Table II Reported number of PID

Category	Total number	Pediatric department	Internal medicine department
I. Combined T and B cell immunodeficiencies	93 (7%)	93 (8%)	0 (0%)
γc deficiency	47	47	0
Adenosine deaminase deficiency	9	9	0
Omenn syndrome	4	4	0
Others	23	23	0
Untested or undetermined	10	10	0
II. Predominantly antibody deficiencies	501 (40%)	434 (38%)	67 (71%)
BTK deficiency	182	173	9
Common variable immunodeficiency disorders	136	107	29
Selective IgG subclass deficiency	66	58	8
Selective IgA deficiency	49	34	15
Hyper IgM syndrome	34	34	0
Transient hypogammaglobulinemia of infancy	7	7	0
Others	11	7	4
Untested or undetermined	16	14	2
III. Other well-defined immunodeficiency syndromes	194 (16%)	189 (17%)	5 (5%)
Wiskott–Aldrich syndrome	60	60	0
DNA repair defects (other than those in category I)	15	15	0
DiGeorge anomaly	38	38	0
Hyper-IgE syndrome	56	52	4
Chronic mucocutaneous candidiasis	17	16	1
Others	5	5	0
Untested or undetermined	3	3	0
IV. Diseases of immune dysregulation	49 (4%)	48 (4%)	1 (1%)
Chediak–Higashi syndrome	9	8	1
Familial hemophagocytic lymphohistiocytosis syndrome	5	5	0
X-linked lymphoproliferative syndrome	8	8	0
Autoimmune lymphoproliferative syndrome	8	8	0
APECED	4	4	0
IPEX syndrome	7	7	0
Others	2	2	0
Untested or undetermined	6	6	0
V. Congenital defects of phagocyte number, function, or both	230 (19%)	223 (19%)	7 (8%)
Severe congenital neutropenia	44	42	2
Cyclic neutropenia	19	17	2
Chronic granulomatous disease	147	144	3
Mendelian susceptibility to mycobacterial disease	5	5	0
Others	9	9	0
Untested or undetermined	6	6	0
VI. Defects in innate immunity	15 (1%)	15 (1%)	0
Anhidrotic ectodermal dysplasia with immunodeficiency	7	7	0
Interleukin-1 receptor-associated kinase 4 deficiency	2	2	0
Others	5	5	0
Untested or undetermined	1	1	0
VII. Autoinflammatory disorders	108 (9%)	101 (9%)	7 (8%)
Familial Mediterranean fever	44	40	4
TNF receptor-associated periodic syndrome	13	12	1
Hyper IgD syndrome	4	4	0
Cryopyrin-associated periodic syndrome	22	22	0

Table II (continued)

Category	Total number	Pediatric department	Internal medicine department
Others	3	3	0
Untested or undetermined	22	20	2
VIII. Complement deficiencies	32 (3%)	29 (3%)	3 (3%)
IX. Undetermined	18 (1%)	14 (1%)	4 (4%)
Total	1,240	1,146	94

APECED Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy, *IPEX* immune dysregulation, polyendocrinopathy, enteropathy, X-linked

increasing age (Fig. 2b). The median age of CID, BTK deficiency, CVID, and CGD patients was 5.2, 12.8, 25.1, and 14.7 years, respectively.

It is well known that PID patients are susceptible to many pathogens and experience community-acquired or opportunistic infections. In this study, we focused on noninfectious complications of PID because they have been less well studied on a large scale and may provide important information for improving the quality of life of PID patients. Twenty-five PID patients developed malignant disorders (2.7%; Table III). Lymphoma, in particular, Epstein–Barr virus-related, and leukemia were dominant, while there were no patients with gastric carcinoma. CVID, Wiskott–Aldrich syndrome (WAS), and ataxia telangiectasia were more frequently associated with malignant diseases among PID patients. A case of Mendelian susceptibility

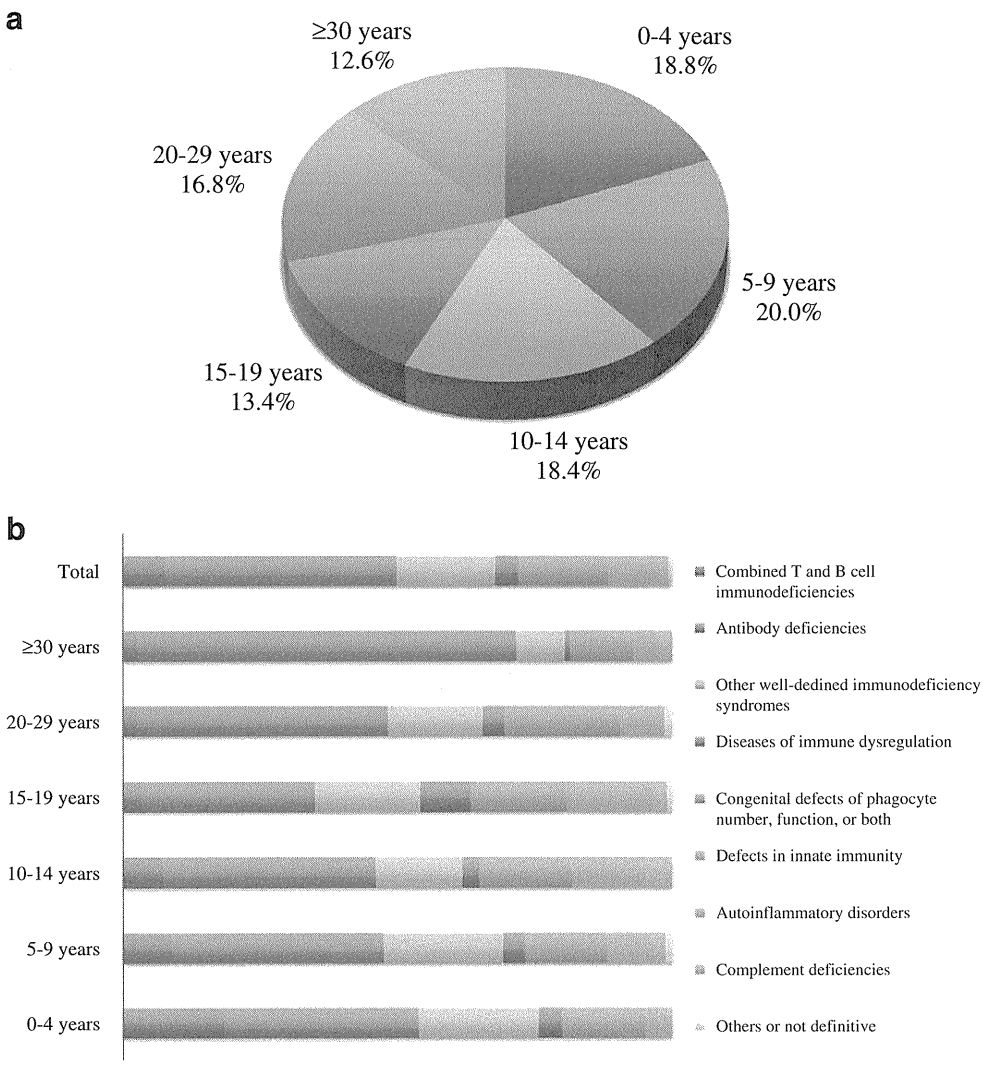

Fig. 2 **a** Age distribution of PID patients. **b** Distribution of PID in each age group

to mycobacterial disease with squamous cell carcinoma was also observed [9] (Table III).

Seventy-eight PID patients had immune-related (autoimmune) diseases (8.5%; Table IVa). Autoimmune lymphoproliferative syndrome, immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, and nuclear factor kappa B essential modulator (NEMO) deficiency were associated with immune-related diseases at a very high incidence. In addition, immune-related diseases were relatively common in CGD and CVID patients (Table IVa). The most commonly observed immune-related disease was inflammatory bowel disease (33 cases), which was most frequently observed in CGD patients, followed by immune thrombocytopenic purpura (13 cases), autoimmune hemolytic anemia (8 cases), and systemic lupus erythematosus (SLE; 8 cases; Table IVa and b). Kawasaki disease occurred in WAS and CGD patients. In addition, this is the first report of Kawasaki disease in patients with complement deficiency (C9) and familial Mediterranean fever (FMF). A patient with warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome and a patient with tumor necrosis factor receptor-associated periodic syndrome (TRAPS) were first reported as cases of type 1 diabetes mellitus and SLE, respectively [10, 11].

Discussion

We conducted a nationwide survey of PID for the first time in 30 years and report the prevalence of PID in Japan. We registered 1,240 PID patients and found that the estimated prevalence of PID (2.3/100,000) is higher than that previously reported (1.0/100,000) in Japan. Our results are equivalent to those reported in Singapore (2.7/100,000) and Taiwan (0.77–2.17/100,000) [12–14]. However, our values are lower than those reported in Middle Eastern countries such as Kuwait (11.98/100,000) or in European countries such as France (4.4/100,000) [5–7, 15]. The high rate of consanguinity may be a cause of the high prevalence rate of PID reported in Middle Eastern countries [6, 15]. There may has been sample selection bias in this study because some asymptomatic cases (SIgAD, etc.), clinically recovered cases (transient hypogammaglobulinemia of infancy, etc.), and cases in which patients were deceased were not registered. In addition, lack of recognition of PID in internal medicine departments, not just the low response rate, might also have influenced the estimated prevalence of PID as well as the age and disease distribution. The regional prevalence of PIDs in Japan was homogenous, unlike in other countries in which a higher prevalence was

Table III Malignancies in PID patients

Primary immunodeficiency	Total	*n*	Malignancy
I. Combined T and B cell immunodeficiencies	75	2	(2.7%)
Ommen syndrome	3	1	NHL (EBV+) 1[a]
Adenosine deaminase deficiency	4	1	Breast carcinoma 1
II. Predominantly antibody deficiencies	378	8	(2.1%)
Common variable immunodeficiency disorders	93	7	HL 2, ML 2, ALL 1, Basal cell carcinoma 1, Cervical carcinoma 1
Good syndrome	4	1	Double primary carcinoma of breast and colon 1
III. Other well-defined immunodeficiency syndromes	165	7	(4.2%)
Wiskott–Aldrich syndrome	57	5	NHL 3, NHL/HL 1, LPD (EBV-) 1
Ataxia telangiectasia	13	2	T-ALL 1, MDS 1
IV. Diseases of immune dysregulation	38	4	(10.5%)
X-linked lymphoproliferative syndrome	5	2	Burkitt lymphoma 2
Autoimmune lymphoproliferative syndrome	6	2	HL (EBV+) 1, Brain tumor 1
V. Congenital defects of phagocyte number, function, or both	153	4	(2.6%)
Severe congenital neutropenia	35	3	MDS 3 (including 2 cases with monosomy 7)
MSMD	7	1	Squamous cell carcinoma of finger 1
VI. Defects in innate immunity	12	0	(0%)
VII. Autoinflammatory disorders	74	0	(0%)
VIII. Complement deficiencies	23	0	(0%)
IX. Undetermined	5	0	(0%)
Total	923	25	(2.7%)

n Number of PID patients who had malignant disorders, *ALL* acute lymphoblastic leukemia, *EBV* Epstein-Barr virus, *HL* Hodgkin lymphoma, *LPD* lymphoproliferative disease, *MDS* myelodysplastic syndrome, *ML* malignant lymphoma, *MSMD* Mendelian susceptibility to mycobacterial disease, *NHL* non-Hodgkin lymphoma

[a] The number of patients

Table IV Immune-related diseases in PID patients

(a) Immune-related diseases with each PID

Primary immunodeficiency	Total	*n*	Immune-related disease
I. Combined T and B cell immunodeficiencies	75	2	(2.6%)
MHC class II deficiency (suspected)	1	1	ITP with AIHA 1[a]
CD4 deficiency	1	1	Hashimoto disease 1
II. Predominantly antibody deficiencies	378	24	(6.3%)
Common variable immunodeficiency disorders	93	16	ITP 3, RA 2, AIHA 2, Hashimoto's disease 2, IBD 2, SLE 1, MG 1, ADEM 1, Autoimmune hepatitis 1, Uveitis 1
Hyper-IgM syndrome	32	3	JIA 1, SLE (complicated with C1q deficiency) 1, IBD 1
Selective IgA deficiency	28	3	SLE 1, SLE with Kikuchi disease 1, RA 1
IgG subclass deficiency	50	2	ITP with AIHA 1, ITP with MS 1
III. Other well-defined immunodeficiency syndromes	165	5	(3.0%)
Wiskott–Aldrich syndrome	57	3	AIHA 2, Kawasaki disease 1
DiGeorge syndrome	33	2	AIHA 1, ITP 1
IV. Diseases of immune dysregulation	38	10	(26.3%)
X-linked lymphoproliferative syndrome	5	1	IBD 1
Autoimmune lymphoproliferative syndrome	6	4	ITP 3, Graves' disease with IBD 1
APECED	5	1	T1DM with Hashimoto's disease and Vogt–Koyanagi–Harada disease 1
IPEX syndrome	6	4	T1DM 1, T1DM with ITP, AIN and IBD 1, Autoimmune enteritis 1, AIHA with Autoimmune enteritis and Hashimoto's disease 1
V. Congenital defects of phagocyte number, function, or both	153	25	(16.3%)
Chronic granulomatous disease	87	25	IBD 20, ITP 2, JIA 1, MCTD 1, Kawasaki disease 1
VI. Defects in innate immunity	12	5	(41.7%)
NEMO deficiency	7	4	IBD 3, IBD with JIA 1
WHIM syndrome	3	1	T1DM 1
VII. Autoinflammatory disorders	74	3	(4.0%)
Familial Mediterranean fever	36	2	SLE 1, Kawasaki disease 1
TNF receptor associated periodic syndrome	9	1	SLE 1
VIII. Complement deficiencies	23	3	(13.0%)
C4 deficiency	1	1	SLE with RA 1
C6 deficiency	1	1	IBD 1
C9 deficiency	11	1	Kawasaki disease 1
IX. Undetermined	5	1	(20%)
Nakajo syndrome	1	1	SLE 1
Total	923	78	(8.5 %)

(b) Immune-related manifestations associated with PID

Immune-related diseases	*n*
IBD (including autoimmune enteritis)	33
ITP	13
AIHA	8
SLE	8
RA/JIA	6
Hashimoto's disease/Graves' disease	5
Kawasaki disease	4
T1DM	4
Uveitis (including Vogt–Koyanagi–Harada disease)	2
ADEM/MS	2
Others	5

n Number of PID patients who had immune-related disorders, *ADEM* acute disseminated encephalomyelitis, *AIHA* autoimmune hemolytic anemia, *AIN* autoimmune neutropenia, *APECED* autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, *IBD* inflammatory bowel disease, *IPEX* immunodysregulation, polyendocrinopathy, enteropathy X-linked, *ITP* immune thrombocytopenic purpura, *JIA* juvenile idiopathic arthritis, *MCTD* mixed connective tissue disease, *MG* myasthenia gravis, *MS* multiple sclerosis, *RA* rheumatoid arthritis, *SLE* systemic lupus erythematosus, *T1DM* type 1 diabetes mellitus, *WHIM* warts, hypogammaglobulinemia, infections, and myelokathexis

[a] The number of patients

observed in urban areas [5, 7, 16]. This may be because many PID patients were treated or followed by PID specialists distributed nationwide in Japan; this is assumed by the location of hospitals with which they were affiliated.

The distribution ratios of BTK deficiency (14.7%) and CGD (11.9%) in Japan were higher than those in a previous report from Europe (5.87% and 4.33%, respectively), while those of CIDs and other well-defined immunodeficiency syndromes were comparable [17]. The prevalence of BTK deficiency was previously reported to be 1/900,000–1,400,000 in a European cohort study [18]. In contrast, this value was estimated to be 1/300,000 in Japan in our study. BTK deficiency appears to be common in Japan, although this may be partially because more patients, including those showing atypical clinical manifestations, were diagnosed more accurately by the recently established genetic diagnostic network in Japan [19]. This is supported by the highest proportion of Japanese patients in the international mutation database for X-linked agammaglobulinemia (BTKbase) [20]. The reason for the low number of registered CGD patients in Europe in a recent report (1/620,000) [17] is unknown; the prevalence of CGD was 1 in 250,000 in a previous European survey [21], which was similar to our results (1 in 380,000 in this study and 1 in 280,000 in our previous study [22]). The percentage of BTK deficiency and CGD would be lower if more adult cases were registered because the prevalence of these disorders is low in adults. CVID was the most commonly reported PID (20.7%) in Europe, and the onset of symptoms was observed most commonly in the third decade of life in these patients [17, 23]. In this study, CVID constituted 11.0% (136 cases) of PID cases, and only 29 cases were reported from internal medicine departments (Table II). A lower number of registered CVID patients may have led to a lower number of reported patients with antibody deficiency and a lower prevalence of PID, although it is still possible that CVID is not as common in Japan as in European countries. There was no significant difference in the distribution rate of SIgAD between Japanese and Europeans, although SIgAD is rare in Japanese (1/18,500) compared with Caucasians (1/330–2,200) according to seroepidemiologic studies [24]. This may be because most SIgAD patients lack clinical manifestations. The distribution ratio of autoinflammatory disorders in Japan (9%) was much higher than that in Europe (1.02%) [17] (Table II). Considering the disease type of the autoinflammatory disorders was not specified in 22 cases (20%), it is possible that many other patients with autoinflammatory disorders remain undiagnosed in Japan as well as in other countries.

The percentage of men (69.7%) with PID is higher in Japan than in Europe (60.8%) or Kuwait (61.8%), but is equivalent to that in Taiwan (70.2%) [6, 13, 17]. The higher ratio of men, particularly in younger generation (<15 years), appears to be due to the larger number of X-linked PID patients (BTK deficiency, X-CGD, γc deficiency, etc.) in this study compared to that in Europe or Kuwait. Adolescents or adults (≥15 years) constituted 42.8% of the patients in this study, which is equivalent to the number in the European study (≥16 years: 46.6%), while those >16 years constituted only 10.9% in the previous survey [3, 17]. In this study, it was found that CVID and SIgAD are common in adults (Table II) and that antibody deficiencies are more common with increasing age (Fig. 2b). A reason for the increased number of adult PID patients may be long-term survival of PID patients due to improved treatments such as immunoglobulin replacement therapy. In addition, an increased likelihood of patients being diagnosed by internists as having late-onset PID, e.g., CVID and SIgAD, may have contributed to these values [17, 25, 26]. Therefore, it is important for internists to be well-informed regarding PID. In contrast, CIDs are fatal during infancy without hematopoietic stem cell transplantation or gene therapy. Because hematopoietic stem cell transplantation has been widely performed in Japan since the 1990s, surviving patients with CID are limited to the younger generation, similar to French patients (Fig. 2b) [5, 27, 28].

It has been reported that PID patients are at increased risk of developing malignant diseases, in particular, non-Hodgkin lymphoma, leukemia, and stomach cancer [29]. Although lymphoma and leukemia were relatively common, stomach cancer was not observed in our study. In the previous survey in Japan, eight of nine PID patients with malignant disorders (including one gastric cancer patient) died [3]. It is possible that some PID patients with malignant disorders were not registered because they were deceased. PID is also associated with immune-related diseases because of a defect in the mechanisms to control self-reactive B and T cells. The frequency of immune-related manifestations varied among individual PID patients, as reported previously [30, 31]. Four PID patients who had developed Kawasaki disease, one patient with WHIM syndrome and type 1 diabetes mellitus, and one patient with TRAPS and SLE in our study may provide new pathophysiological insights of these diseases and the association between PID and autoimmune diseases.

Conclusions

We report the prevalence and clinical characteristics of PIDs in Japan. Although the advances in diagnostic technologies and treatments have improved the prognoses of PID, many patients continue to experience severe complications such as malignancy and immune-related diseases as well as infections. To improve the quality of life of PID patients, it is necessary to pay attention to

complications and treat them appropriately. Web-based PID databases and consultation systems have been created in Japan (Primary Immunodeficiency Database in Japan [4] and Resource of Asian Primary Immunodeficiency Diseases in Asian countries [32]) to reveal precise information regarding PID and to promote cooperation between doctors and researchers [19].

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Conflict of Interest There is no actual or potential conflict of interest in relation to the study.

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In conclusion, the associations among asthma, biofilm-forming bacteria, and revision ESS are strong and robust after adjusting for other factors in patients with CRS from a tertiary medical center. Despite its limitations, this study may improve our understanding of refractory CRS pathogenesis, possibly leading to more effective treatment strategies, such as incorporating the treatments of asthma and biofilm infection into conventional CRS therapies. Prospective cohort studies in diverse populations are needed to assess the causality of these associations.

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Quantification of κ-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects

To the Editor:

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by severely decreased numbers of mature peripheral B lymphocytes as a result of a mutation in the *BTK* gene. Non-XLA is characterized by hypogammaglobulinemia with decreased B-cell counts (less than 2% of mature B cells) in the absence of the *BTK* gene mutation. Both XLA and non-XLA are caused by an early B-cell maturation defect.[1] In patients with XLA and non-XLA, recurrent infections appear between 3 and 18 months of age, whereas the mean age at diagnosis is 3 years.[2] This delayed diagnosis results in frequent hospitalization because of pneumonia, sepsis, meningitis, and other bacterial infections, which frequently require intravenous administration of antibiotics and can be fatal. Frequent pneumonia results in a high incidence of chronic lung diseases.[3] Thus, early diagnosis and early treatment, including periodical intravenous immunoglobulin replacement therapy, is essential to improve the prognosis and the quality of life of patients with XLA and non-XLA.

In the process of B-cell maturation, immunoglobulin κ-deleting recombination excision circles (KRECs) are produced during κ-deleting recombination allelic exclusion and isotypic exclusion of the λ chain.[4] Coding joint (cj) KRECs reside within the chromosome, whereas signal joint (sj) KRECs are excised from genomic DNA. cjKREC levels remain the same after B-cell division, whereas sjKREC levels decrease, because sjKRECs are not replicated during cell division.[5] Because the B-cell maturation defects in XLA and non-XLA occur before κ-deleting recombination, KRECs are not supposed to be produced. Therefore, measurements of KRECs have the potential to be applied to the identification of these types of B-cell deficiencies in patients, which consist of around 20% of all B-cell defects.[6] In addition, some types of combined immunodeficiencies show an arrest in B-cell maturation and can also be identified by this method. The success of newborn screening for T-cell deficiencies by measuring T-cell–receptor excision circles[7] prompted us to develop a newborn screening method for XLA and non-XLA by measuring KRECs derived from neonatal Guthrie cards.

The study protocol was approved by the National Defense Medical College institutional review board, and written informed consent was obtained from the parents of normal controls, the affected children, and adult patients, in accordance with the Declaration of Helsinki.

First, we determined the sensitivity of detection levels of cjKRECs and sjKRECs in Guthrie cards using real-time quantitative PCR.[5] Normal B cells from a healthy adult were isolated from peripheral blood (PB; mean purity, 88.5%). PB was also obtained from 1 patient with XLA (P20) whose B-cell number was 0.09 in 1 μL whole blood and who was negative for sjKRECs ($<1.0 \times 10^2$ copies/μg DNA). Various numbers of normal B cells were serially added to 1 mL whole PB obtained from this patient with XLA. The B-cell–added XLA whole blood was then applied to filter papers, and 3 punches (3 mm in diameter) of dried blood spots were used for DNA extraction. At least 3 DNA samples containing the same B-cell concentrations (0.09-400 B cells/μL) were used for the real-time quantitative PCR of cjKRECs and sjKRECs. The percentages of the positive samples ($>1.0 \times 10^2$ copies/μg DNA) of cjKRECs and sjKRECs increased constantly

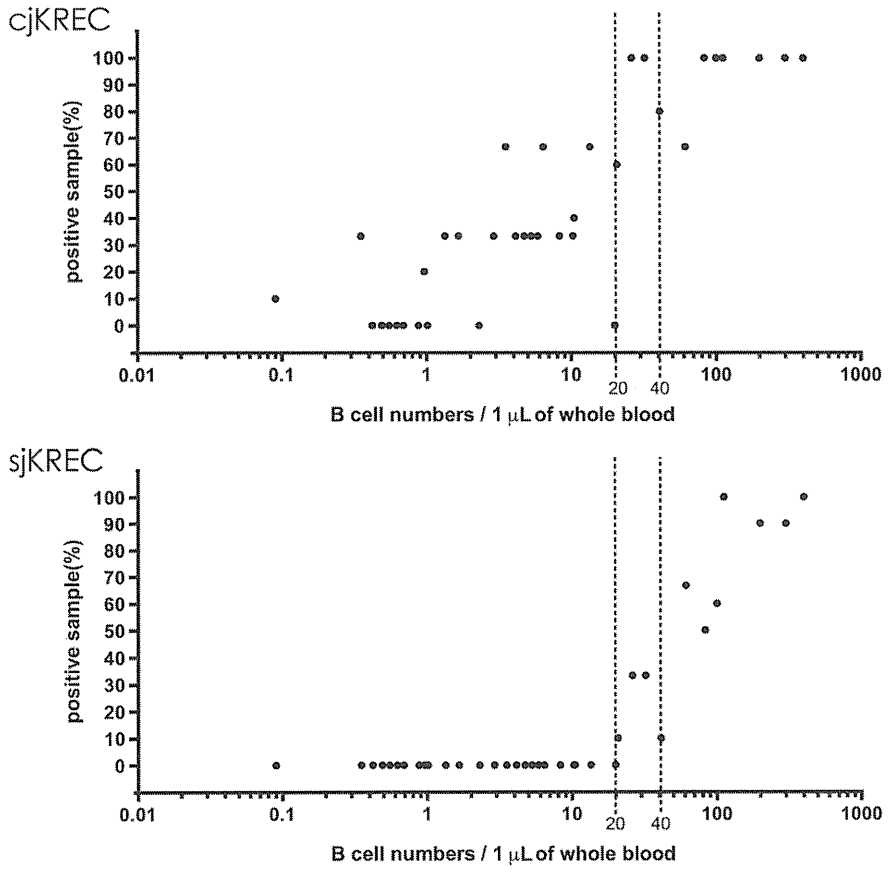

FIG 1. Sensitivity levels of cjKRECs and sjKRECs. Various numbers of purified normal B cells were serially added to whole PB from a patient with XLA (P20) to obtain B-cell–added XLA whole blood. cjKRECs and sjKRECs were measured in 3 to 10 samples of each concentration in triplicate. In all analyses, 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.

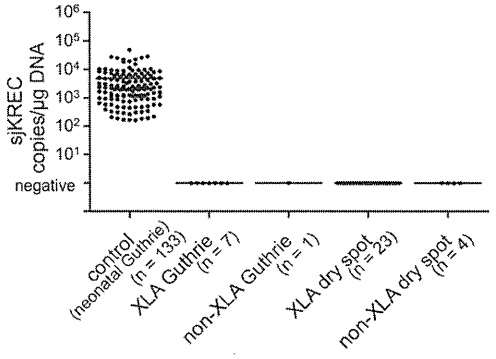

FIG 2. Copy numbers of sjKRECs measured in neonatal Guthrie cards or dried blood spots obtained from B-cell–deficient patients. On all samples from control, neonatal Guthrie cards (n = 133) were sjKREC-positive (4.8 ± 0.6 × 10^3 copies/μg DNA). B-cell–deficient patients were negative for sjKRECs in neonatal 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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TABLE E1. Characteristics of patients with XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL) IgG	IgA	IgM	$CD19^+$ % Lymph	/μL	*BTK* mutation Genomic DNA	cDNA	Amino acid	Source 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.

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

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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

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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.

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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.
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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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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			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.