hospital during this study period (August 2008 to October 2009) and 138 healthy subjects of various ages (1 month to 35 years old) to investigate the validity of this method. The levels of B cells of the patients ranged from 0.0% to 1.1% of total lymphocytes and 0.0 to 35.78/μL. IgG levels were 10 to 462 mg/dL (see this article's Tables E1 and E2 in the Online Repository at www.jacionline.org). Patients with leaky phenotypes[1,10] were included; 1 patient (P30) had more than 1% B cells and 34.22/μL total B cells, and 4 patients had more than 300 mg/dL serum IgG (P12, P30, P31, P33). All of the normal neonatal Guthrie cards were positive for both cjKRECs and sjKRECs ($7.2 \pm 0.7 \times 10^3$ and $4.8 \pm 0.6 \times 10^3$ copies/μg DNA, respectively). All healthy subjects of various ages were also positive for both cjKRECs and sjKRECs (Nakagawa, unpublished data, June 2010). In contrast, specimens from all 35 B-cell–deficient patients were sjKREC-negative ($<1.0 \times 10^2$ copies/μg DNA; Fig 2). All 5 patients with leaky phenotypes were also sjKREC-negative, which might be explained by the hypothesis that leaky B cells of patients with XLA are long-lived B cells that divided several times and have fewer sjKRECs than naive B cells.

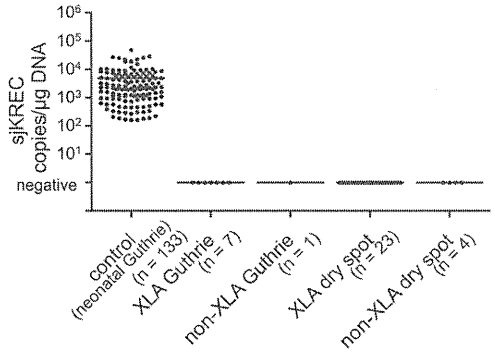

FIG 2. Copy numbers of sjKRECs measured in neonatal Guthrie cards or dried blood spots obtained from B-cell–deficient patients. On all samples from control, neonatal Guthrie cards (n = 133) were sjKREC-positive (4.8 ± 0.6 × 10^3 copies/μg DNA). B-cell–deficient patients were negative for sjKRECs in neonatal Guthrie cards (XLA, n = 7; non-XLA, n = 1) and dried blood spots (XLA, n = 23; non-XLA, n = 4).

One patient (P27) was positive for cjKRECs, but other patients were negative for it. *RPPH1* (internal control) was detectable at the same level as in normal controls in all samples.

These results indicate that sjKRECs are undetectable in XLA and non-XLA and suggest that measurement of sjKRECs in neonatal Guthrie cards has the potential for the use of newborn mass screening to identify neonates with early B-cell maturation defects. Greater numbers of neonatal Guthrie cards should be examined to confirm this potential, and the data obtained from dried blood spots on filter papers must be examined to prove that they truly reflect the data obtained from neonatal Guthrie cards. We should also examine whether screening can reduce the cost of treatment of the bacterial infections and chronic lung diseases in patients with XLA and non-XLA and increase the benefits for these patients. An anticipated pilot study using a large cohort of newborns must address these problems. We also found that T-cell–receptor excision circles and sjKRECs can be measured simultaneously on the same plate. Thus, a pilot study of neonatal screening for both T-cell and B-cell deficiencies could be performed simultaneously.

We thank the patients and their families who participated in this study. We also thank Ms Makiko Tanaka and Ms Kimiko Gasa for their skillful technical assistance and members of the Department of Obstetrics and Gynecology at the National Defense Medical College for collecting umbilical cord blood samples as well as Drs Wataru and Masuko Hirose. We are also indebted to Prof J. Patrick Barron, Chairman of the Department of International Medical Communications of Tokyo Medical University, for his *pro bono* linguistic review of this article.

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Supported in part by grants from the Ministry of Defense; the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science and Technology; and the Kawano Masanori Foundation for Promotion of Pediatrics.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

REFERENCES

1. Conley ME, Broides A, Hernandez-Trujillo V, Howard V, Kanegane H, Miyawaki T, et al. Genetic analysis of patients with defects in early B-cell development. Immunol Rev 2005;203:216-34.
2. Kanegane H, Futatani T, Wang Y, Nomura K, Shinozaki K, Matsukura H, et al. Clinical and mutational characteristics of X-linked agammaglobulinemia and its carrier identified by flow cytometric assessment combined with genetic analysis. J Allergy Clin Immunol 2001;108:1012-20.
3. Plebani A, Soresina A, Rondelli R, Amato GM, Azzari C, Cardinale F, et al. Clinical, immunological, and molecular analysis in a large cohort of patients with X-linked agammaglobulinemia: an Italian multicenter study. Clin Immunol 2002;104:221-30.
4. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of kappa genes in human B cells. Nature 1985;316: 260-2.
5. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med 2007;204:645-55.
6. Eades-Perner AM, Gathmann B, Knerr V, Guzman D, Veit D, Kindle G, et al. ESID Registry Working Party. The European internet-based patient and research database for primary immunodeficiencies: results 2004-06. Clin Exp Immunol 2007; 147:306-12.
7. Morinishi Y, Imai K, Nakagawa N, Sato H, Horiuchi K, Ohtsuka Y, et al. Identification of severe combined immunodeficiency by T-cell receptor excision circles quantification using neonatal Guthrie cards. J Pediatr 2009;155:829-33.
8. Ozyurek E, Cetintas S, Ceylan T, Ogus E, Haberal A, Gurakan B, et al. Complete blood count parameters for healthy, small-for-gestational-age, full-term newborns. Clin Lab Haematol 2006;28:97-104.
9. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood: reference values for lymphocyte subpopulations. J Pediatr 1997;130:388-93.
10. Kaneko H, Kawamoto N, Asano T, Mabuchi Y, Horikoshi H, Teramoto T, et al. Leaky phenotype of X-linked agammaglobulinaemia in a Japanese family. Clin Exp Immunol 2005;140:520-3.

Available online March 11, 2011.
doi:10.1016/j.jaci.2011.01.052

TABLE E1. Characteristics of patients with XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL)			$CD19^+$		*BTK* mutation			Source	
				IgG	IgA	IgM	% Lymph	/μL	Genomic DNA	cDNA	Amino acid	Guthrie	Dry spot
P1	670	0	M	87	<6	10	0.21	12.99	29269G>T	1178-1G>T	Splice acceptor defect	x	
P2	718	0	M	215	<10	<10	0.07	7.04	11593_11594 insA	144_145insA	Arg49 frameshift	x	
P3	722	0	M	80	<1	1	<1.00	NA	25644C>T	763C>T	Arg255X	x	
P4	727	8	M	295	59	57	0.11	3.52	29269G>T	1178-1G>T	Splice acceptor defect		x
P5	732	34	M	1140*	<6	8	0.02	0.24	11631T>A	182T>A	Ile61Asn		x
P6	811	24	M	458*	0	13	0.50	5.32	23570T>G	426T>G	Tyr142X		x
P7	813	18	M	628*	109	6	0.60	6.87	23570T>G	426T>G	Tyr142X		x
P8	814	19	M	260	0	NA	0.20	3.01	16180C>T	344C>T	Ser115Phe		x
P9	815	13	M	600*	<10	<5	0.08	1.72	11590G>T	142-1G>T	Splice acceptor defect		x
P10	816	11	M	12	0	5	0.00	0.00	150kb deletion of *BTK, TIMM8A, TAF7L, DRP2*				x
P11	817	10	M	10	2	24	0.80	35.78	36288C>T	1928C>T	Thr643Ile		x
P12	824	13	M	462	6	27	0.41	14.49	27518C>A	895-11C>A	Splice acceptor defect		x
P13	834	5	M	<237	<37	43	0.00	0.00	25715_26210del	776+57_839+73del	Exon 9 deletion		x
P14	838	21	M	<50	<5	7	0.00	0.00	31596G>C	1631+1G>C	Splice donor defect		x
P15	839	16	M	604*	<1	<2	0.04	0.66	31596G>C	1631+1G>C	Splice donor defect		x
P16	847	11	M	698*	26	11	0.08	1.86	25536delG	655delG	Val219 frameshift		x
P17	877	14	M	20	19	8	0.21	NA	32357T>C	1750+2T>C	Splice donor defect		x
P18	880	5	M	233	39	41	0.06	NA	10941-?_14592+?del	1-?_240+?del	Exon 1-3 deletion		x
P19	888	8	M	<212	<37	150	0.15	6.60	11023G>A	83G>A	Arg28His		x
P20	891	21	M	195	<6	37	0.02	0.09	32243C>G	1638C>G	Cys502Trp		x
P21	958	0	M	<50	<10	9	0.80	27.14	31544_31547 delGTTT	1580_1583del GTTT	Cys527 frameshift		x
P22	701	2	M	115	<2	4	0.09	1.99	16172C>A	336C>A	Tyr112X		x
P23	911	0	M	<10	<6	<4	0.00	0.00	29955A>C	1350-2A>C	Splice acceptor defect	x	
P24	937	0	M	60	<2	58	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P25	938	0	M	<20	<4	<6	0.00	0.00	36269-?_36778+?del	1909-?_2418+?del	Exon 19 deletion	x	
P26	939	0	M	60	<2	22	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P27	890	12	M	<237	<37	<20	0.03	NA	36261G>A	1909-8G>A	Splice acceptor defect		x
P28	944	6	M	12	<1	1	0.02	NA	36281C>T	1921C>T	Arg641Cys		x
P29	948	5	M	<237	<37	<20	0.01	0.70	36261G>A	1909-8G>A	Splice acceptor defect		x
P30	1053	5	M	386	5	113	1.10	34.22	32259A>C	1654A>C	Thr552Pro		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *M,* male; *NA,* not available; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

BTK mutation's reference sequences are NCBI NC_000023.9, NM_000061.2, and NP_000052.1.

*Trough level during intravenous immunoglobulin therapy.

TABLE E2. Characteristics of patients with non-XLA

				Serum Ig (mg/dL)			$CD19^+$			Source	
Patient no.	Unique patient no.	Age (y)	Sex	IgG	IgA	IgM	% Lymph	/μL	*BTK* mutation	Guthrie	Dry spot
P31	596	4	F	386	<6	6	0.42	21.27	Normal		x
P32	719	0	F	<50	<5	<5	0.00	0.00	Normal	x	
P33	835	8	M	311	323	20	0.09	1.88	Normal		x
P34	915	0	M	<212	<37	<20	0.00	0.00	Normal		x
P35	947	0	M	<21	<37	<39	0.00	0.00	Normal		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *F,* female; *M,* male; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

J Clin Immunol (2011) 31:946–951
DOI 10.1007/s10875-011-9583-x

Association of *IRF5* Polymorphisms with Susceptibility to Hemophagocytic Lymphohistiocytosis in Children

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Received: 2 May 2011 / Accepted: 9 August 2011 / Published online: 4 September 2011

Abstract
Introduction Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome and has a varied genetic background. The polymorphism of *interferon regulatory factor 5* gene (*IRF5*) was reported to be associated with susceptibility to macrophage activation syndrome. IRF5 acts as a master transcription factor in the activation of pro-inflammatory cytokines. We assessed associations of *IRF5* gene polymorphisms with susceptibility to secondary HLH.
Methods Three *IRF5* single nucleotide polymorphisms (rs729302, rs2004640, and rs2280714) were genotyped using TaqMan assays in 82 secondary HLH patients and 188 control subjects.
Results There was a significant association of the GT/TT genotype at rs2004640 with secondary HLH susceptibility ($p<0.01$). The *IRF5* haplotype (rs729302 A, rs2004640 T, and rs2280714 T) was associated with secondary HLH susceptibility ($p<0.01$).
Conclusions These findings indicate that *IRF5* is a genetic factor influencing the susceptibility to secondary HLH and that the IRF5-associated immune response contributes to the pathogenesis of HLH.

Keywords Interferon regulatory factor 5 · polymorphisms · hemophagocytic lymphohistiocytosis

Electronic supplementary material The online version of this article (doi:10.1007/s10875-011-9583-x) contains supplementary material, which is available to authorized users.

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Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome that is accompanied by serious morbidity [1, 2]. The incidence of HLH is estimated to be about 1.2 cases per million individuals per year [3]. HLH is characterized by prolonged fever, cytopenias, hepatosplenomegaly, and hemophagocytosis in reticuloendothelial systems. The characteristic laboratory findings include hypertriglyceridemia, hyperferritinemia, hypofibrinogeneima, and increased soluble CD25 [1–4]. These manifestations and laboratory values are described as the result of hypercytokinemia caused by an

ineffective immunological response mediated by histiocytes (macrophages and dendritic cells), natural killer (NK) cells, and cytotoxic T cells (CTL) [1, 5–7]. Increased levels of several pro-inflammatory cytokines, such as interleukin-6 (IL-6), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α have been demonstrated in patients with HLH [8–10]. HLH is classified into primary (genetic) or secondary (acquired) HLH. There are two subtypes of primary HLH, namely, familial HLH (FHL) and other immunodeficiencies such as Chediak–Higashi syndrome, Griscelli syndrome type 2, Hermansky–Pudlak syndrome type 2, and the X-linked lymphoproliferative syndrome [2, 11]. Mutations of *perforin* (*PRF1*), *UNC13D*, *STX11*, and *STXBP2* genes are responsible for 30–70% of FHLH cases [12–16]. It is thought that other unknown genetic defects remain as causes of FHL. Secondary HLH may occur under conditions of severe infections, malignancies, or autoimmune diseases [1, 2]. Many viruses, bacteria, and other infectious agents have been reported to trigger infection-associated HLH (IHLH) [17]. Epstein–Barr virus (EBV) is the most studied virus that trigger IHLH [18]. EBV-associated HLH (EBV-HLH) has a higher prevalence in East Asian countries [18]. Therefore, there may be a genetic variation in susceptibility to EBV-HLH.

Genetic factors other than *PRF1*, *UNC13D*, *STX11*, and *STXBP2* might influence susceptibility even to secondary HLH. Macrophage activation syndrome (MAS) is one form of secondary HLH [1, 2]. MAS occurs in patients with autoimmune diseases, especially with systemic-onset juvenile idiopathic arthritis (systemic JIA) [19, 20]. We recently reported that the *interferon regulatory factor 5* (*IRF5*) gene polymorphism is associated with susceptibility to MAS in systemic JIA patients [21]. IRF5 is a member of the IRF family of transcription factors and is known to have a crucial role in the Toll-like receptor signaling pathway [22, 23]. The activation of the Toll-like receptor is central to innate and adaptive immunity. IRF5 acts as a master transcription factor in the activation of pro-inflammatory cytokine genes especially in the virus-mediated immunological signaling pathway [23]. In *IRF5* knockout mice, a severely impaired induction of IL-6, IL-12, and TNF-α was observed [22].

In the present study, we hypothesized that polymorphisms in the *IRF5* gene may be associated with susceptibility to secondary HLH. We found a close relationship between the *IRF5* gene polymorphism/haplotype and susceptibility to secondary HLH.

Patients and Methods

Study Population

Patients with secondary HLH except for MAS were diagnosed based on the diagnostic criteria used in the HLH-94 Study (for patients who developed HLH before October 2006) and HLH-2004 Study (after October 2006) [4, 24]. The patients who showed known genetic mutations were excluded as primary HLH in this study. Patients under 1 year were also excluded to reduce the possible inclusion of undiagnosed primary HLH because the onset of FHL is below 1 year of age in 70–80% of the cases [25].

Patients with MAS were diagnosed as having systemic JIA based on the International League of Associations for Rheumatology classification criteria for systemic JIA [26]. Because the HLH-94/2004 diagnostic criteria may not always be appropriate when diagnosing MAS in systemic JIA patients who are under inflammatory conditions, patients with systemic JIA were diagnosed as having MAS based on the preliminary diagnostic guidelines for MAS complicating systemic JIA [27], as follows: (1) clinical criteria including central nervous dysfunction, hemorrhage or hepatomegaly and (2) laboratory criteria including decreased platelet counts ($<26.2\times10^9$/l), elevated levels of asparate aminotransferase (>59 U/l), decreased white blood cell counts ($<4.0\times10^9$/l), and hypofibrinogenemia (<2.5 g/l). The diagnosis of MAS requires the presence of two or more of these criteria.

For the diagnosis of EBV-HLH, EBV load in peripheral blood was quantified by real-time PCR as described in our previous study [28]. Patients were diagnosed as having EBV-HLH if they had EBV loads of over 1,000 genome copies per milliliter in whole blood and fulfilled the diagnostic criteria used in the HLH-94/HLH-2004 Study.

A total of 82 patients, 39 males and 43 females, were enrolled in the present study. Among the 82 patients, 48, including 33 having systemic JIA with MAS, were diagnosed as having secondary HLH at Yokohama City University Hospital between November 2000 and December 2009. The remaining 34 patients, who were diagnosed as having secondary HLH between March 2007 and December 2010, were registered in the HLH-2004 as a study of Japanese Pediatric Leukemia/Lymphoma Study Group. In these patients, 32 were diagnosed as having EBV-HLH. The 188 control subjects were recruited from apparently healthy adult volunteers.

Notably, the 33 MAS patients were identical to those analyzed in our previous study, where the significance of *IRF5* polymorphisms was evaluated among systemic JIA patients with or without MAS. In this study, to evaluate the significance of *IRF5* polymorphisms in the susceptibility to secondary HLH as a whole, data were reanalyzed in comparison with healthy controls using the different study population.

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Yokohama City University School of Medicine and each member of the Japan Leukemia/Lymphoma Study Group. Written informed consent was obtained from each patient or his/her guardians as well as the control subjects.

Table I Characteristics entire secondary HLH Study Group and subgroups

	N	Age	Gender
All patients with secondary HLH	82	4.7 (1–16)	39 (47.6%)
Subgroups of HLH patients			
MAS	33	4.8 (1–16)	16 (48.5%)
Non-MAS HLH	49	4.6 (1–15)	23 (46.9%)
EBV-HLH	32	4.3 (1–15)	16 (50.0%)

HLH hemophagocytic lymphohistiocytosis, *MAS* macrophage activation syndrome, *Non-MAS HLH* secondary HLH including EBV-HLH but not MAS, *EBV-HLH* Epstein–Barr virus-associated HLH

Genotyping

Three SNPs—rs729302, rs2004640, and rs2280714—in the *IRF5* gene were selected as described in our previous study [21]. Patients with HLH and control subjects were genotyped using TaqMan SNP Genotyping Assays as described previously [21].

Statistical Analysis

The SNPassoc package using R-language, version 2.8 (The R Foundation for Statistical Computing, http://www.R-project.org) was employed to evaluate the associations between HLH and the SNPs by logistic regression analysis [29]. 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.) [30]. The associations between genotypes under study and laboratory values were analyzed by the Jonckheere–Terpstra test. The following laboratory values were included: levels of hemoglobin, neutrophils, platelets, triglycerides, fibrinogen, ferritin, transaminases, and lactate dehydrogenase. The association between HLH and the *IRF5* haplotypes was evaluated by logistic regression analysis.

Results

Patient characteristics are summarized in Table I. The mean age of the 82 patients with secondary HLH was 4.7 years (1–16 years) at onset. The numbers of patients with MAS and non-MAS HLH were 33 and 49, respectively. In those with non-MAS HLH, 32 with EBV-HLH were included.

The genotype frequencies for the three SNPs of the HLH patients, including their subgroups, and the control subjects were in Hardy–Weinberg equilibrium ($p>0.05$). These results were consistent with the findings of a recent Japanese population study (Table II) [31].

Table II Association of polymorphisms in the *IRF5* gene with susceptibility to secondary HLH

SNP subject subset	*n*	MAF	Allelic association			
			OR	(95% CI)	*p* value	p_c
		rs729302				
All patients with secondary HLH	82	0.20	1.05	0.96–1.15	0.26	n.s.
Subgroups of HLH patients						
MAS	33	0.18	1.04	0.96–1.12	0.32	n.s.
Non-MAS HLH	49	0.20	1.03	0.95–1.12	0.46	n.s.
EBV-HLH	32	0.23	1.00	0.93–1.10	0.90	n.s.
Control subjects	188	0.24	1.0	–	–	
		rs2004640				
All patients with secondary HLH	82	0.49	0.88	0.82–0.95	<0.01	0.006
Subgroups of HLH patients						
MAS	33	0.50	0.92	0.86–0.99	0.02	n.s.
Non-MAS HLH	49	0.49	0.91	0.84–0.98	0.01	0.030
EBV-HLH	32	0.55	0.95	0.88–1.01	0.11	n.s.
Control subjects	188	0.35	1.0	–	–	
		rs2280714				
All patients with secondary HLH	82	0.34	1.1	1.02–1.19	0.02	0.0465
Subgroups of HLH patients						
MAS	33	0.32	1.07	1.00–1.14	0.06	n.s.
Non-MAS HLH	49	0.35	1.07	0.99–1.14	0.09	n.s.
EBV-HLH	32	0.36	1.04	0.98–1.12	0.22	n.s.
Control subjects	188	0.44	1.0	–	–	

IRF5 interferon requlatory factor 5, *SNP* single nucleotide polymorphism, *MAF* minor allele frequency (the C allele at rs729302, T rs2004640, C rs2280714), p_c corrected combined *p* value using the Bonferroni method

Table III Association of polymorphisms in the *IRF5* gene with susceptibility to secondary HLH

SNP	MM/Mm vs. mm			MM vs. Mm/mm		
	OR	(95% CI)	*p* value	OR	(95% CI)	*p* value
rs729302	2.62	0.75–9.19	0.137	1.19	0.69–2.03	0.59
rs2004640	0.43	0.22–0.84	0.18	0.47	0.26–0.83	<0.01
rs2280714	2.54	1.08–5.97	0.03	1.59	0.93–2.71	0.096

Minor allele: the C allele at rs729302, T rs2004640, C rs2280714

SNP single nucleotide polymorphism, *M* major alleles, *m* minor allele

rs2004640 and rs2280714 were associated with susceptibility to secondary HLH as a whole even after Bonferroni correction (Table II). The T allele at rs2004640 was a risk factor for susceptibility to not only secondary HLH as a whole (p_c=0.006, OR=1.13, 95% CI=1.05–1.23) but also to non-MAS HLH (p_c=0.030, OR=1.10, 95% CI=1.02–1.19; Table II). Moreover, the GT/TT genotype at rs2004640 presented a risk for secondary HLH in general (p_c=0.028, OR=2.15, 95% CI=1.21–3.82; Table III). This genotype was also associated with non-MAS HLH (p_c=0.04, OR=2.28, 95% CI=1.12–4.66; Electronic Supplementary Material (ESM) Table 1).

Additionally, a statistically significant association of the ATT haplotype of the *IRF5* gene (rs729302–rs2004640–rs2280714) with susceptibility to secondary HLH was shown ($p<0.001$, OR=1.92, 95% CI=1.21–3.04; Table IV). This haplotype was also associated with susceptibility to subtypes of the MAS and non-MAS HLH, respectively, but not to EBV-HLH (ESM Table 2).

With regard to the laboratory values in the 34 patients with non-MAS HLH registered in the HLH-2004 Study, the low platelet count was associated with the C allele at rs2280714 (p=0.026, Jonckheere–Terpstra test). Other laboratory values were not associated with the IRF5 gene polymorphisms studied (data not shown).

Discussion

HLH is a clinically heterogeneous syndrome presumably because it is associated with a variety of genetic background. Even in primary HLH, there remain about 30% of FHL patients with unknown responsible genes [13]. With regard to secondary HLH, there may be several HLH-susceptible genes. Although mutations of *PRF1*, *UNC13D*, *STX11*, and *STXBP2* genes can be causable for the pathogenesis of FHL, a particular HLH-susceptible gene may contribute to the pathogenesis of secondary HLH cooperatively with other HLH-susceptible genes and may have the potential of influencing the severity of HLH.

In the present study, we revealed that the T allele at rs2004640 and the ATT haplotype in *IRF5* gene are associated with susceptibility to secondary HLH as well as to MAS in systemic JIA patients. The ATT haplotype in the *IRF5* gene was also associated with an increased risk of SLE [32]. The T alleles at both rs2004640 and rs2280714 were related to higher levels of IRF5 mRNA expression [32]. There seems a potentially important role of the IRF5-associated immune response in the pathogenesis of secondary HLH.

In many cases of HLH, viral infections trigger both primary and secondary HLH [18, 33]. Also, IRF5 has a key role in the induction of the antiviral and inflammatory response and controls the production of pro-inflammatory cytokines [22]. Therefore, the association between gene polymorphisms of *IRF5* and susceptibility to HLH is plausible. In order to assess whether there is an influence of *IRF5* gene polymorphisms on IHLH, we analyzed the association between *IRF5* gene polymorphisms and EBV-HLH. The *IRF5* gene polymorphisms tended to be associated with EBV-HLH, but without statistical significance, presumably because of the small number of patients in this study. Ineffective activation of histiocytes, NK cells, and CTL following viral infections is considered important in the pathogenesis of HLH [5–7]. Recently, several research outcomes were reported about the influence of IRF5 on the function of these immune cells [34–37]. For instance, M1 macrophages, which produce pro-inflammatory cytokines and mediate resistance to pathogens, were characterized by large amounts of IRF5 compared with

Table IV Comparison of IRF5 haplotypes in patients with secondary HLH

The order of SNPs in haplotype is rs729302-rs2004640-rs2280714

Haplotype	Haplotype frequencies in control subjects	Haplotype frequencies in secondary HLH patients	*p* value	OR	95% CI
A-G-C	0.405	0.302	0.02	1.0	–
C-G-T	0.208	0.174	0.37	1.19	0.70–2.04
A-T-T	0.333	0.461	<0.001	1.92	1.21–3.04

M2 macrophages, which produce anti-inflammatory cytokines and promote tissue repair [36]. In addition, IRF5 controls the induction of chemokines, such as IL-8, that mediate recruitment of T lymphocytes [34]. Therefore, IRF5 presumably serves as one of the key factors for the pathogenesis of HLH via influencing the function of these immune cells.

The present study still has some limitations. The first issue is the definition of secondary HLH. The patients with the following criteria were excluded from the study: positive defects of known genes (*PRF1*, *UNC13D*, *STX11*, *STXBP2*, and *SAP*), <1 year old at onset, and low or deficient CTL/NK activity. In male patients who had recurrent HLH episodes or were refractory to treatment, mutations in the *SH2D1A* genes were ruled out [38]. With using these criteria, almost all of the patients can be diagnosed with secondary HLH.

The second issue is that we could not perform a validation study. Although a genetic association study should be validated, the incidence of HLH is too low to validate this association in a single institution and even in a nationwide study. Therefore, it is important that the association between the *IRF5* genotype/haplotype and HLH susceptibility is confirmed by other groups.

We found a close relationship between polymorphisms in the *IRF5* gene and susceptibility to secondary HLH in general and its subtypes (MAS and non-MAS HLH), respectively. This finding suggests a potentially important role of the IRF5-associated immune response in the pathogenesis of HLH.

Acknowledgments This work was supported by a grant from Yokohama Foundation for Advancement of Medical Science. We thank the physicians who participated in the HLH-94 and HLH-2004 studies in Japan and all members of the HLH Study Committee of the Japan Leukemia/Lymphoma Study Group. We also thank M Maesato for her secretarial assistance.

References

1. Gupta S, Weitzman S. Primary and secondary hemophagocytic lymphohistiocytosis: clinical features, pathogenesis and therapy. Expert Rev Clin Immunol. 2010;6(1):137–54.
2. Janka GE. Hemophagocytic syndromes. Blood Rev. 2007;21(5):245–53.
3. Henter JI, Elinder G, Soder O, Ost A. Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. Acta Paediatr Scand. 1991;80(4):428–35.
4. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007;48(2):124–31.
5. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): $CD8^+$ T cells and interferon gamma are essential for the disorder. Blood. 2004;104 (3):735–43.
6. Marcenaro S, Gallo F, Martini S, Santoro A, Griffiths GM, Arico M, et al. Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. Blood. 2006;108(7):2316–23.
7. Schneider EM, Lorenz I, Muller-Rosenberger M, Steinbach G, Kron M, Janka-Schaub GE. Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis. Blood. 2002;100(8):2891–8.
8. Akashi K, Hayashi S, Gondo H, Mizuno S, Harada M, Tamura K, et al. Involvement of interferon-gamma and macrophage colony-stimulating factor in pathogenesis of haemophagocytic lymphohistiocytosis in adults. Br J Haematol. 1994;87(2):243–50.
9. Imashuku S, Hibi S, Sako M, Ishii T, Kohdera U, Kitazawa K, et al. Heterogeneity of immune markers in hemophagocytic lymphohistiocytosis: comparative study of 9 familial and 14 familial inheritance-unproved cases. J Pediatr Hematol Oncol. 1998;20(3):207–14.
10. Ohga S, Matsuzaki A, Nishizaki M, Nagashima T, Kai T, Suda M, et al. Inflammatory cytokines in virus-associated hemophagocytic syndrome. Interferon-gamma as a sensitive indicator of disease activity. Am J Pediatr Hematol Oncol. 1993;15(3):291–8.
11. Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. Eur J Pediatr. 2007;166(2):95–109.
12. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003;115(4):461–73.
13. Ishii E, Ohga S, Imashuku S, Yasukawa M, Tsuda H, Miura I, et al. Nationwide survey of hemophagocytic lymphohistiocytosis in Japan. Int J Hematol. 2007;86(1):58–65.
14. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286(5446):1957–9.
15. zur Stadt U, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Hum Mol Genet. 2005;14(6):827–34.
16. zur Stadt U, Rohr J, Seifert W, Koch F, Grieve S, Pagel J, et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. Am J Hum Genet. 2009;85(4):482–92.
17. Fisman DN. Hemophagocytic syndromes and infection. Emerg Infect Dis. 2000;6(6):601–8.
18. Rouphael NG, Talati NJ, Vaughan C, Cunningham K, Moreira R, Gould C. Infections associated with haemophagocytic syndrome. Lancet Infect Dis. 2007;7(12):814–22.
19. Grom AA, Mellins ED. Macrophage activation syndrome: advances towards understanding pathogenesis. Curr Opin Rheumatol. 2010;22(5):561–6.
20. Kelly A, Ramanan AV. Recognition and management of macrophage activation syndrome in juvenile arthritis. Curr Opin Rheumatol. 2007;19(5):477–81.
21. Yanagimachi M, Naruto T, Miyamae T, Hara T, Kikuchi M, Hara R, et al. Association of IRF5 polymorphisms with susceptibility to macrophage activation syndrome in patients with juvenile idiopathic arthritis. J Rheumatol. 2011;38:769–74.
22. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature. 2005;434 (7030):243–9.
23. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. Annu Rev Immunol. 2001;19:623–55.
24. Henter JI, Arico M, Egeler RM, Elinder G, Favara BE, Filipovich AH, et al. HLH-94: a treatment protocol for hemophagocytic lymphohistiocytosis. HLH Study Group of the Histiocyte Society. Med Pediatr Oncol. 1997;28(5):342–7.
25. Arico M, Janka G, Fischer A, Henter JI, Blanche S, Elinder G, et al. Hemophagocytic lymphohistiocytosis. Report of 122 children

from the International Registry. FHL Study Group of the Histiocyte Society. Leukemia. 1996;10(2):197–203.
26. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. J Rheumatol. 2004;31(2):390–2.
27. Ravelli A, Magni-Manzoni S, Pistorio A, Besana C, Foti T, Ruperto N, et al. Preliminary diagnostic guidelines for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. J Pediatr. 2005;146(5):598–604.
28. Matsuda K, Nakazawa Y, Yanagisawa R, Honda T, Ishii E, Koike K. Detection of T-cell receptor gene rearrangement in children with Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis using the BIOMED-2 multiplex polymerase chain reaction combined with GeneScan analysis. Clin Chim Acta. 2011;412:1554–8.
29. Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X, et al. SNPassoc: an R package to perform whole genome association studies. Bioinformatics. 2007;23(5):644–5.
30. Lake SL, Lyon H, Tantisira K, Silverman EK, Weiss ST, Laird NM, et al. Estimation and tests of haplotype–environment interaction when linkage phase is ambiguous. Hum Hered. 2003;55(1):56–65.
31. Shimane K, Kochi Y, Yamada R, Okada Y, Suzuki A, Miyatake A, et al. A single nucleotide polymorphism in the IRF5 promoter region is associated with susceptibility to rheumatoid arthritis in the Japanese population. Ann Rheum Dis. 2009;68(3):377–83.
32. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet. 2006;38(5):550–5.
33. Henter JI, Ehrnst A, Andersson J, Elinder G. Familial hemophagocytic lymphohistiocytosis and viral infections. Acta Paediatr. 1993;82 (4):369–72.
34. Barnes BJ, Kellum MJ, Field AE, Pitha PM. Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes. Mol Cell Biol. 2002;22(16):5721–40.
35. Hao S, Li P, Zhao J, Hu Y, Hou Y. 17beta-estradiol suppresses cytotoxicity and proliferative capacity of murine splenic NK1.1+ cells. Cell Mol Immunol. 2008;5(5):357–64.
36. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, et al. IRF5 promotes inflammatory macrophage polarization and T (H)1–T(H)17 responses. Nat Immunol. 2011;12(3):231–8.
37. Krausgruber T, Saliba D, Ryzhakov G, Lanfrancotti A, Blazek K, Udalova IA. IRF5 is required for late-phase TNF secretion by human dendritic cells. Blood. 2010;115(22):4421–30.
38. Sumazaki R, Kanegane H, Osaki M, Fukushima T, Tsuchida M, Matsukura H, et al. SH2D1A mutations in Japanese males with severe Epstein–Barr virus-associated illnesses. Blood. 2001;98 (4):1268–70.

Association of *IRF5* Polymorphisms with Susceptibility to Macrophage Activation Syndrome in Patients with Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** Systemic-onset juvenile idiopathic arthritis (systemic JIA) and macrophage activation syndrome (MAS), the most devastating complication of systemic JIA, are characterized by abnormal levels of proinflammatory cytokines. Interferon regulatory factor 5 (IRF5) is a member of the IRF family of transcription factors, and acts as a master transcription factor in the activation of genes encoding proinflammatory cytokines. Polymorphisms in the *IRF5* gene have been associated with susceptibility to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis. Our aim was to assess associations of *IRF5* gene polymorphisms with susceptibility to systemic JIA and MAS.

Methods. Three *IRF5* single-nucleotide polymorphisms (rs729302, rs2004640, and rs2280714) were genotyped using TaqMan assays in 81 patients with systemic JIA (33 with MAS, 48 without) and 190 controls.

Results. There were no associations of the *IRF5* gene polymorphisms or haplotypes under study with susceptibility to systemic JIA. There was a significant association of the rs2004640 T allele with MAS susceptibility (OR 4.11; 95% CI 1.84, 9.16; p = 0.001). The *IRF5* haplotype (rs729302 A, rs2004640 T, and rs2280714 T), which was reported as conferring an increased risk of SLE, was significantly associated with MAS susceptibility in patients with systemic JIA (OR 4.61; 95% CI 1.73, 12.3; p < 0.001).

Conclusion. *IRF5* gene polymorphism is a genetic factor influencing susceptibility to MAS in patients with systemic JIA, and IRF5 contributes to the pathogenesis of MAS in these patients. (First Release Jan 15 2011; J Rheumatol 2011;38:769–74; doi:10.3899/jrheum.100655)

Key Indexing Terms:
INTERFERON REGULATORY FACTOR 5 — POLYMORPHISMS
MACROPHAGE ACTIVATION SYNDROME — JUVENILE IDIOPATHIC ARTHRITIS

Systemic-onset juvenile idiopathic arthritis (systemic JIA) is one of the most perplexing diseases in childhood, manifesting as spiking fever, rash, arthritis, pericarditis, and hepatosplenomegaly[1].

The systemic symptoms frequently recur in conjunction with exacerbation of the arthritis symptoms. Some studies have observed that abnormal expression of the proinflammatory cytokines such as interleukin 6 (IL-6) and IL-1ß was characteristic of systemic JIA[2,3].

The most devastating complication of JIA is macrophage activation syndrome (MAS), which is strongly associated with systemic JIA, but rarely with polyarthritis[4]. MAS is accompanied by serious morbidity and sometimes death. The increased levels of several proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and others correlate with the rapid development of clinical symptoms and the progression of abnormal laboratory measurements[4,5]. MAS closely resembles a reactive or an acquired form of familial hemophagocytic lymphohistiocytosis, considered to be caused by diminished natural killer (NK) cell function, and mutations of perforin (*PRF1*), *UNC13D*, and *STX11* genes[6]. Because patients with systemic JIA have decreased levels of perforin in NK cells and diminished NK cell function, it was recently suggested that *PRF1* mutations also play a role in the development of MAS in patients with systemic JIA[7,8,9]. Munc13-4 polymorphism was also associated with MAS in patients with JIA[10]. There

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Supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 16790583).

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Accepted for publication November 16, 2010.

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 × 10^9/l), elevated levels of aspartate aminotransferase (> 59 U/l), decreased white blood cell counts (< 4.0 × 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 × 10^9/l)	27 (81.8)
Elevated levels of aspartate aminotransferase (> 59 U/l)	25 (75.8)
Decreased white blood cell count (< 4.0 × 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)

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			Allelic Association			
				Total	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			Allelic Association				
				Total	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.

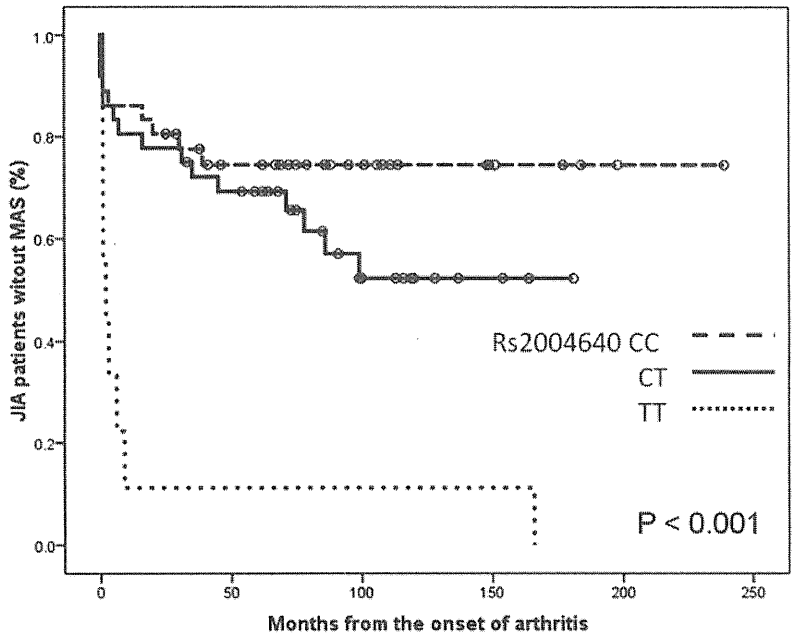

Figure 1. Kaplan-Meier analysis of survival without macrophage activation syndrome and rs2004640 genotypes. JIA: juvenile idiopathic arthritis.

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-

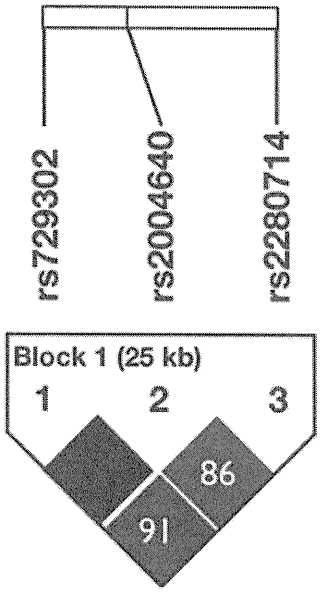

Figure 2. Haplotype blocks of interferon regulatory factor 5 gene polymorphisms.

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.

ACKNOWLEDGMENT

We are grateful to T. Kaneko and S. Morita for advice on statistics. We also thank R. Tanoshima for secretarial assistance.

REFERENCES

1. Cassidy JT, Petty RE. Juvenile idiopathic arthritis. In: Cassidy JT, Petty RE, editors. Textbook of pediatric rheumatology. Philadelphia: WB Saunders; 2005:291-303.
2. De Benedetti F. Targeting interleukin-6 in pediatric rheumatic diseases. Curr Opin Rheumatol 2009;21:533-7.
3. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. J Exp Med 2005;201:1479-86.
4. Kelly A, Ramanan AV. Recognition and management of macrophage activation syndrome in juvenile arthritis. Curr Opin Rheumatol 2007;19:477-81.
5. Ravelli A, Martini A. Macrophage activation syndrome. In: Lehman TJ, Cimaz R, editors. Pediatric rheumatology. Amsterdam: Elsevier; 2008:55-63.
6. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2007;48:124-31.
7. Grom AA, Villanueva J, Lee S, Goldmuntz EA, Passo MH, Filipovich A. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. J Pediatr 2003;142:292-6.
8. Vastert SJ, van Wijk R, D'Urbano LE, de Vooght KM, de Jager W, Ravelli A, et al. Mutations in the perforin gene can be linked to macrophage activation syndrome in patients with systemic onset juvenile idiopathic arthritis. Rheumatology 2010;49:441-9.
9. Grom AA, Mellins ED. Macrophage activation syndrome: advances towards understanding pathogenesis. Curr Opin Rheumatol 2010;22:561-6.
10. Zhang K, Biroschak J, Glass DN, Thompson SD, Finkel T, Passo MH, et al. Macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis is associated with MUNC13-4 polymorphisms. Arthritis Rheum 2008;58:2892-6.
11. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 2005;434:243-9.
12. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet 2006;38:550-5.
13. Han SW, Lee WK, Kwon KT, Lee BK, Nam EJ, Kim GW. Association of polymorphisms in interferon regulatory factor 5 gene with rheumatoid arthritis: a metaanalysis. J Rheumatol 2009;36:693-7.
14. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. J Rheumatol 2004;31:390-2.
15. Ravelli A, Magni-Manzoni S, Pistorio A, Besana C, Foti T, Ruperto N, et al. Preliminary diagnostic guidelines for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. J Pediatr 2005;146:598-604.
16. Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X, et al. SNPassoc: an R package to perform whole genome association studies. Bioinformatics 2007;23:644-5.
17. Lake SL, Lyon H, Tantisira K, Silverman EK, Weiss ST, Laird NM, et al. Estimation and tests of haplotype-environment interaction when linkage phase is ambiguous. Hum Hered 2003;55:56-65.

18. Shimane K, Kochi Y, Yamada R, Okada Y, Suzuki A, Miyatake A, et al. A single nucleotide polymorphism in the IRF5 promoter region is associated with susceptibility to rheumatoid arthritis in the Japanese population. Ann Rheum Dis 2009;68:377-83.
19. Gutierrez-Roelens I, Lauwerys BR. Genetic susceptibility to autoimmune disorders: clues from gene association and gene expression studies. Curr Mol Med 2008;8:551-61.
20. Yanai H, Chen HM, Inuzuka T, Kondo S, Mak TW, Takaoka A, et al. Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. Proc Natl Acad Sci USA 2007;104:3402-7.
21. Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB, Crow MK. Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. Arthritis Rheum 2008;58:2481-7.
22. Yokota S, Mori M, Imagawa T, Takei S, Murata T, Tomiita M, et al. Proposal for juvenile idiopathic arthritis guidance on diagnosis and treatment for primary care pediatricians and nonpediatric rheumatologists. Mod Rheumatol 2007;17:353-63.

BJCP British Journal of Clinical Pharmacology

DOI:10.1111/j.1365-2125.2010.03814.x

Influence of polymorphisms within the methotrexate pathway genes on the toxicity and efficacy of methotrexate in patients with juvenile idiopathic arthritis

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Keywords
5-aminoimidazole-4-carboxamide ribonucleotide transformylase, articular-type juvenile idiopathic arthritis, γ-glutamyl hydrolase, methotrexate

Received
12 June 2010

Accepted
16 September 2010

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Methotrexate (MTX), which causes adverse effects, such as liver and/or renal dysfunction, is the most common disease-modifying antirheumatic drug used for the treatment of rheumatoid arthritis and articular-type juvenile idiopathic arthritis (JIA).
- Pharmacogenetic studies analysing the MTX pathway genes would aid in the development of more personalized therapy.
- Results regarding the influence of gene polymorphisms on the toxicity and efficacy of MTX are conflicting, and there are marked differences between racial groups in pharmacogenetics.

WHAT THIS STUDY ADDS

- The non-TT genotype at γ-glutamyl hydrolase (*GGH*) T16C is associated with a high risk of liver dysfunction due to MTX, even after adjustment for duration of MTX treatment.
- Longer time interval from disease onset to MTX treatment and rheumatoid factor positivity are associated with lower efficacy of MTX in Japanese patients, as reported previously in Caucasian patients with JIA.

AIMS

We investigated whether several polymorphisms within the methotrexate (MTX) pathway genes were related to the toxicity and efficacy of MTX in 92 Japanese patients with articular-type juvenile idiopathic arthritis (JIA).

METHODS

Eight gene polymorphisms within the MTX pathway genes, namely, *RFC*, *BCRP*, *MTHFR* (two), *FPGS*, γ-glutamyl hydrolase (*GGH*; two) and *ATIC*, were genotyped using TaqMan assays. Liver dysfunction was defined as an increase in alanine transaminase to five times the normal upper limit. Non-responders to MTX were defined as patients refractory to MTX and were therefore treated with biologics.

RESULTS

The non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction ($P = 0.028$, odds ratio = 6.90, 95% confidence interval 1.38–34.5), even after adjustment for the duration of MTX treatment. A longer interval from disease onset to treatment (8.5 and 21.3 months, $P = 0.029$) and rheumatoid factor positivity ($P = 0.026$, odds ratio = 2.87, 95% confidence interval 1.11–7.39) were associated with lower efficacy of MTX.

CONCLUSIONS

The non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction, presumably because the C allele of *GGH* C16T may reduce the activity of GGH. The time interval before MTX treatment and rheumatoid factor positivity were associated with the efficacy of MTX treatment. The pharmacogenetics of the MTX pathway genes affects the toxicity and efficacy of MTX in Japanese JIA patients.

Introduction

Juvenile idiopathic arthritis (JIA) is one of the most common forms of paediatric chronic arthritis, with an incidence of approximately 9.7 per 100 000 children (aged 15 years and under) in Japan [1, 2]. Methotrexate (MTX) is the most common disease-modifying antirheumatic drug used for the treatment of articular-type JIA, namely the polyarticular- and oligoarticular-onset types of JIA [2]. Methotrexate is effective in about 75% of cases of the articular-type JIA, but causes adverse effects, such as liver and/or renal dysfunction [2, 3]. The effects of polymorphisms within the MTX pathway genes on the toxicity and efficacy of MTX in patients with rheumatoid arthritis (RA) and JIA have been studied [4–6].

The influence of polymorphisms within the MTX pathway genes encoding solute carrier family 19 member 1 (SLC19A1), also known as reduced folate carrier (RFC), 5,10-methylenetetrahydrofolate reductase (MTHFR), folypolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH), 5-aminomidazole-4-carboxamide ribonucleotide transformylase (ATIC) and breast cancer resistance protein (BCRP/ABCG2) on the toxicity and efficacy of MTX in patients with RA, JIA and other diseases has been studied [4–9]. However, results regarding the influence of these polymorphisms on the toxicity and efficacy of MTX are conflicting, and there are marked differences in pharmacogenetics between racial groups [10]. Therefore, we investigated whether polymorphisms within the MTX pathway genes were related to the toxicity and efficacy of MTX in 92 patients with articular-type JIA in Japan.

Patients and methods

Study population

Patients were eligible if they met the International League of Association for Rheumatology classification criteria for articular-type JIA [11]. A total of 92 children (74 girls and 18 boys; 12 with seronegative polyarticular onset, 46 with seropositive polyarticular onset and 34 with oligoarticular onset) in this study were treated at the Yokohama City University Hospital between December 2007 and December 2009.

All 92 patients had been treated with MTX for at least 3 months without biologics. Initially, MTX was administered orally at a dosage of 4–5 mg m^{-2} per week. Then the dosage was adjusted depending on tolerability and response (maximal dosage, 10 mg m^{-2} $week^{-1}$) [2]. Prednisolone was used concomitantly with MTX in 89 patients (96.7%). Folic acid supplementation was performed in nine patients (9.9%). Clinical data were collected from a patient's medical record without any knowledge of the individual's polymorphisms.

The study was performed in accordance with the Declaration of Helsinki, and approval for it was obtained from the Yokohama City University School of Medicine Ethics Committee. Each patient or his/her guardians gave written informed consent to participate in this study.

Definitions of toxicity and efficacy

For the evaluation of toxicity, liver dysfunction was defined as an increase in serum alanine transaminase (ALT) level to five times the normal upper limit before the addition of biologics.

Responders to MTX were defined as follows: (i) patients in whom the medication was terminated because they had remission of symptoms; (ii) patients who continued the treatment with stable doses of MTX; and (iii) patients who continued MTX treatment with the concomitant use of acceptable doses of prednisolone, without the addition of biologics, such as anti-tumour necrosis factor therapy [12] and anti-interleukin-6 receptor antibody therapy [13, 14].

Non-responders to MTX were defined as patients who were refractory to MTX and thus treated with biologics. Treatment with biologics was conducted according to the following criteria: (i) patients with a history of treatment with nonsteroidal anti-inflammatory drugs and MTX; and (ii) patients who had the active disease for at least 3 months after MTX treatment (up to 10 mg m^{-2} $week^{-1}$). Active disease was characterized by five or more swollen joints and three or more joints with limited range of movement accompanied by pain and/or tenderness, or the use of high doses of corticosteroids (>0.25 mg kg^{-1} daily), with accompanying unacceptable side-effects [12, 13].

Clinical predictors

Clinical predictors that may influence a patient's disease state and the toxicity and efficacy of MTX were selected on the basis of previous reports [5, 6, 15, 16]. The following factors were included: sex; age at disease onset; duration of MTX treatment; time interval from disease onset to MTX treatment; rheumatoid factor (RF) status; anti-cyclic citrullinated peptide (anti-CCP) status; and concomitant use of prednisolone and folic acid.

Genetic predictors

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen K.K., Tokyo, Japan).

The following eight single nucleotide polymorphisms (SNPs) within the MTX pathway genes encoding RFC, MTHFR, FPGS, GGH, ATIC and BCRP were selected according to previous reports [4–9]. Genotyping for the SNPs of *RFC* G80A (rs1051266), *MTHFR* A1298C (rs1801131), *MTHFR* C677T (rs1801133), *FPGS* A1994G (rs10106), *GGH* C452T (rs11545078), *GGH* T16C (rs1800909), *ATIC* C347G (rs2372536) and *BCRP* C421A (rs2231142) was performed using the TaqMan assay (Applied Biosystems, Foster City, CA, USA). TaqMan SNP Genotyping Assays were used for *MTHFR* A1298C and *MTHFR* C677T, and Custom TaqMan SNP Genotyping Assays were used for *RFC* G80A, *FPGS*

A1994G, *GGH* C452T, *GGH* T16C, *ATIC* C347G and *BCRP* C421A [9] (see Supplementary data 1). These SNPs were analysed in real-time PCRs by the AB7500 Real Time PCR system (Applied Biosystems), in the conditions recommended by the manufacturer. Allele discrimination was performed using SDS software version 1.4 (Applied Biosystems).

Statistical analysis

For continuous predictors, such as age and duration of MTX treatment, Student's unpaired *t*-test was used to assess the association between clinical predictors and the toxicity and efficacy. For categorical predictors, such as genetic predictors and sex, a χ^2 test and Fisher's exact test were used to assess the association between predictors and the toxicity and efficacy. Possible confounding effects among the predictors were adjusted using a multiple logistic regression model.

Haplotype phases and haplotype frequencies were estimated using the Expectation-Maximization algorithm (minimum haplotype frequency >0.05). All statistical analyses were carried out using the SAS system version 9 (SAS Institute Inc., Cary, NC, USA).

Results

Distribution of the polymorphisms within the MTX pathway genes

The genotype frequencies for the eight SNPs under study were in Hardy–Weinberg equilibrium ($P > 0.05$). Each result was consistent with the findings of a previous report (see Supplementary data 2) [17].

The toxicity of MTX

Of 92 patients, 10 developed liver dysfunction. Methotrexate treatment of longer duration was a risk factor for liver dysfunction (104.3 months with liver dysfunction, 53.6 months without, $P = 0.005$). No other clinical variables were associated with liver dysfunction (Table 1). None of the patients with folic acid supplementation had liver dysfunction. However, this correlation of folic acid supplementation preventing liver dysfunction was not statistically significant, presumably because of the small study population.

Regarding the association between liver dysfunction and genetic predictors, the TT genotype at *GGH* T16C was a low risk factor for liver dysfunction [$P = 0.031$, odds ratio (OR) = 0.20, 95% confidence interval (CI) 0.03–0.98; Table 2 and Supplementary data 3]. In contrast, the non-TT genotype at *GGH* T16C was a high risk factor for liver dysfunction ($P = 0.031$, OR = 5.10, 95% CI 1.02–25.6), which is of significant clinical interest. This association was statistically significant even after adjustment for duration of MTX treatment ($P = 0.028$, OR = 6.90, 95% CI 1.38–34.5). None of the other SNPs was associated with liver dysfunction.

The *MTHFR* haplotypes and *GGH* haplotypes showed no significant association with liver dysfunction (data not shown).

The efficacy of MTX

Of 92 patients, 67 were non-responders to MTX. Delayed MTX treatment from disease onset (21.3 months with non-responders *vs.* 8.5 months with responders, $P = 0.029$) and RF positivity ($P = 0.026$, OR = 2.87, 95% CI 1.11–7.39) were risk factors for lower efficacy of MTX (Table 3). No other clinical variables were associated with efficacy.

Regarding the association between the efficacy of MTX and genetic predictors, there was no gene polymorphism significantly associated with efficacy (Table 4). The *MTHFR* haplotypes and *GGH* haplotypes showed no significant association with efficacy (data not shown).

In 64 patients treated with MTX within 1 year of disease onset, the CC genotype at *ATIC* C347G tended to be associated with lower efficacy. However, this was not statistically significantly after adjustment for the time interval and RF ($P = 0.106$, OR = 0.38, 95% CI 0.12–1.23) (Table 5).

Discussion

Several studies have shown the influence of polymorphisms within the MTX pathway genes on the toxicity and

Table 1

Association between clinical predictors and liver dysfunction

	ALT >5.0 times normal ($n = 10$)	ALT ≤5.0 times normal ($n = 82$)	*P* value
Age at onset (years, mean)	9.5	7.4	0.138
Sex (male)	20.0%	19.5%	0.971
Time interval from onset to treatment (months, mean)	17.7	17.9	0.987
Prednisolone	90.0%	97.6%	0.204
Folic acid	0.0%	11.0%	0.270
Duration of MTX treatment (months, mean)	104.3	52.6	0.005
MTX efficacy	30.0%	26.8%	0.832

ALT, alanine transaminase.

Table 2

Association between genetic predictors and liver dysfunction

Genotype	Allele model* OR†	*P* value	Dominant model* OR†	*P* value	Recessive model* OR†	*P* value
RFC G80A	1.51	0.414	0.21	0.121	0.59	0.627
BCRP C421A	1.05	0.930	0.80	0.840	0.99	0.988
MTHFR C677T	1.45	0.451	1.12	0.896	2.28	0.214
MTHFR A1298C	0.89	0.852	1.08	0.539	0.74	0.655
FPGS A1994G	0.54	0.249	4.88	0.068	0.70	0.600
GGH T16C	0.42	0.118	0.83	0.475	0.20	0.031
GGH C452T	0.61	0.506	–	–	0.61	0.502
ATIC C347G	1.40	0.560	0.48	0.814	1.17	0.336

M, major allele; and m, minor allele. Major alleles are the A allele at *RFC* G80A, C allele at *BRCP* C421A, C allele at *MTHFR* C677T, A allele at *MTHFR* A1298C, G allele at *FPGS* A1994G, T allele at *GGH* T16C, C allele at *GGH* C452T and C allele at *ATIC* C347G. *Allele model: M *vs.* m; dominant model, (MM or Mm) *vs.* mm; recessive model, MM *vs.* (Mm or mm). †Non-adjusted odds ratio.

Table 3

Association between clinical predictors and methotrexate efficacy

	Responder ($n = 25$)	Non-responder ($n = 67$)	*P* value
Age at onset (years, mean)	6.6	7.9	0.180
Sex (male)	12.0%	22.4%	0.264
Time interval from onset to treatment (months, mean)	8.5	21.3	0.029
Prednisolone	96.0%	97.0%	0.807
Folic acid	4.0%	11.9%	0.254
C-reactive protein at start of treatment (mg dl^{-1}, mean)	2.8	3.3	0.685
Anti-cyclic citrullinated peptide [>4.5 (U ml^{-1})]	32.0%	55.2%	0.062
Rheumatoid factor [>14 (IU ml^{-1})]	40.0%	65.7%	0.026

Table 4

Association between genetic predictors and methotrexate efficacy

Genotype	Allele model* OR†	*P* value	Dominant model* OR†	*P* value	Recessive model* OR†	*P* value
RFC G80A	1.01	0.979	1.32	0.572	1.61	0.435
BCRP C421A	1.28	0.496	0.24	0.151	0.99	0.979
MTHFR C677T	0.75	0.399	0.79	0.708	0.42	0.115
MTHFR A1298C	1.05	0.918	0.36	0.282	0.87	0.775
FPGS A1994G	0.95	0.900	1.37	0.726	1.01	0.984
GGH T16C	1.01	0.986	2.83	0.294	1.24	0.654
GGH C452T	1.15	0.805	–	–	1.15	0.805
ATIC C347G	0.65	0.237	1.08	0.931	0.50	0.139

*Allele model: M *vs.* m.; dominant model, (MM or Mm) *vs.* mm; recessive model, MM *vs.* (Mm or mm). †Non-adjusted odds ratio.

efficacy of MTX in patients with RA [4, 8, 9]. However, results are conflicting, and there are marked differences between racial groups in pharmacogenetic studies [10]. We could find only two studies on the pharmacogenetics of MTX in patients with JIA in Caucasian patients [5, 6], but not one in an Asian population. This is the first reported study on pharmacogenetics of MTX in patients with JIA in an Asian population.

First, we found that the non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction. This should be taken into consideration in treating patients carrying the non-TT genotype at *GGH* T16C with MTX in order to prevent liver dysfunction.

Once inside the cell, MTX undergoes FPGS-catalysed polyglutamation by the addition of two to seven glutamic acid groups. The polyglutamated form is not