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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.

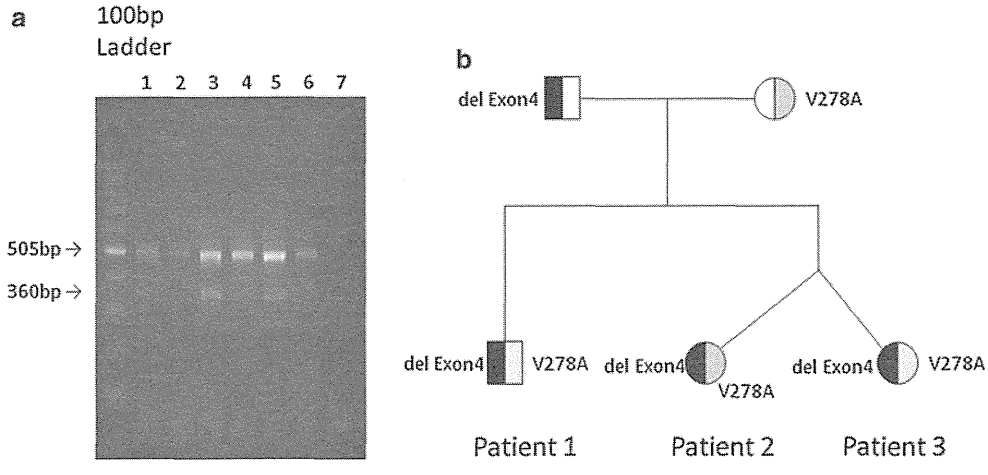

Fig. 1 Molecular genetic findings in the study patients. **a** Agarose gel electrophoresis of RT-PCR products for exon 2 to exon 5 of MVK shows the normal 505-bp alleles in samples from normal healthy control (*lane 6*) and mother (*lane 2*), as well as both the normal allele and the mutant 362-bp allele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

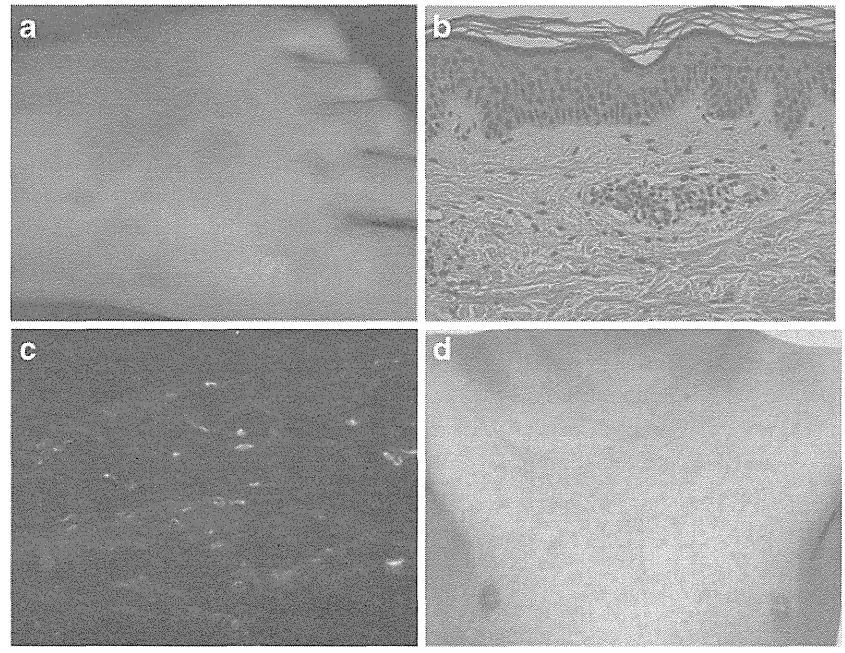

Fig. 1 Presence of skin rash in patients with atypical autoinflammatory syndrome. **a** The clinical appearance of rash on the dorsum of foot in case 1. **b** The histopathological examination of a skin biopsy specimen (hematoxylin and eosin stain, original magnification × 200). Perivascular infiltrate 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,

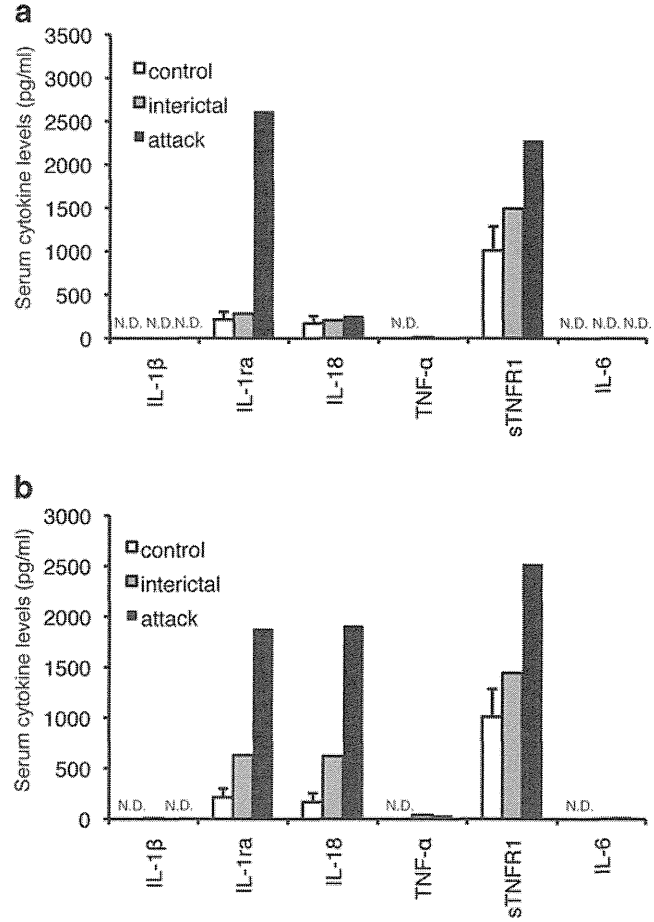

Fig. 2 Inflammatory cytokine levels from two cases during the inter-ictal phase and attack phase. **a** White bars indicate serum inflammatory cytokine levels of control. Grey bars indicate serum inflammatory cytokine levels of case 1 during the inter-ictal period. Black bars indicate serum inflammatory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

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.

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

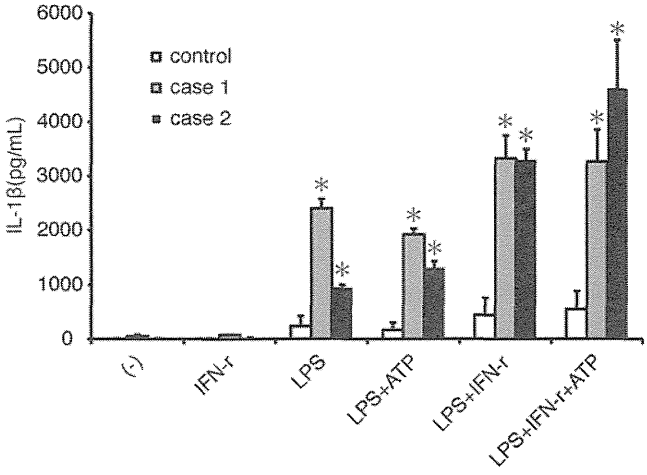

Fig. 3 IL-1β levels from monocytes in case 1 and 2. White bars indicate IL-1β levels in control. Grey bars indicate IL-1β levels in case 1. Black bars indicate IL-1β levels in case 2. IL-1β levels from monocytes from case 1 and 2 were significantly increased compared with controls (n=5). * P<0.05

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-

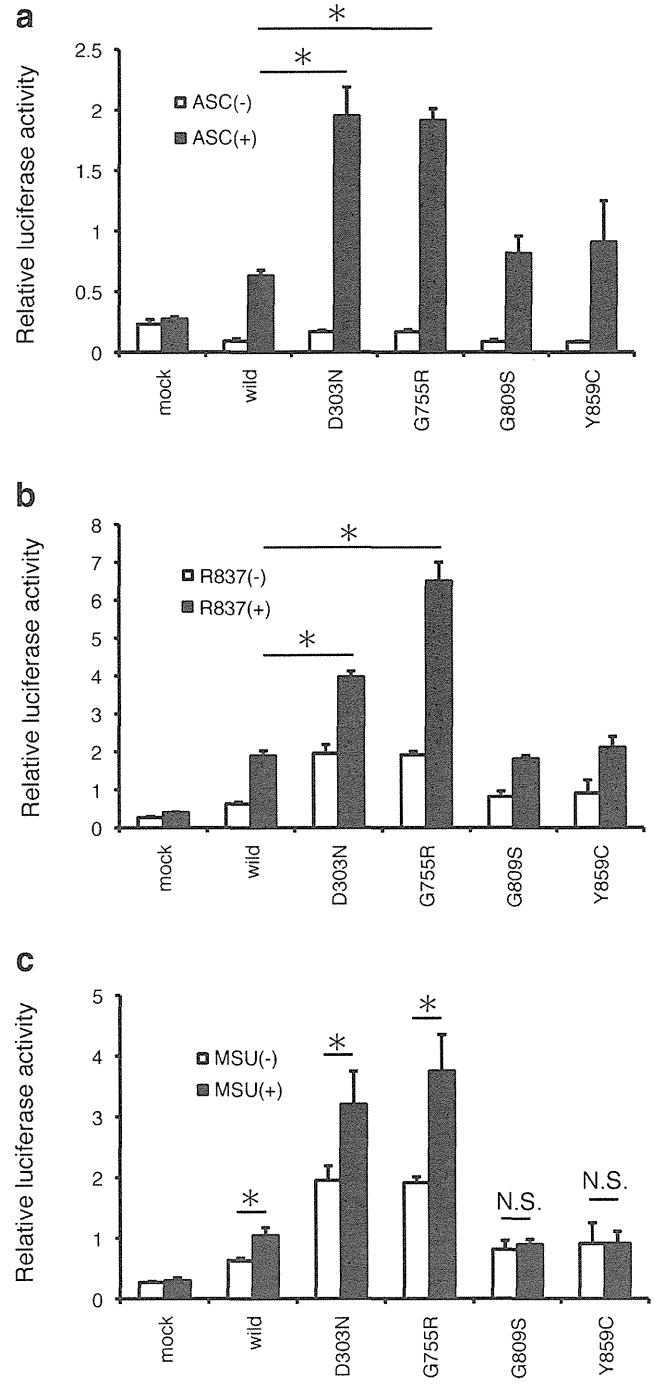

Fig. 4 NF-κB reporter gene activity of *NLRP3* mutants. Bars represent the mean ± SD of triplicate assays. **a** White bars indicate the NF-κB reporter gene activity of the *NLRP3* mutants without co-transfection of ASC. Black bars indicate activity with co-transfection of ASC. ASC-dependent NF-κB reporter 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

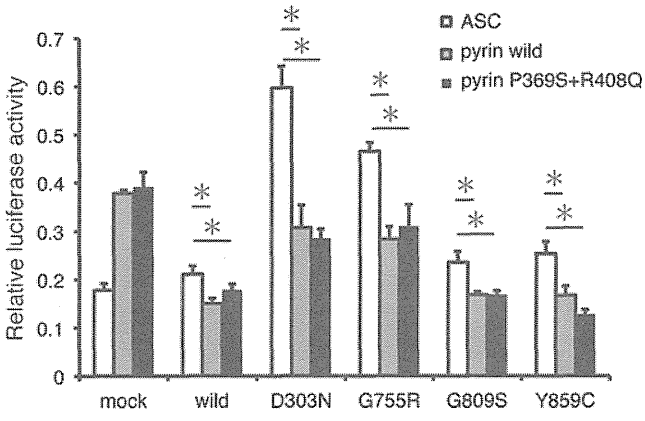

Fig. 5 Pyrin and *NLRP3* mutant-induced NF-κB reporter gene activity. Bars represent the mean ± SD of triplicate assays. White bars indicate NF-κB reporter gene activity with co-transfection of ASC. Grey bars indicate activity with co-transfection of ASC and wild-type pyrin. Black bars indicate activity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

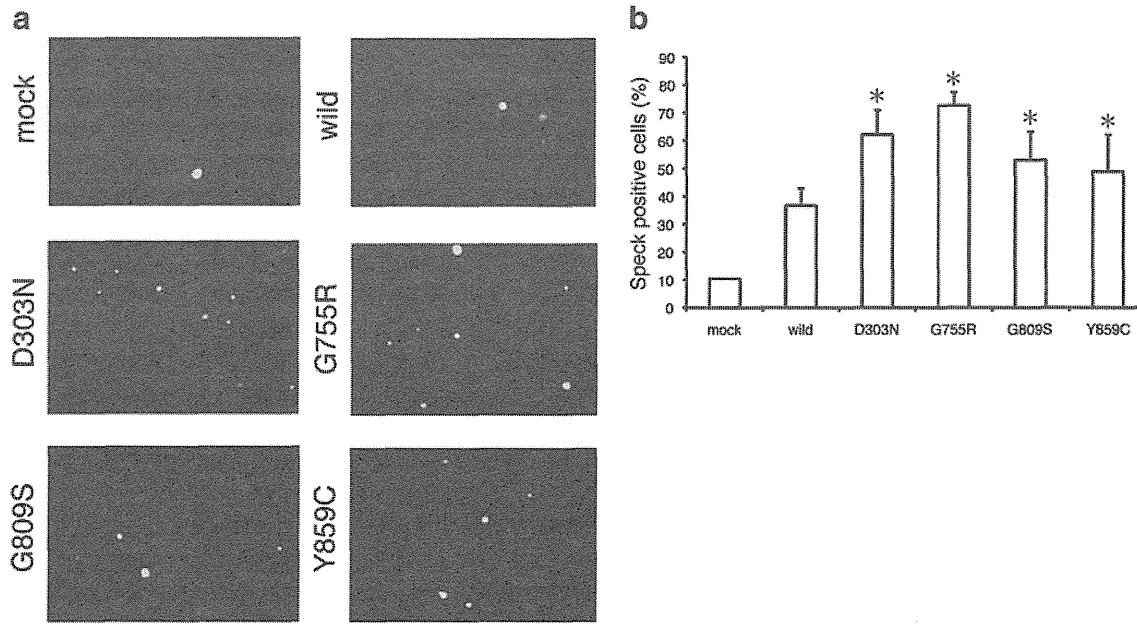

Fig. 6 Effect of the G809S variant on speck formation. Transfection of HEK293-ASC cells with 250 ng each of the *NLRP3* expression plasmids or an empty vector and pyrin expression plasmid was performed. Speck formation was assessed by immunofluorescence microscopy. **a** This panel shows examples of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

G809S may be expected to enhance NF-κB activation in the presence of R837. However, G809S did not increase NF-κB activity like as Y859C [6] (Fig. 4b). Since the *NLRP3* LRR domain plays a central role in mediating inflammation induced by another inflammasome activator, MSU crystals, we examined whether G809S affected NF-κB activation in the presence of MSU [14]. Interestingly, G809S and Y859C mutations did not show any NF-κB activity responses by MSU stimulation. In contrast, wild-type, D303N and G755R mutations significantly increased NF-κB activity following MSU stimulation. These data suggest the G809S LRR missense variant, which may diminish the responsiveness to PAMPs as NOD2 LRR variant reported in Crohn's disease, has a pathogenic effect on these pathways [15–17].

Jéru et al. recently identified a pathogenic Y859C mutation in the LRR domain of *NLRP3*, which increased speck formation and pro-caspase 1 processing, but which had no direct effect on *NLRP3* mediated NF-κB signaling. The G809S variant also increased speck formation relative to wild-type *NLRP*3. These results suggest that G809S, as well as Y859C in the LRR domain, may be a gain of function variant. It should be noted that although the assays used in this study are sensitive, our findings may provide limited evidence to prove that the G809S variant is pathogenic. However, these results indicate that the variant alters the function of NLRP3.

The two case studies presented here consistently showed elevated IL-1-related serum cytokines, IL-1ra, during the attack phase. In addition, monocytes from case 1 and 2 secreted high levels of IL-1β, which may indicate a gain of function variant in *NLRP3*, associated with inflammasome activation. Additionally, we previously reported a CINCA/NOMID patient positive for the compound heterozygous gene mutations, E688K and G809S [9]. This patient developed severe a phenotype compared with her mother, who carried a single mutation, E688K. This genotype-phenotype correlation suggests that the G809S variant may act as an additional genetic factor associated with the severity of CAPS.

However, in this study IL-1β was not detectable in the serum of patients, as IL-1β might be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. Furthermore, although elevated serum IL-18, which is activated by caspase-1 as well as IL-1β, and IL-6 levels were observed in CINCA/NOMID patient [9], the serum IL-18 levels were increased in case 2 but not case 1, and serum IL-6 levels in both cases did not increase during the fever episodes. Thus, it may be considered that the differences of cytokine profiles and disease phenotypes between case 1 and 2 and typical CINCA/NOMID patients result not only from their genetic background, but also environmental factors.

Additional mutation analysis of our patients also revealed heterozygous variant haplotype of *MEFV*, a gene involved in the pathogenesis of FMF, in addition to G809S in *NLRP3*. Case 1 was heterozygous for P369S and R408Q in *cis* and case 2 was heterozygous for E148Q, P369S, and R408Q in *cis*. Allele frequencies of P369S and R408Q in the Japanese population are 3.6 % and 4.8 %, respectively, according to the International HapMap Project (http://www.hapmap.org/). These frequent variant haplotypes were found to be in strong linkage disequilibrium in the Japanese population. In addition, P369S and R408Q variant haplotype are associated with a variable phenotype and are infrequently associated with typical FMF symptoms [18–21]. Heterozygous P369S and R408Q variant haplotype are also associated with other inflammatory diseases, such as Behçet's disease [18], and systemic lupus erythematosus [21]. Moreover, heterozygous E148Q-P369S-R408Q variant haplotype is more rare, which is associated with chronic recurrent multifocal osteomyelitis [20]. In this report, case 1 and case 2 showed the similar phenotypes as FMF or TRAPS, respectively. Although detailed clinical features and cytokine profiles of the two cases are various, they exhibited a long duration of recurrent fever episodes compared with typical FMF. Thus, these findings suggest that P369S and R408Q variant haplotype may have effects on several inflammatory diseases, but the functional evidence of these variant haplotype remains unclear.

The *MEFV* gene codes for pyrin, that can interact with ASC to induce ASC oligomerization and the activation of procaspase-1, which promotes IL-1β and IL-18 processing [12, 22]. In contrast, some reports have described that pyrin inhibited NLRP3-mediated NF-κB activation by disrupting the NLRP3-ASC interaction [23, 24]. In accordance with the reports, co-expression of NLRP3 and pyrin in HEK293T cells indicated less ASC-dependent NF-κB activation than expression of NLRP3 only, whereas there was no difference in the inhibitory capacity of NF-κB activity between pyrin variants and the wild-type protein. Interestingly, a recent study using pyrin deficient and mutated pyrin knock-in mice demonstrated a gain of function with pyrin variants located in B-Box domains, which caused autoinflammatory phenotypes [22]. Thus, research using knock-in mouse experiments with *MEFV* exon3 variants into pyrin deficient mice would help clarify the pathogenic effects of the *MEFV* variant.

In general, hereditary periodic fever syndromes have been considered monogenic diseases. On the other hand, recent reports have described patients with heterozygous low penetrance variants in two recurrent fever genes [2, 25, 26]. These indicate that oligogenic inheritance has been related to pathogenesis of autoinflammatory diseases. In some cases, patients presented with specific symptoms of both diseases or with a more severe phenotypes. Although the patients in this study were positive for the *NLRP3* variant, they did not present with typical symptoms of CAPS, such as deafness or cold-induced rash. In addition, variants in *MEFV* have been detected in both cases, but they also lacked typical FMF symptoms. However,

both cases had obviously periodic fever episodes. These suggest the presence of oligogenicity and that variants in *NLRP3* and *MEFV* synergistically modify the symptoms of the atypical autoinflammatory diseases.

There are two important limitations in this study when discussing the pathogenicity of low penetrance rare variants. The first limitation is the limited number of patients in the study. Further study using a large number of patients is necessary to confirm our results. Secondly, we only analyzed a limited number of genes. In this study, we concluded that the presence of an *NLRP3* variant with the co-existence of *MEFV* variants contributed to atypical autoinflammatory disease. However, the patients may have had alternative genetic mutations or other rare variants of inflammasome related genes such as *CARD8* [27] elsewhere in the genome, which are truly disease causing, and the two variants described in these patients may be unrelated.

Conclusions

This study describes the molecular analysis of two cases with heterozygous low penetrance variants in exon5 of *NLRP3* and exon3 of *MEFV*. The findings provide in vivo and in vitro evidence for the effect of an *NLRP3* missense variant. Importantly the mutations are within the same signaling pathway and are associated with inflammasome activation. Our observations suggest that oligogenic inheritance may occur in patients with atypical autoinflammatory syndrome. It is therefore important to consider that the phenotypes could be modified by synergistic effects with plural autoinflammatory-associated gene mutations when the patients have atypical autoinflammatory disease.

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

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Familial Mediterranean Fever in Japan

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Abstract: Familial Mediterranean fever (FMF) is a hereditary autoinflammatory disease that is prevalent in Mediterranean populations. While it is considered a rare disease in the rest of world, a significant number of FMF patients have been reported in East Asia, including Japan. Our aim was to determine the prevalence of FMF in Japan and elucidate the clinical and genetic features of Japanese patients. A primary nationwide survey of FMF was conducted between January and December 2009. Hospitals specializing in pediatrics and hospitals with pediatric, internal medicine, and rheumatology/allergy departments were asked to report all patients with FMF during the survey year. The estimated total number of Japanese FMF patients was 292 (95% confidence interval, 187–398 people). We evaluated the clinical and genetic profiles of Japanese patients from the data obtained in a secondary survey of 134 FMF patients. High-grade fever was observed in 95.5%, chest pain (pleuritis symptoms) in 36.9%, abdominal pain (peritonitis symptoms) in 62.7%, and arthritis in 31.3%. Of the patients profiled, 25.4% of patients experienced their first attack before 10 years of age, 37.3% in their teens, and 37.3% after age 20 years. Colchicine was effective in 91.8% of patients at a relatively low dose (mean dose, 0.89 ± 0.45 mg/d). AA amyloidosis was confirmed in 5 patients (3.7%). Of the 126 patients studied, 109 (86.5%) were positive for 1 or more genetic mutations and 17 (13.5%) had no mutation detected. Common Mediterranean fever gene (MEFV) mutations were E148Q/M694I (19.8%) and M694I/normal (12.7%). The differences in the prevalence of peritonitis, pleuritis, and a family history of FMF were statistically significant between FMF patients with MEFV exon 10 mutations compared with those without exon 10 mutations.

In conclusion, a significant number of patients with FMF exist in Japan. Although Japanese patients with FMF are clinically or genetically different from Mediterranean patients, the delay in diagnosis is an issue that should be resolved.

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Abbreviations: AA = amyloid A, CI = confidence interval, FMF = familial Mediterranean fever, MEFV = Mediterranean fever, PCR = polymerase chain reaction, SAA = serum amyloid A.

INTRODUCTION

Familial Mediterranean Fever (FMF) is an inherited autoinflammatory disease that is observed in Mediterranean populations, such as Armenians, Arabs, non-Ashkenazi Jews, and Turks.[11,47] The disease is characterized by recurrent febrile episodes and inflammation in the form of sterile polyserositis.[5] The gene responsible for FMF, MEFV, encodes a protein called pyrin/marenostrin and is expressed mainly in neutrophils and monocytes.[13,21] To date, 200 mutations or polymorphisms in the MEFV gene have been associated with the FMF phenotype.[28] The prevalence of FMF varies from 1:400-1000 in Turkey,[9,47] 1:1000 (depending on the ethnic group) in Israel,[10] and 1:500 in Armenia.[35] The various manifestations of FMF in different populations could be caused by a diverse repertoire of mutations specific to their ethnic background.[7] For example, patients carrying exon 2 mutations (such as E148Q) present a milder phenotype.[7] In contrast, patients carrying M694V or M694I mutations are prone to more severe disease.[44] Where the disease is common the diagnosis of FMF is principally based on clinical tests, whereas in countries where FMF is rare, a genetic test is useful.[2] The diagnostic power of the colchicine response, where an FMF patient is expected to respond to colchicine, is still important when monitoring atypical FMF cases.[6]

The genetic homogeneity of Japan has been preserved by national geographic borders, and there has been little inward migration since ancient times. FMF was previously thought to affect people mainly from Mediterranean populations, and was considered a rare disease in Japan. However, a significant number of FMF patients with MEFV gene mutations have been reported in Japan since the identification of the MEFV gene.[22,30,36,38,41,42,46] One severe complication of FMF is the development of amyloid A (AA) amyloidosis.[17] The effect of MEFV genotypes, especially when the M694V mutation is homozygous, is evident in FMF patients with AA amyloidosis.[4] In Japanese patients with rheumatoid arthritis, the SAA1.3 allele was shown to be a risk factor of AA amyloidosis.[31] However, there is a strong positive association of the SAA1.1 allele and M694V homozygosity of the MEFV gene as risk factors for AA amyloidosis in white FMF patients.[16]

Since 1972, the Ministry of Health and Welfare of Japan has promoted research to determine the causes of intractable diseases of unknown etiology.[32] To investigate epidemiologic features of disease (prevalence, age distribution, and clinical phenotypes), the Research Committee on the Epidemiology of Intractable Diseases conducted several surveys in cooperation with various disease research committees.[18] In 2009 the Ministry of Health,

Labour and Welfare of Japan and the Research Committee on the Epidemiology of Intractable Diseases conducted a nationwide survey to elucidate the prevalence of FMF in Japan. We conducted the present study to further estimate the prevalence of FMF and elucidate the clinical features in Japanese patients. Genotype/phenotype correlations were previously reported in Jewish, Turkish, and Armenian patients with FMF.[25,29,48] In the current study we evaluated the genotype/phenotype correlations in Japanese patients with FMF and compared them to those of other ethnic groups.

METHODS

A nationwide survey for FMF was conducted in cooperation with the Japan Research Committee on the Epidemiology of Intractable Diseases in 2009. The target populations were patients with FMF who visited hospitals in 2009. According to the Nationwide Epidemiologic Survey Manual issued by the Research Committee on the Epidemiology of Intractable Disease,[19] we selected 3 types of departments for the targeted survey: pediatrics, internal medicine, and rheumatology/allergy. The hospitals used in the study were selected randomly from a list of all hospitals in Japan. The selection rate was determined according to a stratification based on the number of beds in the hospital. Thus, hospitals with a high number of beds had a greater probability of being selected. The selection rate was 100% for hospitals with 500 beds or more or university hospitals, whereas only 5% of hospitals with fewer than 100 beds were selected at random. In addition, specialized hospitals that had previously reported FMF patients were all selected for the study. After selection, we sent a questionnaire describing the diagnostic criteria for FMF. The primary survey only inquired as to the numbers of patients with FMF who visited the hospital in 2009. Patients were diagnosed clinically according to the modified and simplified diagnostic criteria of Tel-Hashomer,[24] provided with the primary survey. The diagnosis was divided into 1 major criterion: recurrent febrile episodes (3 or more episodes lasting 12 h to 3 d with a fever of 38°C or more) and 8 minor criteria (a febrile attack with 1 of 7 accompanying symptoms including abdominal pain due to peritonitis; chest pain due to pleuritis; monoarthritis of hip, knee, or ankle; pericarditis; scrotum pain due to orchitis; headache due to aseptic meningitis; or a favorable response to colchicine treatment). A diagnosis of FMF was determined if the patient exhibited the major criterion and 1 or more minor criteria. When a suspected FMF patient was identified, a second questionnaire regarding the detailed clinical features for each patient was sent. The present study was approved by the ethical committees of Jichi Medical University (No. 09–20, September 7, 2009).

Using the selection and response rate to the surveys, we estimated the total number of patients with FMF and the 95% confidence intervals (CIs) as described previously.[20,23] The estimate was based on the assumption that department responses were independent of the frequency of patients. The point estimation of prevalence was calculated using the following equation, where SRTk, RRTk, NSk, nk, Nk, and Nki denote the sampling rate, response rate, number of sampling departments, total number of departments, number of responding departments, and the number of departments with i patients in stratum k, respectively.

$$\alpha_k = \frac{1}{SRT_k RRT_k}\sum_i iN_{ki} = \frac{1}{\frac{NS_k}{n_k}\frac{N_k}{NS_k}}\sum_i iN_{ki} = \frac{nk}{Nk}\sum_i iN_{ki}$$

Age and sex distributions of the disease were estimated based on data obtained from the second survey.

Mutation Analysis

Two mL of blood were collected from each subject. Genomic DNA was extracted from whole blood using the Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Mutation analysis was performed by genomic sequencing. Mutations in exon 1–10 of the MEFV gene were tested. Polymerase chain reaction (PCR) was performed using forward and reverse primers for each exon as described previously.[39] PCR products were purified with the ExoSAP-IT (GE Healthcare Japan, Tokyo, Japan) and sequenced directly, using specific primers and BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan). Genetic analysis of the MEFV gene was approved by the Ethics Committee of Nagasaki Medical Center (No. 21003, May 11, 2009).

Determination of SAA1 Alleles

SAA1 genotyping by PCR-RFLP was performed, as previously described.[1] Briefly, a portion specific to SAA1 was amplified using the following primer set: 5′-ATGATGCTGCCAAAAGGGGA-3′ (forward) and 5′-TGGCCAAAGAATCTCTGGAT-3′ (reverse). PCR was carried out with 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The products were digested by Ban I and Bcl I, and genotypes were determined by agarose gel electrophoresis.

Statistical Analyses

Data were analyzed using SPSS (SPSS Inc., Chicago, IL). Results were expressed as the mean ± standard deviation (SD) for continuous variables. For quantitative data, analysis was performed using a Mann–Whitney U rank-sum test for comparison of 2 independent groups. Comparisons for categorical variables were evaluated using the chi-square test. $P < 0.05$ was accepted as significant.

RESULTS

Prevalence

In the primary survey, 2251 hospitals or departments of pediatrics, internal medicine, or rheumatology/allergy were selected. Of them, 1380 (61.3%) responded and 170 patients met the diagnostic criteria for FMF. The results of the questionnaire survey are shown in Table 1. The numbers of patients reported from departments of pediatrics, internal medicine, and rheumatology/allergy were 85 (50.0%), 67 (39.4%) and 18 (10.6%), respectively. The total number of patients was estimated to be 292 (95% CI, 187–398). The estimated numbers from pediatrics, internal medicine, and rheumatology/allergy were 118, 129, and 45, respectively (Table 2).

Demographic Features of Japanese FMF Patients

From 170 FMF patients recruited in the first nationwide survey, detailed clinical data were obtained from 122 patients and from another 12 patients who were diagnosed after the first survey in 2009. We analyzed the clinical and demographic features of these 134 patients. The male to female ratio was 1:1.3. The mean (±SD) age at the time of diagnosis was 28.7 ± 18.5 years, and the mean age at onset of symptoms was 19.6 ± 15.3 years. Thirty-four patients (25.4%) experienced their first attack before the age of 10 years, 50 patients (37.3%) in their teens, and 50 patients (37.3%) after 20 years of age. The mean period from disease onset to diagnosis was 9.1 ± 9.3 years, suggesting a delay in diagnosis.

Clinical Features

Clinical data from 134 patients in the second nationwide survey showed 99 patients (76.2%) with no family history suggestive of FMF. The main clinical findings were present at

TABLE 1. Response Rates and Reported Numbers of FMF Patients

		No. of Departments	Subjects for 1st Survey (n)	(%)	Response Rates (n)	(%)	No. of Departments Reporting FMF Patients	No. of Patients Reported
Internal Medicine	University hospitals	154	154	100.0	83	53.9	9	28
General hospitals	≧500 beds	210	210	100.0	118	56.2	10	14
	400−499 beds	166	133	80.1	74	55.6	1	1
	300−399 beds	327	131	40.1	74	56.5	3	5
	200−299 beds	426	85	20.0	48	56.5	0	0
	100−199 beds	1112	111	10.0	67	60.4	0	0
	≦99 beds	3199	160	5.0	82	51.3	0	0
	Specialized hospitals	26	26	100.0	18	69.2	10	19
	Total	5620	1010	18.0	564	55.8	10	67
Pediatrics	University hospitals	108	108	100.0	82	75.9	17	62
General hospitals	≧500 beds	195	195	100.0	154	79.0	6	6
	400−499 beds	155	123	79.4	93	75.6	4	5
	300−399 beds	298	120	40.3	87	72.5	2	6
	200−299 beds	329	66	20.1	38	57.6	0	0
	100−199 beds	604	60	9.9	37	61.7	0	0
	≦99 beds	1062	53	5.0	35	66.0	0	0
	Specialized hospitals	17	17	100.0	10	58.8	4	6
	Total	2768	742	26.8	536	72.2	33	85
Rheumatology/ Allergy	University hospitals	96	96	100.0	51	53.1	9	9
General hospitals	≧500 beds	46	46	100.0	27	58.7	0	0
	400−499 beds	34	34	100.0	23	67.6	1	4
	300−399 beds	59	59	100.0	32	54.2	1	1
	200−299 beds	80	80	100.0	45	56.3	1	2
	100−199 beds	213	85	39.9	43	50.6	1	0
	≦99 beds	422	85	20.1	49	57.6	1	2
	Specialized hospitals	14	14	100.0	10	71.4	0	0
	Total	964	499	51.8	280	56.1	14	18
Total		9352	2251	24.1	1380	61.3	80	170

the following frequencies: fever (128 patients, 95.5%), abdominal pain (84, 62.7%), chest pain (48, 35.8%), arthritis (42, 31.3%), erysipelas-like erythema (10, 7.5%) and amyloidosis (5, 3.7%). The remaining minor symptoms were pericarditis (3, 2.2%) and headache (17, 12.7%). Febrile attacks, chest pain, and arthritis were comparable between Japanese FMF patients and Mediterranean patients, while abdominal pain and amyloidosis were less prevalent among Japanese FMF patients (Table 3).[3,37,47] AA amyloidosis was confirmed in 5 patients (3.7%) whose genotypes were M694I/M694I, E148Q/E148Q, E148Q/R202Q/P369S/R408Q, and M694I/E148Q/L110P (2 patients) (Table 4).

Colchicine was administered orally to 132 patients, and a favorable therapeutic effect was seen in 122 patients (91.8%). Treatment efficacy was not obtained from the questionnaire survey of 5 patients, and 2 patients had not yet been treated with colchicine. The mean dose of colchicine required to control attacks (0.89 ± 0.45 mg/d) was lower for Japanese FMF patients compared with previous reports of Mediterranean patients.[5]

Mutational Analysis

Among 134 Japanese FMF patients with detailed clinical data, 126 patients underwent MEFV mutation analysis. The MEFV gene mutation was not identified in 17 of 126 patients (13.5%). Of the remaining 109 patients (86.5%), 14 were homozygotes, 66 were compound heterozygotes or had complex alleles, and 29 were heterozygotes. The distribution of the MEFV genotypes in the study group is presented in Table 5. The major detected mutations were homozygous, heterozygous, and compound heterozygous for E148Q, E148Q-L110P, P369S-R408Q, and/or M694I. The most frequent genotype was M694I/E148Q, followed by M694I/normal and M694I/M694I. In consideration of the allelic frequencies, the most common MEFV mutations and polymorphisms among Japanese FMF patients were M694I (29.4%), E148Q (31.3%), L110P (11.5%), P369S (5.6%), and R408Q (5.6%). Moreover, the rare mutations M680I, G304R, R202Q, and E84K were detected in the heterozygous state. It is noteworthy that no

TABLE 2. Estimated Number of FMF Patients in Japan*

Department	No. of Reported Patients	Estimated Patient Number, SE	(95% CI)†
Internal medicine	33	129 ± 45	(40–218)
Pediatrics	33	118 ± 27	(65–172)
Rheumatology/Allergy	14	45 ± 10	(27–64)
Total	80	292 ± 54	(187–398)

Abbreviations: SE = standard error.

*The estimated total number of patients = number of reported patients/(number of responding hospitals/number of target hospitals).

†Ninety-five percent confidence intervals were calculated with an assumption of multinomial hypergeometric distribution.

TABLE 3. Main Clinical Features of Japanese and Mediterranean FMF Patients

Feature	Patients From Japan	Patients From Turkey	Patients From Israel	Arab Patients
No. of patients	134	2838	470	175
Fever, (%)	95.5	92	100	100
Abdominal pain (peritonitis), (%)	62.7	93	95	94
Chest pain (pleuritis), (%)	35.8	31	43	32
Arthritis, (%)	31.3	47	75	33
Skin rash (erysipelas-like erythema), (%)	7.5	21	4	3
Amyloidosis, (%)	3.7	13	27	3
Reference	PR	47	37	3

Abbreviations: PR = present report.

homozygous or heterozygous M694V or V726A mutations were observed in Japanese FMF patients. Mutations of the MEFV gene in exon 10 (M694I, M680I) were detected in 67/126 (53.2%) of FMF patients. These patients showed a significantly higher prevalence of chest and abdominal pain and a lower prevalence of arthritis compared with those without mutations in exon 10. In addition, these patients had a more frequent family history of FMF compared to those without mutations (Table 6). Analysis of the frequency of clinical manifestations between FMF patients without MEFV mutations and those with mutations showed no statistical difference: peritonitis (58.8% vs. 63.3%), pleuritis (23.5% vs. 40.4%) and arthritis (52.9% vs. 29.4%), respectively. Similarly, there was no statistical difference in the dose of colchicine (1.02 ± 0.71 mg/d vs. 0.85 ± 0.40 mg/d) and age at onset (17.3 ± 15.9 yr vs. 19.2 ± 14.7 yr) between FMF patients without MEFV mutations and those with mutations.

DISCUSSION

FMF is considered a common hereditary autoinflammatory disease among Mediterranean populations;[2] however, the true prevalence of FMF has not been elucidated in East Asia. To our knowledge, this was the first nationwide survey of the prevalence of FMF in East Asia. The estimated number of Japanese patients with FMF was approximately 300 (95% CI, 187–398 people). In a second survey, we obtained clinical information from 134 FMF patients, currently the largest survey of Japanese FMF patients. Based on these data, we further identified the spectrum of clinical features, MEFV mutations, and genotype/phenotype correlations in Japanese FMF. The age of onset among Japanese FMF patients was significantly different from Mediterranean populations, where 90% of patients developed FMF before 20 years of age compared with 63% of Japanese patients.[4] The incidences of clinical symptoms during febrile attack were relatively similar between Japanese and Mediterranean FMF patients except for peritonitis and amyloidosis. The prevalence of abdominal pain (62.7%) and amyloidosis (3.7%) was lower in Japanese patients than in Mediterranean FMF patients.[3,37] Genetic factors (ethnicity and spectrum of MEFV mutations) and related disease severity could contribute to the differences in the age at onset and incidence of abdominal pain and amyloidosis between Japanese and Mediterranean FMF patients.

Two patients with AA amyloidosis (40%) exclusively carried MEFV gene mutations in exon 2 or 3, which are considered low-risk mutations for FMF-related AA amyloidosis.[43] This supports the concept that the phenotype or genotype of FMF does not necessarily predict the development of amyloidosis.[45] However, recurrent or long-standing subclinical inflammation may have contributed to the association of amyloidosis in these patients. The Turkish FMF Study Group reported that the mean period from disease onset to diagnosis was 6.9 ± 7.7 years.[47] The mean delay from disease onset to diagnosis of Japanese FMF was 9.0 years, thus putting undiagnosed patients at risk of secondary amyloidosis due to recurrent inflammation, which is a significant public health issue. The mainstay of treatment for FMF is daily oral colchicine, which decreases the frequency and intensity of attacks and prevents the development

TABLE 4. Demographic Features of Patients With AA Amyloidosis

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex	M	F	M	M	M
Current age (yr)	46	34	51	50	44
Age at onset (yr)	23	20	20	30	15
Age at diagnosis of FMF (yr)	43	28	51	50	43
Delay in diagnosis of FMF (yr)	20	8	31	20	28
ESRD	(+)	(−)	(−)	(−)	(−)
Current treatment	Colchicine (1 mg/d)	Prednisolone (colchicine was discontinued due to AE)	Colchicine (1 mg/d)	Colchicine (1 mg/d)	Colchicine (1 mg/d)
MEFV genotype	M694I/E148Q/L110P	E148Q/E148Q	E148Q/R202Q/P369S/R408Q	M694I/M694I	M694I/E148Q/L110P
SAA1 genotype	1.3/1.5	NT	1.3/1.5	1.5/1.5	1.1/1.1

Abbreviations: AE = adverse effect, ESRD = end-stage renal disease, NT = not tested.

TABLE 5. Genotype of 126 Japanese FMF Patients

Mutation	Patients No.	Patients (%)
M694I/M694I	8	6.3
M694I/normal	16	12.7
M694I/E148Q	25	19.8
M694I/L110P	2	1.6
M694I/E148Q/L110P	14	11.1
M694I/E148Q/E148Q/L110P/L110P	1	0.8
M680I/E148Q/L110P	1	0.8
E148Q/E148Q	1	0.8
E148Q/E148Q/L110P	2	1.6
E148Q/E148Q/P369S/R408Q	2	1.6
E148Q/normal	8	6.3
E148Q/L110P	7	5.6
E148Q/R202Q	2	1.6
E148Q/G304R	1	0.8
E148Q/S503C	1	0.8
E148Q/L110P/R202Q	1	0.8
E148Q/P369S/R408Q	5	4.0
E148Q/R202Q/P369S/R408Q	1	0.8
E148Q/G304R/P369S/R408Q	1	0.8
R202Q/normal	1	0.8
S503C/normal	1	0.8
E84K/normal	3	2.4
P369S/R408Q	5	4.0
(−)	17	13.5
Total	126	

of amyloidosis. The normal adult dose of colchicine is 1.2–1.8 mg/d and leads to clinical improvement in more than 90% of patients.[12] Pras et al[33] reported that 30% of North African Jewish patients required a minimum colchicine dose of 2 mg/d to control symptoms. In the current study, Japanese patients with FMF were treated with a relatively low dose of colchicine (mean, 0.89 ± 0.45 mg/d), which had a therapeutic efficacy of 91.8% of Japanese FMF patients.

In the current study we investigated the spectrum of MEFV mutations and the genotype/phenotype correlation in Japanese FMF patients registered in the first nationwide survey. The mutations associated with the most severe phenotype were located in exon 10 of the MEFV gene. This encodes the C-terminal pyrin domain, B30-2/SPRY, the binding site of caspase-1.[8] The mutations in exon 10 of the MEFV gene, M694V, V726A, and M694I, are predominant in Mediterranean FMF patients.[15] Our data showed heterogeneous MEFV genotypes consisting of M694I, E148Q, L110P/E148Q, and P369S/R408Q in Japanese FMF patients. M694V and V726A are the most common mutations of Mediterranean FMF patients.[45] However, these mutations were not found in Japanese FMF patients. The founder effect is likely to be related to the biased MEFV mutation spectrum of Japanese FMF patients. An intriguing finding in FMF is the differing penetrance associated with certain mutations or polymorphisms. The E148Q mutation has low penetrance and is described as a polymorphism due to its high carrier rate, but demonstrates a lack of phenotype in some homozygous patients.[40] However, in our nationwide survey, patients carrying the E148Q mutation exhibited the typical FMF phenotype. The incomplete penetrance of mutations in exon 2 or exon 3 of the MEFV gene suggests the presence of other genetic factors or environmental factors that could influence the disease expression. Although the classical MEFV mutations in exon 10 are characterized by high penetrance, the carrier rate of MEFV exon 10 mutations among Japanese healthy controls is extremely rare.[38] Therefore, the diagnostic significance of their presence, even when heterozygous, is very pronounced in Japanese patients.

In the present study we also attempted to evaluate the genotype/phenotype correlation in Japanese FMF patients. FMF patients with exon 1, 2, or 3 mutations or no mutations comprised a genetically distinct phenotype compared to those with MEFV exon 10 mutations. These patients were positive for various mutated alleles, such as E84K, E148Q, P369S, R202Q, and were more likely to have nonspecific musculoskeletal symptoms including arthralgia. In contrast, the frequency of serositis (chest pain and abdominal pain) was significantly lower compared to those carrying MEFV exon 10 mutations. MEFV gene mutations located in exon 3 have been shown to be responsible for a variant form of FMF or atypical clinical manifestations of FMF.[6,34] These findings may partly explain the observation that FMF patients with mutations in other exons of the MEFV gene present with diverse clinical manifestations compared to FMF patients with exon 10 mutations. Previous genotype/phenotype correlation studies have suggested that mutations located within exon 10, that is, M694V, are associated with severe disease and the frequent occurrence of amyloidosis.[14,16] In contrast, mutations in exon 2, such as E148Q, were associated with milder disease with no amyloidosis.[43] The frequencies of exon 2 (E148Q, E148Q/L110P) mutations are relatively high in Japanese patients compared to white patients,[27] and may reflect the milder form of phenotype observed. We also demonstrated a relationship between the M694I mutation and a higher prevalence of serositis and familial aggregation in Japanese patients with FMF.

TABLE 6. Genotype-Phenotype Correlation in Japanese FMF Patients

Clinical Feature	Total (n = 126)	Patients With *MEFV* Exon 10 Mutations (n = 67) No. (%)	Patients Without *MEFV* Exon 10 Mutations (n = 59) No. (%)	P
Abdominal pain	79 (62.7)	50 (74.6)	29 (49.2)	0.001
Chest pain	48 (38.1)	40 (59.7)	8 (13.6)	0.0001
Arthritis	41 (32.5)	15 (22.4)	26 (44.1)	0.021
Myalgia	15 (11.9)	7 (10.4)	8 (13.6)	0.641
Amyloidosis	5 (4.0)	3 (4.5)	2 (3.4)	0.560
Age at onset (yr)	19.1 ± 15.1	17.9 ± 11.6	20.6 ± 18.3	0.915
Male/female	53/73	34/33	19/40	0.035
Family history	32 (25.4)	24 (35.8)	8 (13.6)	0.004