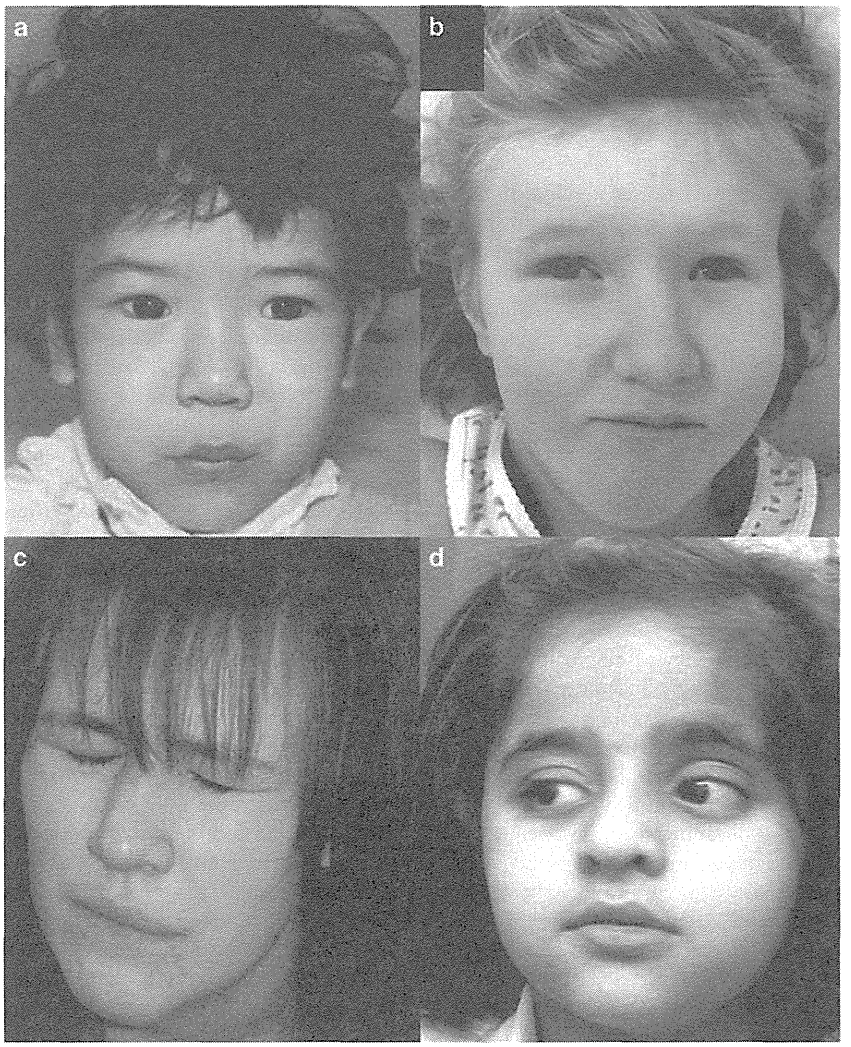

Figure 2 Facial findings of the patients in this study. (**a**) Patient 1 at 3 years of age, (**b**) patient 2 at 7 years of age, (**c**) patient 3 at 19 years of age and (**d**) patient 5 at 8 years of age. Similar facial findings including triangle face and a prominent jaw are shared among patients 1–3. Written informed consents for publication of the patients' photographs were obtained from the guardians of all patients.

DISCUSSION

In the present study, we conbined the genetic and clinical information of five female patients with deletions within Xq22, and studied the correlation between their genotypes and phenotypes (Figure 1). Three of the five patients (patients 1–3) have similar large deletions, ranging from Xq22.1 to Xq22.3, over 3 Mb in size, and commonly manifested severe intellectual disability, hypotonia and behavioral problems (Table 1). Although patient 2 had a family history of an unknown neuromuscular disease, she did not show any muscular phenotypes and rather showed phenotypes shared with the other two patients (patients 1 and 3). Similar facial features, including a triangular face and a prominent jaw, were also noted among three of the patients (Figures 2a–c). The most striking similarity among them are the behavioral problems, including poor eye contact, self-injuries and sleep abnormality. The shortest region of overlap (SRO) of the deletions in these three patients is between the proximal and the distal breakpoints of patients 2 and 3 respectively (chrX: 101 365 862–103 982 269), and includes *PLP1*.

Previously, a male patient with Sotos syndrome and Marfanoid features has been reported.[12] This patient had a typical 5q35 deletion, which is responsible for the Sotos syndrome, and an additional deletion of Xq22.3. This Xq22.3 deletion was thought to be responsible for his Marfanoid feature. His healthy mother was an asymptomatic carrier of the Xq22.3 deletion. This indicates that the 862 kb deletion at position chrX: 105 167 104–106 028 458 is not pathogenic in females and can be excluded from the region thought to be responsible for the severe neurological manifestations in the patients discussed here. Shimojima *et al.*[12] suggested that their patient's severe developmental delay may result from a positional effect of the deletion on *interleukin 1 receptor accessory protein-like 2* (*IL1RAPL2*). IL1RAPL2 is a member of the interleukin 1 receptor family, and the similar protein IL1RAPL1, located on Xp21.3–p21.2, is implicated in the development of autism.[13] Because the first two exons and the promoter region of this gene are involved in SRO, this gene is one of the candidate genes that may be involved in the symptoms observed in the patients discussed in the present study.

Previously, Grillo *et al.*[14] identified a 1.1-Mb deletion in a female child and her mother with mild intellectual disability that overlaps a portion of the proposed SRO (Figure 1). They discussed the genes responsible for intellectual disability and suggested that some of the nuclear RNA export factor genes that cluster in this region might be responsible for the phenotype. The *nuclear RNA export factor 5*

(*NXF5*) gene is the most powerful candidate, as it was disrupted by an inversion in a patient with intellectual disability.[15,16] However, *NXF5* is not included in the proposed SRO in this study. Furthermore, this mother and daughter did not show severe intellectual disability and any behavioral abnormalities that are typical of the present three patients (patients 1–3). Therefore, the overlapping region designated as a possible candidate region 'A' in Figure 1 can be eliminated.

The deletions in two patients (patients 4 and 5) in the present study are included in the proposed SRO. A 0.25-Mb deletion was identified in patient 4 who showed mild intellectual disability and bilateral sensorineural hearing loss. These are recognized features of Martin–Probst syndrome caused by mutations and deletions in *the RAB40A member RAS oncogene family-like* (*RAB40AL*) gene.[17] This gene is contained within the deletion in patient 4. This patient did not exhibit hypotonia or behavioral abnormalities. Thus, the deletion region identified in this patient can be excluded from the region thought to be responsible for the severe intellectual disability, hypotonia and behavioral abnormalities observed in the three patients with larger Xq22 deletions (patients 1–3). Furthermore, patient 5 showed a small deletion, 85 kb in size, involving *PLP1* and only two further genes, the *transmembrane protein 31* (*TMEM31*) gene and the *glycine receptor α4* (*GLRA4*) gene, about which there is very limited information (Supplementary Table S1). This patient showed severe intellectual disability and behavioral abnormalities, resembling the typical phenotype of the three other patients (patients 1–3).

Although a large number of duplications involving *PLP1* have been identified previously, the reciprocal deletion of this region is rarely observed. The previously reported deletions identified in patients with PMD were much smaller than the duplications, with involvement of only two neighboring genes, as seen in patient 5 in the present study (Figure 1 and Supplementary Table S1).[4,18] Presumably, larger deletions of this region would cause lethality or other diverse syndromes.[18] Inoue *et al.*[4] observed symptomatic carriers with mild late-onset spastic diplegia. This paradoxical presentation could be explained by mosaicism or random XCI. Figure 1 displays the microdeletions involving *PLP1* in the patients in the present study and those reported by Inoue *et al.*[4] and Torisu *et al.*[5] The deletions reported by Inoue *et al.*[4] and Torisu *et al.*[5] were maternally inherited, indicating that females with deletions of this region would not necessarily show severe neurological features. Because the deletion identified in patient 5 overlapps completely with the region reported by Inoue *et al.*,[4] this deletion alone is insufficient to explain her severe intellectual disability and behavior abnormalities. Thus, there must be another reason why patient 5 shows a similar phenotype to the other three patients. We can speculate that there may be very small and very complex chromosomal rearrangements in the neighboring regions of the deletion in patient 5. Microarray technology cannot detect copy-neutral chromosomal rearrangements. Because the clinical features of patient 5 are similar to the other three patients, the critical region for the neurological features common to the other three patients may be disrupted by balanced inversions or translocations in Xq22 region. A coincidental genetic alteration in another chromosomal region is also a possibility. Whole genome sequencing using next-generation sequencing may be the only way to reveal the underlying genomic mechanism in this patient. This is an area for future study. Because the facial features of patient 5 differ from those of the other three patients, the genes responsible for this phenotype can be assumed to be located in SRO, with exception of the deletion region of patient 5.

The possible candidate region for the severe intellectual disability, hypotonia and behavioral abnormalities can therefore be now narrowed down to regions 'B' and 'C'. Region 'C' (chrX: 102 993 719–103 982 269) is demarcated by the distal ends of the deletions in patient 3 and Inoue *et al.* (A).[4] Because the only protein-coding gene in this segment is *IL1RAPL2*, this gene remains a candidate gene. The remaining possible candidate region is 'B' that is 724 kb in size (chrX: 102 233 526–102 957 289) and rests between the distal and proximal ends of the deletions in patient 4 and Inoue *et al.*[4] (B), respectively (Figure 1). The *brain expressed X-linked* (*BEX*) genes are clustered in this region. This includes *BEX1*, *BEX4*, *BEX2* and *BEX3* (also known as the *nerve growth factor receptor-associated protein 1* (*NGFRAP1*) gene). The Bex gene family members are highly homologous and highly expressed in the brain, but differ in their expression pattern.[19] Among them, the most highly validated human Bex ortholog is *BEX3* (*NGFRAP1*) that encodes p75NTR-associated cell death executor (NADE). This interacts with p75 neurotrophin receptor (p75NTR). Neurotrophins are growth factors that play critical roles in the development, maintenance, survival and death of the nervous system.[20] Its interactions with p75NTR initiate signal transduction systems that mediate diverse biological functions,[21] limiting quantities of neurotrophins during development and controling the numbers of surviving neurons to ensure a match between neurons and the requirement for a suitable density of target innervation.[22] Thus, there is a possibility that brain dysfunction may be caused by allelic loss of *BEX3* (*NGFRAP1*) in females. The other *BEX* genes may also be involved in the neurological features.

As mentioned above, chromosomal deletions encompassing *PLP1* are rarely observed in PMD patients, indicating that large deletions in this region in males are likely to be lethal. The sizes of the previously reported deletions are much smaller than the duplications in PMD patients and their carrier mothers and are restricted to the region between regions 'B' and 'C'. This indicates that the gene(s) located on the most proximal neighboring regions of *PLP1* would cause lethality in males and cause severe intellectual disability in females, when those were deleted; and finally narrowed the deletion regions in PMD patients. From this perspective, *BEX3* (*NGFRAP1*), located in the region nearest *PLP1*, is the most powerful candidate gene.

In the present study, three patients showed skewed XCI. Because no patient showed a PMD phenotype, the X chromosome with the deletion involving *PLP1* is assumed to be predominantly inactivated in these patients. In spite of this, these patients showed severe intellectual disability. One possible explanation for this phenomenon is that the candidate gene in this region causing severe intellectual disability may escape XCI and that haploinsufficiency of this gene may cause intellectual disability in females. Delayed myelination seen in patient 2 may be because of mildly skewed XCI pattern in this patient. Further cases are likely to be reported in the future and may help clarify the contributions of the genes discussed to the phenotype in these patients. Identifications of nucleotide alterations of these genes would provide further support for this hypothesis.

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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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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

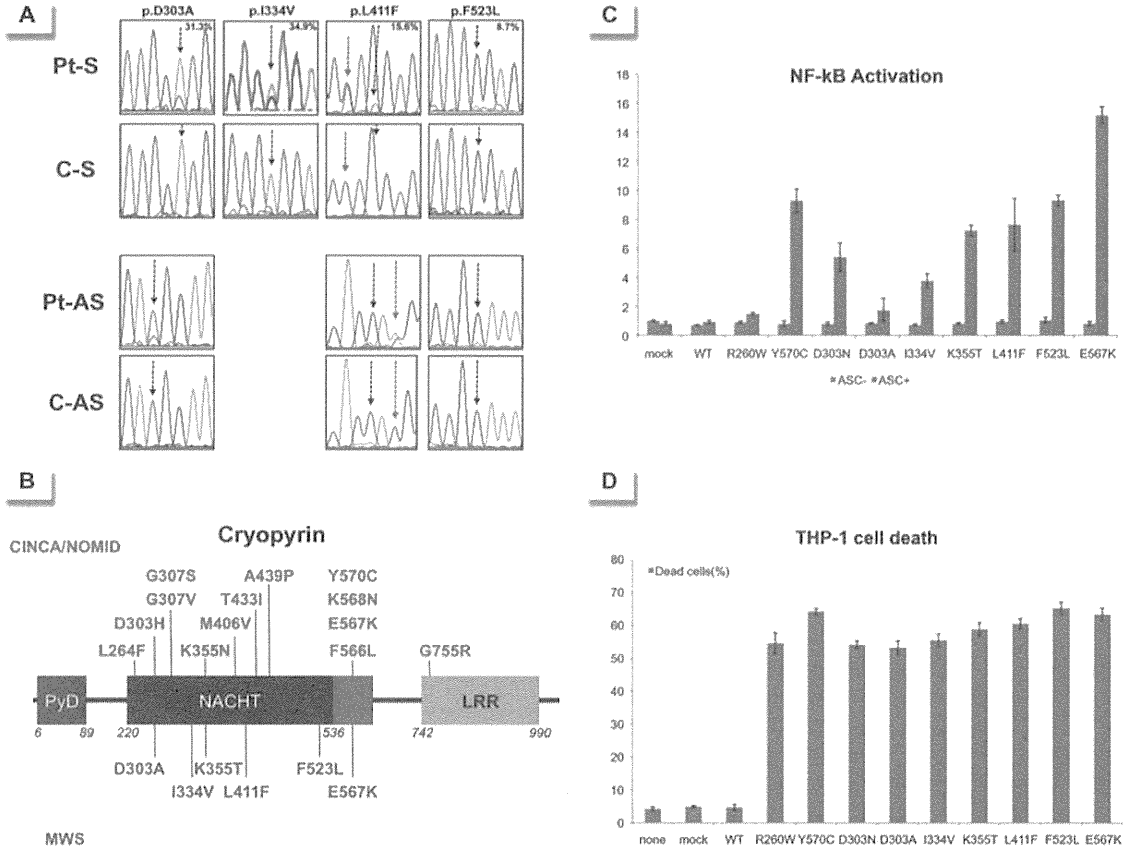

Figure 1 (A) Sense (upper rows) and antisense (bottom rows) chromatograms from four patients with somatic *NLRP3* mosaicism and controls obtained by Sanger sequencing using genomic DNA extracted from whole blood. The black arrows show the *NLRP3* positions where the somatic mutations were detected. The 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	Nucleotide exchange*	Amino acid exchange	Massively parallel DNA sequencing: Mutated allele frequency	Massively parallel DNA sequencing: Coverage	Bioinformatics analyses: SIFT	Bioinformatics analyses: PolyPhen-2	Population genetics†	Reference	Analysed relatives: Kinship	Analysed relatives: 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 Mother	Negative§ 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

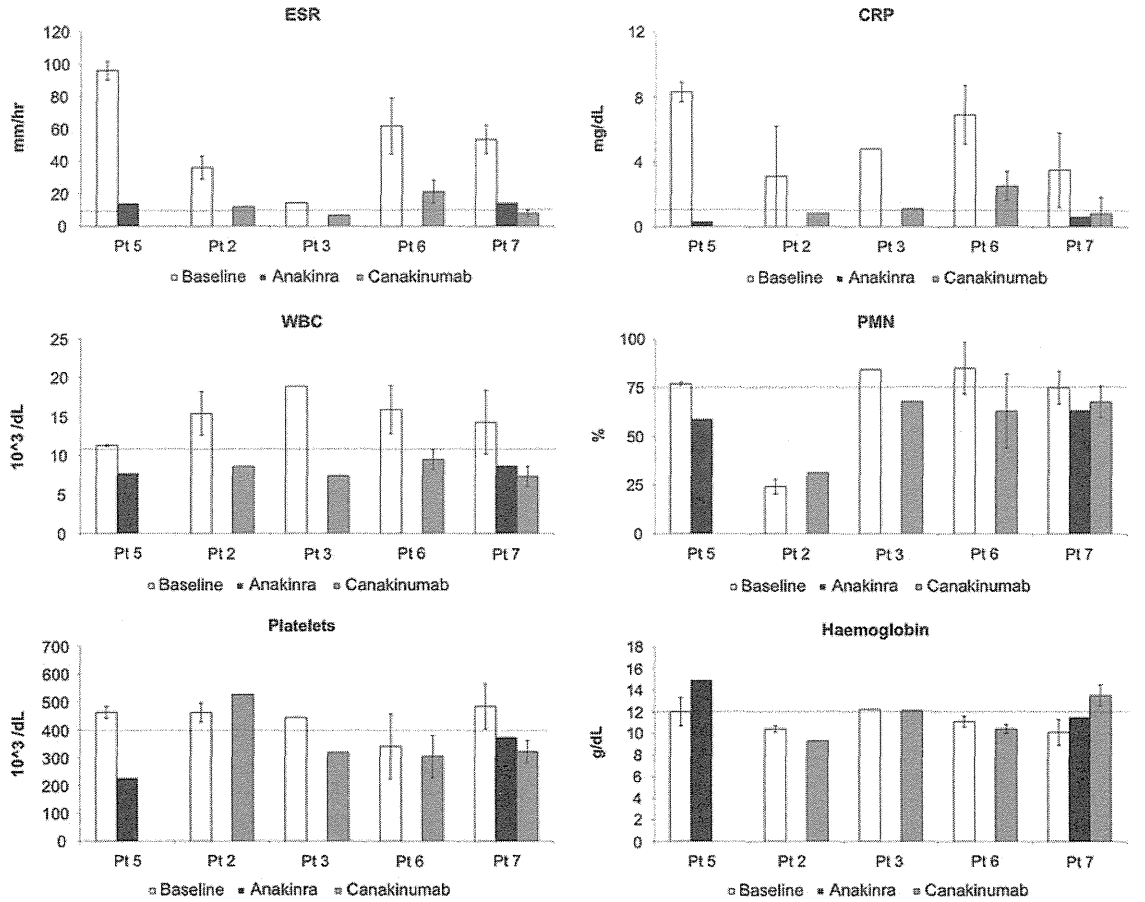

Figure 2 Laboratory values obtained in the five patients treated with different anti-interleukin 1 drugs. Patient's graphics were ordered as follows: First, those graphics from the patient who only received treatment with anakinra (Pt 5), followed by those from patients who only received treatment with 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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