研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
原　寿郎	免疫疾患	原寿郎/高橋孝雄/細井創	標準小児科学　第8版	医学書院	東京	2014	258-79
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井田弘明	自己炎症症候群	最新医学	68	2561-2569	2013
中野倫代, 神戸直智	Blau症候群／若年発症サルコイドーシス研究の現状と展望	日本臨床	71	737-41	2013
江原瑞枝, 神戸直智	序 ～自己炎症症候群の概説と現在提唱されている定義・分類～	アレルギー・免疫	20	1395-8	2013
神戸直智	皮膚から診断する全身疾患 皮膚所見から考える自己炎症症候群	日皮会誌	123	2826-8	2013
荻野篤彦、金澤伸雄、古江増隆	皮膚を編む―小児掌蹠丘疹性皮膚炎（砂かぶれ様皮膚炎）や自己炎症性症候群の臨床と病態	ラジオNIKKEI マルホ皮膚科セミナー特別番組「明日の治療指針」		印刷中	
金澤伸雄	中條－西村症候群：和歌山発・プロテアソーム不全による新しい自己炎症疾患	日本臨床皮膚科医会近畿ブロック会誌		印刷中	
金澤伸雄	中條 - 西村症候群	分子リウマチ治療	7	25-29	2014
金澤伸雄	サルコイドーシス	Monthly Book Derma「肉芽腫のすべて」	204	15-23	2013
金澤伸雄	日本で見出された自己炎症疾患―中條－西村症候群―	皮膚アレルギー・接触皮膚炎学会雑誌	7	158-168	2013
金澤伸雄	中條 - 西村症候群	アレルギー・免疫	20	1456-1462	2013
森尾友宏	好中球過剰活性化制御機構と炎症	炎症と免疫	21	345-351	2013
武井修治	自然免疫と適応免疫のクロストーク～SLEにおける自然免疫の機能不全	臨床とウイルス		印刷中	2014
武井修治	若年発症サルコイドーシス/Blau症候群	アレルギー・免疫	30	1438-1446	2013
右田清志, 和泉泰衛, 野中文陽, 江口勝美	日本人における自己炎症疾患関連遺伝子の異常. 特集<Basic Science> リウマチ・膠原病のゲノム解析update.	炎症と免疫.	21(5)	401-409	2013
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今井耕輔	原発性免疫不全症の最新国際分類	アレルギー科	58	446-466	2012
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研究成果の刊行物・別冊

ARD Online First, published on December 10, 2013 as 10.1136/annrheumdis-2013-204361

EXTENDED REPORT

Somatic *NLRP3* mosaicism in Muckle-Wells syndrome. A genetic mechanism shared by different phenotypes of cryopyrin-associated periodic syndromes

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Handling editor Tore K Kvien

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2013-204361).

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Received 27 July 2013
Revised 16 October 2013
Accepted 24 November 2013

To cite: Nakagawa K, Gonzalez-Roca E, Souto A, *et al. Ann Rheum Dis* Published Online First: [*please include* Day Month Year] doi:10.1136/annrheumdis-2013-204361

ABSTRACT

Familial cold autoinflammatory syndrome, Muckle-Wells syndrome (MWS), and chronic, infantile, neurological, cutaneous and articular (CINCA) syndrome are dominantly inherited autoinflammatory diseases associated to *gain-of-function NLRP3* mutations and included in the cryopyrin-associated periodic syndromes (CAPS). A variable degree of somatic *NLRP3* mosaicism has been detected in ≈35% of patients with CINCA. However, no data are currently available regarding the relevance of this mechanism in other CAPS phenotypes.

Objective To evaluate somatic *NLRP3* mosaicism as the disease-causing mechanism in patients with clinical CAPS phenotypes other than CINCA and *NLRP3* mutation-negative.

Methods *NLRP3* analyses were performed by Sanger sequencing and by massively parallel sequencing. Apoptosis-associated Speck-like protein containing a CARD (ASC)-dependent nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) activation and transfection-induced THP-1 cell death assays determined the functional consequences of the detected variants.

Results A variable degree (5.5–34.9%) of somatic *NLRP3* mosaicism was detected in 12.5% of enrolled patients, all of them with a MWS phenotype. Six different missense variants, three novel (p.D303A, p.K355T and p.L411F), were identified. Bioinformatics and functional analyses confirmed that they were disease-causing, *gain-of-function NLRP3* mutations. All patients treated with anti-interleukin1 drugs showed long-lasting positive responses.

Conclusions We herein show somatic *NLRP3* mosaicism underlying MWS, probably representing a shared genetic mechanism in CAPS not restricted to CINCA syndrome. The data here described allowed definitive diagnoses of these patients, which had serious implications for gaining access to anti-interleukin 1 treatments under legal indication and for genetic counselling. The detection of somatic mosaicism is difficult when using conventional methods. Potential candidates should benefit from the use of modern genetic tools.

Cryopyrin-associated periodic syndromes (CAPS) are a group of autoinflammatory diseases that include familial cold autoinflammatory syndrome, Muckle-Wells syndrome (MWS), and chronic, infantile, neurological, cutaneous and articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease (NOMID).[1] Some clinical features are shared by almost all CAPS phenotypes (ie, onset during childhood, an urticaria-like skin rash) whereas others are restricted to certain phenotypes (ie, serum amyloid A protein (AA) amyloidosis in MWS, destructive arthropathy in CINCA-NOMID).[1] CAPS are caused by dominantly inherited or de novo *NLRP3* mutations.[2–4] This gene encodes for cryopyrin, a component of one of the cytosolic complexes named inflammasomes that generate the active form of interleukin 1ß (IL-1ß).[5] Previous studies showed a *gain-of-function* behaviour for those *NLRP3* mutations associated with CAPS because they provoke an uncontrolled IL-1ß overproduction, representing the basis from which to treat these patients with anti-IL-1 drugs.[3 6] Genetic heterogeneity was suggested in CINCA-NOMID because only ≈55% of patients was *NLRP3* mutation-positive.[3 4] The use of novel genetic methods recently detected somatic *NLRP3* mosaicism in ≈35% of patients with CINCA-NOMID.[7 8] However, no data are currently available about the role of this genetic mechanism in other CAPS phenotypes because genetic heterogeneity has hitherto been scarcely reported in previous studies.

We herein show the causal role of somatic *NLRP3* mosaicism in patients with MWS, in whom previous studies did not detect *NLRP3* mutations, suggesting that this genetic mechanism is shared among the different CAPS phenotypes.

PATIENTS AND METHODS

Patients

For this study we enrolled patients with a clinical suspicion of CAPS, with a phenotype of MWS and overlapping syndromes, and *NLRP3* mutation-negative in previous studies. The clinical inclusion criteria were the presence of an urticaria-like skin rash and at least one of the following symptoms: recurrent fever, recurrent arthritis, recurrent aseptic meningitis, sensorineural deafness or AA amyloidosis (see online supplementary table S1 for details). All patients with a CINCA-NOMID phenotype were excluded. The patients' data were collected by direct interviews and chart reviews. Written informed consent from patients (or patients' parents if younger than 18-years-old) was obtained at each institution. The ethics committees of Hospital Clinic, Barcelona and the Graduate School of Medicine, Kyoto University approved this study, which was conducted in accordance with the Helsinki Declaration.

NLRP3 analyses

These analyses were performed in the Graduate School of Medicine, Kyoto University or in the Hospital Clínic, Barcelona. Genomic DNA was obtained from whole peripheral blood using QIAmp DNA Blood Mini Kit (QIAgen, Germany). For Sanger sequencing all exons of *NLRP3* gene were amplified by PCR using the primers and conditions previously described.[2] The PCR amplicons were purified with Illustra ExoStar 1-Step kit (GE Healthcare, USA), bidirectional fluorescence sequencing using ABI BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and run on an automated ABI 3730XL DNA analyzer. For massively parallel DNA sequencing, all exons of *NLRP3* gene were amplified as previously described.[8] Library preparation and emulsion PCR were performed according to manufacturer's instructions. All sequencing runs were performed on the GS Junior 454 Sequencer using the GS Junior Titanium Sequencing kits (Roche, Switzerland). The obtained sequences were analysed using the Amplicon Variant Analyzer software.

Bioinformatics analyses

In silico sequence analyses were performed using two different algorithms. The Sorting Intolerant from Tolerant is a sequence homology based tool that predicts whether the amino acid substitution is or is not probably damaging by reporting a score. The PolyPhen-2 is a tool for prediction of the possible impact of an amino acid substitution on the structure and function of a protein, and qualitatively appraised as benign, possibly damaging or probably damaging.[9 10]

Functional studies

The functional consequences of the novel *NLRP3* variants were evaluated in two in vitro assays.[11] Wild type and mutant *NLRP3* cDNA, obtained by mutagenesis PCR, were subcloned into the expression vectors pEF-BOSEX and pcDNA5/TO (Invitrogen, USA). The Apoptosis-associated Speck-like protein containing a CARD (ASC)-dependent nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) activation was evaluated using a dual-luciferase reporter assay in HEK293FT cells transfected with *NLRP3*-pEF-BOSEX plasmids with a NF-kB reporter construct (pNF-kB-luc, BD Biosciences) and an internal control construct (pRLTK, Toyo Ink) in the presence or absence of ASC-expression plasmid. To evaluate the necrosis-like cell death, the THP-1 cell line (a human monocytic cell line derived from a patient with acute monocytic leukemia) was transfected with green fluorescent protein (GFP)-tagged *NLRP3*-pcDNA5/TO plasmids. After 4 h, cells were stained with 7-aminoactinomycin D and cell death of GFP positive cell was analysed by FACS Caliber (Becton-Dickinson).

Statistical analyses

Continuous variables are presented as the mean±SD or as the median and IQR, while categorical variables are presented as numbers, ratios and/or percentages. To detect potential differences among patients with germline mutations and with somatic mutations, the Mann-Whitney U test was used for continuous variables and Fisher's exact test was used for categorical variables.

RESULTS

Genetic analyses

Fifty-six patients (23 Japanese and 33 Spanish) who fulfilled the inclusion criteria were enrolled. Sanger sequencing of the *NLRP3* gene did not identify mutations in any patients. However, small peaks with reduced signal intensities compared with controls were detected in two patients: the A-to-C transversion at c.908 position in Patient 1 and the A-to-G transition at c.1000 position in Patient 2, which encode for the p.Asp303Ala and p.Ile334Val cryopyrin variants, respectively (figure 1A and table 1). Massively parallel DNA sequencing was performed in all patients and revealed somatic *NLRP3* mosaicism in seven patients (7/56; 12.5%). Six different nucleotide changes, all of them located in the exon 3, were detected, and their frequency varied notably among patients, ranging from 5.5% to 34.9% (table 1). All *NLRP3* variants encode for non-synonymous amino acid changes, three of them being novel (p.Asp303Ala, p.Lys355Thr and p.Leu411Phe) and the remainder already described (p.Ile334Val, p.Phe523Leu and p.Glu567Lys) (figure 1B). In Patient 4 the frequency of the mutated *NLRP3* allele remained identical in blood samples obtained over an 8-year period (table 1).

Bioinformatics and functional analyses

All missense *NLRP3* variants were predicted to be possibly or probably damaging to cryopyrin structure and/or function according to at least one of the two algorithms employed, with the only exception of p.Glu567Lys variant (table 1). Interestingly, this *NLRP3* variant was twice detected in the unrelated patients with somatic mosaicism, and has also been reported in other patients with CAPS, reasonably supporting its pathogenic effect.[7 11] We did not find any of the detected *NLRP3* variants in two groups of ethnically matched healthy individuals (Japanese controls n: 200 chromosomes; Spanish controls n: 500 chromosomes) nor in the database National Center for Biotechnology Information (NCBI) single nucleotide polymorphism database (dbSNP) Build 137 (table 1), reasonably ruling out that they could be rare gene polymorphisms.

Finally we evaluated their functional consequences by two different in vitro assays. The results showed that all *NLRP3* variants induced ASC-dependent NF-κB activation (figure 1C) and necrosis-like programmed cell death of THP-1 cell line (figure 1D) at a similar or higher level than those induced by other well-known disease-causing mutations (p.Arg260Trp, p.Asp303Asn and p.Tyr570Cys). Altogether, these data clearly support a pathogenic effect for all *NLRP3* mutations detected as somatic mutations in the enrolled patients.

Clinical features of patients with somatic *NLRP3* mosaicism

At the time of inclusion in the study, the clinical diagnosis of patients with somatic *NLRP3* mosaicism was compatible with MWS. Neither consanguinity nor familial history of the disease

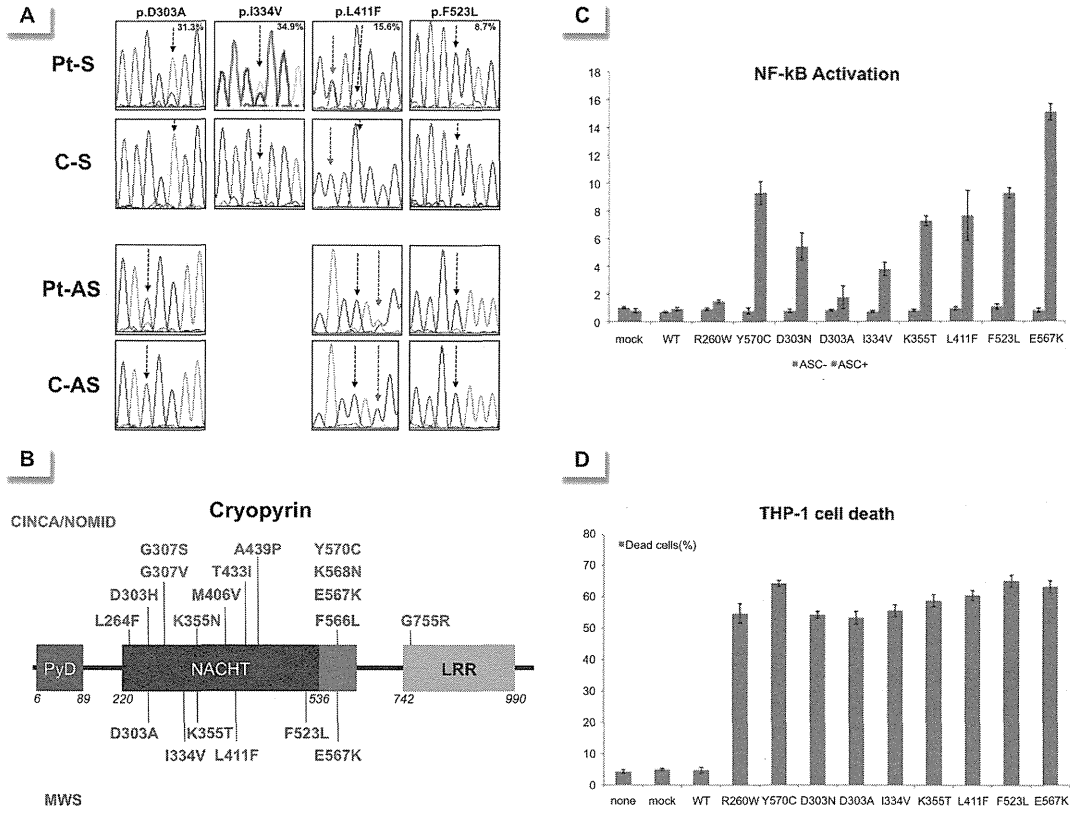

Figure 1 (A) Sense (upper rows) and antisense (bottom rows) chromatograms from four patients with somatic *NLRP3* mosaicism and controls obtained by Sanger sequencing using genomic DNA extracted from whole blood. The black arrows show the *NLRP3* positions where the somatic mutations were detected. The percentage in the upper panels represents the frequency of the mosaicism obtained by massively parallel DNA sequencing in each patient. The red arrows indicate the c.1231 C>T *NLRP3* polymorphism (rs#148478875). (B) Structural organisation of cryopyrin. Above the protein structure are indicated all missense cryopyrin variants that have been detected as somatic mutations in patients with chronic, infantile, neurological, cutaneous and articular (CINCA)-neonatal-onset multisystem inflammatory disease (NOMID) in previous reports, and those below the protein structure are the missense variants detected as somatic mutations in the present study. (C) ASC-dependent NF-kB activation and (D) necrotic THP-1 cell death, induced by the detected *NLRP3* mutations. Values are the mean±SD of triplicate experiments, and data are representative of two independent experiments. AS, antisense; ASC, Apoptosis-associated Speck-like protein containing a CARD; C, control; LRR, leucine-rich repeat; mock, vector without *NLRP3*; MWS, Muckle-Wells syndrome; NACHT, a family of NTPases that originally included the NAIP, CIITA, HETE-E and TP-1 proteins; NF-kB, nuclear factor kappa-light chain-enhancer of activated B cells; None, nothing transfected; Pt, patient; PyD, pyrin domain; S, sense; WT, wild type *NLRP3*.

was reported in any of them. The inflammatory disease started during their infancy or childhood (median: 4 years; IQR: 1.3–9.0 years), with an urticaria-like skin rash and a marked inflammatory acute response as the main features at that time (see table 2 for clinical details at the disease onset).

All patients referred to the chronic course of their disease, with variable disease evolution (median: 20 years; IQR: 12–26 years). During this time, recurrent arthritis (6/7; 85.7%), headache (5/7; 71.4%) and recurrent conjunctivitis (4/7; 57.1%) mainly added to those features detected at the disease onset. None of these patients developed AA amyloidosis, whereas five of them (71.4%) developed progressive bilateral sensorineural deafness (see table 3 for a detailed summary of clinical features detected during the course of the disease).

Outcome of anti-IL-1 blockade

Five patients with somatic *NLRP3* mosaicism were treated with anti-IL-1 drugs. Only Patient 5 was treated with anakinra (100 mg/24 h subcutaneous for a duration of 20 months). Three patients only received canakinumab: Patient 2 (150 mg/8 weeks subcutaneous for a duration of 13 months), Patient 3 (2 mg/kg/8 weeks subcutaneous for a duration of 16 months) and Patient 6 (initial dose of 150 mg/4 weeks, subsequently increased up to 300 mg/4 weeks, for a duration of 14 months). Patient 7 was first treated with anakinra (1 mg/kg/24 h subcutaneous for a duration of 24 months) and subsequently switched to canakinumab (150 mg/8 weeks subcutaneous for a duration of 14 months). All patients showed a marked and sustained improvement while treated with anti-IL-1 drugs, with a complete remission of urticaria-like skin rash (5/5), fever (3/3), conjunctivitis (2/2) and aseptic meningitis (1/1), and marked benefits for arthritis (complete response in 75%) and headache (complete response in 75%, and marked improvement in 25%). Inversely, IL-1 blockade did not improve the sensorineural deafness (0/4). The clinical improvement was associated with sustained reductions of erythrocyte sedimentation rate and C reactive protein level, and normalisation of white blood cell, neutrophil and platelets counts, and haemoglobin level (see figure 2 for details).

Comparative phenotype analyses

To identify potential clinical differences among patients with germline or with somatic *NLRP3* mutations two cohorts of

Table 1 Summary of genetic data of patients with somatic *NLRP3* mosaicism

Pt (Country)	Phenotype	Massively parallel DNA sequencing: Nucleotide exchange*	Amino acid exchange	Mutated allele frequency	Coverage	Bioinformatics analyses: SIFT	PolyPhen-2	Population genetics†	Reference	Analysed relatives: Kinship	Results
1 (Spain)	MWS	c.908 A>C	p.D303A	31.3%‡	622×‡	Damaging	Probably damaging	Absent	Present Study	n.d.	n.d.
2 (Japan)	MWS	c.1000 A>G	p.I334V	34.9%‡	1060×‡	Damaging	Benign	Absent	12	Father	Negative§
										Mother	Negative§
3 (Japan)	MWS	c.1064 A>C	p.K355T	20.2%‡	100×‡	Tolerated	Probably damaging	Absent	Present Study	n.d.	n.d.
4¶ (Spain)	MWS	c.[1231 C>T; 1233 G>T]	p.L411F	14.4%‡	590×‡	Tolerated	Possibly damaging	Absent	Present Study	Mother	Negative§
4** (Spain)	MWS	c.[1231 C>T; 1233 G>T]	p.L411F	15.6%‡	870×‡	Tolerated	Possibly damaging	Absent	Present Study	Mother	Negative§
5 (Spain)	MWS	c.1569 C>A	p.F523L	8.7%††	569×††	Tolerated	Possibly damaging	Absent	3	Daughter	Negative§
6 (Japan)	MWS	c.1699 G>A	p.E567K	5.6%‡	1211×‡	Tolerated	Benign	Absent	11	n.d.	n.d.
7 (Japan)	MWS	c.1699 G>A	p.E567K	5.5%‡	724×‡	Tolerated	Benign	Absent	11	n.d.	n.d.

*NCBI Reference Sequence NM_001243133.1.
†Data of population genetics obtained from NCBI dbSNP Build 137.
‡Mean of two independent experiments.
§Analyses performed by Sanger sequencing.
¶Blood sample collected in 2002.
**Blood sample collected in 2009.
††Mean of four independent experiments.
MWS, Muckle-Wells syndrome; n.d., not done; Pt, patient; SIFT, Sorting Intolerant from Tolerant.

patients with MWS were compared. The group of patients with MWS with somatic *NLRP3* mosaicism included the seven patients described here whereas the cohort of patients with MWS with germline mutations included 41 patients (13 Japanese and 28 Spanish) from our databases. In this last group the germline status was established by means of pedigree analyses and/or by massively parallel sequencing. As expected, the familial history of the disease was a significant variable between the two groups. No significant differences were detected among the main clinical features (fever, urticaria-like rash, joint, neurological and ocular involvements, and deafness) despite their variable frequency in each group (see table 4 for details). However, patients with somatic *NLRP3* mosaicism seemed to have late onsets of the disease and of the sensorineural deafness, an increased incidence of arthritis and a reduced risk of developing AA amyloidosis, when compared with patients with germline mutations.

DISCUSSION

CINCA-NOMID syndrome represents the severest CAPS phenotype, and is usually a consequence of de novo *NLRP3* mutations. Recent works have established its genetic basis, with ≈55% of patients carrying germline *NLRP3* mutations and ≈35% carrying somatic *NLRP3* mosaicism.[3–4 7 11–16] However, no studies addressing the presence of somatic *NLRP3* mosaicism have been undertaken in other CAPS phenotypes because genetic heterogeneity has been poorly described in them, with only five reported patients with *NLRP3* mutation-negative MWS.[17–19] This scenario prompted us to hypothesise that somatic *NLRP3* mosaicism might be an underlying genetic mechanism in patients with other CAPS phenotypes. For this proposal two ethnically different cohorts of candidates were screened, and 12.5% of them (7/56) carried variable degree of somatic *NLRP3* mosaicism in peripheral blood. Additional evidences, as shown here, definitively support that the detected *NLRP3* variants are pathogenic

Table 2 Summary of clinical features of patients with somatic *NLRP3* mosaicism at the onset of the disease

Pt	Age at disease onset	Cold-exposure trigger	Urticaria-like skin rash	Fever	Joint involvement	CNS involvement	Acute inflammatory response*	First diagnoses
1	18 years	–	Yes	Yes	Arthralgias	–	Yes	
2	2 years	–	Yes	–	Arthralgias	–	Yes	JIA
3	1 week	–	Yes	–	–	–	Yes	Chronic urticaria, So-JIA
4	14 years	–	Yes	Yes	–	–	Yes	Erythema nodosa
5	4 years	Yes	Yes	Yes	Arthralgias	–	Yes	
6	4 years	Yes	Yes	Yes†	Oligoarthritis	–	Yes	Oligo-JIA
7	7 months	-	Yes	Yes	Oligoarthritis	–	n.a.	So-JIA, TRAPS

*Defined by increased values of white blood cells (normal range 4.00–11.00×10^3/dL), circulating neutrophils (normal range 45–75%), platelets (normal range 130–400×10^3/dL), C reactive protein (normal range <1 mg/dL) and/or erythrocyte sedimentation rate (normal <10 mm/h).
†Low-grade fever.
–, absent; CNS, central nervous system; JIA, juvenile idiopathic arthritis; n.a., not available; Pt, Patient; So-JIA, systemic-onset juvenile idiopathic arthritis; TRAPS, TNF receptor-associated periodic syndrome.

Table 3 Summary of clinical manifestations detected in patients with somatic *NLRP3* mosaicism during the course of the disease

Pt	Sex (Age)	Cold-exposure trigger	Urticaria-like skin rash	Joint involvement					CNS involvement			Deafness (age at onset)	Ocular involvement	AA amyloidosis
				Fever	Type of arthritis	Involved joints	Symmetric	Erosive	Arthropathy	Headache	Aseptic meningitis	Papilloedema			
1	M (39 years)	–	Yes	Yes	Polyarthritis	Large and small	–	–	–	–	–	–	Yes (38 years)	Conjunctivitis	–
2	M (14 years)	–	Yes	–	–	–	–	–	–	Yes	Yes	–	Yes (7 years)	–	–
3	F (12 years)	–	Yes	–	Monoarthritis	Large	–	–	–	Yes	–	–	Yes (6 years)	–	–
4	F (41 years)	–	Yes	Yes	Polyarthritis	Small	–	–	–	Yes	–	–	–	Conjunctivitis	–
5	M (64 years)	Yes*	Yes	Yes†	Polyarthritis	Large and small	–	–	–	–	–	–	Yes (45 years)	–	–
6	F (16 years)	Yes†	Yes	Yes	Oligoarthritis	Large	–	–	–	Yes	–	–	–	Conjunctivitis	–
7	M (16 years)	–	Yes	Yes	Oligoarthritis	Large	–	–	–	Yes	–	–	Yes (13 years)	Conjunctivitis	–

*Always.
†Occasionally.
–, No or absent; AA, serum amyloid A protein; CNS, central nervous system; F, female; M, male; Pt, Patient.

and include their absence in panels of ethnically matched controls and in a database of genomic diversity, in silico analyses that predict their damaging effect for the function and/or structure of cryopyrin, and in vitro functional studies that clearly showed its *gain-of-function* behaviour. Taken together these evidences support that somatic *NLRP3* mosaicism is a genetic mechanism shared by different CAPS phenotypes, and it is not restricted to CINCA-NOMID syndrome.

Among *NLRP3* mutations detected 50% (3/6) were novel, representing an unexpected high proportion for a small cohort. Taking into account their consequences on the cryopyrin function it is conceivable to hypothesise that, in germline status, they could be incompatible with life. We have also found a marked variability in the degree of somatic mosaicism among patients, which may have important consequences. For diagnostic purposes the level of somatic mosaicism could be the determining factor in achieving a definitive genetic diagnosis. Those patients with mosaicism around, or higher than, 15% will probably be detected in conventional studies using Sanger's method by means of careful analyses, as we have shown in the patients' chromatograms. However, those patients with frequencies of less than 15% are probably missed by Sanger sequencing and will only be detected by using new technologies that are not currently widely available. The differences of disease severity observed among patients with somatic mosaicism, including those from this study and those from previous reports, could be explained by different and cumulative factors, which probably cannot be independently analysed. These factors might include, at least, the type of amino acid exchange, its location in the cryopyrin, its functional consequence in the normal cryopyrin function, and the degree and tissue distribution of somatic mosaicism. We must also note that all known somatic *NLRP3* mutations seem to be located in some few amino acid residues (303, 355, 567) or in small regions of cryopyrin (303–307, 433–439 and 566–570), probably representing hot spots for these types of mutations. Consequently these regions should be carefully analysed when using Sanger sequencing to identify potential carriers of somatic mosaicism.

All patients with somatic *NLRP3* mosaicism were sporadic patients, with no affected relatives, which is notably different from patients with germline mutations (positive familial history in 65.9%). Their main clinical features were compatible with a MWS phenotype and similar to those previously described in patients with germline mutations, with the potential exceptions of a reduced incidence of AA amyloidosis, an increased incidence of recurrent arthritis, and slightly older ages at the disease onset and also at onset of sensorineural deafness. It is interesting to note that most patients (4/7; 57.1%) were misdiagnosed as having juvenile idiopathic arthritis when the disease started, a similar misdiagnosis previously reported in different inherited autoinflammatory diseases.[20–23] Despite the evidence shown here, the actual frequency of somatic *NLRP3* mosaicism is unknown and probably underestimated. In our study a potential bias in the selection of patients could exist because they were selected on the basis of the presence of an urticaria-like skin rash associated with other symptoms. Recent studies have described atypical CAPS presentations in patients with germline *NLRP3* mutations in whom urticaria-like skin rash was nearly absent.[24 25] These data suggest that clinical diversity of CAPS is probably wider than previously described and further studies are necessary to delineate the profile of potential candidates to carry somatic *NLRP3* mosaicism.

The evidence obtained may have serious implications for patients, especially with regards to treatment and genetic

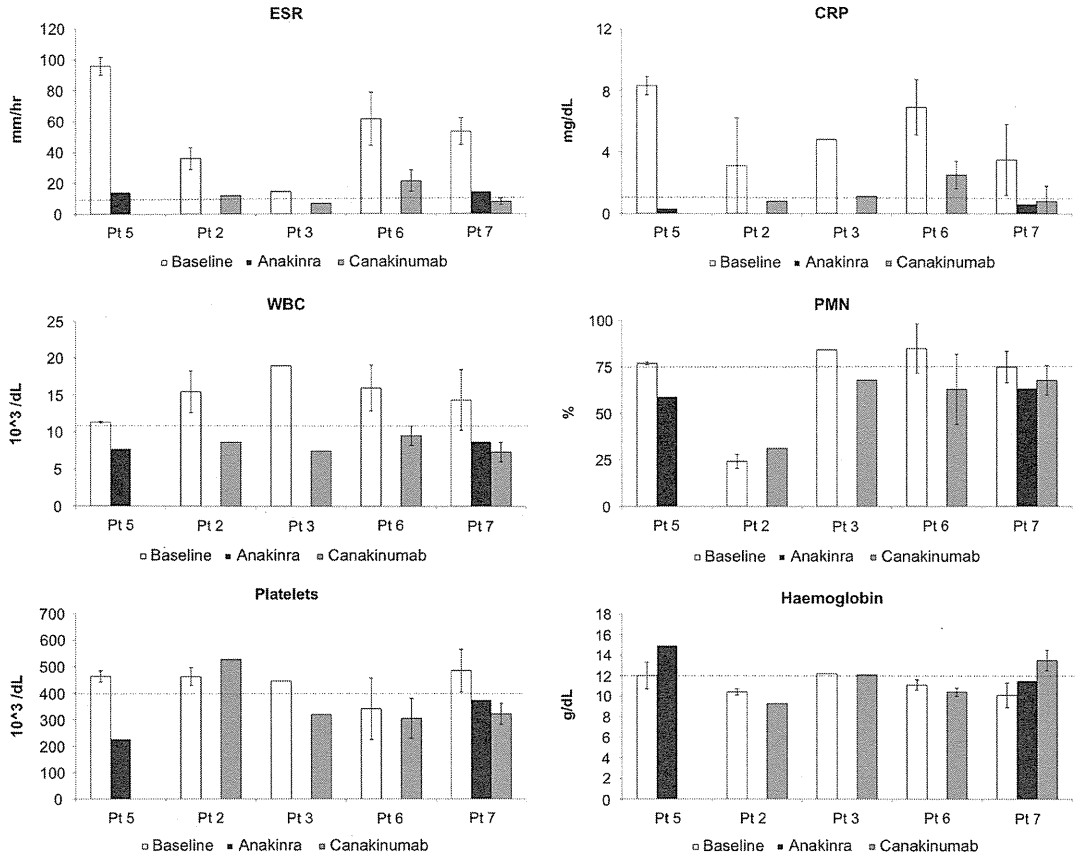

Figure 2 Laboratory values obtained in the five patients treated with different anti-interleukin 1 drugs. Patient's graphics were ordered as follows: First, those graphics from the patient who only received treatment with anakinra (Pt 5), followed by those from patients who only received treatment with canakinumab (Pt 2, 3 and 6) and finally those from the patient who received both treatments (Pt 7). Vertical bars represent the mean±SD of values obtained during treatment periods. Horizontal discontinued lines represent the upper limit of the normal range, with the only exception of the haemoglobin box, in which this line represents the lower limit of the normal range. CRP, C reactive protein; ESR, erythrocyte sedimentation rate; PMN, polymorphonuclears; WBC, white blood cell count.

Table 4 Comparison of main clinical data of patients carrying germline versus somatic *NLRP3* mutations

Clinical features	Patients with germline *NLRP3* mutations (n:41)	Patients with somatic *NLRP3* mutations (n:7)	p Value
Age at disease onset (years)—median (IQR)	0.5 (0.0–4.4)	4.0 (1.3–9.0)	n.s. (p=0.223)
Delay of diagnosis (years)—median (IQR)	33.0 (10–49)	20 (12–26)	n.s. (p=0.416)
Presence of familial history of the disease (%)	65.9	0	p=0.002
Cold exposure as disease triggering factor (%)	36.6	28.6	n.s. (p=1.000)
Fever (%)	63.4	71.4	n.s. (p=1.000)
Urticaria-like skin rash (%)	87.8	100	n.s. (p=1.000)
Joint involvement			
Arthralgias (%)	80.5	85.7	n.s. (p=1.000)
Arthritis (%)	53.7	85.7	n.s. (p=0.214)
Neurological involvement			
Headache (%)	56.1	71.4	n.s. (p=0.683)
Aseptic meningitis (%)	29.3	14.3	n.s. (p=0.656)
Papilloedema (%)	12.2	0	n.s. (p=1.000)
Ocular involvement			
Conjunctivitis (%)	61.0	57.1	n.s. (p=1.000)
Uveitis (%)	17.1	0	n.s. (p=0.573)
Sensorineural deafness (%)	68.3	71.4	n.s. (p=1.000)
Age at onset of deafness (years)—median (IQR)	7.0 (5.5–11)	13.0 (7–38)	n.s. (p=0.210)
AA amyloidosis (%)	17.1	0	n.s. (p=0.573)

Patients with germline mutations were carriers of one of the next *NLRP3* mutations: p.R170S (c.508 C>A), p.R260W (c.778 C>T), p.V262A (c.785 T>C), p.D303N (c.907 G>A), p.H312P (c.935 A>C), p.T348M (c.1043 C>T), p.A439T (c.1315 G>A), p.A439V (c.1316 C>T), p.F443L (c.1329 C>G), p.E567A (c.1700 A>C) and p.Y859C (c.2576 A>G).
AA, serum amyloid A protein; n.s., not significant differences.

counselling. The outcome of IL-1 blockade in patients with somatic *NLRP3* mosaicism was nearly identical to those reported in patients with germline mutations.[26 27] The only symptom that did not improve with IL-1 blockade was the sensorineural deafness. In this regard, apparently contradictory responses have been reported, with improvement or amelioration in some patients and no response in others.[14 17 28–30] It has been suggested that the time of evolution of deafness previous to starting anti-IL-1 drugs could be a determining factor for the type of response, but probably additional and unknown factors could also play a role in this particular manifestation. We have also observed a notable delay in gaining access to anti-IL-1 drugs with respect to the disease onset (median: 20 years; IQR: 12–26 years), because these treatments were administered under legal indication once the definitive CAPS diagnosis was established by means of the identification of somatic *NLRP3* mosaicism. Taking into account the excellent response observed to IL-1 blockade, it is reasonable to hypothesise that if this was started earlier it should have provoked the non-appearance of some severe complications such as deafness.

For an appropriate genetic counselling the scenario is extremely different in patients with CAPS with germline or with somatic mutations. In the case of germline mutations, the risk of transmission to future pregnancies is 50%. Inversely, the prediction of the risk of transmission in cases of somatic mosaicism is more complex, because it may vary in the different tissues, it is not usually determined in gonadal tissues, and its detection probably requires new sensitive genetic methods that are not widely available. The vertical transmission of a somatic mutation is an extremely rare event, with only one case recently described in MWS.[31] Consequently, this possibility should be considered during the genetic counselling of these patients, although one of the main messages to patients is that its probability remains low.

We show that somatic *NLRP3* mosaicism underlies MWS and is probably a shared genetic mechanism in different CAPS phenotypes, and not restricted to CINCA/NOMID syndrome. Its detection was achieved by using massively parallel sequencing, and functional studies confirmed the *gain-of-function* behaviour of the detected variants. The detection of somatic mosaicism has had serious clinical implications for patients, including access to treatment under legal indication, adequate follow-up and ensuring appropriate genetic counselling. Further studies are necessary to delineate the clinical phenotype of candidates to looking for somatic mosaicism, in which new sensitive genetic technologies should be used.

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Acknowledgements The authors thank the patients and their families for their participation in this study.

Contributors KN, TH, JY, RN and JIA designed research, discussed data and wrote the paper. EG-R, ER-O, FR, EI, TY, KI, TK and OO performed genetic and functional investigations, discussed data and reviewed the manuscript. AS, TK, HU, JMC, JC, ST, NK, JLC-R, NO-C, JA, SJ-T, CV, JF-M, IC, JH-R, MM, MTD, MB, SB, MY, TK, RK, NA, KS, NI, MKS and NK provided clinical data and blood samples, discussed data and reviewed the manuscript.

Funding Supported by the Spanish Ministry of Health (FIS PS09/01182), by the Japan's Ministry of Health, Labor and Welfare, and by the Japan's Ministry of Education, Culture, Sports, Science and Technology.

Competing interests None.

Patient consent Obtained.

Ethics approval The ethics committees of Hospital Clinic, Barcelona and the Graduate School of Medicine, Kyoto University approved this study.

Provenance and peer review Not commissioned; externally peer reviewed.

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Somatic *NLRP3* mosaicism in Muckle-Wells syndrome. A genetic mechanism shared by different phenotypes of cryopyrin-associated periodic syndromes

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Ann Rheum Dis published online December 10, 2013
doi: 10.1136/annrheumdis-2013-204361

Notes

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22 *The Open Rheumatology Journal*, 2013, 7, 22-25

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MEFV Variants in Patients with PFAPA Syndrome in Japan

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Abstract: *Background*: The pathogenesis of PFAPA (periodic fever, aphthous stomatitis, pharyngitis, adenitis) syndrome is unknown as yet. In order to understand whether genes implicated in other auto-inflammatory diseases might be involved in the pathogenesis of PFAPA, all variants in the genes causing familial Mediterranean fever (FMF), tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), and Hyper IgD syndrome were analyzed in children with PFAPA.

Patients and Methods: All variants in *MEFV*, *TNFRSF1A*, and *MVK* were analyzed in 20 patients with PFAPA. PFAPA were diagnosed by previous published criteria. The findings of all analyses in PFAPA patients were compared with those of unaffected normal subjects (n=62).

Results: In the 13 children of 20 with PFAPA, the heterozygous variants of *MEFV* (5 patients: *E148Q-L110P*, 2 patients: *E148Q*, 1 patient: *E148Q-L110P/E148Q*, 1 patient: *E148Q-P369S-R408Q-E84K*, 1 patient: *E148Q-L110P-P369S-A408G*, 1 patient: *R202Q*, 1 patient: *P115R*) were found. No variants belonging to *TNFRSF1A* or MVK were detected in children with PFAPA. The frequency of the *E148Q-L110P* variants in children with PFAPA was significantly higher than that observed in unaffected normal subjects (7/20 versus 8/62). The duration of the episodes of illness in PFAPA children with *MEFV* variants was shorter than that of patients without variants.

Conclusion: Genes involved in the development and progression of *MEFV* may affect the incidence and the phenotype of PFAPA in children.

Keywords: PFAPA, *MEFV*, FMF, Variant, Japanese.

INTRODUCTION

In 1987 [1], Marshall firstly described, the PFAPA (periodic fever, aphthous stomatitis, pharyngitis and adenitis) syndrome, which is characterized by recurrent episodes of fever associated with cervical adenitis, pharyngitis and aphthous stomatitis. The prognosis of this disease has been reported to be better than that of another autoinflammatory diseases [2]. Corticosteroids are effective in controlling episodes of illness in PFAPA, but they do not cure the ailments or prevent recurrence of the symptoms of this syndrome [3]. Interventions like tonsillectomy and administration of H_2 blockers have been reported to be partially effective for prophylaxis [3]. However the complete pathogenesis of PFAPA is unknown yet, and hence the therapeutic regimens have not yet been established for PFAPA [4].

Familial Mediterranean fever (FMF) is a recessively inherited disorder characterized by acute attacks of fever, and serositis usually lasting for 1–3 days. FMF is caused by mutations in the ME diterranean FeVer gene (*MEFV),* which encodes the protein pyrin (marenostrin)1[5,6]. Colchicine has been shown to be effective for prophylaxis in only 90% of the patients with FMF [7,8].

Recent studies have described that heterozygous variants of the *MEFV* gene were found in patients with PFAPA [9-11]. However, it still remains unclear whether these variants are the causative factors of PFAPA.

The purpose of this study was to understand whether heterozygous variants of *MEFV* may be associated with the onset of PFAPA. We have also tried to understand whether these mutations act as accessory factors and modify the phenotype of patients with PFAPA.

PATIENTS AND METHODS

Twenty children with frequent PFAPA episodes who visited our pediatric outpatient clinic were consecutively selected over a 5-year period (from January 2005 to January 2010). The diagnosis of PFAPA syndrome was established using previously established criteria [1,3,9]. These criteria include recurring fevers associated with exudative tonsillitis, negative throat culture, and possibly, aphthous stomatitis and cervical lymphadenopathy. The additional clinical criteria included completely asymptomatic intervals between the episodes, normal growth and development and exclusion of

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1874-3129/13

FMF, Behcet' s disease, and cyclic neutropenia. Oral low dose of prednisolone (0.3-0.5mg/kg/dose, 1 or 2 doses per day) was effective on all enrolled patients. *MEFV*, mevalonate kinase (*MVK*), and tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*) genes of all enrolled patients were sequenced. After obtaining a written informed consent approved by the Institutional Review Board of Kyoto University, peripheral blood was collected from all patients, and, if needed, their family members. Genomic DNA was extracted, and all the exons including exon-intron junctions of the *MVK, MEFV,* and *TNFRSF1A* genes were amplified by polymerase chain reaction and then sequenced using the ABI3130.

The results are shown as a mean ± SD or proportion, as appropriate. Differences between the groups in discrete variables were evaluated using Fisher's exact test at 5% significance.

Two-sided *P* values were adjusted for multiplicity using Hochberg's method.

Comparisons of continuous variables were done using unpaired Student's *t*-test. All *P* values given are 2-sided. *P* values less than 0.05 were considered significant. Statistical calculation was conducted by SAS version 9.1.3.

RESULTS

Twenty patients (9 boys, 11 girls) diagnosed with PFAPA were followed up in our clinic. Thirteen of these patients had a single *MEFV* (M^+ group). No variant of *TNFRSF1A* and *MVK* was detected in all patients. The genotypes of the *MEFV* gene in the 13 patients are seen in Table **1**. The most common *MEFV* variant patterns seen were *E148Q-L110P* (5 patients) and *E148Q* (2 patients). One patient was homozygous of *E148Q* and heterozygous of *L110P* of *MEFV*. One patient was heterozygous of *E148Q-P369S-R408Q*. One patient was heterozygous for *E148Q-P369S-R408Q-E84K*. One patient had *E148Q-L110P-P369S-R408Q*. The minor variants, *P115R* and *R202Q*, were detected in 2 patients. More than 2 *MEFV variants* were on 1 allele in all PFAPA patients. In 7 patients, no *MEFV* mutations were found. The allele frequencies of *E148Q*, *L110P*, *P369S, R408Q* and G304R in 20 PFAPA patients were 0.3, 0.175, 0.075, 0.075 and 0, respectively (Table **3**).

Table 1. The Genotypes of *MEFV* Genes of 13 Patients with PFAPA

MEFV Variant	No. of Patients
E148Q-L110P	5
E148Q	2
E148Q-L110P/E148	1
E148Q-P369S-R408Q	1
E148Q-P369S-R408Q-E84K	1
E148Q -L110P-P369S-R408Q	1
R202Q	1
P115R	1

Clinical and laboratory data were compared between *MEFV* positive group (n=13) and negative group (n=7) and are presented in Table **2**. Patients carrying an *MEFV* variant showed shorter duration of episodes of illness than patients without variants (3.6±0.86 days versus 5.3±1.89 days,

Table 2. Clinical Characteristics of PFAPA Patients with Variants in the *MEFV* Gene Compared with those of PFAPA Patients without *MEFV* Variants

	Patients with *MEFV* Variants (n=13)	Patients without *MEFV* Variants (n=7)	*P* Value
Age at onset (years)	2.8±1.9	3.2±1.9	NS
Age at Diagnosis (years)	4.3±2.2	4.9±1.9	NS
Male: female ratio	5/8	4/3	NS
Family history of PFAPA	4/9	3/4	NS
Attack duration (days)	3.6±0.86	5.3±1.89	*P*=0.0174
Interval between attacks (weeks)	4.9±1.59	5.5±0.96	NS
Cyclic periodic attacks	5/13	4/7	NS
Pharyngitis	13/13	7/7	NS
Aphthae	7/13	5/7	NS
Enlarged tonsillitis	13/13	7/7	NS
Abdominal pains	1/13	2/7	NS
Musculoskeletal Pains	1/13	2/7	NS
Headaches	4/13	5/7	NS
WBC/μL	143±41	142±41	NS
ESR mm/h	87±23	72±14	NS
CRP levels mg/dL	6.52±3.53	5.87±2.85	NS

NS: not significant.

p=0.0174). No significant differences in all other clinical and laboratory data were found between the 2 groups (Table **2**).

We also analyzed all sequences of *MEFV* genes in normal Japanese subjects (n=62). These individuals were healthy adult volunteers and had no recurrent episodes of fever. There was no difference in recruitment between the PFAPA patients and the control group. A comparison of these results between normal and PFAPA subjects is shown in Table **3**. In normal individuals, no significant allele frequencies were observed for the 4 variants found in PFAPA patients. In addition, the frequencies of *E148Q-L110P* and *P369S-R408Q* in the 2 groups were compared. A significant difference in the frequency of these variants was observed between the 2 groups (Table **4**, p = 0.043 and p = 0.026, respectively).

Table 3. Allele Frequencies of *MEFV* Variants in PFAPA Subjects and Normal Unaffected Subjects

Variant	PFAPA Subjects (n=40)	Unaffected Subjects (n=124)	*P* Value
E148Q	30.0%	18.5%	NS
L110P	17.5%	6.5%	NS
G304R	0.0%	3.2%	NS

NS: not significant.

Table 4. Frequencies of *MEFV* Variants in PFAPA Subjects and Normal Unaffected Subjects

Variant	PFAPA Subjects (n=20)	Unaffected Subjects (n=62)	*P* Value
E148Q-L110P	35%	13%	*P*=0.043

DISCUSSION

We studied 20 patients with PFAPA who were diagnosed by Marshall criteria [1]. Our aim was to access the roles of the predominant variants in genes that cause other febrile illnesses like FMF, TNF receptor-associated periodic syndrome (TRAPS) and the MVK deficiency. We did not find any incidence of variants of TRAPS and MVK deficiency in PFAPA patients. However several heterogeneous variants of *MEFV* were detected in 13 out of 20 patients with PFAPA. We analyzed the frequency of the *E148Q-L110P* and *P369S-R408Q* variants in PFAPA and control subjects. Our analyses indicate that the incidence of these 2 variants is significantly higher in patients with PFAPA than in normal individuals.

Amongst autoinflammatory disease, only PFAPA syndrome has been described as a non-inherited syndrome, since familial inheritance has not been reported in previous studies [3,12,13]. However some studies have reported familial cases that included siblings or a sibling and the sibling's mother [14-16]. Therefore, the hereditary nature of this syndrome is still a matter of debate. With respect to the genetic factors that may cause the PFAPA syndrome, one study has strongly argued against the involvement of the *MEFV* gene [10], but another article [11] has shown that mutations of the *MEFV* genes were found in 27% of cases diagnosed with PFAPA syndrome on the basis of Marshall's clinical criteria. Our observations of the high frequency (65%) of *MEFV* variants are in agreement with that reported by Dagan [11]. The differences in the findings may be attributed to the ethnic differences between the individuals studied, the small sized of the study, and the study population that was selected.

The *L110P* variant, which is located in exon 2, was first reported in FMF patients in 2000 [17], and to date, several compound heterozygotes with other variants have been reported even in Japan [18,19]. In contrast, although the role of the *E148Q* variant, which is located in exon 2, in FMF patients was controversial, a recent study concluded that the variant is just a benign polymorphism [20]. In a Japanese study [18] of FMF patients, the most frequently observed *MEFV* variants observed were *E148Q/M694I* (25.0%), *M694I* (17.5%), and *L110P/E148Q/M694I* (17.5%). However, no patients had the *M694V* variant. These patterns are quite different from those in Mediterranean patients with FMF. The study also reported that the allele frequencies of *E148Q*, *M694I*, and *L110P* were 0.44, 0.35, and 0.31, respectively, and that these frequencies were significant difference from those seen in healthy controls. In our study, the allele frequencies of *E148Q*, *L110P*, and *G304* were 0.3, 0.175, and 0, respectively, and these frequencies did not differ significantly between patients and healthy controls. No mutation at the *M694I* was detected in our cohorts of patients and controls. The allele frequency of *L110P* is higher in patients with PFAPA than in healthy controls; however, the difference is not significant. The frequency of the *E148Q-L110P* variant combination is significantly higher in the PFAPA group than in the healthy control group. If the *E148Q* variant is non-functional, the L110P variant may be associated with the onset of PFAPA syndrome.

In several types of inflammatory such as Behcet's disease [21], Crohn's disease [22], ulcerative colitis [23], Henoch-Schönlein purpura [24,25] and the co-incidence of FMF variants has been investigated. These studies show increased incidence of genes involved in FMF in patients with these autoinflammatory diseases as well as the increased severity of the symptoms of each disease. On the other, in the patients with asthma, the incidence of FM mutation was decreased and the lower incidence correlated with reduced severity of symptoms [26]. Thus, FMF variants may affect the transition from Th2 to Th1 polarity in each disease. According to Berkun's study [27], PFAPA episodes in carriers of *MEFV* variants were shorter compared to those in patients without variants. In *MEFV* variant-positive patients, the regular cyclic pattern of attacks and the occurrence of oral aphthae was lower than those in patients without *MEFV* variants. In the present study, we found that the only affected variable was the duration of PFAPA episodes. Although no significant differences were observed in the regular cyclic pattern of attacks and the occurrence of oral aphthae between the 2 groups, the duration of PFAPA episodes was shorter in the variant-positive group than in the variant-negative group.

Taken together, these results show that the *MEFV* gene may not affect the onset of several autoinflammatory diseases, but is likely to modify the intensity and the displayed phenotype in terms of disease symptoms.