Table 5

Association between *ATIC* 347CC genotype and methotrexate efficacy in patients with the early phase of juvenile idiopathic arthritis

(a)	OR†	95% Confidence interval	P value
ATIC 347CC genotype	0.32	0.11–0.93	0.033

(b)	OR‡	95% Confidence interval	P value
ATIC 347CC genotype	0.38	0.12–1.23	0.106
Rheumatoid factor [>14 (IU ml⁻¹)]	0.22	0.07–0.72	0.012
Time interval*	0.85	0.70–1.04	0.12

*Time interval, time interval from disease onset to methotrexate treatment. †Non-adjusted odds ratio. ‡Adjusted odds ratio.

readily transported across the cell membrane, and thus, the intracellular half-life of MTX is increased. This polyglutamation process is reversed via GGH-catalysed removal of the glutamic acid groups. Therefore, the amount of intracellular MTX-polyglutamates (MTX-PGs) depends on the net rate of polyglutamation determined by the opposing activities of FPGS and GGH [8].

It was reported that *GGH* T16C, which results in a Cys6Arg substitution, was associated with the efficacy of MTX in patients with RA. The variant C allele may cause a loss of GGH activity, resulting in decreased efflux of MTX and thus increased intracellular MTX-PG levels [8]. This result was consistent with ours. Although we did not address the MTX-PG levels in hepatic cells, it is possible that the C allele at *GGH* T16C was associated with reduced GGH activity and thereby increased the MTX-PG levels in hepatic cells. As a result, the risk of liver dysfunction rises. The AA genotype at *FPGS* A1994G tended to be associated with liver dysfunction ($P = 0.068$, OR = 4.88, 95% CI 0.78–30.9). Future research using large study populations to address the effects of the combination of *GGH* and *FPGS* polymorphisms on MTX toxicity is needed.

The MTX dosage was probably associated with the toxicity and efficacy of the drug. In this cohort study, some patients underwent MTX treatment at other hospitals and had liver dysfunction before being referred to our institution. For these patients, we did not have access to previous medical records concerning the exact dosage of MTX at the time of liver dysfunction. As a general rule, non-responders to MTX received higher dosages of MTX (up to 10 mg m⁻²) before the introduction of biologics than the responders. We therefore used MTX efficacy as the clinical predictor instead of MTX dosage. The MTX efficacy tended to be associated with liver dysfunction ($P = 0.083$), although the effect of MTX dosage on the

toxicity and efficacy of this drug should be evaluated directly in the future.

Second, we found that the longer time interval from disease onset to MTX treatment and RF positivity were associated with lower efficacy of MTX. This was consistent with previous research results. Time to treatment was reported as an important factor in the response to MTX in patients with JIA [6], and RF positivity was associated with worse disease activity [18, 19].

Paediatric rheumatologists have recently been able to use MTX for patients with earlier phases of JIA, because MTX has become well known as a first-line drug in the treatment of RA and JIA [2, 3]. Therefore, we analysed the subgroup of early JIA patients. In those who were treated with MTX within 1 year of disease onset, the CC genotype at *ATIC* C347G tended to be associated with the lower efficacy of MTX. Methotrexate-polyglutamates inhibit ATIC, the last enzyme in the *de novo* purine synthesis pathway. Methotrexate achieves part of its anti-inflammatory effect through inhibition of ATIC, which results in the release of the anti-inflammatory agent, adenosine [9].

It was reported that RA patients with the G allele at *ATIC* C347G, resulting in a Thr116Ser substitution, were likely to have a good response to MTX [9]. Although the effect of the C347G polymorphism on ATIC enzyme activity is unknown, *ATIC* C347G may be in linkage disequilibrium with an unknown functional variant, which is associated with the activity of the purine synthesis pathway and with the level of adenosine production. Future basic and clinical prospective studies on a large number of patients are needed to elucidate this association.

There are some limitations to the present study. The incidence of RF positivity in the patient population studied was higher than generally seen (~10%) [18], presumably because our institution is one of the very few paediatric rheumatology centres in Japan, and many severe cases with RF positive are referred to our institution for highly specialized treatment with biologics [13, 20]. The efficacy rate of MTX in this study (28%) was significantly lower than those in previous Japanese reports [2, 3]. This may be due to the use of a new second-line choice of biologics, as well as the characteristics of our institution and the lower limit of the maximal MTX dosage (10 mg m⁻²) for the treatment of JIA in Japan [2].

In summary, we found an association between the non-TT genotype at *GGH* T16C and liver dysfunction due to MTX. We also found an influence of time interval from disease onset to MTX treatment on the efficacy of MTX in Japanese patients with JIA. Our study showed the importance of early use of MTX for patients with JIA as well as the possibility of more personalized therapy for patients with JIA based on pharmacogenetic study of the MTX pathway genes.

Competing Interests

REFERENCES

1 Cassidy JT, Petty RE. Juvenile idiopathic arthritis. In: Textbook of Pediatric Rheumatology, 5th edn. eds Cassidy JT, Petty RE. Philadelphia, PA: WB Saunders, 2005; 206–60.

2 Mori M, Naruto T, Imagawa T, Murata T, Takei S, Tomiita M, Itoh Y, Fujikawa S, Yokota S. Methotrexate for the treatment of juvenile idiopathic arthritis: process to approval for JIA indication in Japan. Mod Rheumatol 2009; 19: 1–11.

3 McKendry RJ, Dale P. Adverse effects of low dose methotrexate therapy in rheumatoid arthritis. J Rheumatol 1993; 20: 1850–6.

4 Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y, Akama H, Kitamura Y, Kamatani N. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 2002; 12: 183–90.

5 Schmeling H, Biber D, Heins S, Horneff G. Influence of methylenetetrahydrofolate reductase polymorphisms on efficacy and toxicity of methotrexate in patients with juvenile idiopathic arthritis. J Rheumatol 2005; 32: 1832–6.

6 Albers HM, Wessels JA, van der Straaten RJ, Brinkman DM, Suijlekom-Smit LW, Kamphuis SS, Girschick HJ, Wouters C, Schilham MW, le Cessie S, Huizinga TW, Ten Cate R, Guchelaar HJ. Time to treatment as an important factor for the response to methotrexate in juvenile idiopathic arthritis. Arthritis Rheum 2009; 61: 46–51.

7 Laverdière C, Chiasson S, Costea I, Moghrabi A, Krajinovic M. Polymorphism G80A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia. Blood 2002; 100: 3832–4.

8 van der Straaten RJ, Wessels JA, de Vries-Bouwstra JK, Goekoop-Ruiterman YP, Allaart CF, Bogaartz J, Tiller M, Huizinga TW, Guchelaar HJ. Exploratory analysis of four polymorphisms in human GGH and FPGS genes and their effect in methotrexate-treated rheumatoid arthritis patients. Pharmacogenomics 2007; 8: 141–50.

9 Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, Kremer J. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate

synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum 2004; 50: 2766–74.

10 Toffoli G, De Mattia E. Pharmacogenetic relevance of MTHFR polymorphisms. Pharmacogenomics 2008; 9: 1195–206.

11 Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, He X, Maldonado-Cocco J, Orozco-Alcala J, Prieur AM, Suarez-Almazor ME, Woo P. International League of Associations for Rheumatology. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. J Rheumatol 2004; 31: 390–2.

12 Mori M, Takei S, Imagawa T, Imanaka H, Maeno N, Kurosawa R, Kawano Y, Yokota S. Pharmacokinetics, efficacy, and safety of short-term (12 weeks) etanercept for methotrexate-refractory polyarticular juvenile idiopathic arthritis in Japan. Mod Rheumatol 2005; 15: 397–404.

13 Yokota S, Imagawa T, Mori M, Miyamae T, Aihara Y, Takei S, Iwata N, Umebayashi H, Murata T, Miyoshi M, Tomiita M, Nishimoto N, Kishimoto T. Efficacy and safety of tocilizumab in patients with systemic-onset juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled, withdrawal phase III trial. Lancet 2008; 371: 998–1006.

14 Nishimoto N, Ito K, Takagi N. Safety and efficacy profiles of tocilizumab monotherapy in Japanese patients with rheumatoid arthritis: meta-analysis of six initial trials and five long-term extensions. Mod Rheumatol 2010; 20: 222–32.

15 Gossec L, Dougados M, Goupille P, Cantagrel A, Sibilia J, Meyer O, Sany J, Daurès JP, Combe B. Prognostic factors for remission in early rheumatoid arthritis: a multiparameter prospective study. Ann Rheum Dis 2004; 63: 675–80.

16 Wessels JA, van der Kooij SM, le Cessie S, Kievit W, Barerra P, Allaart CF, Huizinga TW, Guchelaar HJ. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. : Pharmacogenetics Collaborative Research Group. Arthritis Rheum 2007; 56: 1765–75.

17 International HapMap Consortium. The International HapMap Project. Nature 2003; 426: 789–96.

18 Petty RE, Cassidy JT. Polyarthritis. In: Textbook of Pediatric Rheumatology, 5th edn. eds Cassidy JT, Petty RE. Philadelphia, PA: WB Saunders, 2005; 261–73.

19 Adib N, Silman A, Thomson W. Outcome following onset of juvenile idiopathic inflammatory arthritis: II. predictors of outcome in juvenile arthritis. Rheumatology 2005; 44: 1002–7.

20 Yokota S, Mori M, Imagawa T, Murata T, Tomiita M, Itoh Y, Fujikawa S, Takei S. Guidelines on the use of etanercept for juvenile idiopathic arthritis in Japan. Pediatric Rheumatology Association of Japan. Mod Rheumatol 2010; 20: 107–13.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary data 1
TaqMan® SNP Genotyping Assays.
Supplementary data 2
Distribustion of gene polymorpshisms under the study.
Supplementary data 3
Distribustion of gene polymorpshisms in patiets with or without liver dysfunction.

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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 ($<26.2 \times 10^9$/l), elevated levels of asparate aminotransferase (>59 U/l), decreased white blood cell counts ($<4.0 \times 10^9$/l), and hypofibrinogenemia (<2.5 g/l). The diagnosis of MAS requires the presence of 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 regulatory 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 *SH2D1*A 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.

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.

Novel mutations of MVK gene in Japanese family members affected with hyperimmunoglobulinemia D and periodic fever syndrome

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Abstract Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS) is a recessively inherited recurrent fever syndrome. We describe a family of eldest son and monozygotic twin younger sisters with characteristic syndrome of HIDS, but normal level of IgD. Mevalonate kinase (MK) activity was deficient in all of them, and analysis of the MVK gene revealed compound heterozygosity for 2 new mutations, one of which was the disease-causing splicing mutation and the other was a novel missense mutation. All the patients had the same compound heterozygous mutations c.227-1 G > A and c.833 T > C, which resulted in exon 4 skipping and p.Val278Ala. This is the first case in which exon skipping mutation of the MVK gene has been certainly identified at the genomic DNA level. In each case, in which HIDS is clinically suspected, despite normal IgD level, analysis of MK activity and the MVK gene should be performed.

Introduction

Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) is a rare autosomal recessive auto-inflammatory disorder characterized by recurrent febrile attacks with lymphadenopathy, abdominal distress, skin eruptions, and joint involvement [1–3]. Febrile attacks usually last for 3–7 days and are interrupted by asymptomatic intervals of several weeks' duration [4–6]. Symptoms appear in early infancy and may persist throughout life with gradual increases in serum IgD [7, 8]. The diagnostic hallmark of HIDS is a constitutively elevated level of serum IgD, although parts of the patients have been reported to have normal amount of serum IgD levels.

The HIDS is caused by mutations on mevalonate kinase gene (MVK), which encodes an enzyme involved in cholesterol and non-sterol isoprenoid biosynthesis. We present herein a Japanese family, eldest son and monozygotic twin younger sisters, with HIDS that had compound heterozygous mutations on MVK gene, one of which was the disease-causing splicing mutation and the other was a novel missense mutation. Serum concentrations of IgD were repeatedly within the normal range. These cases demonstrate that detail analysis with more specific diagnostic tests such as urinary excretion of mevalonic acid and MVK genetic analysis should be performed not to miss the correct diagnosis in patients, especially younger children with HIDS.

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Case reports

Patients are the eldest son and monozygotic twin younger sisters of parents of Japanese origin. The eldest son (patient 1) had presented with recurrent fever from 5 months of age. The twin younger sisters (patient 2 and 3) presented with fever from 1 month of age. Vomiting and diarrhea were presented in the younger sister (patient 3). Febrile episodes appeared every 4–8 weeks and lasted for 3–5 days on all the three patients. During febrile episodes, peripheral blood leukocytosis and CRP elevations (more than 10 mg/dl) were observed. In intermittent period between fever episodes, serum CRP levels decreased, but did not always become negative. Their parents had no history of recurrent fever. Sepsis work-up did not show any foci and any pathogens causing the febrile episodes. The repeated bacterial cultures resulted in negative, and administration of the antimicrobial agents did not change the clinical courses of the febrile episodes, indicating that the fever was not induced by pathogen. In addition, immunological analysis such as serum IgA, IgM, IgG, and IgD, lymphocytes counts including T, B, NK cells, and mitogen proliferation assays of peripheral blood mononuclear cells (PBMCs) were normal.

Due to the recurrent high fevers caused most unlikely by pathogen and the heavy family history of the periodic fevers, we suspected hereditary periodic fever syndromes and performed genetic study. After written informed consents approved by institutional review board of the Kyoto University Hospital were obtained, peripheral blood samples were collected from the patients and their parents for isolating genomic DNA and total RNA.

First, we performed genomic DNA sequencing for MEFV gene for familial Mediterranean fever, MVK gene for HIDS, NLRP3 for cryopyrin-associated periodic syndrome, and TNFRSF1A for TNF receptor-associated periodic syndrome. Genomic DNA sequencing analysis of the MVK gene revealed the presence of heterozygous mutations of c.227-1 G > A at the exon/intron border of exon 4 and c.833T > C (p.Val278Ala). Subsequent amplification of the cDNA by RT-PCR showed that the former mutation caused deletion of exon 4 (Fig. 1a). Genomic DNA sequence analysis on their parents revealed that the parents inherited c.227-1 G > A from their father and c.833T > C from their mother, indicating that the three patients were compound heterozygous for MVK gene (Fig. 1b). The patients had markedly elevated excretion of mevalonic acid in urine, especially in febrile periods, and their mevalonate kinase enzyme activities were very low, which confirmed that all the three patients suffered from HIDS (Table 1).

While the patients did not have any mutations on TNFRSF1A and NLRP3, we identified MEFV non-synonymous nucleotide alterations on the elder brother, who was a heterozygote for L110P, E148Q, and R202Q, and the younger twin, who was a heterozygote for R202Q in addition to MVK gene mutations. These MEFV gene nucleotide alterations were regarded as SNPs, and the clinical diagnosis of FMF was not compatible with the patients, although the complex MEFV gene alterations of L110P/E148Q/R202Q have been reported on the clinically-diagnosed FMF patients.
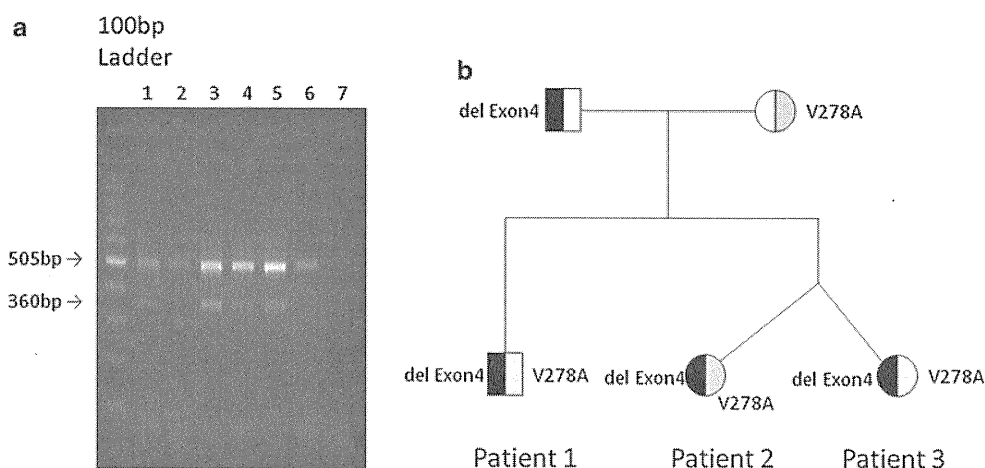

Fig. 1 Molecular genetic findings in the study patients. **a** Agarose gel electrophoresis of RT-PCR products for exon 2 to exon 5 of MVK shows the normal 505-bp alleles in samples from normal healthy control (*lane 6*) and mother (*lane 2*), as well as both the normal allele and the mutant 362-bp allele in the sample from father (*lane 1*), patient 1 (*lane 3*), patient 2 (*lane 4*), and patient 5 (*lane 5*). Subsequent cDNA sequencing confirmed that this 144-bp deletion in cDNA corresponds to codon 303–407 (exon 4). The molecular size marker was a 100-bp ladder. *Lane 7* represents PCR with distilled water added but not with DNA, indicating that there was no background amplification. **b** Pedigree of the affected family. The three patients are heterozygous for del exon 4 and V278A

Table 1 Urinary mevalonic acid and mevalonate kinase levels in the study patients

Patient no.	Mevalonic acid in urine (μg/mgCr)		Mevalonate kinase (pmol/minute/mg)
	Febrile period	Intermittent period	
1	67.9	11.3	3
2	55.6	17.7	2
3	58.8	18.5	2
Control		0.078 ± 0.012^a	214 ± 62^a

Control data are given as mean ± SD

[a] Values form healthy subjects were used to obtain a control range for urinary mevalonic acid levels (mean ± SD) and mevalonate kinase levels (mean ± SD)

Discussion

We present herein a sibling of HIDS that demonstrated compound heterozygous for two novel mutations of MVK gene. All the patients had the same compound heterozygous mutations c.227-1 G > A and c.833T > C, which resulted in exon 4 skipping and p.Val278Ala. The mutations are novel, especially the splicing mutation of MVK gene was identified at the genomic DNA level.

Cuisset et al. [9] reported that HIDS mutations were evenly distributed along the coding region of the MVK gene, in contrast to mutations causing MA, which clustered between 243 and 334. The sequence variations seen in MA are missense mutations that are in the same region as the variants described in HIDS. Further studies will be needed to clarify the association of phenotypical differences with MVK gene mutations. Over 80% of patients with HIDS were reported to have compound heterozygous mutation in the MVK gene. To our knowledge, both the skipping of exon 4 and V278A mutation have not been reported previously in HIDS. Moreover, this is the first case in which exon skipping mutation of the MVK gene has been certainly identified at the genomic DNA level. Only few groups reported HIDS patients with the skipping of exon in the cDNA of the MVK gene [10, 11]. They suggested that these exon skipping was probably due to the presence of a potential splice site mutation, but could not identify mutations responsible for these altered splicing through the sequence analysis at the genomic level. Most MVK mutations in patients with HIDS and MA have only been determined at the cDNA level; however, analysis of cDNA sometimes appeared troublesome, probably due to instability of the MVK mRNA. More detailed studies through the sequence analysis at the genomic level lead us to elucidate the role of MVK mutations in HIDS and MA, and expression studies in *E. coli* will be necessary to evaluate the effect of each mutation.

HIDS is classically defined as a high concentration of mevalonic acid in the urine and is characterized by a high serum IgD concentration during each febrile episode, but some reports from the Netherlands stated that high levels of serum IgD levels were not seen and affirmed that other diseases also showed high serum IgD levels [12]. In our cases, the analysis of enzymes and molecular genetics of MVK gene yielded the correct diagnosis, although serum concentrations of IgD were within the normal range. Thus, it should be now common practice to examine the MVK gene in order to diagnose this disease.

In conclusion, we present a Japanese family with HIDS that appeared to have novel mutations of MVK gene. Most of the HIDS cases were reported from European, especially Dutch, whereas only one HIDS case of Japanese patient was reported by Naruto et al. [13], which is only one report of Asian patient. Cases of HIDS may so far have been overlooked or misdiagnosed as infectious diseases or autoimmune disorders in Japan, besides there may be difference in race. It is necessary that accumulation of case in hereditary mutation and in other race leads to solve a detailed cause of HIDS.

References

1. van der Meer JW, Vossen JM, Radl J, Van Nieuwkoop JA, Meyer CJ, Lobatto S et al (1984) Hyperimmunoglobulinaemia D and periodic fever: a new syndrome. Lancet 1:1087–1090
2. Church LD, Churchman SM, Hawkins PN, McDermott MF (2006) Hereditary auto-inflammatory disorders and biologics. Semin Immunopathol 27:494–508
3. Simon A, van der Meer JW (2007) Pathogenesis of familial periodic fever syndromes or hereditary autoinflammatory syndromes. Am J Physiol Regul Integr Comp Physiol 292:R86–R98
4. Fenkel J, Houten SM, Waterham HR, Wanders RJ, Rijkers GT, Duran M et al (2001) Clinical and molecular variability in childhood periodic fever with hyperimmunoglobuninaemia D. Rheumatology (Oxford) 40:579–584
5. Stojanov S, Lohse P, Lohse P, Hoffmann F, Renner ED, Zellerer S et al. (2004) Molecular analysis of the MVK and TNFRSF1A genes in patients with a clinical presentation typical of the hyperimmunoglobulinemia D with periodic fever syndrome: a low-penetrance TNFRSF1A variant in a heterozygous MVK carrier possibly influences the phenotype of hyperimmunoglobulinemia D with periodic fever syndrome or vice versa. Arthritis Rheum 50:1951–1958
6. Touitous I, Lesage S, McDermott M, Cuisset L, Hoffman H, Dode C et al (2004) Infevers: an evolving mutation database for autoinflammatory syndromes. Hum Mutat 24:194–198
7. Pouchot J, Sampalis JS, Beaudet F, Carette S, Decary F, Salusinsky-Sternbach M et al (1991) Adult Still's disease: manifestations, disease course, and outcome in 62 patients. Medicine (Baltimore) 70:118–136

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8. Haas D, Hoffmann GF (2006) Mevalonate kinase deficiencies: from mevalonic aciduria to hyperimmunoglobulinemia D syndrome. Orphanet J Rare Dis 1:13

9. Cuisset L, Drenth JP, Simon A, Vincent MF, Van der Velde Visser S, Van der Meer JW (2001) Molecular analysis of MVK mutations and enzymatic activity in hyper-IgD and periodic fever syndrome. Eur J Hum Genet 9(4):260–266

10. Drenth JP, Cuisset L, Grateau G, Vasseur C, van de Velde-Visser SD, de Jong JG et al (1999) Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome. International Hyper-IgD Study Group. Nat Genet 22:178–181

11. Takada K, Aksentijevich I, Mahadevan V, Dean JA, Kelley RI, Kastner DL (2003) Favorable preliminary experience with etanercept in two patients with the hyperimmunoglobulinemia D and periodic fever syndrome. Arthritis Rheum 48:2645–2651

12. Drenth JP, Haagsma CJ, van der Meer JW (1994) Hyperimmunoglobulinemia D and periodic fever syndrome. The clinical spectrum in a series of 50 patients. International Hyper-IgD Study Group. Medicine (Bartimore) 73:133–144

13. Naruto T, Nakagishi Y, Mori M, Miyamae T, Imagawa T, Yokota S (2009) Hyper-IgD syndrome with novel mutation in Japanese girl. Mod Rheumatol 19(1):96–99

CASE REPORT

Patient with neonatal-onset chronic hepatitis presenting with mevalonate kinase deficiency with a novel MVK gene mutation

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Abstract A Japanese girl with neonatal-onset chronic hepatitis and systemic inflammation was diagnosed with hyper-immunoglobulinemia D and periodic fever syndrome (HIDS). However, she lacked the typical HIDS features until the age of 32 months. She had compound heterozygous MVK mutations, H380R and A262P, the latter of which was novel. These findings suggest that HIDS patients could lack typical episodes of recurrent fever at the onset and that HIDS should be considered as a possible cause of neonatal-onset chronic hepatitis.

Abbreviations

HIDS	Hyper-immunoglobulinemia D and periodic fever syndrome
IgD	Immunoglobulin D
MVK	Mevalonate kinase
FMF	Familial Mediterranean fever
MEFV	Familial Mediterranean fever gene
AIH	Autoimmune hepatitis
CRP	C-reactive protein

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Introduction

Mevalonate kinase deficiency is an autosomal recessive metabolic disorder caused by mevalonate kinase (MVK) gene mutations. The disorder presents as a phenotypic spectrum in which mevalonic aciduria is the more severe form, with neurological complications, and hyperimmuno-globulinemia D and periodic fever syndrome (HIDS) is the milder form. HIDS is characterized by recurrent febrile attacks, with lymphadenopathy, abdominal symptoms, skin eruptions, and joint involvement [1]. In this report, we describe a patient with a severe form of HIDS caused by a novel MVK mutation; the patient had presented with neonatal-onset chronic hepatitis that was temporarily diagnosed as autoimmune hepatitis (AIH). The lack of typical recurrent fever and rashes at the onset of the disease delayed the diagnosis of HIDS, which alerted the clinicians that HIDS could exist in patients with continuous inflammatory episodes even with atypical clinical courses.

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Case report

A Japanese girl was born at 36 weeks' gestation with a weight of 2,240 g. Her parents were non-consanguineous and the family history was unremarkable. At birth she had no symptoms, but physical examination revealed hepatomegaly (1.5 cm below the right costal margin) without splenomegaly. No jaundice, ascites, or coagulation abnormalities were present. Laboratory examinations showed increased white blood cell count (45,700/mm^3) and serum C-reactive protein (CRP) (15.8 mg/dl), as well as increased transaminase levels (aspartate aminotransferase [AST] 207 IU/l, alanine aminotransferase [ALT] 96 IU/l), lactate dehydrogenase (LDH) (6,575 IU/l), and biliary enzyme levels (γ-guanosine triphosphate [GTP] 61 IU/l). An increased immunoglobulin M level (53.0 mg/dl) caused us to work on congenital infections, with bacterial cultures of blood, cerebrospinal fluid (CSF), and gastric fluid, and determination of serum β D-glucan, and measurements of serum antibodies against cytomegalovirus, toxoplasmosis, syphilis, rubella, herpes simplex type I and type II, listeriosis, Epstein-Barr virus, adenovirus, hepatitis A and B and C viruses, *Chlamydia trachomatis*, and mycoplasma, all of which were negative. Radiographic work-up with computed tomography (CT), magnetic resonance imaging (MRI), and gallium scintigraphy, as well as bone-marrow aspirate examination, did not reveal any congenital neoplasm. A search for metabolic disorders by measurements of blood amino acids and urinary organic acids was negative.

Without any specific treatments, the hepatomegaly gradually increased, although abdominal MRI revealed diffuse inflammation of the liver. To explore further the cause of the hepatomegaly, a needle liver biopsy was performed at the age of 6 months. The biopsied liver specimen showed the presence of mild lymphocytic infiltration and fibrosing lesions in the portal area, and short septa extending from a slightly enlarged portal tract (Fig. 1a, b), which indicated a diagnosis of chronic hepatitis without specific causes.

At the age of 14 months, splenomegaly appeared, and elevated serum IgG (2,299 mg/dl) as well as anti-smooth muscle antibodies (1:160) were detected, which led us to diagnose the patient as having AIH [2]. The patient received methylprednisolone pulse therapy, followed by prednisolone and azathioprine for the presumed AIH [3]. Serum transaminase levels normalized in response to the treatment, although cervical lymphadenopathy, hepatosplenomegaly, and elevated serum CRP persisted.

The continuous elevation of CRP prompted us to consider autoinflammatory diseases; thus, we performed genetic analysis for familial Mediterranean fever (FMF), tumor necrosis factor (TNF) receptor-associated periodic syndrome, and cryopyrin-associated periodic syndrome, at the age of 26 months. After obtaining written informed consent from the parents and approval from the Institutional Review Board of Kyoto University, peripheral blood samples were collected from the patient and her parents for genetic analysis. The analysis was done by sequencing all the exons, including exon–intron junctions, which showed heterozygous L110P and E148Q missense mutations on the familial Mediterranean fever (MEFV) gene (Fig. 2a) without any mutations of the TNFRSF1A and NLRP3

Fig. 1 Liver biopsy specimen showing chronic hepatitis. **a** The portal tract is infiltrated with lymphocytes (H&E, ×200). **b** Short septa extend from the slightly enlarged portal tract (reticulum, ×100)
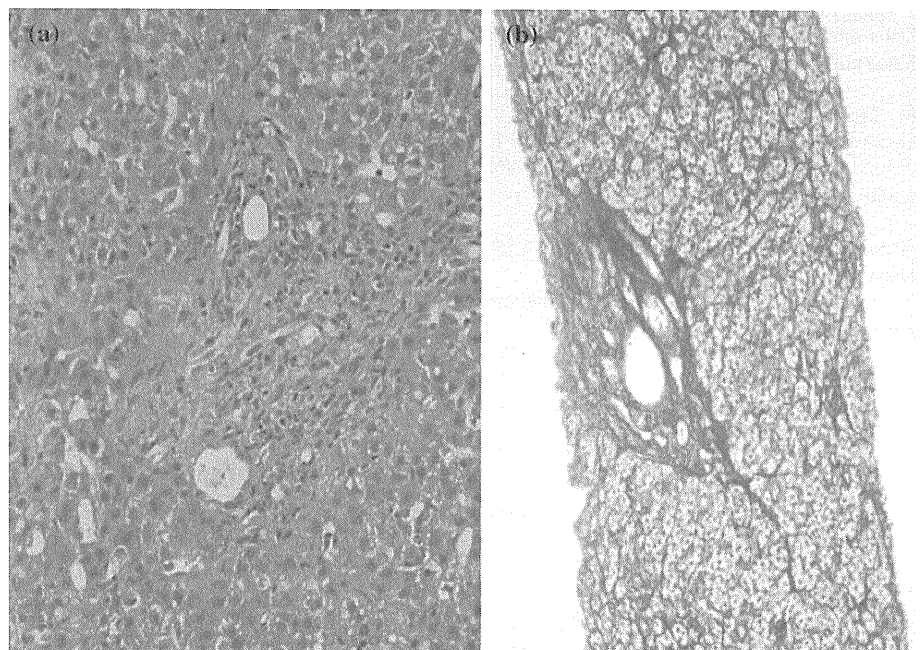
genes. The L110P and E148Q mutations on MEFV were considered to be single-nucleotide polymorphisms (SNPs), based upon the prevalence of the mutations, as well as their weak association with FMF in Japan. Because none of periodic fever, rashes, gastrointestinal symptoms, or elevated serum IgD was observed at that time, the MVK gene was not examined.

The patient continued to show a good response to the AIH treatments, although tapering off the prednisone induced periodic fever with maculopapular rashes approximately once a month, shown for the first time at the age of 32 months. The fever episodes persisted for 3–5 days and the duration of the fever was reduced to 1–2 days by temporarily increasing the dose of prednisone. Serum CRP levels were around 20 mg/dl at the onset of fever, and 1–4 mg/dl in the asymptomatic period. The newly emerged clinical symptoms and the good response to the systemic steroid prompted us to consider HIDS. Full examination for HIDS showed: (1) elevated serum IgD (19.2 mg/dl) (control 0–9 mg/dl); (2) increased urinary

mevalonic acid (49.1 µg/mg creatinine) (control 0.091 ± 0.028 µg/mg creatinine); and (3) a significant decrease in the mevalonate kinase activity of peripheral blood mononuclear cells (PBMCs; below the detection limit). Genetic analysis of the MVK gene revealed compound heterozygous mutations, A262P and H380R, the former of which was a novel mutation (Fig. 2b). The MVK mutations were not identified in 100 healthy Japanese controls. Finally we diagnosed the patient with HIDS, at the age of 6 years. We treated the patient with simvastatin (0.07 mg/kg/day), which was partially effective in reducing the frequency of the periodic fever. Although no decline in urinary mevalonic acid has been produced by simvastatin (33.7–107.8 µg/mg creatinine), the patient has had a benign course, without mental retardation or neurological impairments (Fig. 3).

To see if the patient's liver abnormalities were due to either AIH or HIDS, we performed an immunohistological analysis of the biopsied liver specimen. It was stained for CD68, and unstained for CD3 and CD79 (Fig. 4). These

Fig. 2 Genetic analysis. a Genetic analysis of the MEFV gene. The patient had heterozygous amino acid changes of L110P and E148Q. b Genetic analysis of the MVK gene. The patient had heterozygous mutations of A262P and H380R
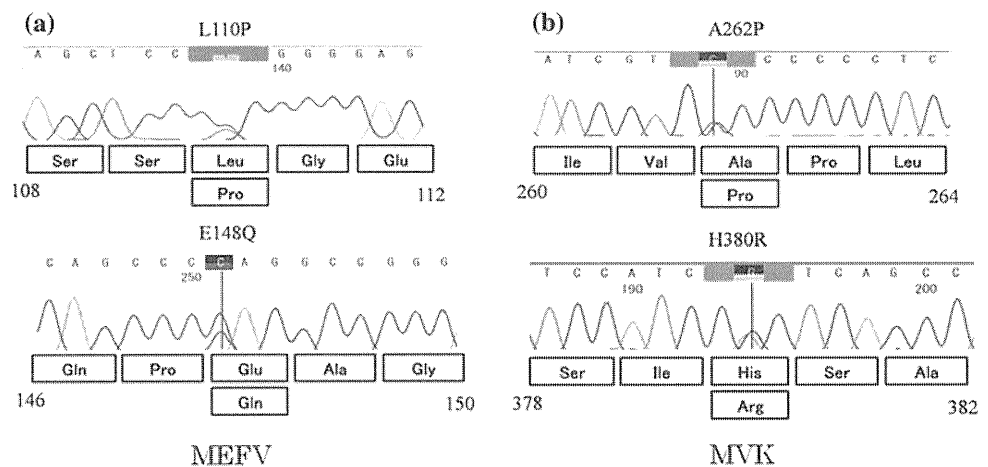
Fig. 3 Clinical course. Hepato Hepatosplenomegaly, Lymph cervical lymphadenopathy, MP methylprednisolone, PSL prednisolone, AZ azathioprine, ASMA anti-smooth muscle antibody, U-MVA urinary mevalonic acid (µg/mg creatinine)

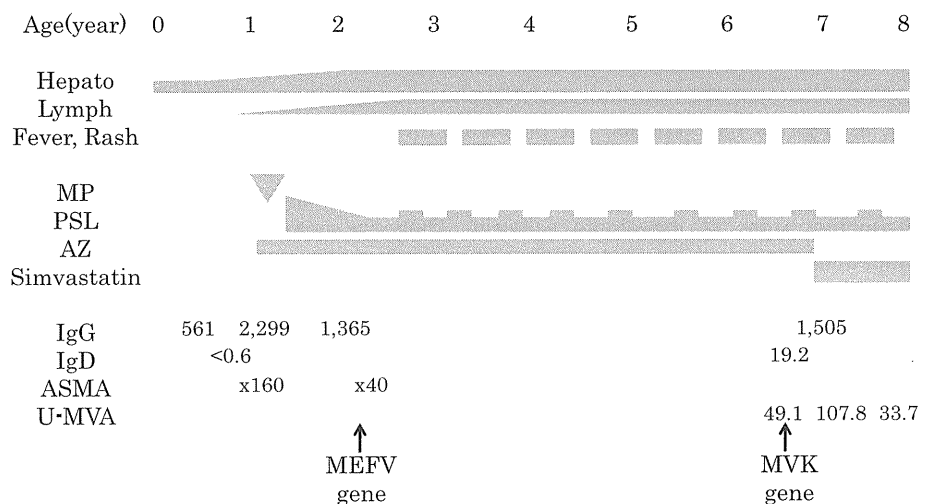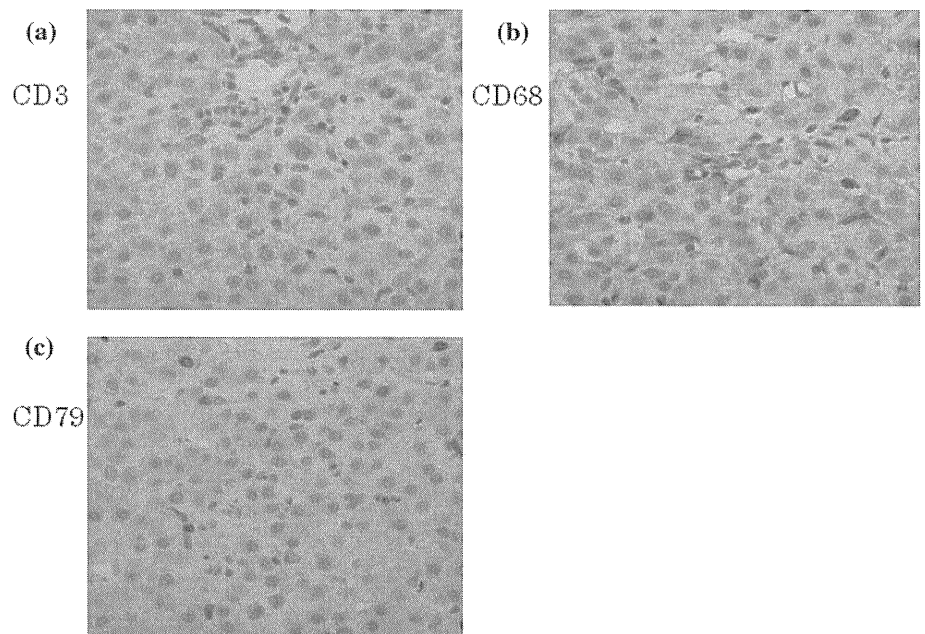**Fig. 4** Immunohistochemical
analysis of the biopsied liver
tissues. **a** CD3 (×400), **b** CD68
(×400), **c** CD79 (×400)

data led us to conclude that the hepatitis seemed to be a manifestation of HIDS, rather than resulting from an autoimmune response.

Discussion

We have reported here a Japanese girl who was diagnosed with HIDS by genetic analysis, as well as by laboratory examinations such as mevalonate kinase activity and urinary excretion of mevalonate. According to the report of the Japanese HIDS registry, the 4 most prevalent MVK mutations (V377I, I268T, H20P/N, and P167L) accounted for 71.5% of the mutations found. Our patient had a very rare genotype among the patients on the HIDS registry, as the H380R mutation was identified in 1.5% of the patients and A262P was a novel mutation. Because mevalonate kinase activity was below the detection limit, mevalonic aciduria could have been considered as the diagnosis in our patient. However, the mevalonic acid level in the urine was not as high as that reported for patients with mevalonic aciduria [4] and the clinical features of our patient lacked the neurological and developmental abnormalities that are distinctive signs of mevalonic aciduria. Thus, we concluded that the patient suffered from a severe form of HIDS, although we note that mevalonate kinase deficiency presents as a phenotypic continuum in which disease severity ranges from mevalonic aciduria to HIDS [5].

Serum transaminase levels in our patient were elevated since birth, which is relatively rare for HIDS, and liver biopsy showed chronic non-specific hepatitis [6]. Although the serum transaminase levels were improved by the

treatment for AIH, the histological and immunohistochemical findings were not typical of AIH [6], which is a generally unresolving inflammation of the liver of unknown cause [7]. There have been some reports of HIDS patients with liver abnormalities. Topaloglu et al. [8] reported a similar case of HIDS in a patient who had neonatal hepatosplenomegaly without fever at the beginning, and they performed liver biopsy which showed portal fibrosis. Hinson et al. [9] reported two patients with mevalonate kinase deficiency who had neonatal hepatosplenomegaly and elevated transaminase levels; liver biopsy showed chronic active cholestatic hepatitis and portal fibrosis, respectively.

Neonatal hepatitis is a syndrome associated with a history that includes any type of infectious, genetic, toxic, or metabolic causation. Neonatal hepatitis is characterized by clinical and laboratory findings of liver dysfunction, particularly conjugated hyperbilirubinemia. In our patient, the clinical course in early childhood was not typical of neonatal hepatitis. But the clinical course in our patient suggests that it is important to include HIDS in the differential diagnosis of neonatal hepatitis or neonatal-onset chronic hepatitis.

Genetic analysis of autoinflammatory disease genes in our patient revealed 2 heterozygous amino acid changes, L110P and E148Q, in the MEFV gene, which were shared with the patient's asymptomatic mother. It has been reported that the allele frequency of E148Q in the Japanese population was high (16.38%), and both E148Q and L110P are considered as SNPs [10]. On the other hand, Touitou et al. [11] demonstrated that E148Q may have an exacerbating effect on FMF when it is part of complex alleles. In addition, there are other reports that mutations in 2 autoinflammatory

genes cause more severe diseases [8, 12]. Thus, the hetero-zygous E148Q and L110P amino acid changes in the MEFV gene may cause a more severe form of HIDS.

The name 'HIDS' was given to the disorder because of the observed elevation in serum IgD, before the identification of the disease-causing mutations in the MVK gene. In a study of 103 HIDS patients, 22% had normal serum IgD, particularly during infancy [13], which indicates that serum IgD is not sensitive enough for diagnosing HIDS. In Asian countries like Japan, HIDS is so rare that clinicians do not know the clinical relevance of IgD in relation to the diagnosis of HIDS. Therefore, it is very important to let clinicians know that more specific and more sensitive diagnostic tests; namely, measurement of urinary meva-lonic acid and/or genetic analysis of the MVK gene are necessary to diagnose HIDS. It should also be pointed out that both the measurements of urinary mevalonic acid and the genetic tests of the MVK gene require special laboratory equipment, which makes it difficult to access such diagnostic tests.

In conclusion, we have reported a patient with a severe form of HIDS who presented with neonatal-onset chronic hepatitis with a novel MVK mutation. HIDS should be included in the differential diagnosis of neonatal-onset chronic hepatitis, even if the serum IgD is within the normal range and typical recurrent fever is not identified.

References

1. Drenth JP, Denecker NE, Prieur AM, van der Meer JW. Hyper-immunoglobulin D syndrome. Presse Med. 1995;24:1211–3.

2. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, et al. International Autoimmune Hepatitis Group report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol. 1999;31:929–38.

3. Sogo T, Fujisawa T, Inui A, Komatsu H, Etani Y, Tajiri H, et al. Intravenous methylprednisolone pulse therapy for children with autoimmune hepatitis. Hepatol Res. 2006;34:187–92.

4. Houten SM, Wanders RJA, Waterham HR. Biochemical and genetic aspects of mevalonate kinase and its deficiency. Biochim Biophys Acta. 2000;1529:19–32.

5. Simon A, Kremer HPH, Wevers RA, Scheffer H, de Jong JG, van der Meer JWM, et al. Mevalonate kinase deficiency: evidence for a phenotypic continuum. Neurology. 2004;62:994–7.

6. Kage M. Pathology of autoimmune liver diseases in children. Hepatol Res. 2007;37:S502–8.

7. Manns MP, Czaja AJ, Gorham JD, Krawitt EI, Mieli-Vergani G, Vergani D, et al. Diagnosis and management of autoimmune hepatitis. Hepatology. 2010;51:2193–213.

8. Topaloglu R, Ayaz NA, Yuce A, Gumruk F, Sanal O. Hyperimmunoglobulinemia D and periodic fever syndrome; treatment with etanercept and follow-up. Clin Rheumatol. 2008;27:1317–20.

9. Hinson DD, Rogers ZR, Hoffmann GF, Schachtele M, Fingerhut R, Kohlschtter A, et al. Hematological abnormalities and cholestatic liver disease in two patients with mevalonate kinase deficiency. Am J Med Genet. 1998;78:408–12.

10. Komatsu M, Takahashi T, Uemura N, Takada G. Familial Mediterranean fever medicated with an herbal medicine in Japan. Pediatr Int. 2004;46:81–4.

11. Touitou I. The spectrum of familial Mediterranean fever (FMF) mutations. Eur J Hum Genet. 2001;9:473–83.

12. Stojanov S, Lohse P, Lohse P, Hoffmann F, Renner ED, Zellerer S, et al. Molecular analysis of the MVK and TNFRSF1A genes in patients with a clinical presentation typical of the hyperimmu-noglobulinemia D with periodic fever syndrome: a low-penetrance TNFRSF1A variant in a heterozygous MVK carrier possibly influences the phenotype of hyperimmunoglobulinemia D with periodic fever syndrome or vice versa. Arthritis Rheum. 2004;50:1951–8.

13. Van der Hilst JC, Bodar EJ, Barron KS, Frenkel J, Drenth JP, van der Meer JW, International HIDS Study Group, et al. Long-term follow-up, clinical features, and quality of life in a series of 103 patients with hyperimmunoglobulinemia D syndrome. Medicine (Baltimore). 2008;87:301–10.

CLINICAL TRIALS AND OBSERVATIONS

Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4 protein

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Familial hemophagocytic lymphohistiocytosis (FHL) is a potentially lethal genetic disorder of immune dysregulation that requires prompt and accurate diagnosis to initiate life-saving immunosuppressive therapy and to prepare for hematopoietic stem cell transplantation. In the present study, 85 patients with hemophagocytic lymphohistiocytosis were screened for FHL3 by Western blotting using platelets and by natural killer cell lysosomal exocytosis assay. Six of these patients were diagnosed with FHL3. In the acute disease phase requiring platelet transfusion, it was difficult to diagnose FHL3 by Western blot analysis or by lysosomal exocytosis assay. In contrast, the newly established flow cytometric analysis of intraplatelet Munc13-4 protein expression revealed bimodal populations of normal and Munc13-4–deficient platelets. These findings indicate that flow cytometric detection of intraplatelet Munc13-4 protein is a sensitive and reliable method to rapidly screen for FHL3 with a very small amount of whole blood, even in the acute phase of the disease. (*Blood.* 2011;118(5):1225-1230)

Introduction

The granule-dependent cytotoxic pathway is a major immune effector mechanism used by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.[1] The pathway involves a series of steps, including cell activation, polarization of the lysosomal granules to the immunologic synapse, exocytosis of lytic proteins such as perforin and granzymes, and induction of apoptosis in the target cells.[2] In addition to its central role in the defense against intracellular infections and in tumor immunity, this pathway also plays an important role in the regulation of immune homeostasis. Defects in the granule-dependent cytotoxic pathway result in a catastrophic hyperinflammatory condition known as hemophagocytic lymphohistiocytosis (HLH).[1,3]

HLH is a life-threatening syndrome of immune dysregulation resulting from the uncontrolled activation and proliferation of CTLs, which leads to macrophage activation and the excessive release of inflammatory cytokines.[4,5] Clinical diagnosis of HLH is made on the basis of cardinal signs and symptoms including prolonged fever and hepatosplenomegaly, and by characteristic laboratory findings such as pancytopenia, hyperferritinemia, hypofibrinogenemia, increased levels of soluble IL-2 receptor, and low or absent NK cell activity.[5,6] HLH can be classified into primary (genetic) or secondary (acquired) forms according to the underlying etiology, although this distinction is difficult to make in clinical practice.[4,5]

Familial hemophagocytic lymphohistiocytosis (FHL) encompasses major forms of primary HLH for which mutations in the genes encoding perforin (*PRF1*; FHL2),[7] Munc13-4

(*UNC13D*; FHL3),[8] syntaxin-11 (*STX11*; FHL4),[9] and syntaxin-binding protein 2 (also known as Munc18-2) (*STXBP2*; FHL5)[10,11] have been identified to date. Perforin is a cytolytic effector that forms a pore-like structure in the target cell membrane. Munc13-4, syntaxin-11, and Munc18-2 are involved in intracellular trafficking or the fusion of cytolytic granules to the plasma membrane and the subsequent delivery of their contents into target cells.[1,12] Consequently, defective cytotoxic activity of CTLs and NK cells is one of the hallmark findings of FHL,[7,8,13-16] although NK cell activity is also decreased in some cases of secondary HLH.[15,17-20]

Prompt and accurate diagnosis of FHL is mandatory to initiate life-saving immunosuppressive therapy and to prepare for hematopoietic stem cell transplantation. Detection of perforin expression in NK cells with flow cytometry is a reliable method to screen for FHL2.[21] Another test analyzes the expression of CD107a on the surface of NK cells, which marks the release of cytolytic granules.[22] Reduced expression of CD107a implies impaired degranulation of NK cells and predicts a likelihood of FHL3.[23] However, this analysis is not available in some patients with extremely reduced NK cell numbers, such as during the acute phase of HLH.[19] In addition, NK-cell degranulation is also impaired in FHL4[24] and FHL5,[10,11] making it impossible to differentiate these disorders.

We reported previously that Munc13-4 protein is expressed in platelets and regulates the secretion of dense core granules.[25] Herein we report that Munc13-4 is expressed far more abundantly in platelets than in PBMCs. We also describe the development of a

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new method to screen for FHL3 rapidly by detecting intraplatelet Munc13-4 expression through flow cytometry.

Methods

Patients

Between January 2008 and March 2010, whole blood samples from 85 patients were screened for FHL3. The patients had been clinically diagnosed with HLH by their referring physicians and were suspected of possible FHL. Characteristics of the enrolled patients are summarized in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). As a control, blood obtained from healthy adults at the time of patient sampling was shipped for screening along with the patient samples. Before the laboratory studies were performed, informed consent was obtained from the patients and their parents, in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Preparation of PBMCs and platelet samples

Whole blood samples treated with EDTA were centrifuged gently at 100*g* for 10 minutes, and platelets were collected from the supernatant plasma layer. Alternatively, platelets were prepared from small aliquots of blood samples by lysing red blood cells with ammonium chloride. PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation from the remaining sample. CD4$^+$, CD8$^+$, CD14$^+$, CD19$^+$, and CD45$^+$ cells were separated from PBMCs using an AutoMACS Pro (Miltenyi Biotec) and magnetic bead–conjugated mAbs according to the manufacturer's instructions. Flow cytometric analysis revealed that each cell population contained > 95% CD4$^+$, CD8$^+$, CD14$^+$, CD19$^+$, and CD45$^+$ cells (data not shown).

Mutation analysis

Genomic DNA was isolated from the PBMCs of patients with defective Munc13-4 expression using standard procedures. Primers were designed for the amplification and direct DNA sequencing of the *UNC13D*-coding exons, including the adjacent intronic sequences for the identification of splice-site variants. Primer sequences are available upon request. Products were sequenced directly with an ABI3130 genetic analyzer (Applied Biosystems).

Antibodies

Rabbit polyclonal antibodies raised against the N-terminal region (residues 1-262)[25] and full-length human Munc13-4 protein were used as primary antibodies for Western blot and flow cytometric analysis, respectively. Rabbit polyclonal anti-integrin αIIb (Santa Cruz Biotechnology) and mouse polyclonal anti–β-actin (Sigma-Aldrich) antibodies were used as primary antibodies for Western blotting. The mAbs used in the flow cytometric analysis were FITC-conjugated anti-CD3 (SK7; BD Pharmingen), phycoerythrin (PE)–conjugated anti-CD41a (HIP8; BD Pharmingen); allophycocyanin-conjugated anti-CD56 (N901; Beckman Coulter), and PE-conjugated anti-CD107a (H4A3; eBioscience).

Western blot analysis

Cell extracts were fractionated by SDS-PAGE, and the fractionated proteins were electrotransferred onto polyvinylidene fluoride membranes. The membranes were blocked overnight in blocking buffer (5% skim milk) and incubated for 1 hour at room temperature with the primary antibodies, followed by HRP-conjugated anti–rabbit or anti–mouse IgG polyclonal antibodies (Santa Cruz Biotechnology). Specific bands were visualized by the standard enhanced chemiluminescence method.

Flow cytometric analysis of Munc13-4 protein

After surface staining with anti-CD41a mAbs, platelets were fixed and permeabilized by Cytofix/Cytoperm (BD Biosciences) and washed 3 times with Perm/Wash buffer (BD Biosciences). After nonspecific reactions were blocked with Chrome-Pure human IgG (Jackson ImmunoResearch Laboratories), rabbit polyclonal antibody against the full-length human Munc13-4 protein was added, followed by FITC-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Platelets were gated on the basis of their appearance on forward- and side-scatter plots in log/log scale and by CD41a expression. The gated platelets were analyzed for Munc13-4 expression by flow cytometry (FACSCalibur; BD Biosciences).

Lysosomal degranulation assays

To quantify lysosome exocytosis by NK cells, 2×10^5 PBMCs were mixed with 2×10^5 human erythroleukemia cell line K562 cells and incubated for 2 hours in complete medium (RPMI 1640 medium supplemented with 2mM L-glutamine and 10% FCS) at 37°C in 5% CO$_2$. Cells were resuspended in PBS supplemented with 2% FCS and 2mM EDTA; stained with anti-CD3–FITC, anti-CD56–allophycocyanin, and anti-CD107a–PE mAbs; and analyzed by flow cytometry.

Platelet exocytosis of the lysosomal granules was analyzed as described previously[26] but with a minor modification. Briefly, platelets were suspended in PBS containing 2mM EDTA and PE-conjugated anti-CD107a mAb, stimulated with 5 U/mL of thrombin (Wako Pure Chemical Industries) for 10 minutes at 25°C, and immediately analyzed by flow cytometry. The degranulation index of platelets was calculated as: (mean fluorescence value of stimulated sample − mean fluorescence value of nonstimulated sample)/mean fluorescence value of nonstimulated sample.

Statistical analysis

Statistical analyses were performed with 1-way ANOVA followed by the Tukey post hoc test to compare multiple groups, with a $P < .05$ level considered to be significant.

Results

Diagnosis of FHL3 by Western blot analysis using platelets

Before screening for FHL3, the Munc13-4 expression level was compared between platelets and PBMCs. Munc13-4 expression in platelets was approximately 10 times higher than that in PBMCs (Figure 1A). CD8$^+$ cells expressed a similar level of Munc13-4 protein as other PBMC cell types (Figure 1B). Similar amounts of platelet- and PBMC-derived proteins could be obtained from a sample (data not shown). Therefore, platelets were used to perform Western blotting to screen for Munc13-4 deficiency. Of the 85 patients screened, 6 patients were diagnosed with FHL3 (Figure 1C). Munc13-4 protein was barely detected in the platelets of each FHL3 patient regardless of the gene mutation (Table 1). For each sample, no more than 1 mL of whole blood was required to perform the analysis.

Difficulty in diagnosing FHL3 in the acute phase of the disease

Patients in the acute phase of the disease who require screening for FHL often receive platelet transfusions because of thrombocytopenia.[4-6] To study the effect of transfused platelets on screening results, FHL3 screening was attempted in a patient receiving platelet transfusions. As expected, Western blotting using platelets could not detect Munc13-4 deficiency because of the normal expression of the protein in the transfused platelets (Figure 2A left column). Surprisingly, Western blotting using PBMCs also could not clearly identify Munc13-4 deficiency because a substantial number of platelets were present in the PBMCs obtained by the standard method (Figure 2A right column). By positively selecting CD45$^+$ cells and removing platelets, it was found that a considerable amount of the Munc13-4 protein detected in PBMC samples