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

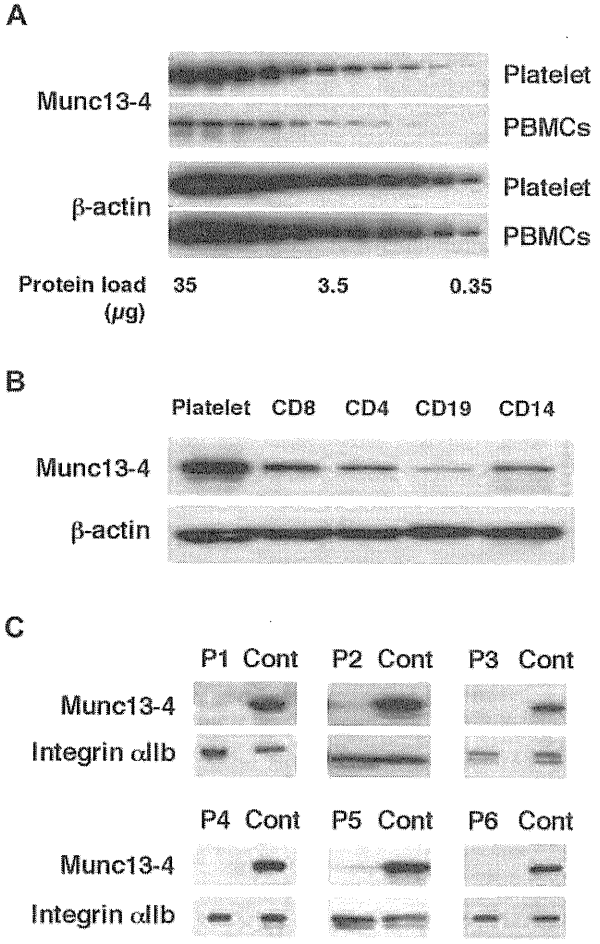

Figure 1. Diagnosing FHL3 by Western blotting using platelet protein. The amount of Munc13-4 protein expression was compared between platelets and PBMCs (A) and among platelets, $CD8^+$, $CD4^+$, $CD19^+$, and $CD14^+$ cells (B) by Western blotting. A representative result of 5 independent experiments is shown. (C) Six FHL3 patients were diagnosed by Western blotting for Munc13-4 protein using platelets.

obtained by standard density gradient centrifugation was actually derived from the contaminating platelets (Figure 2B).

We performed a NK-cell degranulation assay for every referred sample and found the assay to be defective for every FHL3 patient identified (data not shown). All of the other patients showed a normal release of lysosomal granules by NK cells; however, the analysis could not be performed in some patients because of the extremely low NK-cell number during the acute phase of the disease (data not shown).

We also examined the lysosomal granule release of platelets in 31 patients to determine whether this assay could be used as a screening method for FHL3. Lysosomal exocytosis of FHL3 platelets was partially impaired at steady state, but profound impairment was observed during the acute phase of the disease (Figure 3A-C). This profound impairment was also observed in platelets obtained from some secondary HLH patients during the acute phase (Figure 3B-C). These results indicate that it is difficult to diagnose FHL3 during the acute phase of HLH either by Western blot or by lysosomal degranulation assay.

Table 1. UNC13D gene mutations of FHL3 patients

Patient	Age at onset	Gender	Mutation	Genotype	Predicted effect
P1	14 days	Female	c.1596 + 1G → C	Homo	Splice error
P2	2 months	Male	c.322–1G → A	Hetero	Splice error
			c.990G → C	Hetero	p.Q330H
			c.3193C → T	Hetero	p.R1065X
P3	12 months	Female	c.754–1G → C	Hetero	Splice error
			c.2485delC	Hetero	p.L829fs
P4	4 months	Female	c.754–1G → C	Hetero	Splice error
			c.1799C → T	Hetero	p.T600M
			c.1803C → A	Hetero	p.Y601X
P5	2 months	Female	c.754–1G → C	Hetero	Splice error
			c.1596 + 1G → C	Hetero	Splice error
P6	5 months	Male	ND	ND	ND

Mutations were checked for single nucleotide polymorphisms using the dbSNP Build 132 database from the National Center for Biotechnology Information.

X indicates stop; fs, frame shift; and ND, not determined.

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

To overcome the difficulty in diagnosing FHL3 during the acute phase of HLH, antibodies were raised against the full-length human Munc13-4 protein (supplemental Figure 1) and a new method was developed to detect Munc13-4 protein in platelets by flow cytometry. A total of 35 patients, including 4 with FHL3 (P3-P6), were

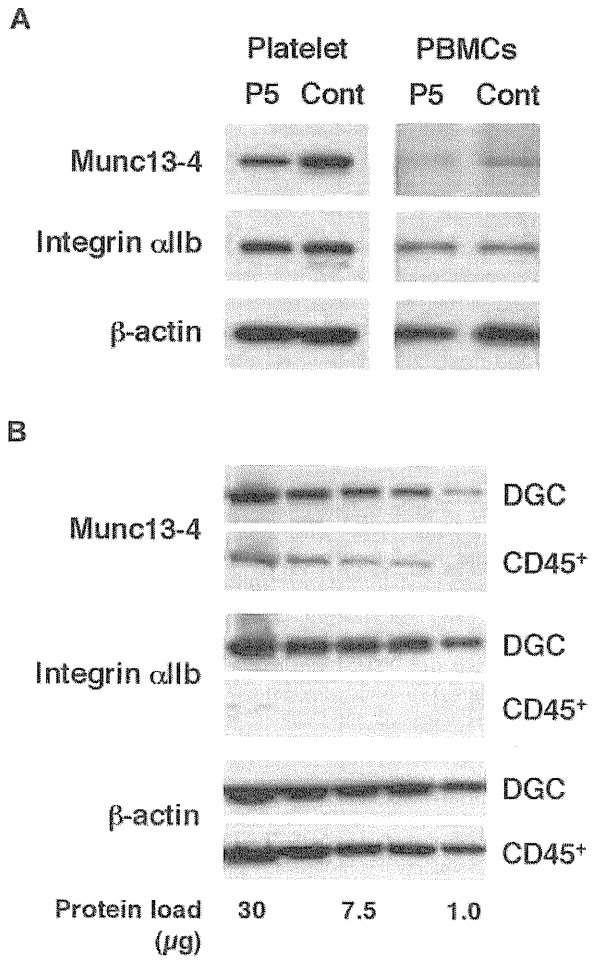

Figure 2. Effect of platelet transfusion on Western blot analysis. (A) Western blotting analysis for Munc13-4 expression using platelets and PBMCs from an FHL3 patient (P5) receiving platelet transfusions during the acute phase of the disease. (B) The expression of Munc13-4 was compared between PBMCs obtained by density gradient centrifugation (DGC) and $CD45^+$ cells obtained by magnetic sorting from healthy controls. A representative result of 3 independent experiments is shown.

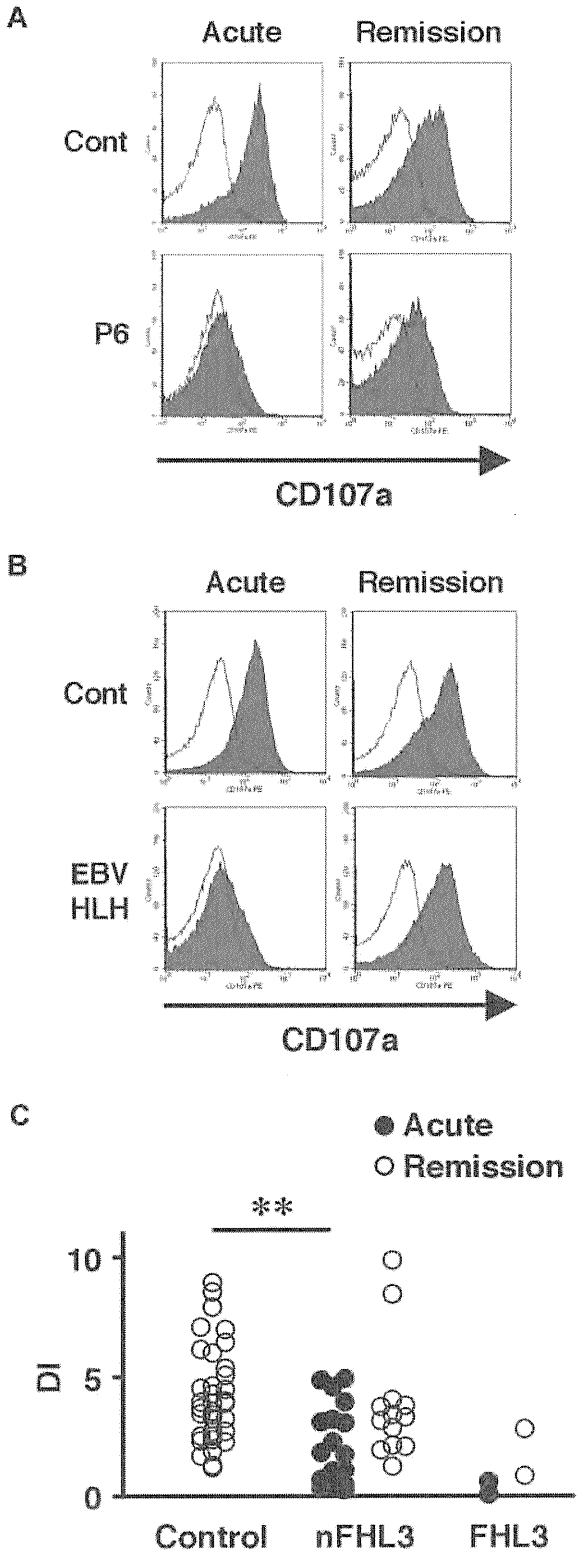

Figure 3. Analysis of lysosomal exocytosis using platelets from HLH patients. Platelets from an FHL3 patient (P6; A) and from a secondary (EBV-associated) HLH patient (B) along with healthy controls were left untreated (open histogram) or were stimulated with thrombin (closed histograms), and the surface expression of CD107a was analyzed by flow cytometry. Analysis was performed during the acute phase of the disease (left column) and after clinical remission (right column). (C) Degranulation index (DI) of platelets from HLH patients during the acute phase (●) and after clinical remission (○). HLH patients with normal NK-cell degranulation and Munc13-4 protein expression by Western blot analysis were defined as non-FHL3 (nFHL3). $^{**}P < .01$ by the Tukey post hoc test.

analyzed using this method. Munc13-4 deficiency was readily detected in all of the FHL3 patients, with a sample volume of < 100 μL of whole blood (Figure 4A-C). Munc13-4 protein was expressed at normal level in the platelets of parents and siblings of FHL3 patients carrying heterozygous *UNC13D* mutations (data not shown). In the FHL3 patient receiving platelet transfusions, flow cytometric analysis revealed bimodal populations of normal and Munc13-4–deficient platelets (P5 in Figure 4A). As shown in Figure 4B, the method was able to clearly identify Munc13-4–deficient platelets in whole blood samples stored at room temperature for 1 week.

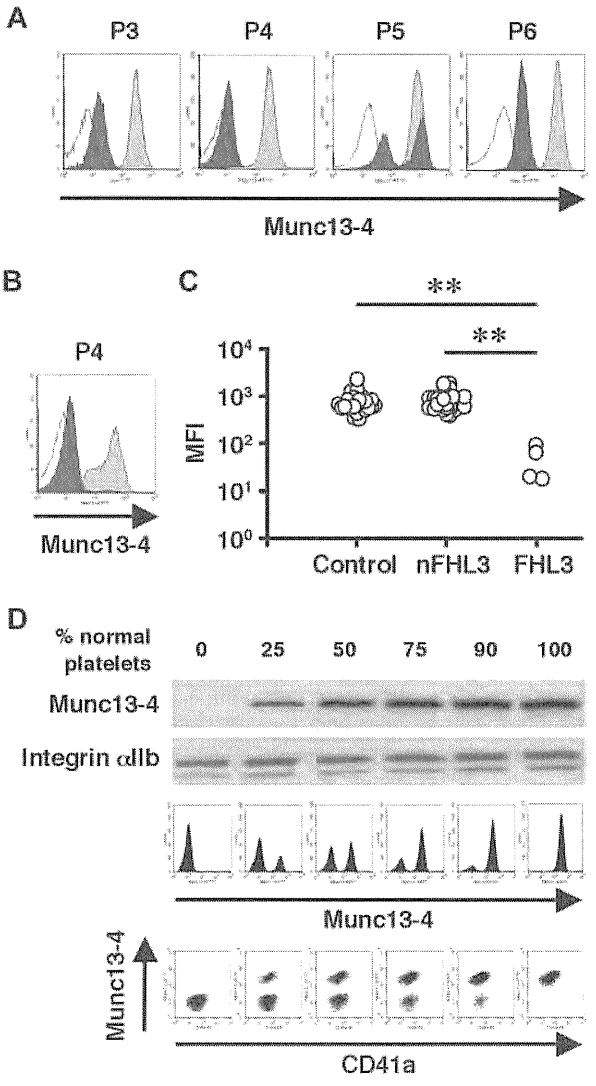

Figure 4. Flow cytometric detection of intraplatelet Munc13-4 protein. Flow cytometric analysis of intraplatelet Munc13-4 expression in 4 FHL3 patients and healthy controls using whole blood samples shipped overnight (A) and in an FHL3 patient (P4) and a healthy control using samples stored at room temperature for a week (B). Dark closed histograms represent platelets from FHL3 patients, whereas light closed histograms represent platelets from healthy controls. Open histograms represent staining with isotype controls. (C) Mean fluorescence intensity (MFI) of intraplatelet Munc13-4 staining for HLH patients and healthy controls. All of the healthy controls (n = 35) were adults. Non-FHL3 (nFHL3) patients (n = 31), as defined in Figure 3, varied in age (2 days-39 years) and included 2 patients with FHL2. Age-related variations in the MFI of Munc13-4 staining were not observed. $^{**}P < .01$ by the Tukey post hoc test. (D) The sensitivities of Western blot and flow cytometric analyses for detecting Munc13-4–deficient platelets were compared.

To determine the sensitivity of the new method, Munc13-4–deficient platelets were mixed with normal platelets at varying ratios. Western blot analysis could not detect Munc13-4–deficient platelets easily, even when the proportion of normal platelets was as low as 25% (Figure 4D). In contrast, flow cytometric analysis easily identified 10% Munc13-4–deficient platelets among 90% normal platelets (Figure 4D), which proved the high sensitivity of the method in diagnosing FHL3.

Discussion

FHL is a rare but life-threatening inherited immune disorder for which mutations in 4 genes have been identified as causative factors. *PRF1* encodes the cytolytic effector protein perforin that forms a pore-like structure in the target cell membrane.[1,12] A mutation in *PRF1* results in FHL2,[7] which accounts for 20%-50% of FHL cases.[4,5] *UNC13D* encodes the protein Munc13-4, which is crucial for the fusion of cytolytic granules to the plasma membrane and the subsequent release of perforin and granzymes.[1,12] Mutations in *UNC13D* result in FHL3,[8] which accounts for 20%-30% of FHL cases.[4,12] FHL4 is caused by mutations in *STX11*, which encodes syntaxin-11.[9] Mutations in *STXBP2*, which encodes Munc18-2, were recently reported to cause FHL5.[10,11] Syntaxin-11 and Munc18-2 also mediate the fusion of cytolytic granules to the plasma membrane.[1,5,12] The ability to screen for FHL2-5 rapidly would facilitate the initiation of life-saving immunosuppressive therapy and the preparation of FHL patients for hematopoietic stem cell transplantation.

In the present study, we found that the Munc13-4 protein is expressed abundantly in platelets (Figure 1A-B). The detection of Munc13-4 protein in platelets by Western blotting (Figure 1C) or flow cytometry (Figure 4A-B) was a reliable screening method to identify FHL3 patients. Munc13-4–deficient platelets were identified easily among normal transfused platelets by flow cytometry, which indicated that this method could be applied to patients who are receiving platelet transfusions during the acute phase of the disease (P5 in Figure 4A). Detection of intraplatelet Munc13-4 was enabled by the use of highly specific antibodies against the full-length human Munc13-4 (supplemental Figure 1).

There is a possibility that FHL3 patients with residual Munc13-4 protein expression could be overlooked by the screening methods described in this study. Most FHL3 patients have mutations that result in the absence or significant reduction of Munc13-4 protein expression,[16,23] as was the case with the patients screened in this study (Figure 1C), which suggests that the mutated Munc13-4 protein is unstable. The NK-cell degranulation assay, which was performed for every referred sample with a sufficient number of NK cells, revealed defective degranulation only in the identified FHL3 patients (date not shown). These results indicate that the majority of mutations in *UNC13D* are likely amenable to rapid detection by the new methods described in this study. Comparative studies on the *UNC13D* genotype, Munc13-4 protein expression, and the lysosomal exocytosis assay must be performed to confirm the reliability of these methods.

It was also investigated whether the analysis of lysosomal release by platelets could be used as an alternative method to screen for FHL3. Profound impairment of lysosomal exocytosis by platelets during the acute phase of the disease and restoration of this impairment after clinical remission was observed in FHL3 and in some secondary HLH patients (Figure 3). It is not clear whether this transient impairment of platelet degranulation is involved in HLH pathogenesis or if it merely reflects in vivo platelet activation by diffuse endothelial damage during the acute phase of the disease that renders them unresponsive to ex vivo stimulation. The release of lysosomal granules by Munc13-4–deficient platelets was impaired only minimally at steady state (Figure 3A and 3C), which is in contrast to a recent study showing the involvement of the Munc13-4 protein in the release of lysosomal granules in mouse platelets.[27] Although the precise reason for this discrepancy is unclear, platelet degranulation is likely to be regulated differentially between species; for example, Munc13-4–deficient mice have bruising and bleeding tendencies[27] that are not commonly associated with human FHL3. Further studies are warranted to elucidate the exocytosis pathways of platelets and their role in the pathophysiology of HLH.

With the development of tools for rapid screening, the diagnostic approach for FHL has changed over the years. Impaired NK cytotoxicity was the first reported signature clinical finding of FHL patients.[13,14] Defective CTL activity was subsequently reported as another hallmark of FHL.[7,8,16,28] However, NK-cell activity is also decreased in some cases of secondary HLH,[15,17-20] and the CTL cytotoxicity assay is not readily accessible to most clinicians. The NK-cell lysosomal exocytosis assay is a comprehensive method to identify patients with a degranulation defect.[10,11,22-24] However, this analysis is not available in some patients with extremely reduced NK-cell numbers, which are often observed during the acute phase of HLH.[19] Although CTLs can be an alternative tool to perform the lysosomal exocytosis assay,[24,28,29] it remains impossible to differentiate FHL3-FHL5.[10,11,23,24] Impairment in these assays warrants the genetic confirmation of FHL, but sequencing all of the candidate genes is not a suitable approach for rapid diagnosis. Flow cytometric detection of perforin expression in NK cells is a reliable and rapid way of identifying patients with FHL2,[21] and the new method described in this study for the detection of Munc13-4 expression in platelets would add to the rapid diagnosis of FHL3.

Platelets could also be used for the screening of FHL4 and FHL5 because they share some granule-transport mechanisms with other types of hematopoietic cells, including CTLs and NK cells.[2,30,31] Indeed, in the present study, both syntaxin-11 and Munc18-2 were expressed abundantly in platelets (data not shown). We are currently using platelet proteins to screen for FHL4-FHL5 by Western blot analysis, although no cases have been found so far because of the extreme rarity of these disorders.

In summary, platelets abundantly express Munc13-4 protein and are a useful tool to screen for FHL3. By detecting intraplatelet Munc13-4 expression by flow cytometry, it is now possible to rapidly screen for FHL3 with a very small sample of whole blood, even in the acute disease phase requiring platelet transfusion. Because platelets share some of their granule transport systems with other types of hematopoietic cells, they could also be used to screen for other types of immune disorders, including FHL4 and FHL5.

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Authorship

Contribution: T.Y., R.N., T.N., H.H., and H.T. designed the research; Y.M., K.I., and M.S. performed the Western blot and flow cytometric analyses; K.O. and O.O. performed the genetic analyses; R.S. and H.H. prepared the anti–Munc13-4 antibodies and started the FHL3 screening; Y.M., T.Y., R.S., K.I., H.S., J.A., N.T., T.K., R.N., E.I., T.N., H.H., and T.H. analyzed and discussed the results; and Y.M., T.Y., and T.H. wrote the manuscript.

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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.

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Keywords Autoimmune hepatitis · Hyper-IgD syndrome · Liver biopsy · MVK gene · 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

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 hyperimmunoglobulinemia 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.

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

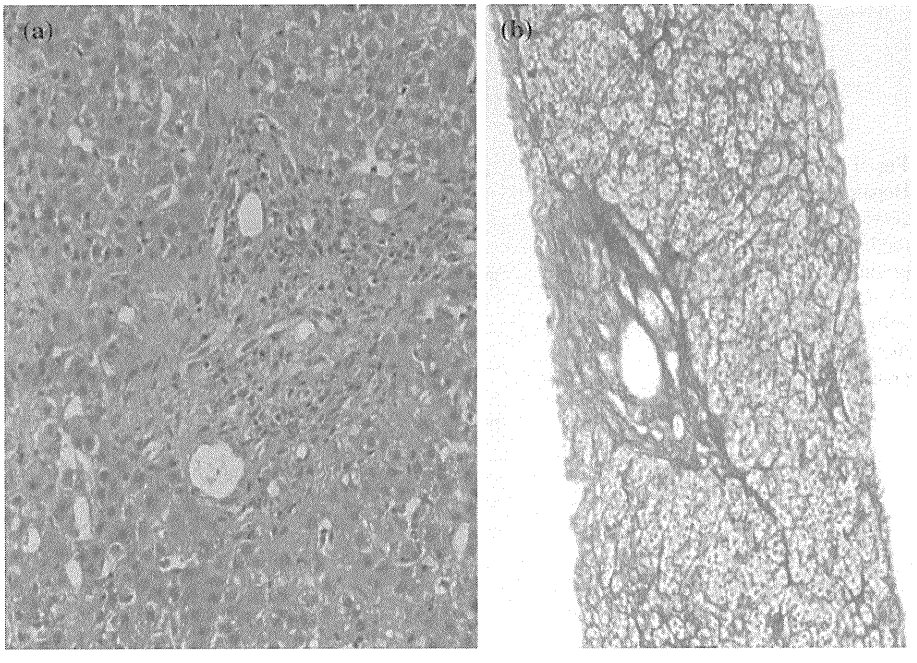

Fig. 1 Liver biopsy specimen showing chronic hepatitis. **a** The portal tract is infiltrated with lymphocytes (H&E, ×200). **b** Short septa extend from the slightly enlarged portal tract (reticulum, ×100)

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

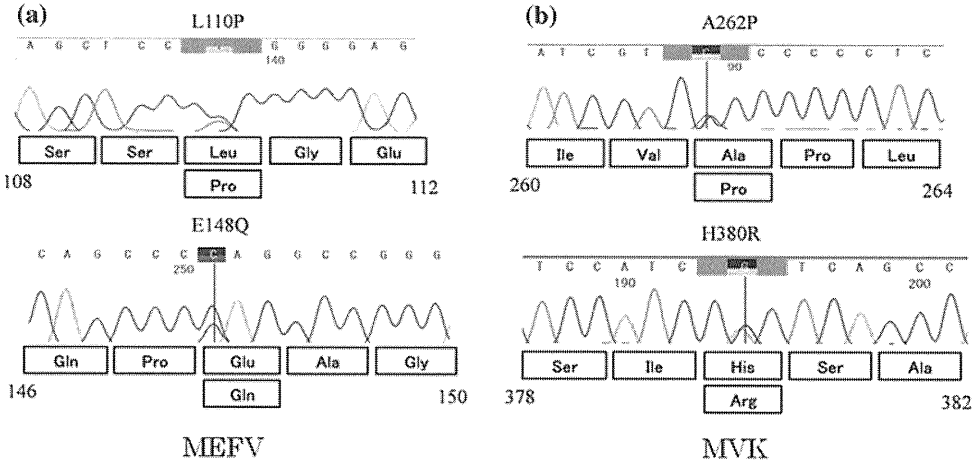

Fig. 2 Genetic analysis. **a** Genetic analysis of the MEFV gene. The patient had heterozygous amino acid changes of L110P and E148Q. **b** Genetic analysis of the MVK gene. The patient had heterozygous mutations of A262P and H380R

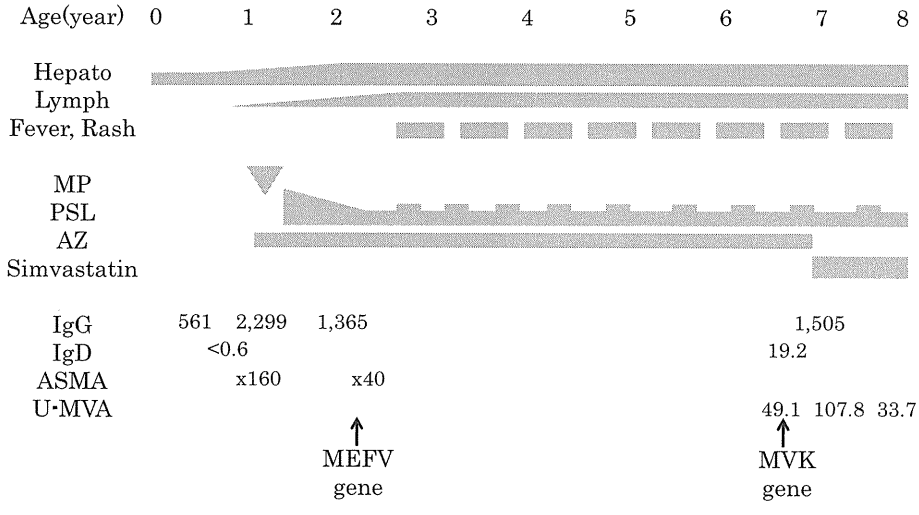

Fig. 3 Clinical course. *Hepato* Hepatosplenomegaly, *Lymph* cervical lymphadenopathy, *MP* methylprednisolone, *PSL* prednisolone, *AZ* azathioprine, *ASMA* anti-smooth muscle antibody, *U-MVA* urinary mevalonic acid (μg/mg creatinine)

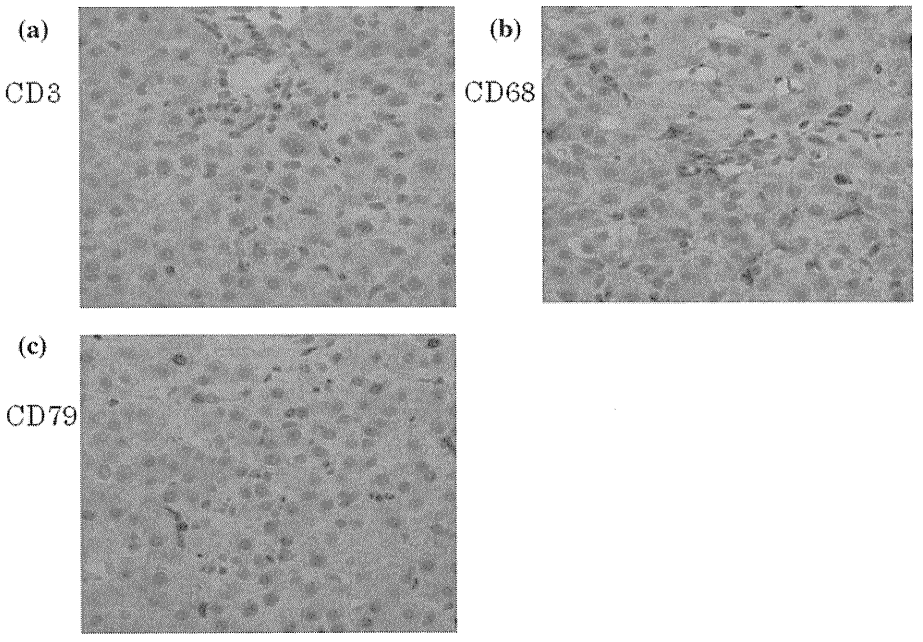

Fig. 4 Immunohistochemical analysis of the biopsied liver tissues. **a** CD3 (×400), **b** CD68 (×400), **c** CD79 (×400)

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 heterozygous 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 mevalonic 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.

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Conflict of interest The authors have no conflicts of interest to declare.

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Familial Cases of Periodic Fever with Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis Syndrome

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We report three familial cases of periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis syndrome, including a pair of monozygotic twins and their mother. It suggests that periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis syndrome may have a certain monogenetic background. *(J Pediatr 2011;158:155-9)*

Periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA) syndrome was first described in 1987 by Marshall et al.[1] Several subsequent reports have confirmed this syndrome as a distinct clinical entity[2-4] taking the form of periodic fever occurring at intervals and associated with aphthous stomatitis, pharyngitis, and cervical adenitis, beginning usually before the age of 5 years. Diagnostic criteria for PFAPA are shown in a previous report.[3] PFAPA syndrome has been described as a noninfectious, nonautoimmune, and autoinflammatory disease that shows dramatic response to corticosteroid therapy. Most cases of PFAPA syndrome are sporadic, but some previous reports have described cases of nontwin siblings[5] and of siblings and their mother,[6] suggesting that the syndrome may be induced by environmental or genetic factors. We treated three familial cases of PFAPA syndrome, namely, a mother and her monozygotic twins.

Methods

Patients

Case 1. The elder twin was a 2-year-old girl who was referred to our hospital at 1 year and 4 months of age. She was born from healthy and nonconsanguineous parents without any prenatal or postnatal problems. Her neurodevelopment was normal. Her first episode of fever occurred at 11 months of age and was not associated with any other complaints. After this episode, high fevers occurred suddenly and periodically, always lasting for 3 to 5 days. The patient was routinely diagnosed with "pharyngitis or tonsillitis" but always had no upper respiratory tract symptoms and no abdominal complaints (Table I). Each episode of refractory fever continued despite treatment with antibiotics but eventually resolved spontaneously. At first, the intervals between febrile episodes were irregular, but they gradually settled into a regular schedule and then occurred "like clockwork," with about 15 to 20 days between episodes. During the interval periods, the patient exhibited no clinical symptoms.

At 1 year and 4 months of age, the patient visited our hospital during an episode of high fever and tonsillitis. Laboratory examinations (Table I) during febrile episodes revealed elevation of C-reactive protein (CRP) and erythrocyte sedimentation rates (ESR), mild leukocytosis without neutropenia; these results were normal during nonfebrile periods. There were no positive findings in any culture samples or in any virus antigen tests or serum titers. Levels of other inflammatory agents (C3, C4, CH50, antinucleotide and anti-DNA antibody, MMP-3, and PRO-/MPO-ANCA) were all normal. Analysis of immunoglobulin components revealed only immunoglobulin D (IgD) mildly to moderately elevated throughout febrile and symptom-free periods. Only a part of fevers lasted for more than 10 days, but all fevers and some characteristic symptoms (Table I) eventually disappeared spontaneously and completely regardless of treatment with systemic antibiotics.

After about 10 similar clinical episodes of periodic fever attack, we diagnosed the patient with PFAPA syndrome at 2 years of age and introduced oral low-dose prednisolone ([L-PSL] dosage 0.3-1 mg/kg/dose, 1 or 2 doses per day) at the beginning of every fever. The introduction of prednisolone dramatically decreased the duration of each fever to remission in less than 3 hours. After the introduction of L-PSL, the patient's and her family's quality of life improved remarkably, but periodic fever attacks still recur at the same interval of about 15 to 18 days. Cimetidine therapy was refused because of its bad taste, and the parents refused adenotonsillectomy.

CRP	C-reactive protein
ESR	Erythrocyte sedimentation rates
FMF	Familial Mediterranean fever
HIDS	Hyperimmunoglobinemia D syndrome
IgD	Immunoglobulin D
L-PSL	Low-dose prednisolone
MEFV	Familial Mediterranean fever
MVK	Mevalonate kinase
PFAPA	Periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A

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Table I. Clinical features and laboratory findings in familial cases of PFAPA

	Case 1 23 clinical episodes	Case 2 26 clinical episodes	Case 3 10 clinical episodes
Clinical features			
High-grade fever (≥38.5° C)	100%	100%	100%
Pharyngitis	100%	100%	100%
Tonsillitis	100%	100%	100%
Aphthous stomatitis	34.8%	30.8%	60%
Cervical adenitis	56.5%	73.1%	80%
Headache	0%	0%	60%
Abdominal complaints (pain)	0%	0%	0%
Joint pain/muscle pain	0%	0%	0%
Skin rash	0%	0%	0%
Laboratory data			
White blood cell count (/mm^3)*			
No.	12	12	3
Range	8180-23 230	6230-20 000	6570-7500
Mean ± SD	13 475 ± 1170	13 466 ± 1103	
CRP (mg/dL)*			
No.	12	12	3
Range	1.1-18.3	1.4-15.2	0.01-0.5
Mean ± SD	5.0 ± 1.3	6.4 ± 1.1	
ESR at 60 min (mm)*			
No.	12	12	2
Range	15-102	17-102	6-7
Mean ± SD	50.2 ± 6.9	70.5 ± 7.6	
IgM (mg/dL)*			
No.	9	12	2
Range	45-116	51-112	72-102
Mean ± SD	66.4 ± 7.4	68.2 ± 6.1	
IgA (mg/dL)*			
No.	9	12	2
Range	30-79	49-117	98-125
Mean ± SD	47.4 ± 4.9	75.2 ± 6.2	
IgG (mg/dL)*			
No.	9	12	2
Range	633-940	625-970	992-1042
Mean ± SD	775.7 ± 39.1	811.5 ± 28.2	
IgD (mg/dL)*			
No.	2	3	1
Range	35.5-71.6	33.5-41.7	18.2
IgD (mg/dL) (nonfebrile periods)			
No.	3	3	2
Range	46.4-58.2	39.9-66.1	16.5-17.1
Urinary mevalonolactone (during febrile periods)	Normal	Normal	Normal
MK activity (versus normal control)	98%	58%	88%
MVK gene	No mutation detected	No mutation detected	No mutation detected
MEFV gene	Hetero P369S/E148Q#	Hetero P369S/E148Q#	No mutation detected
TNFRSF1A gene	No mutation detected	No mutation detected	No mutation detected

No., Number of times of blood sampling; *#*, not compound single nucleotide polymorphism.
*Measured during febrile periods.

Case 2. The younger patient was the second-born of monozygotic twins from the same parents. Her neurodevelopment also was normal. She had periodic abrupt fevers that occurred only in association with pharyngitis and cervical adenitis beginning at 12 months of age. Antibiotic therapy was not effective; instead, each episode resolved spontaneously about 4 to 5 days after its onset. Aphthous stomatitis appeared late in some episodes, around the time that the fever resolved (Table I).

Clinical examinations (Table I) in our hospital revealed elevated levels of the inflammatory agents including CRP and ESR, and leukocytosis without cyclic neutropenia, but, as in the case of the elder sister (Case 1), no indication of infection or autoimmune disease. This patient exhibited mild to moderate elevation in IgD throughout febrile and nonfebrile periods. After about 12 similar febrile episodes, starting of oral L-PSL resolved each fever dramatically and promptly in 2 to 4 hours after treatment. Although prednisolone has improved the patient's quality of life, she is still experiencing periodic febrile episodes, and the intervals between episodes have gradually shortened to about 14 to 18 days. Cimetidine therapy was discontinued because of its unpleasant taste, and the parents refused adenotonsillectomy.

Case 3 (Cases 1 and 2). The biological mother of twins was a healthy 29-year-old woman. In an interview, she related that she had frequently experienced recurring abrupt high

Table II. Summary of reported familial cases of PFAPA syndrome

	Sampaio et al[5]	Valenzuela et al[6]		Present cases
Patients' background				
Familial history	Two siblings	Two sisters and two brothers in different families		Monozygotic twins and mother
Consanguinity	None	None	None	None
Age/sex	(1) 10 years/boy (2) 4 years/girl	(1) 9 years/girl (2) 7 years/girl	(1) 7 years/boy (2) 3 years/boy	(1) 2 years/girl (2) 3 years/boy (3) 29 years/mother
Race				
Paternal	Unknown	German-Italian-Chilean	Spanish	Japanese
Maternal	Unknown	Jewish Ashkenazi-Spanish-Chilean	Spanish	Japanese
Predisposing factor	Emotional, psychologic factors	None (both)	None (both)	None (all)
Age of onset	(1) 18 months (2) 3 years	(1) 18 months (2) 2 years	(1) 3 years (2) 2 years	(1) 11 months (2) 12 months (3) 2 years
Growth and development	Normal	Normal	Normal	Normal
Clinical symptoms				
Periodicity of fever	Monthly (both)	(1) 5-6 weeks (2) 4 weeks	(1) 3 weeks (2) 16-20 days	(1) 15-18 days (after PSL) (2) 14-18 days (after PSL) (3) 30 days (before PSL)
Aphthous stomatitis	+ (Both)	+ (Both)	+ (Both)	+ (All)
Pharyngitis	+ (Both)	+ (Both)	+ (Both)	+ (All)
Cervical adenitis	+ (Both)	+ (Both)	+ (Both)	+ (All)
Respiratory complaints	None (both)	None (both)	None (both)	None (all)
Abdominal complaints	None (both)	Vomit (both)	(1) None (2) Vomit, diarrhea	None (all)
Joint pain	None (both)	None (both)	None (both)	None (all)
Laboratory findings				
Leukocytosis	+ (Both)	Mild (both)	Mild (both)	+ (All)
Neutropenia	None (both)	None (both)	None (both)	None (all)
Elevated CRP (or ESR)	+ (Both)	+ (Both)	+ (Both)	+ (All)
Elevated immunoglobulin	None (both)	None (both)	(1) Not described (2) None	Igd (all)
Throat cultures	Negative (both)	Negative (both)	Negative (both)	Negative (all)
Treatment				
Antibiotics	No response (both)	No response (both)	No response (both)	Poor response (all)
Corticosteroids	Dramatic response (all)	Not prescribed (both)	Not prescribed (both)	Dramatic response (all)
Cimetidine	Unknown (both)	Not described (both)	Not described (both)	Discontinued (twins)
Tonsillectomy	Not done (both)	Not done (both)	Not done (both)	Not done (all)
Prognosis				
Age of last attacks	Unknown	(1) 6 years (2) 6 years	(1) 7 years (2) Not described	Continued (all)
Present status	No remission	Cr (both)	Cr (both)	(1) (2) No remission (3) Relapse in adulthood

Cr, Complete remission; *PSL*, prednisolone.

fevers, which were diagnosed as "acute pharyngitis and aphthous stomatitis," between 2 and 10 years of age. Each time, she visited a pediatric clinic and received oral antibiotics, but her fevers failed to respond. After refractory fever had lasted for 3 to 5 days, the patient recovered spontaneously and was symptom-free during nonfebrile periods. After her first pregnancy and delivery of a twins' sister now 5 years of age, abrupt and periodic febrile episodes began again and repeated at about 30-day intervals, always with the same symptoms, including pharyngitis, aphthous stomatitis, and cervical adenitis, and with elevated CRP ranging from 4 to 10 mg/dL. Each episode lasted for 3 to 5 days, did not respond to oral antibiotic therapy, and eventually resolved spontaneously. Serum IgD level during both a febrile and a non-febrile period and found it mildly elevated (Table I). L-PSL (6 mg/doses, only a dose per day) was very effective against her abrupt high fevers, diminishing them dramatically and promptly improved her general condition, although her PFAPA syndrome was an especially unusual type in that her episodes began in childhood, stopped, and then began again in adulthood. In this case, cimetidine therapy has not yet been prescribed.

Genetic Analysis of the Mevalonate Kinase, Familial Mediterranean Fever, and Tumor Necrosis Factor Receptor Superfamily, Member 1A Genes

After written informed consent approved by Institutional Review Board of Kyoto University was obtained, peripheral blood was collected from all the patients and their family members. Genomic DNA was extracted, and all the exons including exon-intron junctions of mevalonate kinase (MVK), familial Mediterranean fever (MEFV), and tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A) genes were amplified by polymerase chain reaction and sequenced by ABI3130. No defect was found.

Analysis of Mevalonate Kinase Activity

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of the patients by using Lymphoprep (Axis-Shield PoC, Norton, Massachusetts). Then the PBMCs

were stimulated by PHA to obtain T-lymphocyte, which were harvested to measure mevalonate kinase (MK) activity as previously described by Gibson et al.[7]

Discussion

PFAPA syndrome has recently been identified as a new clinical entity, typically occurring in childhood, characteristically consisting of periodic fever, pharyngitis, aphthous stomatitis, and cervical adenitis, which responds dramatically to corticosteroid treatment, although it is unaffected by antibiotic treatment.[2-4] Among the various autoinflammatory diseases (eg, cyclic neutropenia, hyperimmunoglobinemia D syndrome [HIDS], familial Mediterranean fever [FMF], and tumor necrosis factor–receptor-associated periodic syndrome), only PFAPA syndrome still has an unknown genetic background and pathogenesis. One report has indicated that PFAPA syndrome is associated with abnormal cytokine regulation.[8] The only cytokine measured in these 3 cases was soluble interleukin-2 receptor (s-IL2-R), which was mildly elevated.

The other periodic fever syndrome from which PFAPA syndrome must be differentiated on the basis of these clinical symptoms, and serum IgD levels is HIDS, which consists of nonperiodic or periodic fever, chest complaints, joint pain, abdominal pain, diarrhea, hepatosplenomegaly, and skin rash, with elevation of urinary mevalonolactone levels and serum immunoglobulin A levels during fever periods; none of these conditions are true for cases 1 to 3. We performed immunoassay of MK activity and screening of MVK gene mutation in cases 1 to 3; all results were normal (Table I). Most researchers have reported normal levels of serum IgD in patients with PFAPA, but one report[3] describes mild elevation of IgD levels in 12 of 18 clinical PFAPA cases. Thus it appears that mild elevation of IgD may be characteristic of PFAPA syndrome, but this is not a criterion for diagnosis with PFAPA syndrome.

Among the autoinflammatory diseases listed above, only PFAPA syndrome has been described as a noninherited syndrome; this is because several review articles[2-4] on PFAPA syndrome have included no familial cases. Recently, however, familial cases in which patients are siblings[5] or siblings and their mother[6] have been reported. Thus, this poorly understood syndrome is suspected, but not proven, to be heritable (Table II).

With regard to genetic background of PFAPA syndrome, one article[9] strongly argued against the involvement of MEFV, but another article[10] described that 27% of cases diagnosed as PFAPA syndrome on the basis of clinical criteria[3] exhibited MEFV gene mutations, which are mainly responsible for FMF syndrome. The latter study suggests the involvement of MEFV in PFAPA syndrome and some clinical overlap with FMF syndrome. So we additionally demonstrated screening of MEFV gene in cases 1 to 3, resulting in no significant mutations, except for heterozygous P369S and E148Q (both were variants) only in cases 1 and 2 (Table I). Isolated and typical cases of this syndrome, such as these cases, should be differentiated from those of other monogenic periodic fevers by detecting responsible genes.

The monozygotic twinning of cases 1 and 2 was established through gynecologic findings of their mother at the time of their birth and polymorphisms in the MEFV gene. There are no seasonal or environmental factors triggering the onset of their febrile episodes (data not shown); this observation suggests that the episodes are autoinflammatory responses occurring in the absence of infection. Case 3, their mother, represents an interesting clinical course, because the clinical features of her febrile episodes in childhood were extremely similar to those of her daughters and because she has experienced a recurrence of febrile episodes in adulthood. Recently, one report[11] has demonstrated that adult patients with PFAPA syndrome can be classified into two types: the "early-onset type," which begins in childhood, appears to resolve and then recurs in adulthood (2 of 15 cases), and the "late-onset type," which begins in adulthood (13 of 15 cases). Case 3 in this investigation is believed to belong to the former type.

It is worth noting that another 5-year-old sister born from the same mother as these twins (cases 1 and 2) has frequent episodes of high fever with pharyngitis and highly elevated CRP that resolve spontaneously (no elevation in IgD level). In addition, the elder brother of case 3 and uncle of cases 1 and 2, at 36 years of age also has the same episodes as those of case 3, consisting of periodic fevers and laboratory data (highly elevated inflammatory agents; mildly elevated IgD 9 mg/dL (measured once), and no mutations in MEFV, MVK genes) improving dramatically with L-PSL (6 mg/d) treatment.

Finally, we additionally demonstrated screening of TNFRSF1A gene, recently discussed in HIDS, FMF, and tumor necrosis factor–receptor-associated periodic syndrome, all resulting in the absence of mutations, in cases 1 to 3 and the elder brother of case 3 (Table I).

We report monozygotic twins cases of PFAPA and their family, speculating on the existence of genetic background in PFAPA syndrome. Familial cases of PFAPA syndrome require genetic testing for differential diagnosis and understanding the mechanism of this perplexing syndrome. ■

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Characterization of *NLRP3* Variants in Japanese Cryopyrin-Associated Periodic Syndrome Patients

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Abstract The etiology of cryopyrin-associated periodic syndrome (CAPS) is caused by germline gene mutations in NOD-like receptor family, pryin domain containing 3 (*NLRP3*)/cold-induced autoinflammatory syndrome 1 (*CIAS1*). CAPS includes diseases with various severities. The aim of this study was to characterize patients according to the disease severity of CAPS. Five Japanese patients with four kinds of gene variations in *NLRP3* were found and diagnosed as CAPS or juvenile idiopathic arthritis. Two mutations in *NLRP3*, Y563N and E688K, found in CAPS patients exhibit significant positive activities in the nuclear factor-κB reporter gene assay. Increased serum interleukin (IL)-18 levels were only observed in severe cases of CAPS. In mild cases of CAPS, the serum IL-18 levels were not increased, although lipopolysaccharide- or hypothermia-enhanced IL-1β and IL-18 production levels by their peripheral blood mononuclear cells were detectable. This series of case reports suggests that a combination of in vitro assays could be a useful tool for the diagnosis and characterization of the disease severity of CAPS.

Keywords Autoinflammatory disease · cryopyrin · familial cold autoinflammatory syndrome · interleukin-18 · *NLRP3*

Abbreviations

CAPS	Cryopyrin-associated periodic syndrome
CIAS1	Cold-induced autoinflammatory syndrome 1
CINCA	Chronic infantile neurologic cutaneous and articular
CRP	C-reactive protein
FCAS	Familial cold autoinflammatory syndrome
HEK	Human embryonic kidney
IL	Interleukin
JIA	Juvenile idiopathic arthritis
LPS	Lipopolysaccharide
MWS	Muckle–Wells syndrome
NLRP3	NOD-like receptor family, pryin domain containing 3
NF-κB	Nuclear factor-κB
NOMID	Neonatal-onset multisystem inflammatory disease
PBMCs	Peripheral blood mononuclear cells
TNF	Tumor necrosis factor

Introduction

Cryopyrin-associated periodic syndrome (CAPS) is an autoinflammatory syndrome [1] caused by germline gene mutations in NOD-like receptor family, pryin domain containing 3 (*NLRP3*)/cold-induced autoinflammatory syndrome 1 (*CIAS1*) [2–4]. The diagnosis of CAPS is based on its

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characteristic clinical phenotypes and examination of gene mutations in *NLRP3*. A hotspot of gene mutations in *NLRP3* is located on exon 3. On the other hand, approximately 40% of cases with the clinically diagnosed severe form of CAPS, chronic infantile neurologic cutaneous and articular (CINCA)/neonatal-onset multisystem inflammatory disease (NOMID) syndrome, have no detectable germline gene mutations in *NLRP3* [5, 6]. Some of these patients have gene mutations in *NLRP3* outside of exon 3, *NLRP12*, or somatic mosaicism of *NLRP3* [5, 7–10]. In some of the remaining typical CAPS patients, the disease-causing mutations cannot be confirmed. Thus, the clinical phenotypes are very important for diagnosing CAPS patients.

Familial cold autoinflammatory syndrome (FCAS) shows the mildest clinical phenotypes in the spectrum of CAPS, such as cold-induced urticaria-like skin rash, while CINCA/NOMID syndrome shows additional severe phenotypes, such as severe arthritis, patella overgrowth, aseptic meningitis, mental retardation, and progressive sensory neural hearing loss [1]. The diagnosis of FCAS is relatively difficult owing to its mild phenotypes compared with the more severe phenotypes of CAPS (CINCA/NOMID syndrome or Muckle–Wells syndrome (MWS)). On the other hand, and similar to other autoinflammatory syndromes such as familial Mediterranean fever, it is important for CAPS treatment to prevent the onset of renal amyloidosis for consideration of the prognosis. Interleukin (IL)-1β inhibitory drugs, such as anakinra, rilonacept, and canakinumab, can prevent the clinical phenotypes of CAPS including renal amyloidosis [11]. However, the usage of IL-1 blockade for the severe form of CAPS may sometimes be an overtreatment for FCAS because the clinical symptoms are relatively mild and the frequency of onset of renal amyloidosis was reported to be low in FCAS patients [11]. Therefore, precise evaluation of the disease severity of CAPS may contribute to a reduction in the usage of IL-1 blockade. Consequently, a convenient objective standard is anticipated for discrimination between the mild and severe forms of CAPS.

In this study, to diagnose CAPS and characterize the differences between the mild and severe forms of CAPS, we evaluated the serum inflammatory cytokine levels, cytokine production levels by peripheral blood mononuclear cells (PBMCs), and cell-based nuclear factor (NF)-κB reporter gene activities of *NLRP3* variants in patients. Our results provide new insights into the characterization of the severity of CAPS.

Methods

Case Reports

The five clinical cases evaluated in this study are described below, and their characteristics are summarized in Table I.

Table I Genotypes and clinical profiles of the patients

	Analyzed age	Onset age	Gender	Diagnosis	Genotype (*NLRP3*)	CNS		Skin	Joint	Others		The inflammatory markers	
						Meningitis	Mental retardation	Urticaria like rash	Arthritis	Hearing loss	Renal amyloidosis	WBC (/μl)	CRP (mg/dl)
Case 1	3 months	3 months	Female	FCAS	Y563N	–	–	+	–	–	–	14,890	0.48
Case 2	34 years	Unknown	Male	FCAS	Y563N	–	–	+	+	–	–	13,120	3.05
Case 3	14 years	11 months	Male	CINCA/NOMID	E688K, G809S	+	+	+	+	–	–	22,500	12.30
Case 4	45 years	Unknown	Female	MWS	E688K	–	–	+	+	+	–	13,640	4.17
Case 5	3 years	3 years	Female	JIA	E378K	–	–	+	+	–	–	15,200	11.69

CNS central nervous system, *FCAS* familiar cold inflammatory syndrome, *CINCA* chronic infantile neurologic cutaneous and articular syndrome, *MWS* Muckle–Wells syndrome, *JIA* juvenile idiopathic arthritis, *WBC* the count of white blood cells, *CRP* the serum C-reactive protein level

All of the patients' family members and healthy control subjects provided informed consent to participate in the study, and the ethical principles of the Declaration of Helsinki were followed.

Case 1 The onset of disease (FCAS) in this patient occurred at 3 months of age. She exhibited a recurrent generalized urticaria-like skin rash upon exposure to cold temperatures (Fig. 1a). Progressive sensory neural hearing loss and renal amyloidosis were not seen. Her serum C-reactive protein (CRP) levels were continuously and slightly increased (0.24–2.1 mg/dl).

Case 2 Case 2 was the father of case 1. He was a 34-year-old male with a recurrent urticaria-like skin rash, fever, conjunctivitis, and arthralgia that developed following fatigue or exposure to cold temperatures. The precise time of his disease onset was unknown. Progressive sensory neural hearing loss and renal amyloidosis were not seen [12]. His CRP levels were continuously increased (1.52–3.98 mg/dl).

Case 3 The onset of disease (CINCA/NOMID) in this patient occurred at 11 months of age. Continuous aseptic meningitis, urticaria-like skin rash, arthritis at the end of the fingers, and Raynaud's symptoms were observed. Arteriosclerosis of the ophthalmic artery was found. However, severe patella overgrowth was not seen. At 14 years of age, he had heart failure with myocarditis, which was considered to be a rheumatic characteristic. The patient died suddenly at 19 years of age (the detailed clinical case will be described elsewhere by Teramoto et al.).

Case 4 Case 4 was the mother of case 3. The precise time of her disease (MWS) onset was unknown. Initially, she was diagnosed with rheumatic arthritis and received oral prednisolone therapy. She suffered progressive sensory neural hearing loss at 30 years of age and underwent artificial cochlea replacement therapy at 48 years of age. This was greatly effective in improving her hearing ability. Meningitis and renal amyloidosis were not seen.

Case 5 The onset of disease in this patient occurred at 3 years of age. Fever that continued for more than 2 weeks, severe polyarthritis (serum matrix metalloproteinase-3 of >800 ng/ml), and recurrent urticaria-like non-itchy skin rash (Fig. 1b) were observed. Lymphadenopathy, hepatosplenomegaly, and serositis were not seen. Patella overgrowth, aseptic meningitis, progressive sensory neural hearing loss, and renal amyloidosis were not seen. Rheumatoid factor was negative. Other autoantibodies, including anticyclic citrullinated peptide antibody, were not detected. Her serum CRP and ferritin levels were increased (11.69 mg/dl and 255.1 ng/ml, respectively). Based on the below-mentioned hereditary traits and the results of in vitro functional assays, we diagnosed this patient as juvenile idiopathic arthritis (JIA), according to the criteria for JIA from the International League of Associations for Rheumatology [13]. A combination therapy with steroid and tocilizumab was effective.

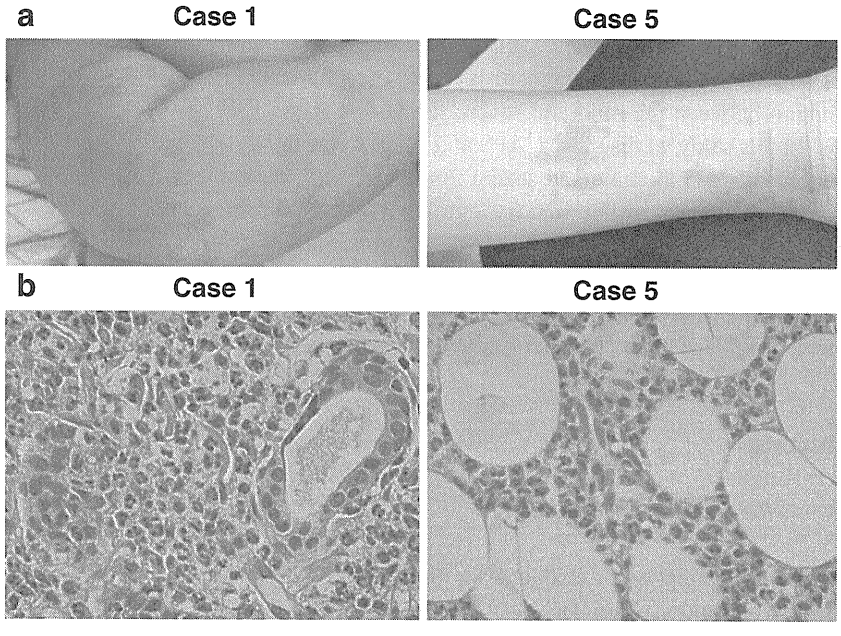

Fig. 1 Urticaria-like skin rash of cases 1 and 5. **a** Clinical appearances of the urticaria-like rash of cases 1 and 5. **b** Histopathological examinations of biopsy specimens from the skin rash of cases 1 and 5. Both skin biopsies show a recurrent cold-induced non-itchy urticaria-like skin rash and also show neutrophil infiltration

DNA Sequencing

Genomic DNA was extracted from leukocytes using Sepa-Gene (Eidia, Tokyo, Japan). A DNA fragment of the *NLRP3* gene was amplified by PCR and analyzed using Big Dye Terminator Bidirectional Sequencing (Applied Biosystems, Foster City, CA, USA).

Cell Culture

PBMCs were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (GE Healthcare, Uppsala, Sweden). The PBMCs were cultured in medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 pg/ml). Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (high glucose-containing DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Vector Preparations

A cDNA encoding *NLRP3* tagged at the C terminus with a FLAG epitope (NLRP3-FLAG) was cloned into the plasmid vector pcDNA3.1+ (Invitrogen). Mutants of *NLRP3* (E378K, Y563N, E688K, and G809S) were generated using a GeneEditor In Vitro Site-Directed Mutagenesis System (Promega, Madison, WI, USA). An ASC variant 1 tagged at the C terminus with a myc epitope (ASC1-myc) was also cloned into pcDNA3.1+. An NF-κB luciferase reporter vector (pGL4.32-luc2P/NF-kappaB-RE/Hygro) and a *Renilla* luciferase reporter vector (pGL4.74-hRluc/TK) were purchased from Promega.

NF-κB Reporter Gene Activity

HEK293T cells in 96-well plates were transfected with 16 ng/well of pcDNA3.1+ control vector or pcDNA3.1+ NLRP3-FLAG vector (wild-type or mutant-type) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The pcDNA3.1+ ASC1-myc vector, NF-κB luciferase reporter vector, and *Renilla* luciferase reporter vector were cotransfected. After transfection, the cells were cultured for 24 h. The luciferase reporter gene activities were analyzed using a Dual-Luciferase Reporter Assay System (Promega). The statistical significance of differences in the luciferase activities between the wild-type and mutant genes in the NF-κB gene reporter assays was analyzed by the Kruskal–Wallis test, and further analysis was performed by the Bonferroni/Dunn test. Statistical significance was assumed for values of $P<0.05$.

Lipopolysaccharide- or Hypothermia-Induced Assays

PBMCs were suspended at 1×10^6 cells/ml in culture medium and cultured in the presence or absence of 10 or 100 ng/ml of LPSO127 (Sigma) for 24 h in six-well plates at 30°C or 37°C in a humidified atmosphere containing 5% CO_2.

Measurements of Tumor Necrosis Factor-α, IL-6, IL-1β, IL-1ra, and IL-18

Sera from the patients and healthy control subjects ($n=10$; age range, 1–35 years) were stored at −80°C until analysis. The sera of cases 1 and 2 were collected when they had the cold-induced rash, but not fever. The sera of cases 3, 4, and 5 were collected during a fever episode as an autoinflammatory symptom. Culture supernatants in test tubes or microtest plates were centrifuged to remove the cells and then stored at −80°C until analysis. The tumor necrosis factor (TNF)-α, IL-6, IL-1β, IL-1ra, and IL-18 concentrations were measured using a Human TNF-α Immunoassay Kit (BioSource, Camarillo, CA, USA), Human IL-6 Immunoassay Kit (BioSource), Human IL-1β Immunoassay Kit (BioSource), Quantikine Human IL-1ra/IL-1F3 ELISA Kit (R&D Systems, Minneapolis, MN, USA), and Human IL-18 ELISA Kit (MBL, Nagoya, Japan), respectively. The detection limits of the cytokine measurement kits were as follows: TNF-α, 1.7 pg/ml; IL-6, 2.0 pg/ml; IL-1β, 1.0 pg/ml; IL-1ra, 6.26 pg/ml; IL-18, 12.5 pg/ml. Values under the detection limits were shown as not detected. The serum cytokine levels were measured at two points at least, and the average values were calculated. The cytokine production levels by PBMCs were measured in duplicate and the average values were calculated. We defined cytokine levels of more than the mean+2 SD as increasing.

Results

Detection of Gene Variations in *NLRP3*

In the five patients, four heterozygous missense variations (E378K, Y563N, E688K, and G809S) of the *NLRP3* gene were identified (Table I). Interestingly, case 3 showed compound heterozygous gene variations, E688K and G809S, while his mother (case 4) had only one mutation, E688K, of *NLRP3*. The G809S allele was inherited from his asymptomatic father. In case 5, a novel missense variation, E378K, in *NLRP3* was identified. In addition, a heterozygous mutation, E148Q, in *MEFV* was identified. Gene mutations in *TNFRSF1A*, *MVK*, *NLRP12*, and *NOD2* were not found.