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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

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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

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.

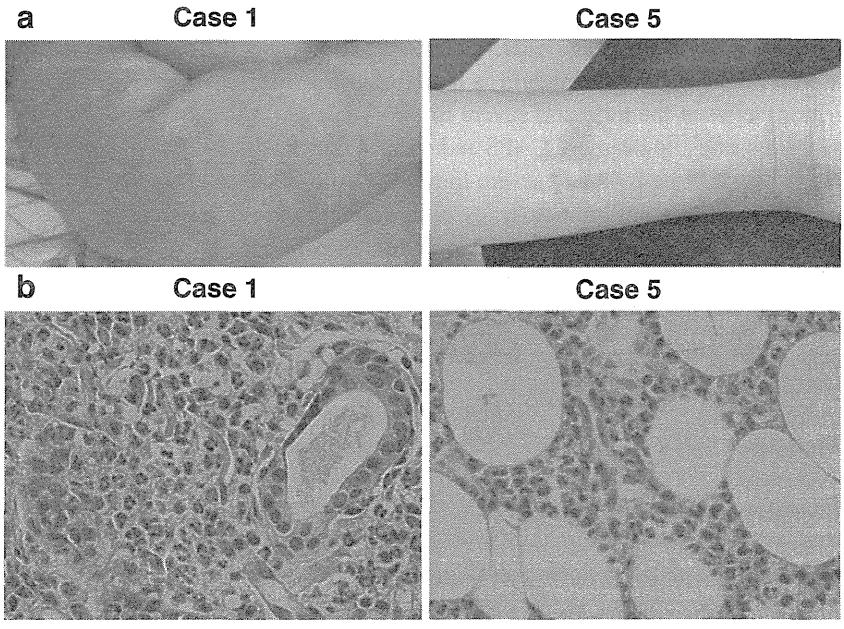

Fig. 1 Urticaria-like skin rash of cases 1 and 5. **a** Clinical appearances of the urticaria-like rash of cases 1 and 5. **b** Histopathological examinations of biopsy specimens from the skin rash of cases 1 and 5. Both skin biopsies show a recurrent cold-induced non-itchy urticaria-like skin rash and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

The genotypes of *NLRP3* and *MEFV* in her asymptomatic mother were the same. It should be noted that E378K and G809S were not present in the *INFEVERS* database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) [14] and were confirmed as rare variants that were not identified in the 100 ethnically matched control subjects.

NF-κB Reporter Gene Activities of the *NLRP3* Variants

Figure 2 shows the ASC-dependent NF-κB activities of the *NLRP3* variants in vitro. The NF-κB reporter gene activities were increased by the Y563N and E688K mutations in *NLRP3*. The activities were higher for D303N (as a positive control *NLRP3* mutation that was previously identified in a CINCA/NOMID patient [5]) and E688K than for the FCAS mutation, Y563N. E378K and G809S did not cause any significant increases in the activities. Initially, we suspected that case 5 had CAPS. However, based on these results, we were able to confirm the diagnosis of case 5 as JIA, rather than CAPS.

Cytokine Profiles of the Patients

The serum IL-1β, IL-6, and TNF-α levels were not detected in the sera of the healthy control subjects. Although we were unable to detect IL-1β in the patients' sera, we clearly detected the serum IL-18 and IL-1ra levels in all cases (Fig. 3a, b). The serum IL-18 levels were extremely high in the CINCA/NOMID (case 3), MWS (case 4), and JIA (case 5) patients compared with the control subjects. The serum IL-1ra and IL-6 levels were increased in cases 2, 3, 4, and 5 (Fig. 3b, c). The serum TNF-α levels were increased in cases 1, 2, and 3 (Fig. 3d).

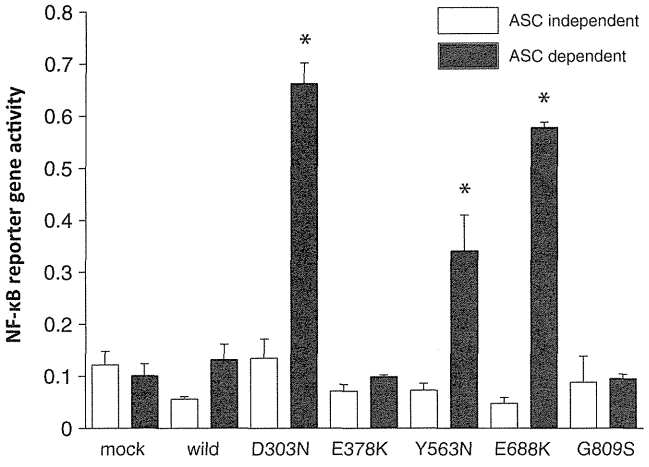

Fig. 2 NF-κB reporter gene activities of the *NLRP3* variants. The *white bars* indicate the NF-κB reporter gene activities of the *NLRP3* variants without cotransfection of ASC, while the *black bars* indicate these activities with cotransfection of ASC. The data shown are the means±SD of triplicate assays. The ASC-dependent NF-κB reporter gene activities are increased for the variants with D303N, Y563N, and E688K. The activities for the CINCA/NOMID mutations, D303N and E688K, are higher than those for the FCAS mutation, Y563N. The variants with E378K and G809S do not show any significant increases in the activities. *$P<0.05$

Interestingly, the serum IL-18 levels in the FCAS patients (cases 1 and 2) did not show any increases compared with the control subjects (Fig. 3a). Furthermore, the levels of spontaneous IL-1β production by PBMCs from the CINCA/NOMID (case 3) and MWS (case 4) patients were increased, whereas those of the control subjects, FCAS patients, and JIA patient (cases 1, 2, and 5) did not show any increases (Fig. 4a).

The lipopolysaccharide (LPS)-induced cytokine production levels by PBMCs from the FCAS and JIA patients are shown in Fig. 4b–d. The IL-1β and IL-18 production levels were increased in the FCAS patients compared with the control subjects. However, TNF-α did not show any significant changes. Comparisons of the cytokine production levels by the PBMCs cultured at 30°C and 37°C are shown in Fig. 5. The PBMCs from the FCAS patients showed obvious increases in the IL-1β and IL-18 production levels after culture at the lower temperature with no stimulation.

Discussion

The diagnosis of CAPS is still based on the clinical symptoms and recognition of a syndrome. Detection of a pathogenic *NLRP3* mutation can confirm the CAPS diagnosis. However, to confirm the diagnosis of CAPS patients with novel identified *NLRP3* variations, some functional experiments regarding the effects of the *NLRP3* mutations, such as the NF-κB luciferase reporter gene assay used in this study, are necessary because of the existence of nonfunctional missense variations of *NLRP3* [7]. Furthermore, although there are many previously reported missense mutations of *NLRP3* associated with CAPS in the *INFEVERS* database [14], the mutations with confirmed functional evidence are limited. In this study, we identified *NLRP3* gene mutations in five patients who were suspected of having autoinflammatory syndromes. Two mutations of *NLRP3*, Y563N and E688K, were previously reported to be disease-causing mutations [15, 16], although in vitro functional assays were not performed. Y563N was first identified in FCAS patients who were diagnosed based on the clinical criteria of FCAS [16, 17]. Our FCAS patients (cases 1 and 2) showed a skin rash, occasional fever, and mild arthritis and did not show any severe symptoms, such as neurological disorders, hearing loss, and renal amyloidosis. On the other hand, E688K was first identified in an Italian male CINCA/NOMID patient [15] who was described as having a skin rash, hearing loss, fever, and transient arthritis without persistent deformities of the involved joints. Our patients with E688K

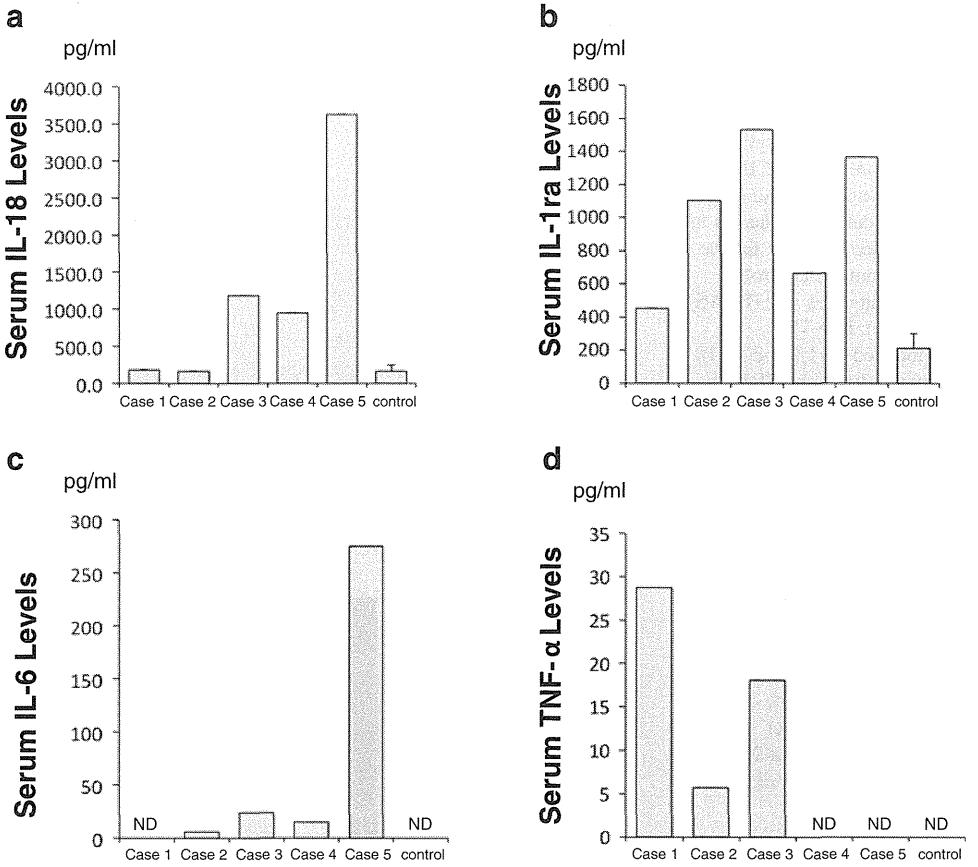

Fig. 3 Serum inflammatory cytokines in the four CAPS cases. IL-1β, IL-6, and TNF-α were not detected in the sera of the control subjects. The means ±D of the serum IL-18 and IL-1ra levels of the healthy control subjects were 169.2±85.7 and 213.4±87.1 pg/ml, respectively (n=10)

(cases 3 and 4) also had no strong deformities of the joints, but had obviously more severe phenotypes than FCAS, such as aseptic meningitis and hearing loss. In the present study, the E688K mutation in the MWS and CINCA/NOMID syndrome patients showed significantly stronger NF-κB activities than the Y563N mutation identified in the FCAS patients. Our findings indicate that the clinical phenotypes and values of the ASC-dependent NF-κB activity assay are well correlated with the genetic mutations, consistent with a previous report [18]. However, the artificial reporter gene assay system used may have little to do with the function of the CAPS pathophysiology, and limited numbers of *NLRP3* variants have been assessed using the assay in the present and previous studies, thereby making it difficult to prove this hypothesis at the present time. Consequently, further experiments including large amounts of pathogenic mutations and accumulation of detailed clinical information about the disease severity of CAPS are necessary to confirm this hypothesis. It should be noted that low-penetrance mutation, G809S, did not show positive activity with this in vitro assay system. But the clinical phenotype of case 3 was obviously more severe than case 4, although the father of case 3, who also was found to have G809S, was as symptomatic. Because of the discrepancy between the patient and the father, it remains unclear whether G809S is a pathogenic mutation or, alternatively, if there is an alternative genetic explanation for disease in the patient not detected by genomic DNA sequencing.

On the other hand, it requires time to build the above-mentioned in vitro experimental system. For the rapid diagnosis and characterization of CAPS, a simple screening system is necessary. In this study, we measured several serum inflammatory cytokine levels in our patients (Fig. 3). The serum IL-6 level is usually used for evaluating the disease severity of rheumatoid arthritis [19]. Moreover, the serum IL-18 level was recently reported to reflect the disease severity of not only JIA but also other diseases such as allergic diseases [20, 21]. In our CAPS patients, the serum levels of IL-18, but not IL-1β, seemed to be correlated with the disease phenotypes. Although the precise reason for this dissociation between the IL-18 and IL-1β levels in the sera is unknown, IL-1β may be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. In fact, serum IL-1ra, which is the counter-regulator of IL-1, was increased in our CAPS patients. Thus, the serum IL-18 levels may be used as an appropriate marker for the evaluation of treatments, although it is unlikely that serum IL-18 can contribute to the differential diagnosis between CAPS and other diseases.

The diagnosis of FCAS seems to be relatively difficult because of its mild phenotypes compared with the other more severe phenotypes of CAPS. The serum inflammatory

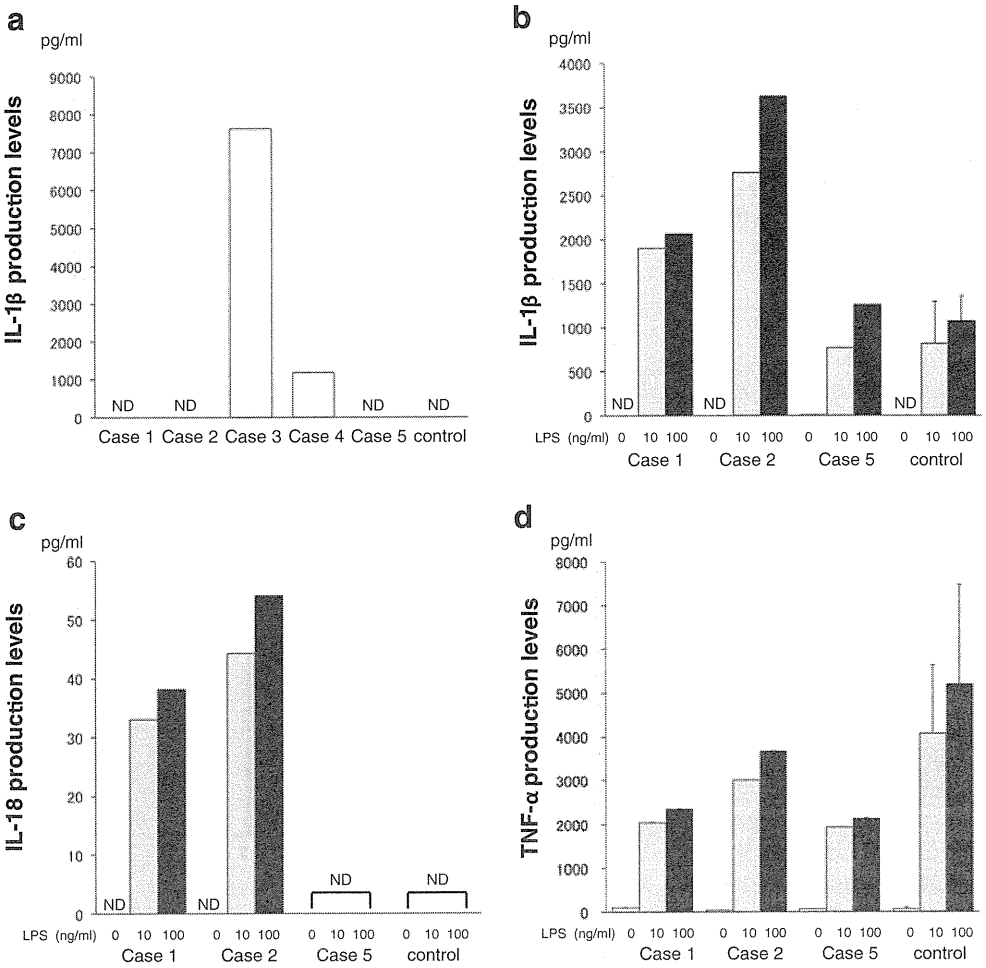

Fig. 4 LPS-induced cytokine production levels in the patients. **a** The *white bars* indicate the spontaneous IL-1β production levels by PBMCs. Increased IL-1β production by PBMCs from case 3 (CINCA/NOMID syndrome) and case 4 (MWS) is detected, whereas no increases are observed for the PBMCs from the control subjects and cases 1, 2 (FCAS), and 5 (JIA). **b**, **c** The LPS-induced IL-1β and IL-18 production levels by PBMCs from the FCAS patients are increased compared with PBMCs from the control subjects. **d** The TNF-α production levels by PBMCs from the FCAS and JIA patients do not show any significant changes. In **b**–**d**, the *white bars* indicate the cytokine production levels without stimulation and the *gray* and *black bars* indicate the cytokine production levels after stimulation by 10 and 100 ng/ml LPS, respectively

cytokine levels in our FCAS patients did not show any typical increases, unlike the case for the CINCA/NOMID patient (Fig. 3), indicating that the establishment of an effective and easy screening method is important for the diagnosis of FCAS. Therefore, we focused on the cytokine production levels in these patients' blood cells. First, IL-1β production by nonstimulated PBMCs was observed in our CINCA/NOMID and MWS patients (cases 3 and 4, respectively), as reported previously [5]. However, no enhancement of spontaneous IL-1β production was observed in our FCAS patients (cases 1 and 2) (Fig. 4a), suggesting that this method may not be suitable for screening of FCAS.

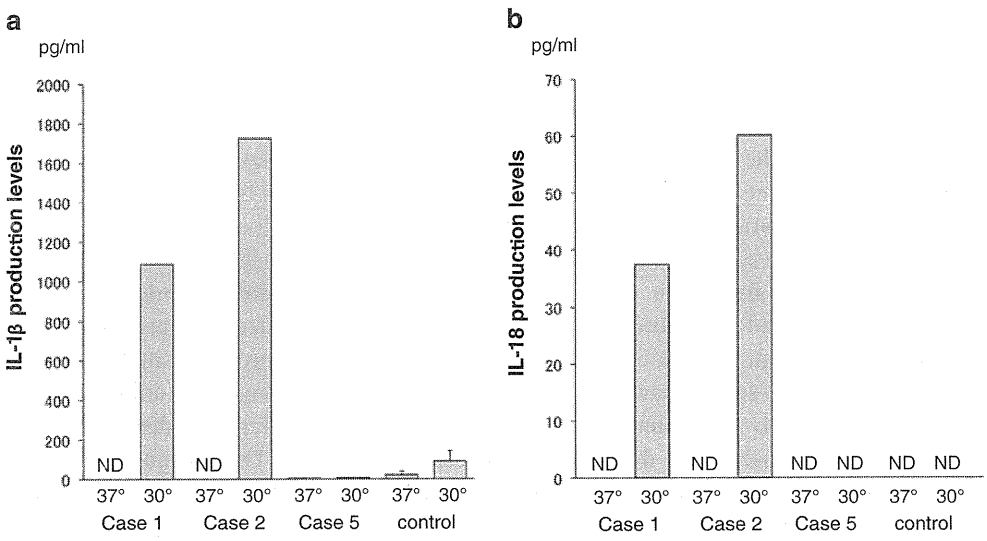

Fig. 5 Hypothermia-induced cytokine production levels by PBMCs from the FCAS and JIA patients. **a**, **b** Comparisons of the cytokine production levels by PBMCs cultured at 30°C and 37°C. The PBMCs from the FCAS patients (cases 1 and 2) show obvious increases in the IL-1β and IL-18 production levels after culture at lower temperature with no stimulation

Furthermore, the LPS- or hypothermia-induced cytokine production levels by the PBMCs showed marked elevation of IL-1β or IL-18 (Figs. 4a–c and 5b), as reported previously [16, 22]. The phenomena for hypothermic culture were similar to the findings in our recent report that NF-κB activity induced by LPS stimulation through TLR4 is enhanced in low-temperature cultures [23], although the precise mechanism of the association between the *NLRP3* variations and the low-temperature stimulation requires further clarification. These findings suggest that the cytokine production assays induced by LPS or hypothermia stimulation should be helpful for the diagnosis of FCAS. It should be noted that the serum IL-18 levels could be detected in all of the non-CAPS subjects, although the production levels of IL-18 from their PBMCs were lower than the detection limit. This might be dependent on the long half-life of IL-18 in human blood compared with the above-mentioned half-life of IL-1β.

The discrimination between CAPS and JIA cases is sometimes difficult because of their similar clinical characteristics. Interestingly, although case 5 had a rare missense variation in *NLRP3* (E378K) and some of her clinical symptoms were similar to those of CAPS (Table I), the E378K variant did not show enhancement of NK-κB activity (Fig. 2). This gene variation was inherited from her mother who did not show any inflammatory symptoms. Case 5 showed strong polyarthritis, continuous fever, and a recurrent generalized urticaria-like erythema as well as symptoms of CAPS. In particular, histopathological examination of a biopsy specimen from her skin rash revealed infiltration of neutrophils and mononuclear cells, representing similar findings to case 1 (Fig. 1). Thus, it was difficult to discriminate CAPS by the clinical symptoms alone in this case.

Therefore, to discriminate between CAPS and JIA in this case, we focused on her cytokine profiles. Her serum IL-6 and IL-18 levels were extremely high compared with not only the healthy controls but also the other CAPS patients (Fig. 3a, c). These observations resembled the serum cytokine pattern of systemic-onset JIA [21, 24]. Furthermore, the LPS-induced and hypothermia-induced IL-1β and IL-18 production levels by PBMCs from case 5 showed no increases compared with the control subjects (Figs. 4b, c and 5a, b). Recently, Saito et al. [5] reported that another screening method, LPS-induced monocyte cell death, was effective for diagnosing CAPS. The monocytes in case 5 did not show LPS-induced cell death. These objective results also supported the diagnosis of case 5 as JIA, rather than CAPS.

In this study, we evaluated several methods for the limited genotypes of patients with *NLRP3* variants. According to comparisons of the clinical phenotypes of previous case reports and our cases, the disease severity seems to be correlated with the serum cytokine levels and the ex vivo and in vitro responses and is almost completely determined by the specific mutations, which appear to suggest that other genetic or epigenetic determinants or environmental factors do not play a significant role.

Conclusions

A precise and easy method for the diagnosis of CAPS has not yet been established. The characteristics of the clinical phenotypes and the identification of proven gene variations of *NLRP3*, as the etiology of CAPS, are very important for diagnosing CAPS. In addition, the serum IL-18 levels and NF-κB activities of patients with the *NLRP3* variants reflect the phenotypes of disease severity. Evaluation of the cytokine profile is also a useful tool for diagnosing and discriminating the severity of CAPS.

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Conflicts of Interest The authors have declared no conflicts of interest.

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ORIGINAL ARTICLE

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.

Keywords HIDS · MVK gene · Novel mutation · Compound heterozygous mutation · Splicing mutation · Inherited recurrent fever syndrome

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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.

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.

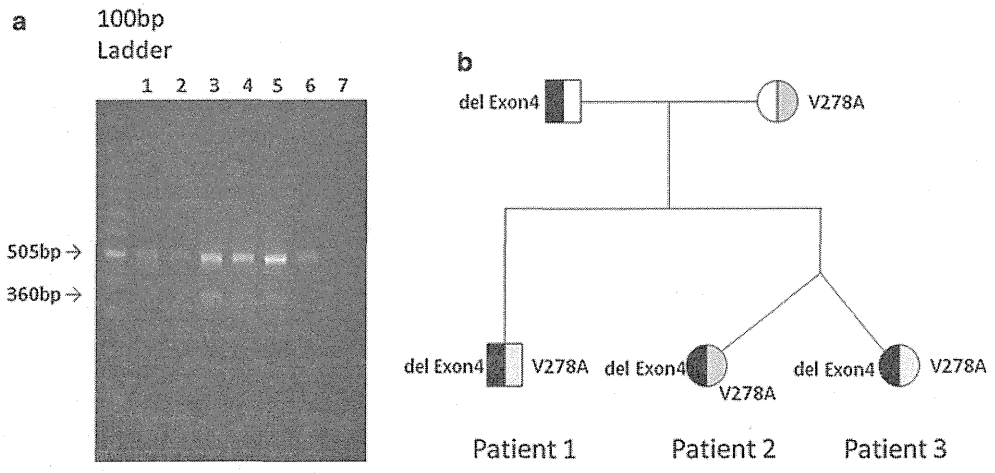

Fig. 1 Molecular genetic findings in the study patients. **a** Agarose gel electrophoresis of RT-PCR products for exon 2 to exon 5 of MVK shows the normal 505-bp alleles in samples from normal healthy control (*lane 6*) and mother (*lane 2*), as well as both the normal allele and the mutant 362-bp allele 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.

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Conflict of interest There is no financial or other potential conflict of interest for each author.

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ORIGINAL RESEARCH

In Vitro Analysis of the Functional Effects of an *NLRP3* G809S Variant with the co-Existence of *MEFV* Haplotype Variants in Atypical Autoinflammatory Syndrome

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Abstract
Purpose Hereditary periodic fever syndromes have been considered monogenic diseases. However, some recent reports have described patients with co-existence of recurrent fever responsible genes. This study assessed whether a rare variant, found in Japanese children showing atypical autoinflammatory syndrome, located in the leucine-rich repeat domain of Nod-like receptor family, pyrin domain containing 3 (*NLRP3*) with co-existence of Mediterranean fever (*MEFV*) haplotype variants may contribute to a proinflammatory phenotype using a systematic approach.
Methods Cytokine production in serum or from peripheral blood monocytes was measured by ELISA. DNA sequence analysis of genes including *NLRP3*, *MEFV*, mevalonate kinase (*MVK*), and tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*) were performed on patient samples. In vitro functional assays determined the effects of the NLRP3 variants and pyrin using NF-κB activation and speck formation assays.
Results A heterozygous genetic variant of *NLRP3*, G809S, was found in samples from both patients. Additionally the previously reported heterozygous *MEFV* variants (P369S-R408Q or E148Q-P369S-R408Q) were also detected in both patients. Serum IL-1ra and sTNFR1 levels increased in the attack phase of the disease in both patients. The production levels of IL-1β from monocytes isolated from both cases were elevated following LPS and IFN-γ stimulation. The *NLRP3* G809S variant demonstrated no increase of NF-κB activity following monosodium urate stimulation, whereas it significantly increased speck formation by interacting with apoptosis-associated speck-like protein with caspase recruitment domain.
Conclusions The phenotype of atypical autoinflammatory disease in patients could be modified by a synergistic effect with two other variants of autoinflammatory-associated genes.

Keywords NLRP3 · leucine-rich repeat domain · autoinflammatory disease · ASC

Abbreviations

ASC	Apoptosis-associated speck-like protein containing a CARD
CAPS	Cryopyrin-associated periodic syndrome
CINCA	Chronic infantile neurologic cutaneous, articular
FCAS	Familial cold-induced autoinflammatory syndrome
FMF	Familial Mediterranean fever
HEK	Human embryonic kidney
IL	Interleukin
MEFV	Mediterranean fever

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MVK	Mevalonate kinase
MWS	Muckle–Wells syndrome
MSU	Monosodium urate
NBS	Nucleotide-binding site
NLRP3	Nod-like receptor family pyrin domain containing 3
NOMID	Neonatal-onset multisystem inflammatory disease
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A
TRAPS	Tumor necrosis factor receptor-associated periodic syndrome

Introduction

Autoinflammatory syndromes are characterized by systemic inflammation without the presence of antigen-specific T cells or high-titers of autoantibodies [1]. Many autoinflammatory syndromes are clinically characterized by recurrent or persistent features that include fever, elevation in the levels of acute-phase reactants, and organ-specific complications such as skin rashes and osteoarticular, serosal, neurologic, and ocular manifestations [2]. To date, well-known hereditary periodic fever syndromes are familial Mediterranean fever (FMF), hyperimmunoglobulinemia D with periodic fever syndrome, cryopyrin-associated periodic syndromes (CAPS), and tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS). These syndromes are discriminated by some characteristic phenotypes such as varying age of onset, duration of fever, development of cutaneous manifestations, and several other features.

CAPS include familial cold-induced autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID), also known as chronic infantile neurologic, cutaneous, articular (CINCA) syndrome. FCAS exhibits cold-induced urticaria-like skin rash whereas MWS develops severe phenotypes, such as periodic fever, neural progressive hearing loss and renal amyloidosis. CINCA/NOMID syndrome shows additional more severe phenotypes, such as severe arthritis, patella overgrowth, aseptic meningitis, and mental retardation [3]. CAPS are caused by mutations in the Nod-like receptors (NLRs) family, pyrin domain containing 3 (*NLRP3*) gene, and more than 80 variants are associated with CAPS, in addition to over 50 variants of unclear significance that have been reported in the INFEVERS database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) to date [4].

NLRs recognize microbial molecules such as pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns, which trigger inflammation as well as Toll-like receptor immune responses. NLRP3 protein contains an N-terminal pyrin domain, a central nucleotide-binding site (NBS) domain, and C-terminal leucine-rich repeats (LRR) [5]. Most pathogenic mutations associated with autoinflammatory syndromes are located in exon 3 of *NLRP3*, which encodes the NBS domain. In addition, several mutations outside exon 3 on the LRR domain of *NLRP3*, such as G755R, G755A, and Y859C have been found in patients with CINCA syndrome or atypical autoinflammatory disorders [6–8].

This study reports two cases of Japanese children who presented with atypical periodic fever episodes and who had the variants in the LRR domain of *NLRP3* with co-existence of Mediterranean fever (*MEFV*) haplotype variants. The patients showed periodic prolonged fever and erythema, but lacked symptoms typical of CAPS, FMF, and other common autoinflammatory syndromes. By genetic analysis and functional assays of these variants, the data from this study suggest that the phenotype of atypical autoinflammatory disease in patients could be modified by a synergistic effect with other autoinflammatory-associated genes.

Methods

Subjects

Case 1

The first case was a 9-year-old girl who had experienced recurrent fever episodes approximately three times a year for 6 years from onset at 3 years of age. Although she underwent a tonsillectomy at the age of 5, she still experienced recurrent fever episodes. She presented with mild abdominal pain without signs of peritoneal irritation, peritonitis or pleuritis as typically observed in FMF. High serum C-reactive protein (CRP) levels were observed in the attack phase. She presented with pigmented macules with erythema, which persisted for 6 months, and bilateral petechiae on her legs and dorsa of feet (Fig. 1a). Histological examination of the skin lesion revealed perivascular infiltrate with mononuclear cells in the upper and middle dermis, but vasculitis was not observed (Fig. 1b). Direct immunofluorescence analysis revealed deposits of complement component 3 (C3) at the capillary walls in the upper to middle dermis, but not the presence of immunoglobulin (Ig)A or IgM (Fig. 1c). Rheumatoid factor and autoantibodies were not detected. Colchicine treatment (0.5 mg per day) was effective in treating the erythema and alleviating fever with elevating CRP. Both parents had experienced lasting recurrent fever episodes during their childhood although it was likely that their symptoms were not so severe. The fever episodes of parents resolved spontaneously without specific

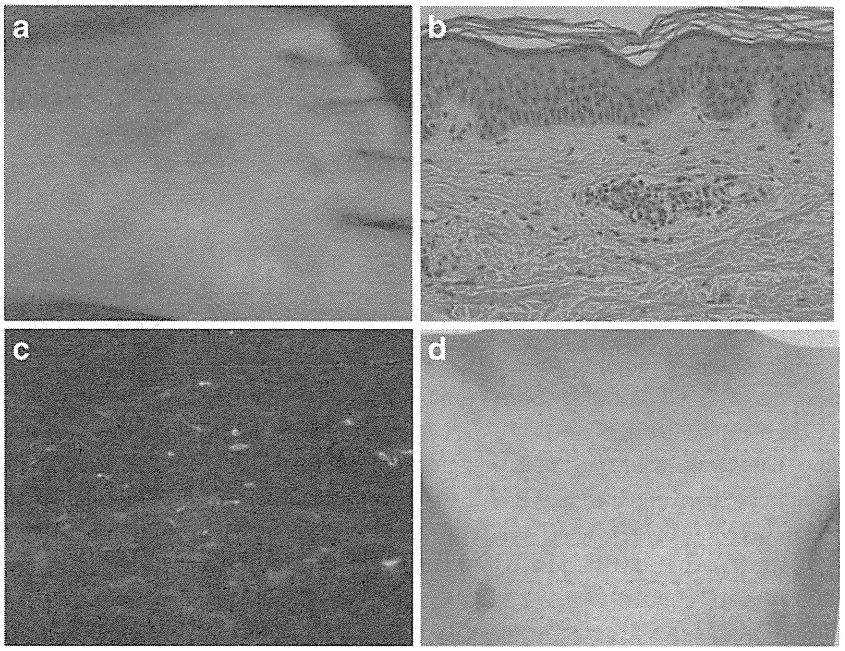

Fig. 1 Presence of skin rash in patients with atypical autoinflammatory syndrome. **a** The clinical appearance of rash on the dorsum of foot in case 1. **b** The histopathological examination of a skin biopsy specimen (hematoxylin and eosin stain, original magnification × 200). Perivascular infiltrate with mononuclear cells was observed in the upper and middle dermis. **c** Direct immunofluorescence demonstrates C3 deposits in the capillary walls (original magnification × 50). **d** The clinical appearance of the skin rash on the breast in case 2

medications such as colchicines and corticosteroids or tonsillectomy when they were about 10 years old. However, they do not remember their childhood in detail as it was over 30 years ago. Their episodes may represent autoinflammatory disease.

Case 2

The second case involved a 4-year-old boy, presenting with recurrent episodes of fever of various duration from a few days to weeks, with or without mild liver dysfunction and multiple erythema without skin itch. The frequency of episodes was at least twice a year. The skin erythema was observed during the fever episodes at 18 months old and at 4 years old (Fig. 1d). The cervical lymphadenopathy and diarrhea were observed in almost all of the fever attack episodes. Although fever duration was 1 week, it resolved immediately following oral administration of 1 mg/kg prednisolone. Rheumatoid factor and autoantibodies were not detected. His parents had no symptoms like periodic fever syndromes or rheumatic diseases. The fever did not recur for a few months after the cessation of oral prednisolone treatment. From 3 years old, colchicine treatment was started because of recurrent fever attacks. However, currently this treatment is not effective.

The genotypes and the clinical profiles of these cases are summarized in Table I. This study was performed according to the Helsinki Declaration. All subjects provided informed consent to participate in the study.

DNA Sequencing

Genomic DNA was extracted from leukocytes using SepaGene (EIDIA, Tokyo, Japan). DNA fragments of the *NLRP3*, *MEFV*, mevalonate kinase (*MVK*), and TNF receptor superfamily, member 1A (*TNFRSF1A*) genes were amplified by polymerase chain reaction (PCR), and analyzed using big dye terminator bidirectional sequencing (Applied Biosystems, Foster City, CA, USA).

Table I Genotype and clinical profiles of cases

	Case 1	Case 2
Initial diagnosis	FMF	TRAPS
Gender	Female	Male
Clinical features		
Age at onset of attacks	3 years	6 months
Duration of episodes	3–5 days	>1 week
Fever	Yes	Yes
Abdominal signs	Yes	Yes
Arthralgia	No	No
Lymphatic signs	No	Yes
Cutaneous manifestations	persistence of rash (pigmented erythema with petechiae)	Two episodes of rash
Hearing loss	No	No
Neurologic signs	No	No
Proteinuria	No	No
Laboratory findings		
WBC (/μl)	11,800	14,620
CRP (mg/dl)	10.1	3.1
ESR (mm/h)	45	32
NLRP3 Genotype	G809S	G809S
MEFV Genotype	P369S, R408Q	E148Q, P369S, R408Q

CRP the serum C-reactive protein level. *WBC* white blood cells. *ESR* erythrocytes sedimentation rate. Laboratory findings were the data in the attack phase

Genotyping

Allelic frequency of NLRP3 G809S (rs141389711) was investigated on a Step One Real-Time PCR System using Custom TaqMan SNP Genotyping assays (Applied Biosystems) in 421 healthy subjects. Further, genotype was confirmed by direct sequence analysis.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of control donors and from patients by gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). The CD14-positive cells were cultured in medium consisting of RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 μg/ml). Human embryonic kidney (HEK) 293 T cells and HEK293-ASC cells were cultured in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Analysis of Serum Cytokine Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples of patients and controls were stored at −80 °C until assayed. TNF-α concentrations were measured with an Immunoassay Kit (BioSource International, Carlsbad, CA, USA) with a detection limit of 1.7 pg/ml. Similarly, interleukin (IL)-6 and IL-1β concentrations were measured by immunoassay Kit (BioSource) with detection limits of 1.7 pg/ml and 1.0 pg/ml, respectively. IL-1ra and sTNFR1 concentrations were measured by ELISA (R&D Systems) with detection limits of 6.26 pg/ml and 0.77 pg/ml, respectively. IL-18 was assayed by ELISA (MBL, Nagoya, Japan), with a detection limit of 25.6 pg/ml. We defined serum cytokine levels of more than the mean + 2 SD as increasing. Values below the detection limit are shown as not detected.

IL-1β Production from Monocytes

CD14-positive cells were purified from PBMCs using CD14 MACS MicroBeads and MACS magnetic columns according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). The CD14 positive cells were seeded to a density of 3.0×10^5 per ml and cultured with the addition of 1.0 μg/mL LPS O127 (Sigma-Aldrich) and 20 μg/ml IFN-γ (R&D Systems, Minneapolis, MN, USA) for 24 h at 37 °C in a humidified atmosphere at 5 % CO_2 and pulsed with 5 mM ATP (Sigma-Aldrich) for 30 min before harvesting. The cell-culture supernatants were harvested, and stored at −80 °C until assayed. The IL-1β was measured with ELISA. The assay was performed at two different times. The statistical significance between control and each case in the IL-1β production was analyzed using Dunnett's multiple comparison test. *P*-value of <0.05 was considered statistically significant.

Vector Preparations

cDNA encoding *NLRP3* tagged at the C-terminus with a FLAG-epitope (NLRP3-FLAG) was cloned into plasmid pcDNA3.1+ (Invitrogen). NLRP3 mutants (D303N, G755R, G809S and Y859C) were generated using the GeneEditor *In vitro* Site-Directed Mutagenesis System (Promega, Madison, WI, USA). A cDNA encoding pyrin tagged at the C-terminus with an HA-epitope (pyrin-HA) was cloned into plasmid pcDNA3.1+. Pyrin variants (P369S+R408Q) were generated using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). The apoptosis-associated speck-like protein containing a CARD (ASC) variant 1 tagged at the C-terminus with a myc-epitope (ASC1-myc) construct was cloned into pcDNA3.1+. The NF-κB luciferase reporter vector (pGL4.32-luc2P/NF-kappaB-RE/Hygro) and the Renilla luciferase reporter vector (pGL4.74-hRluc/TK) were purchased from Promega.

NF-κB Reporter Gene Activity

HEK293T cells were transfected with 16 ng per well of pcDNA3.1+ control vector or pcDNA3.1+ NLRP3-FLAG (wild type or mutant) or pcDNA3.1+ pyrin-HA (wild type or mutant) in 96-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The pcDNA3.1+ ASC1-myc, NF-κB luciferase reporter, and Renilla luciferase reporter were co-transfected. After transfection, cells were incubated for 24 h. Cells were stimulated with R837 at a concentration of 10 μg/ml (InvivoGen, San Diego, CA, USA) or monosodium urate (MSU) at 250 μg/ml (InvivoGen) for 8 h. Luciferase reporter activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega). The statistical significance of differences in luciferase activity between wild-type and mutant gene activity in the NF-κB reporter assays was analyzed using Dunnett's multiple comparison test. A *P*-value of <0.05 was considered statistically significant.

Speck Quantification Assay

HEK293 cells were transfected with ASC-myc and positively selected using 1 mg/ml G418 for 4 weeks. HEK293-ASC cells (1×10^5) were co-transfected with 250 ng of each NLRP3 expression plasmid and pyrin expression plasmid using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. After 24-h incubation, cells were fixed with 3.7 % paraformaldehyde in PBS for 10 min,

and washed with 10 mM glycine in PBS. Fixed cells were permeabilized using PBS containing 0.2 % Triton X-100 for 1 h at room temperature. Cells were then incubated with an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and anti-myc antibody (Invitrogen). Primary antibody binding was detected by incubation with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen) secondary antibodies. Fixed cells were incubated with 4'-6-diamidino-2-phenylindole, a nuclear stain, and mounted using Vectashield Mounting Medium (Vector Laboratories Burlingame, CA, USA). The percentage of cells containing ASC specks in the cells expressing *NLRP3* was calculated by randomly selecting at least 10 fields. Differences were analyzed using Dunnett's multiple comparison test. A *P*-value of <0.05 was considered statistically significant.

Results

Detection of NLRP3 and MEFV Mutations in Two Patients with Autoinflammatory Syndrome

In case 1, a heterozygous c.2425G>A (p.Gly809Ser) on LRR in exon5 of *NLRP3* and heterozygous P369S-R408Q in exon3 of *MEFV* were identified (Table I). There are 17 individuals who have the allele of G809S in 421 healthy control subjects. The allele frequency of this variant was 0.02. There were no control subjects carrying P369S-R408Q in *MEFV* in addition to the G809S variant. Interestingly, the same *NLRP3* and *MEFV* haplotype variants were identified in the father of case 1. The heterozygous *MEFV* variant haplotype P369S-R408Q were also observed in the mother of case 1.

Case 2 expressed the same heterozygous *NLRP3* variant found in case 1. In addition, heterozygous E148Q-P369S-R408Q in exon2 and exon3 of *MEFV* were identified (Table I). The G809S variant of *NLRP3* was inherited from his asymptomatic father. His asymptomatic mother was positive for homozygous E148Q and heterozygous P369S-R408Q sequences.

MVK and *TNFRSF1A* mutations were not detected in either case.

The Cytokine Profile of Patients

Serum IL-1β, IL-6 and TNF-α levels were not detected in the sera of healthy control subjects. The mean concentration ± SD of serum IL-18 and IL-1ra in healthy control subjects were 169.2±85.7 pg/ml and 213.4±87.1 pg/ml, respectively [9]. The mean concentration ± SD of serum sTNFR1 in healthy control subjects was 1009±276.4 pg/ml. Figure 2a and b show the serum cytokine profiles from the patients. The serum cytokine concentrations were measured at two different points at least during fever and inter-ictal periods respectively, and average values were calculated. In both cases, serum IL-1β, TNF-α, and IL-6 did not increase during the fever episodes, whereas serum IL-1ra and sTNFR1 levels were increased. IL-18 levels during the fever episodes were increased in case 2, not in case 1. Interestingly, the serum IL-1ra and IL-18 levels from case 2 were elevated during the inter-ictal period.

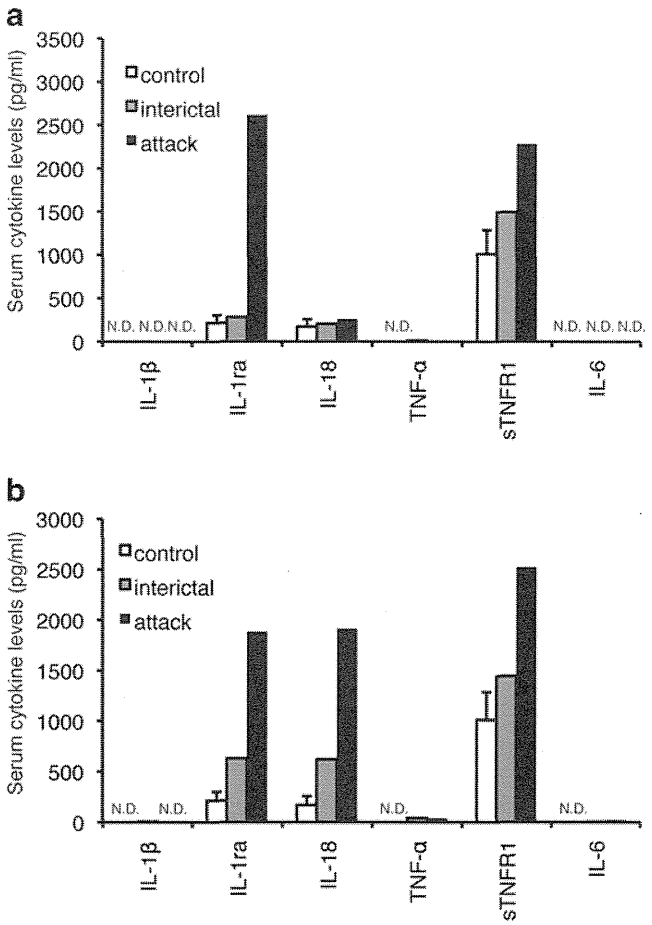

Fig. 2 Inflammatory cytokine levels from two cases during the inter-ictal phase and attack phase. **a** White bars indicate serum inflammatory cytokine levels of control. Grey bars indicate serum inflammatory cytokine levels of case 1 during the inter-ictal period. Black bars indicate serum inflammatory cytokine levels of case 1 during the attack phase. **b** White bars indicate serum inflammatory cytokine levels of control. Grey bars indicate serum inflammatory cytokine levels of case 2 during the inter-ictal period. Black bars indicate serum inflammatory cytokine levels of case 2 during the attack phase

Figure 3 shows the production of IL-1β from monocytes with LPS, IFN-γ and/or ATP stimulation. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects ($n=5$) without stimulation were 5.54±4.40 pg/ml. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects stimulated with 20 ng/ml IFN-γ or 1 μg/ml LPS were 7.74±9.81 pg/ml and 236.0±188.4 pg/ml, respectively. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects stimulated with 1 μg/ml LPS added 5 mM ATP was 166.0±138.3 pg/ml. The mean concentration ± SD of IL-1β from monocytes of healthy

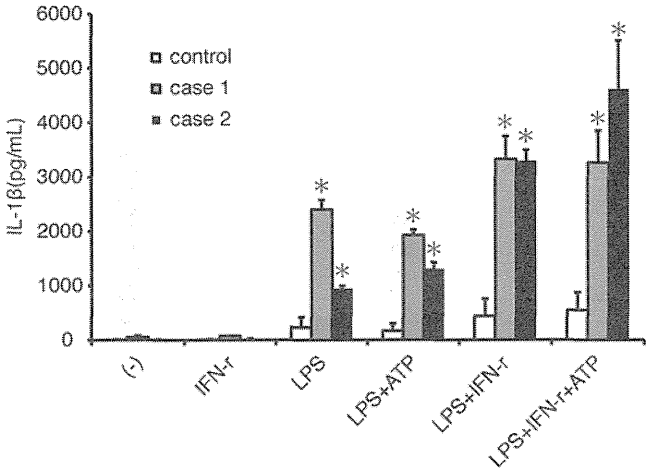

Fig. 3 IL-1β levels from monocytes in case 1 and 2. White bars indicate IL-1β levels in control. Grey bars indicate IL-1β levels in case 1. Black bars indicate IL-1β levels in case 2. IL-1β levels from monocytes from case 1 and 2 were significantly increased compared with controls (n=5). * P<0.05

control subjects stimulated with both 20 ng/ml IFN-γ and 1 μg/ml LPS were 441.3±316.5 pg/ml. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects stimulated with both 20 ng/ml IFN-γ and 1 μg/ml LPS added 5 mM ATP was 549.2±327.3 pg/ml. In both cases, IL-1β secretion was increased compared with the healthy controls when the monocytes were stimulated with LPS and IFN-γ. Additionally, IL-1β from monocytes in case 2 stimulated with LPS and IFN-γ was increased in response to ATP. This was not observed for monocytes from case 1.

NF-κB Reporter Gene Activity of NLRP3 and Pyrin Variants

To assess the function of the *NLRP3* variant G809S on NF-κB signaling, we compared the G809S sequence with those of wild-type and three *NLRP3* mutations (D303N, G755R and Y859C). D303N, G755R, and Y859C were identified in CAPS patients [6, 10, 11] (Fig. 4). When ASC was co-expressed, D303N and G755R mutations increased NF-κB reporter gene activity. However, G809S and Y859C did not lead to significant activation of NF-κB. In the presence of R837, an *NLRP3* inflammasome activator, *NLRP3* D303N and G755R mutations showed enhanced NF-κB activation, whereas G809S and Y859C did not induce any increase in activity. Subsequently, the evaluation of G809S enhanced NF-κB activation in the presence of MSU was measured. MSU induced NF-κB activation of wild-type, D303N and G755R *NLRP3*. However, both G809S and Y859C mutations significantly inhibited NF-κB activation mediated by MSU.

To investigate the role of mutational effect of pyrin in the NF-κB signaling pathway, wild-type or variant pyrin (P369S+R408Q) was expressed in HEK293 cells and co-

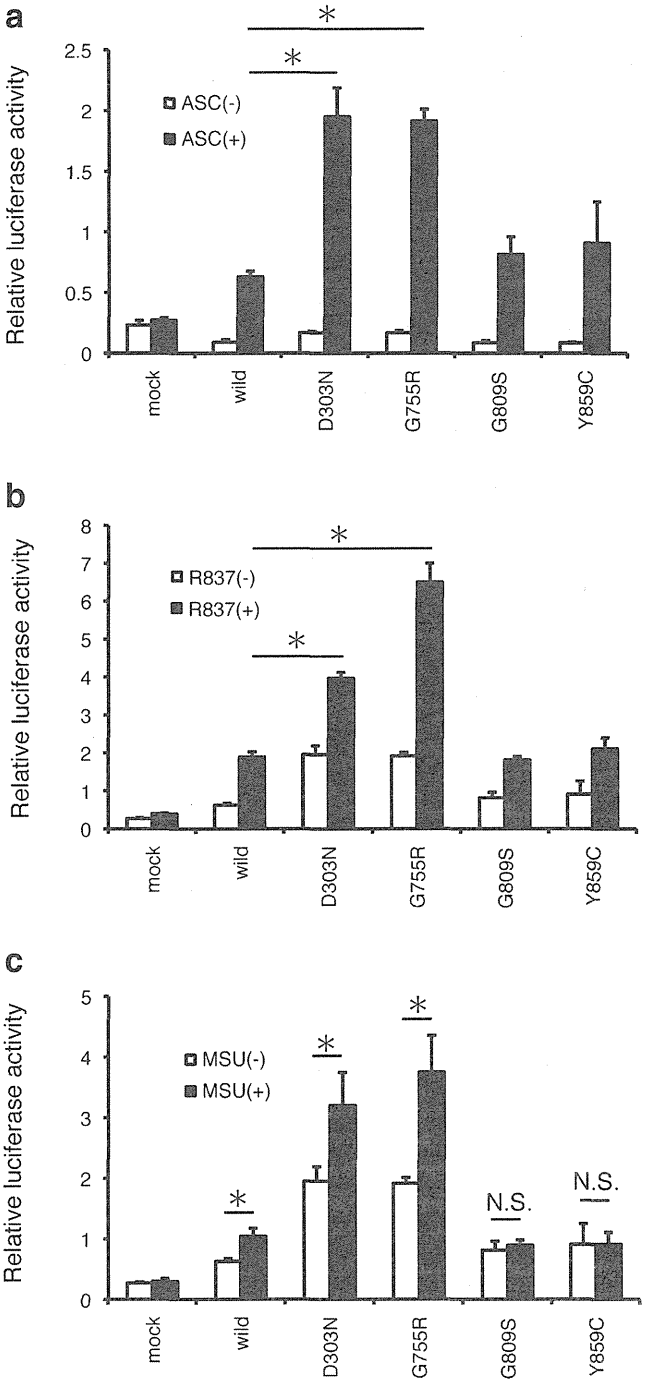

Fig. 4 NF-κB reporter gene activity of *NLRP3* mutants. Bars represent the mean ± SD of triplicate assays. **a** White bars indicate the NF-κB reporter gene activity of the *NLRP3* mutants without co-transfection of ASC. Black bars indicate activity with co-transfection of ASC. ASC-dependent NF-κB reporter gene activity was increased by mutants D303N and G755R. G809S and Y859C did not induce NF-κB reporter gene activity. **b** White bars indicate NF-κB reporter gene activity with co-transfection of ASC. Black bars indicate activity after stimulation with 10 μg/ml R837. **c** White bars indicate NF-κB reporter activity following co-transfection of ASC. Black bars indicate activity after stimulation with 250 μg/ml MSU. * P<0.05

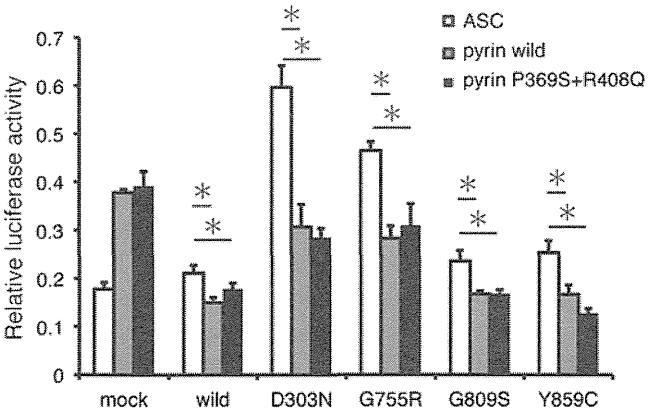

Fig. 5 Pyrin and *NLRP3* mutant-induced NF-κB reporter gene activity. Bars represent the mean ± SD of triplicate assays. White bars indicate NF-κB reporter gene activity with co-transfection of ASC. Grey bars indicate activity with co-transfection of ASC and wild-type pyrin. Black bars indicate activity with co-transfection of ASC and pyrin variant P369S+R408Q. * $P<0.05$

transfected with ASC (Fig. 5). Although both wild-type and variant pyrin inhibited NF-κB activation with co-transfection of wild-type or mutant NLRP3 protein, there was no significant difference in inhibitory capacity between the wild-type and variant pyrin.

Speck Quantification Assay

Previous studies have shown that *NLRP3* LRR variants have an increased ability to induce speck formation in the presence of ASC [6, 12]. To test the effect of G809S on *NLRP3*-ASC interactions and speck formation, wild-type, *NLRP3* variants or empty vectors and pyrin were transiently transfected with cells stably expressing ASC. Cells transfected with *NLRP3* wild-type displayed speck formation (mean ± SD, 36.7± 6.1 %). In comparison, the *NLRP3* D303N, G755R, G809S and Y859C mutants induced significantly higher numbers of speck formation (62.1±8.8 %, 72.6±4.8 %, 53.1±10.1 % and 48.8±13.2 % respectively, Fig. 6).

Discussion

The current study identified a G809S variant within the LRR domain of *NLRP3* with the co-existence of *MEFV* haplotype variants in two unrelated patients with atypical autoinflammatory syndrome. Although we recently reported a CINCA/NOMID patient with the compound heterozygous gene mutations E688K and G809S, it is unclear whether G809S is a pathogenic mutation [9]. To confirm a functional role for the G809S variant, its effect on the NF-κB signaling pathway was investigated in vitro. Although several variants of *NLRP3* show significant increases of ASC dependent NF-κB reporter gene activity in a previous report and as data presented here, no significant increase was observed owing to the *NLRP3* G809S variant in this assay. Kambe et al. demonstrated that the *NLRP3* G755R mutation located within the LRR domain could induce significant NF-κB activation in the presence of an *NLRP3* inflammasome activator, R837 [13]. Therefore,

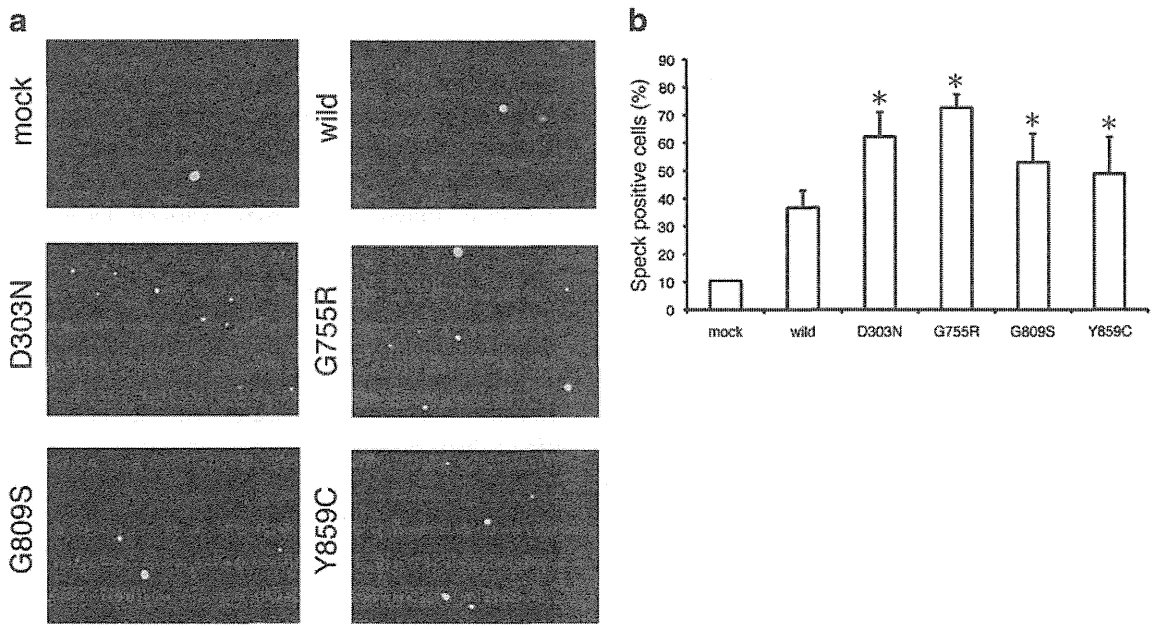

Fig. 6 Effect of the G809S variant on speck formation. Transfection of HEK293-ASC cells with 250 ng each of the *NLRP3* expression plasmids or an empty vector and pyrin expression plasmid was performed. Speck formation was assessed by immunofluorescence microscopy. **a** This panel shows examples of fields obtained by immunofluorescence microscopy. **b** The percentage of cells containing ASC-myc specks was calculated as the mean ± SD percentage of cells. * $P<0.05$