secreted form (sST2) [10]. sST2 acts as a decoyreceptor for IL-33 [11].

IL-33 affects the function of cells that express ST2 molecule. IL-33 polarizes naive T cells to produce Th2-associated cytokines IL-4, IL-5 and IL-13 [7] and functions as a chemoattractant for Th2 cells in vitro and in vivo [12], but also induces secretion of proinflammatory cytokines and chemokines bymast cells [13], basophils [14] and Th1 type cytokines from NK and NKT cells [14,15]. Also, IL-33 amplifies polarization of alternatively activated M2 macrophages [16], induces maturation of dendritic cells [17] and may promote Th1-type response [18].

Besides the regulation of disease outcome through the modulation of Th1/Th2 bias, there is some evidence to suggest that ST2 may also be involved in inflammatory responses. A previous report revealed that the sST2-Fc fusion protein suppressed inflammatory responses that were induced by lipopolysaccharide (LPS) both in vitro and in vivo [19]. In normal conditions, the serum concentration of sST2 is below the detectable level, but elevated level of sST2 has been reported in patients with autoimmune diseases [20], asthma [11], idiopathic pulmonaryfibrosis [23]. The sST2 levels were found to correlate with the activity and severity of these conditions [21–24].

In this way, IL-33 and ST2 have important functions in host defense, immune regulation, and inflammation. However, its role in the pathogenesis of s-JIA and a causal relationship with disease activity are still unclear. To assess the role of IL-33 and ST2, in the pathogenesis of s-JIA, we sequentially measured serum levels of IL-33 and sST2 in patients with s-JIA and determined their correlation with measures of disease activity and severity.

2. Methods

2.1. Patients and samples

Serum samples were obtained from 24 patients with s-JIA, 5 patients with RF + poly-JIA, and 20 age- and sex-matched healthy controls (HC) [age, s-JIA: 8.9 ± 6.5 years and HC: 10.5 ± 7.4 years]. Eleven patients were evaluated longitudinally on a second occasion when their disease was in an inactive phase. Four patients with MAS were evaluated serially from the phase of MAS to remission. The clinical characteristics of the patients with s-JIA are shown in Table 1.

Diagnoses of s-JIA and RF + poly-JIA were based on the International League of Associations for Rheumatology criteria [1]. MAS was diagnosed based on the combination of cytopenias affecting at least two cell lines, coagulopathy, and liver dysfunction, according to the guidelines proposed by Ravelli et al. [24]. The criteria defining the active phase of s-JIA were active arthritis, fever, rash,

Table 1
Clinical characteristics of patients with systemic juvenile idiopathic arthritis during the active phase. CRP, C-reactive protein; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; PSL, prednisolone; MTX, methotrexate.

	S-JIA
Patients	24
Sex (male:female)	11:13
Age (years)	10 (1–26)
Disease duration (years)	0.1 (0–11)
Laboratory finidings	
CRP (mg/dl) ($n = 24$)	8.41 (1.6–25.8)
AST (IU/l) ($n = 24$)	39 (11–136)
LDH (IU/l) ($n = 24$)	344 (162–1359)
Ferritin (ng/mL) ($n = 17$)	864 (250–17,484)
Treatment	
PSL (mg/day) ($n = 9$)	17.5 (5–37.5)
MTX (mg/m^2) ($n = 2$)	10

hepatosplenomegaly, generalized lymphadenopathy, and serositis as well as increased erythrocyte sedimentation rates and C-reactive protein (CRP) levels. The criteria for the inactive phase of s-JIA on medication were as follows: the first time after the recovery from MAS with no clinical symptoms that were observed in the active phase, as well as normal erythrocyte sedimentation rates (<5 mm/h) and CRP levels (<0.1 mg/dL). The criterion for remission of patients with s-JIA on medication was six continuous months of inactive disease while on medication [25].

Serum was separated from cells, divided into aliquots, frozen, and stored at −80 °C until use. This study was approved by the Institutional Review Board at Kanazawa University, and all specimens were used after informed consent was obtained.

2.2. Quantification of serum cytokines

Levels of IL-33 were evaluated by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Human IL-33 DuoSet® ELISA Development System, R&D Systems, Inc., Minneapolis, MN, USA). Levels of sST2 were evaluated by ELISA according to the manufacturer's instructions (Human ST2/IL-1R4DuoSet® ELISA Development System, R&D Systems, Inc., Minneapolis, MN, USA). The range of ELISA for IL-33 was from 3.6 to 1500 pg/mL. The range of ELISA for sST2 was from 31.5 to 2000 pg/mL. RF positivity did not interfere with the ELISA assay.

2.3. Statistical analysis

Within-group comparisons were analyzed by the Mann–Whitney test. Correlations were expressed using the Spearman rank correlation coefficient. For the analyzed measures, $P < 0.05$ was considered significant.

3. Results

3.1. Serum levels of IL-33 and sST2

We determined the serum levels of IL-33 and sST2 in patients with s-JIA and compared them with the levels in patients with RF + poly-JIA and HC. Serum IL-33 levels in most patients with active s-JIA were found to lie below the lowest detection limit of the assay (Fig. 1A). IL-33 was detected in 4 out of 24 s-JIA patients (17%) 9 out of 20 control subjects (45%). The differences in the serum IL-33 levels among these s-JIA patients (median, 68; range, 4–702 ng/mL), and HC (median, 50; range, 4–169 ng/mL) were not statistically significant. On the other hand, serum IL-33 levels in RF + poly-JIA patients (median, 155; range, 61–533 ng/mL) were significantly elevated compared with those in active s-JIA patients and HC ($P < 0.01$). These patients did not demonstrate symptoms that were suggestive of allergic diseases or atopy at the time of the study.

In contrast, serum sST2 levels in patients with MAS (median, 19,500; range, 3680–61,000 pg/mL) and in patients in the active phase of s-JIA (median, 2205; range, 496–43,000 pg/mL) were much higher than those in patients with RF + poly-JIA (577, 338–1120 pg/mL) and HC (354, 102–1052 pg/mL)(Fig. 1B). Serum sST2 levels in patients with s-JIA were significantly elevated even in the inactive phase (839, 360–4550 pg/mL), and they normalized in the remission phase (402, 267–512 pg/mL). The serum sST2 levels in RF + patients were not significantly different compared to HC.

3.2. Markedly elevated concentrations of serum sST2 in patients in the active phase of s-JIA and MAS

To investigate the relevance of sST2 to the pathogenesis of s-JIA and MAS, serum sST2 levels were serially monitored in four cases

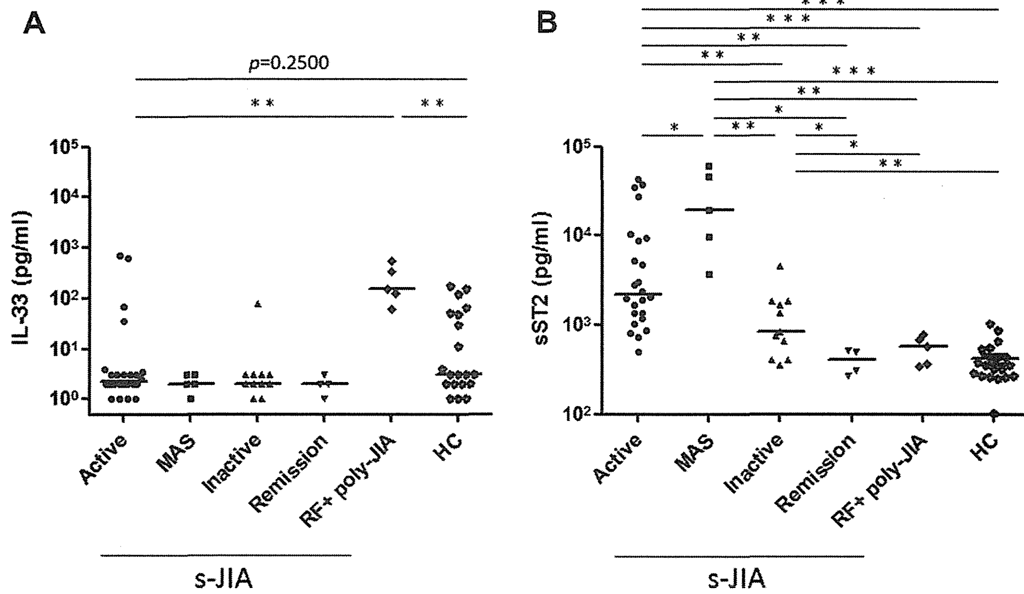

Fig. 1. Serum levels of IL-33 (A) and sST2 (B) in patients with s-JIA. Bars represent median values. Statistically significant differences between each patient group are shown as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. IL, interleukin; sST2, soluble ST2; s-JIA, systemic juvenile idiopathic arthritis; RF + poly-JIA, rheumatoid factor + polyarticular juvenile idiopathic arthritis; HC, healthy control.

Fig. 2. Longitudinal assessment of serum sST2 levels and LDH in four patients with MAS. Changes in serum sST2 (solid lines) and LDH (dotted lines) levels are shown in the upper panels and the details of the therapeutic interventions are shown in the lower panels. Time points of blood draw are shown with arrows. sST2, soluble ST2; LDH, lactate dehydrogenase; M, MAS; A, active phase; I, inactive phase; R, remission; mPSL, methylprednisolone; DEX-P, dexamethasone palmitate; PSL, prednisolone; CyA, cyclosporine.

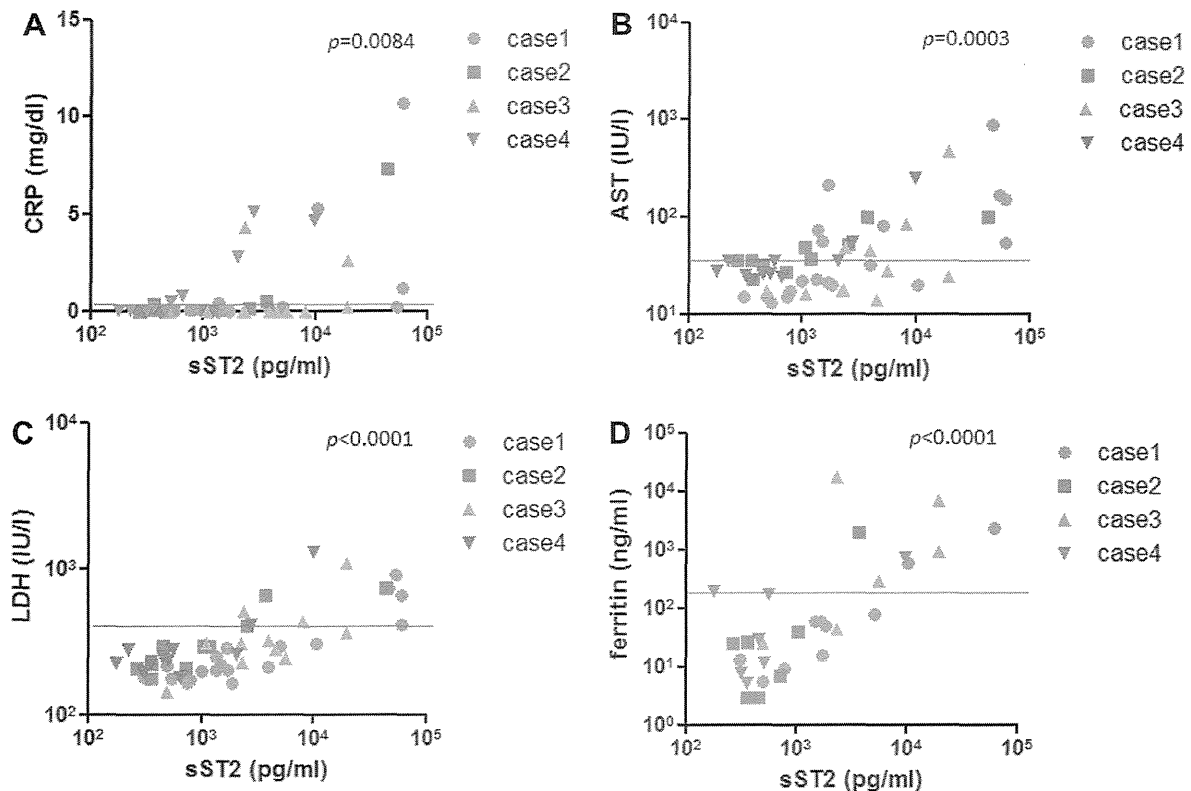

Fig. 3. Positive correlations between serum sST2 levels and measures of disease activity, including the other cytokines, during the clinical course of s-JIA. (A, CRP; B, AST; C, LDH; D, ferritin). IL, interleukin; CRP, C-reactive protein; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

of s-JIA (Fig. 2). The serum sST2 levels were both rapidly and markedly elevated when the complication of MAS occurred, but gradually reduced after such manifestations disappeared with corticosteroid and cyclosporine therapy. Even a few weeks after normalization of the indicators of an inflammatory reaction, such as lactate dehydrogenase (LDH) levels, the sST2 levels were still well above the values of HCs. Disease relapse of s-JIA with MAS was complicated in 1 case (Fig. 2A) during this phase of high serum sST2 levels.

3.3. Correlation between serum sST2 and measures of disease activity in the clinical course of four cases of s-JIA

Because the levels of serum CRP, aspartate aminotransferase, LDH, and ferritinare clinically used as indicators of disease activity in s-JIA, their concentrations were compared with those of sST2. The serum sST2 levels correlated positively with each of these indicators (Fig. 3A–D). However, even during the clinically inactive phase after remission from MAS, the serum sST2 levels remained elevated, although other clinical parameters were normalized.

4. Discussion

In this study, we demonstrated that serum sST2 levels in patients with s-JIA were markedly elevated during the active phase of s-JIA and correlated with measures of disease activity. These findings indicate that serum sST2 levels may have a potential role as a promising indicator of disease activity. Furthermore, serum sST2 levels in patients with s-JIA were significantly elevated even during the inactive phase, and they were normalized in the remission phase. High serum sST2 levels persisted in patients for about six continuous months of inactive disease while on medication. These findings support the appropriateness of the Wallace criteria for the clinical remission of JIA (six continuous months of inactive disease while on medication) [25]. In this way, monitoring serum sST2 levels may be also useful for assessing treatment effects and the clinical remission of s-JIA.

Elevated IL-33 levels were infrequently observed in s-JIA patients, and their levels were found to be comparable to those of controls. Serum IL-33 levels were not found to be related to sST2 levels or the disease activity of s-JIA. It is possible that IL-33 may have formed immune complexes with sST2 or was downregulated by sST2 through negative regulatory mechanisms because sST2 functions as a negative regulator and is an antagonistic decoy receptor for IL-33 [11].

Other possibility is that biological action of increased sST2 in serum might not be mediated via IL-33 neutralization. There is some evidence to suggest that sST2 may also be involved in inflammatory responses. A previous report revealed that sST2 suppressed the expression of toll-like receptor (TLR) 1 and 4 [19] and that this may contribute to the anti-inflammatory effects of sST2 in macrophages. The role of TLRs in s-JIA has been consistently demonstrated [26]. For example, signaling through the IL-1 and IL-18 receptors shares the downstream portion of the TLR4 signaling pathway, and IL-1 and IL-18 provide positive feedback loops that further contribute to the perpetuation of the inflammatory responses in s-JIA [26]. These findings indicate that sST2 may have a role in resolving inflammatory responses by regulating macrophage activation in the pathogenesis of s-JIA. Furthermore, sST2 may provide a novel approach for treating s-JIA by inhibiting the release of proinflammatory cytokines. In fact, a previous study revealed the therapeutic effects of sST2-Fc in a murine model of collagen-induced arthritis [27].

The alternative activation of monocytes and macrophages may play a role in resolving inflammatory responses in the

pathogenesis of s-JIA. It has been well described that IL-33 and ST2 are modulators of inflammation and mediate Th2 immune responses [12]. IL-33 has been shown to induce the production of Th2 cytokines [7] and to possess a chemoattractant effect for human Th2 cells [12]. In this study, serum sST2 levels in patients with s-JIA were elevated not only in the active phase but also in the inactive phase. These findings indicate that sST2 may have a role in resolving inflammatory responses by promoting monocyte differentiation into M2 macrophages through Th2 immune responses in the pathogenesis of s-JIA, as previously reported [16,28].

In this study, serum IL-33 levels were significantly elevated in RF + poly-JIA patients. A previous study revealed that serum IL-33 levels were elevated in sera and synovial fluid samples from patients with rheumatoid arthritis (RA), and the levels correlated with disease activity [29–37]. IL-33 is produced mainly in inflamed joints [29]. A recent study showed that the serum IL-33 levels were correlated with the production of IgM and RA-related autoantibodies, including RF and anticitrullinated protein antibodies [30]. Our results were consistent with these findings. Although the number of RF + poly-JIA patients in this study was small and a larger study may help define the true diagnostic value of these markers, our study indicates that IL-33 may play an important role in the joint inflammation of human RF + poly-JIA.

We have not determined the source of IL-33 and sST2 in patients with s-JIA in this study. A previous study showed that various cell types, including smooth muscle cells, epithelial cells, fibroblasts, keratinocytes, dendritic cells, and activated macrophages, express IL-33 mRNA [7]. IL-33 mRNA was induced in fibroblasts and keratinocytes by stimulation with tumor necrosis factor (TNF)-α and IL-1 [7]. However, IL-33 mRNA was only modestly induced in dendritic cells and macrophages by stimulation with LPS [7]. Furthermore, the expression of IL-33 mRNA has been found in endothelial cells from chronically inflamed tissues from patients with Crohn's disease and RA [38]. sST2 was induced in fibroblasts, macrophages, and monocytes by stimulation with LPS, TNF-α, or IL-1 [39]. Furthermore, previous studies showed that epithelial and endothelial cells, and cardiac myocytes can secrete sST2 [40,41]. These findings indicate that sST2 and/or IL-33 may be released from activated macrophages, dendritic cells, or endothelial cells from inflamed tissues in patients with s-JIA.

sST2 levels obtained in HC were again 10-fold higher than many other papers measuring sST2 in serum. Some reasons of this discrepancy might be due to the difference of kit and/or operation difference.

The limitation of the present study was the small number of s-JIA patients. A larger study may help to define the true diagnostic value of these markers. Despite this limitation, our results indicate that ST2 may be an important mediator in s-JIA. Serum sST2 levels in s-JIA patients correlated with disease activity, suggesting a potential role of ST2 as a promising indicator of disease activity.

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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.

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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

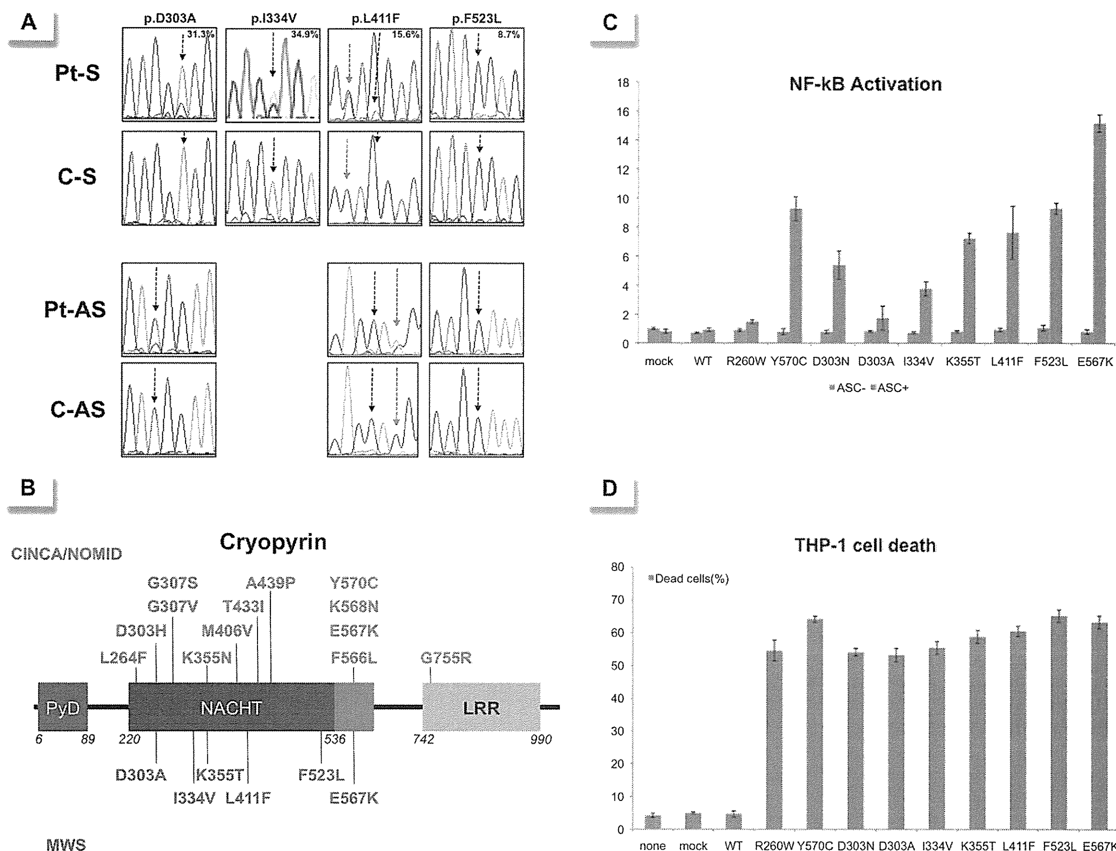

Figure 1 (A) Sense (upper rows) and antisense (bottom rows) chromatograms from four patients with somatic *NLRP3* mosaicism and controls obtained by Sanger sequencing using genomic DNA extracted from whole blood. The black arrows show the *NLRP3* positions where the somatic mutations were detected. The 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	Fever	Joint involvement: Type of arthritis	Involved joints	Symmetric	Erosive	Arthropathy	CNS involvement: Headache	Aseptic meningitis	Papilloedema	Deafness (age at onset)	Ocular involvement	AA amyloidosis
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

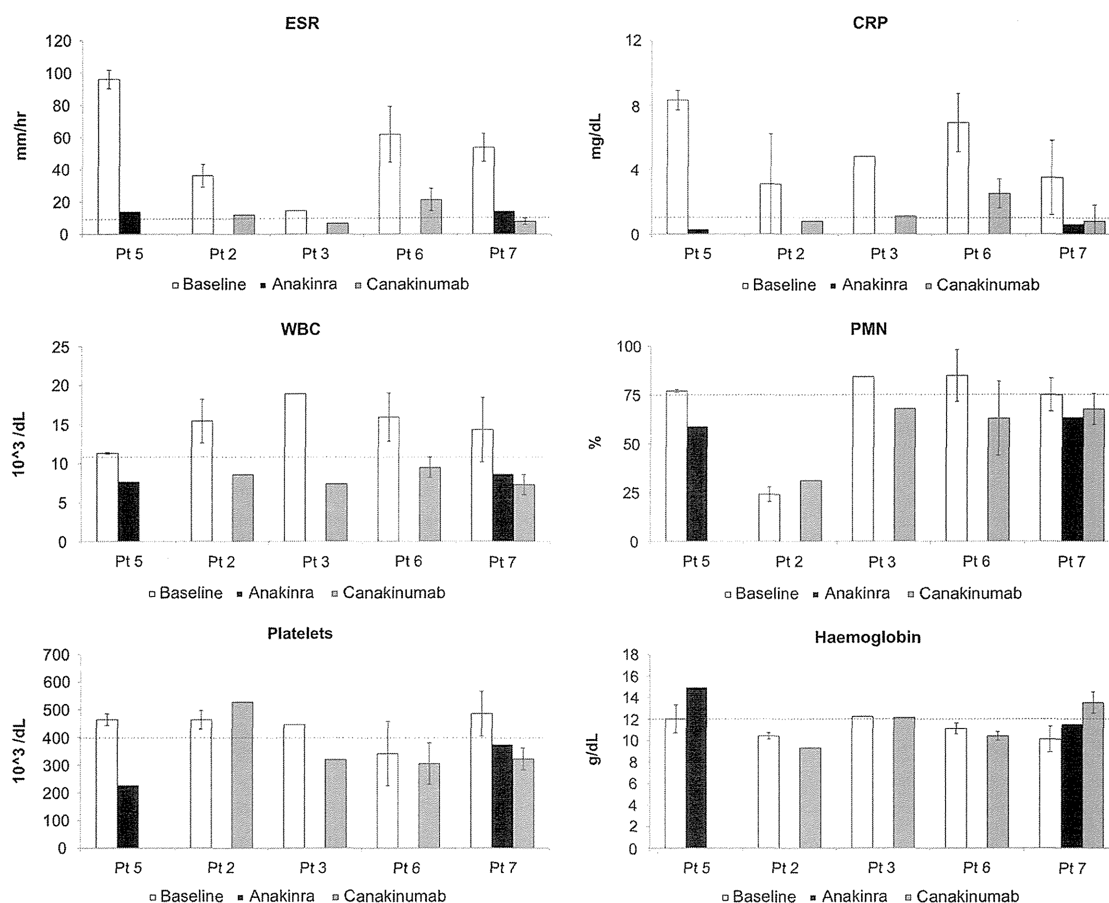

Figure 2 Laboratory values obtained in the five patients treated with different anti-interleukin 1 drugs. Patient's graphics were ordered as follows: First, those graphics from the patient who only received treatment with anakinra (Pt 5), followed by those from patients who only received treatment with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

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A nationwide survey of Aicardi-Goutières syndrome patients identifies a strong association between dominant *TREX1* mutations and chilblain lesions: Japanese cohort study

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Abstract

Objectives. Aicardi-Goutières syndrome (AGS) is a rare, genetically determined, early onset progressive encephalopathy associated with autoimmune manifestations. AGS is usually inherited in an autosomal recessive manner. The disease is rare, therefore the clinical manifestations and genotype-phenotype correlations, particularly with regard to autoimmune diseases, are still unclear. Here we performed a nationwide survey of AGS patients in Japan and analysed the genetic and clinical data.

Methods. Patients were recruited via questionnaires sent to paediatric or adult neurologists in Japanese hospitals and institutions. Genetic analysis was performed and clinical data were collected.

Results. Fourteen AGS patients were identified from 13 families; 10 harboured genetic mutations. Three patients harboured dominant-type *TREX1* mutations. These included two *de novo* cases: one caused by a novel heterozygous p.His195Tyr mutation and the other by a novel somatic mosaicism resulting in a p.Asp200Asn mutation. Chilblain lesions were observed in all patients harbouring dominant-type *TREX1* mutations. All three patients harbouring *SAMHD1* mutations were diagnosed with autoimmune diseases, two with SLE and one with SS. The latter is the first reported case.

Conclusion. This study is the first to report a nationwide AGS survey, which identified more patients with sporadic AGS carrying *de novo* dominant-type *TREX1* mutations than expected. There was a strong association between the dominant-type *TREX1* mutations and chilblain lesions, and between *SAMHD1* mutations and autoimmunity. These findings suggest that rheumatologists should pay attention to possible sporadic AGS cases presenting with neurological disorders and autoimmune manifestations.

Key words: Aicardi-Goutières syndrome, *TREX1*, *SAMHD1*, dominant-type, mosaicism, chilblain, autoimmunity.

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Introduction

Aicardi–Goutières syndrome (AGS) is a rare, genetically determined, early onset progressive encephalopathy [1]. Patients with AGS typically suffer from irritability, inconsolable crying and progressive microcephaly associated with severe neurological symptoms, such as hypotonia, dystonia, seizures, spastic quadriplegia and severe developmental delay [2]. On brain imaging, AGS is characterized by calcifications of basal ganglia, white matter abnormalities and cerebral atrophy [3, 4]. Cerebrospinal fluid (CSF) analysis shows chronic lymphocytosis and elevated IFN-α and neopterin levels [3–5]. AGS patients are often misdiagnosed as having intrauterine infections, such as TORCH (toxoplasmosis, other infections, rubella, cytomegalovirus infection and herpes simplex) syndrome, because of the similarities in the clinical findings, particularly the intracranial calcifications [1]. As extraneural findings, chilblain lesions are seen in approximately 40% of patients [3]. Some patients also show bouts of mild fever, hepatosplenomegaly, abnormal liver function and thrombocytopenia [1, 3, 4].

Mutations in five genes—*TREX1*, *RNASEH2B*, *RNASEH2C*, *RNASEH2A* and *SAMHD1*—are linked with AGS [6–8]. Approximately 90% of patients with characteristic clinical and radiologic findings of AGS harbour aetiological mutations in one of these five genes [1]. The *ADAR1* gene was recently identified as a sixth gene linked with AGS [9]. AGS is often inherited in an autosomal recessive manner, although a few cases show an autosomal dominant pattern of inheritance [2, 10–12]. Mutations in *TREX1* are often associated with early onset neonatal AGS, which presents with more severe neurological features, whereas *RNASEH2B* mutations are related to a later-onset presentation that is associated with less severe neurological problems and lower mortality rates [2, 3].

The main pathophysiological feature of AGS is the overproduction of type I IFN, caused by the accumulation of nucleic acids within cells stimulating the pattern recognition receptors and the innate immune system [1]. Therefore AGS can be regarded as an interferonopathy [13]. SLE is also an interferonopathy and shares many clinical features with AGS, including skin lesions, neurological abnormalities and the expression of type I IFN-related genes [13, 14]. Some SLE patients harbour *TREX1* mutations, suggesting that AGS and SLE are both allelic disorders that share a common pathophysiology, overproduction of type I IFN [15]. AGS is a disease of monogenic autoimmunity and an important disorder that must be considered when making a differential diagnosis of SLE; indeed, some molecularly proven AGS patients fulfil the diagnostic criteria for SLE [2, 16, 17]. In addition, AGS is similar, particularly with regard to skin lesions, to familial chilblain lupus (FCL), a rare cutaneous form of SLE that shows Mendelian inheritance [18]. We recently reported a family containing AGS and FCL cases caused by a heterozygous p.Asp18Asn mutation in *TREX1*, and proved the clinical continuity of these two conditions [19]. Although the similarities between SLE and AGS are known, the autoimmune aspects of AGS are still unclear because of the rarity of the disorder.

As a rare monogenic disorder, AGS reports have predominantly examined individual AGS families. Alternatively, some genetic analysis centres have recruited AGS patients from around the world. Here we report the first nationwide survey of Japanese AGS patients, which was conducted using questionnaires designed to identify the clinical manifestations and genotype–phenotype correlations in detail, with a particular focus on autoimmune symptoms.

Materials and methods

Patients

Questionnaires were sent to 1852 hospitals and institutions that specialize in paediatric and adult neurology, and 760 replies (41.0%) were received. Detailed questionnaires were then sent to the hospitals or institutions with positive replies and clinical information regarding suspected AGS cases was obtained. In addition, some patients enrolled in the study were referred directly to our institution by paediatric neurologists and paediatric rheumatologists. Clinical information, patient histories and laboratory data were collected from medical records and by direct interviews with patients, their families and their attending physicians. Neuroimaging data included CT scans and/or MRI scans. AGS was diagnosed according to the following criteria: (i) neurological abnormalities of encephalopathy, (ii) intracranial calcifications, (iii) the absence of prenatal infection and (iv) at least one CSF abnormality, such as a white cell count $\geqslant 5$ cells/mm^3, elevated IFN-α levels (>6 IU/ml or >12.5 pg/ml), or raised neopterin levels (reference ranges 8.0–25.0 nM at an age of 2–12 years and 7.3–31.6 nM in adults). In cases with no available CSF data, a diagnosis of AGS was made when the patients fulfilled criteria 1–3 and either had mutations in the genes responsible for AGS or had siblings who had been diagnosed with AGS.

Laboratory data

IFN-α levels were measured using a cytopathic effect inhibition assay (SRL, Tokyo, Japan). In the present study, a value >6 IU/ml was considered elevated due to the limit of detection of the assay, although most previous reports considered >2 IU/ml as the cut-off value for AGS. In some cases, IFN-α was measured by ELISA (SRL). The CSF neopterin level was measured as previously described [20].

Genetic analysis

The nationwide survey was approved by the ethical committee of the National Hospital Organization, Sagamihara National Hospital, and genetic analyses were approved by the ethical committees of Kyoto University Hospital and the National Hospital Organization, Sagamihara National Hospital, in accordance with the Declaration of Helsinki. After obtaining written informed consent from all study subjects (or their parents or guardians), genetic analyses

of *TREX1*, *RNASEH2B*, *RNASEH2C*, *RNASEH2A* and *SAMHD1* were performed. Genetic analysis of the *ADAR1* gene was performed for those patients clinically diagnosed with AGS but who did not harbour a mutation in any of these genes. Genomic DNA was extracted from the whole blood samples taken from the patients and their parents (if available) and all exons, including the exon-intron boundaries, were sequenced as previously described [21]. *TREX1* gene mosaicism was analysed using massively parallel DNA sequencing as previously described [22]. Leucocyte subpopulations were isolated and sorted using an autoMACS Pro Separator (Miltenyi Biotec, Gladbach, Germany) as previously described [23]. The purity of the isolated subpopulations was >90%.

Exonuclease assays

The pFN18A HaloTag T7 Flexi Vector system (Promega, Madison, WI, USA) with a HaloTag at the N-terminus was used to generate a recombinant human TREX1 protein. Recombinant protein was produced in Single Step (KRX) Competent Cells (Promega) and isolated according to the manufacturer's protocol. The recombinant proteins were then immunoblotted with an anti-TREX1 antibody (Sigma-Aldrich, St Louis, MO, USA) as previously described [21]. The concentrations of the purified TREX1 proteins were determined using the Flamingo Fluorescent Gel Stain (Bio-Rad, Hercules, CA, USA) assay with a Molecular Imager FX Pro Plus (Bio-Rad).

The ssDNA and dsDNA exonuclease assays were performed according to the method of Orebaugh *et al.* [24]. Briefly, for the ssDNA assays, a 30-mer oligonucleotide with 5′-fluorescein (6-FAM) (Sigma-Aldrich) and TREX1 proteins were incubated at 37°C for 30 min, followed by separation on 23% denaturing polyacrylamide gels. Using the visualized fluorescein-labelled bands, the fraction of oligomer at each position was multiplied by the number of excised deoxynucleoside monophosphates (dNMPs) to determine the activities of the recombinant TREX1 proteins (fmol of dNMP/s/fmol of enzyme). For the dsDNA assays, the p3xFLAG CMV-14 plasmid (Sigma-Aldrich), which contains a single Nt.BbvCI site (New England Biolabs, Ipswich, MA, USA), was digested by Nt.BbvCI. The nicked plasmid DNA was then incubated with the TREX1 proteins at 25°C for 30 min, followed by separation in electrophoresis on 0.8% agarose gels. Using SYBR Green I (Lonza, Basel, Switzerland), the excised dNMPs and activities of the recombinant TREX1 proteins (fmol of dNMP/s/fmol of enzyme) were calculated by the amount of degraded dsDNA.

Statistical analysis

Fisher's exact test was used to examine differences in categorical variables among the cohort in the present study or between the cohort in the present study and cohorts from other studies. Statistical significance was set at $P < 0.05$.

Results

Genetic and molecular findings

We identified 14 AGS patients from 13 families. All patients were Japanese and none were consanguineous. Of the 13 families, 10 harboured mutations and 3 did not (Table 1). Altogether, we identified 15 mutations in four genes, 10 of which were novel. All of the novel mutations were absent from at least 100 Japanese control alleles and from the 1000 Genomes Project database. Six of the novel mutations were missense mutations. PolyPhen-2 analysis predicted five to be probably damaging and the other, a p.Gly258Val mutation in *SAMHD1*, to be possibly damaging; all were predicted to be deleterious by sorting intolerant from tolerant (SIFT) analysis. The median age of the patients at the time of genetic analysis was 6 years (range 6 months-18 years).

Of the 10 families harbouring mutations, 5 harboured mutations in *TREX1*, 3 in *SAMHD1*, 1 in *RNASEH2B* and 1 in *RNASEH2A*. None of the families harboured mutations in *RNASEH2C* or *ADAR1*. No families harboured homozygous mutations. The *RNASEH2B* mutation was heterozygous; however, the mother who showed no symptoms of AGS shared the same heterozygous

Table 1 Genetic findings of Aicardi-Goutières syndrome patients

Patient	Gene	Nucleotide change	Protein change
1	AGS1/*TREX1*	c.[52G>A];[=]	p.[Asp18Asn];[=]
2	AGS1/*TREX1*	c.[**583C>T**];[=], *de novo*	p.[**His195Tyr**];[=], *de novo*
3	AGS1/*TREX1*	c.[=/598G>A], *de novo*	p.[=/Asp200Asn], *de novo*
4	AGS1/*TREX1*	c.[**667G>A**];[**839delG**]	p.[**Ala223Thr**];[**Gly280Glufs*18**]
5	AGS1/*TREX1*	c.[**839delG**];[859_876del18]	p.[**Gly280Glufs*18**];[Leu287_Gly292del]
6	AGS2/*RNASEH2B*	c.[**155T>G**];[=]	p.[**Leu52Trp**];[=]
7	AGS4/*RNASEH2A*	c.[**557G>A**];[**703C>T**]	p.[**Arg186Gln**];[**Arg235Trp**]
8	AGS5/*SAMHD1*	c.[368A>C];[**1567A>T**]	p.[His123Pro];[**Lys523***]
9	AGS5/*SAMHD1*	c.[**773G>T**];[**1141_1143delATT**]	p.[**Gly258Val**];[**Ile381del**]
10	AGS5/*SAMHD1*	c.[428G>A(;)**1435G>T**]	p.[Arg143His(;)**Glu479***]

Novel mutations are indicated in bold. Consanguinity was not present in any of the families of these AGS patients. = denotes normal sequence, and * denotes nonsense variant.

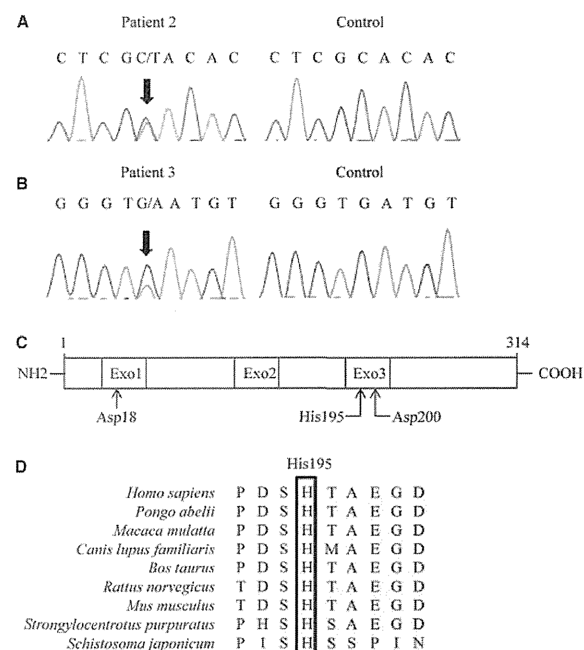
mutation and further analyses were not performed due to a lack of additional samples of the patient or the family.

Three families (patients 1–3) harboured *TREX1* mutations on one allele only. The patient harbouring the heterozygous p.Asp18Asn mutation (patient 1) had a maternal family history of FCL, which was caused by the same mutation [19]. Parental genotyping showed that the other two mutations, p.His195Tyr and p.Asp200Asn (patients 2 and 3), were *de novo* (Fig. 1A and B). RT-PCR analyses also confirmed a lack of additional mutations in *TREX1* of the three patients.

Since dominant-type *TREX1* mutations are a very rare cause of AGS [2, 10–12], we performed additional biochemical analyses of the three dominant mutations identified in our cohort, particularly the novel p.His195Tyr mutation (patient 2), to confirm their mutational effects. The TREX1 protein contains three exonuclease domains that form the active site of the enzyme, and His195, located in the third exonuclease domain, is the catalytically important histidine (Fig. 1C) [25–28]. First, we confirmed that His195 was highly conserved among different species (Fig. 1D). Next, we generated recombinant human TREX1 proteins (Fig. 1E) and compared them with wild-type TREX1 proteins ($TREX1^{WT}$). The ssDNA and dsDNA exonuclease assays revealed that TREX1 proteins harbouring the p.His195Tyr mutation ($TREX1^{p.His195Tyr}$) showed a marked reduction in enzymatic activity, similar to TREX1 proteins harbouring the p.Asp18Asn ($TREX1^{p.Asp18Asn}$) and p.Asp200Asn ($TREX1^{p.Asp200Asn}$) mutations, which are reported to cause dominant-type AGS (Fig. 1F and G) [10, 11].

In addition, we identified a patient harbouring *TREX1* with a p.Asp200Asn mutation (patient 3), in which the signal intensity of the mutated A at position 598 was lower than that of the reference G (Fig. 1B). Since this result suggested that a somatic mosaicism can cause AGS, we performed additional genetic analyses. Massively parallel DNA sequencing showed that neutrophils, monocytes, T cells, B cells and cells of the buccal mucosa harboured the same mutation at frequencies of approximately 20–30%, suggesting that the mutation occurred at an early stage of development and that approximately half of the mutated and normal cells co-existed in the patient.

Fig. 1 Genetic and molecular analyses of the *TREX1* mutations



(**A**) Chromatograms for the direct sequencing of the *TREX1* gene from patient 2 and from a healthy control. The arrow indicates the heterozygous c.583C>T substitution, which leads to the p.His195Tyr mutation. (**B**) Chromatograms for the direct sequencing of the *TREX1* gene from patient 3 and from a healthy control. The arrow indicates the mutant A allele: the signal intensity of this mutation, at position 598, is lower than that of the reference G, suggesting somatic mosaicism of c.598G>A, which leads to the p.Asp200Asn mutation. (**C**) Schematic representation of the human TREX1 protein. Exo1–3 shows the three exonuclease domains that form the active site. The arrows indicate the positions of Asp18, His195 and Asp200. Mutations in these amino acid residues can cause dominantly inherited AGS. (**D**) Alignment of the TREX1 protein sequences. His195 is highly conserved from *Schistosoma japonicum* to *Homo sapiens*.

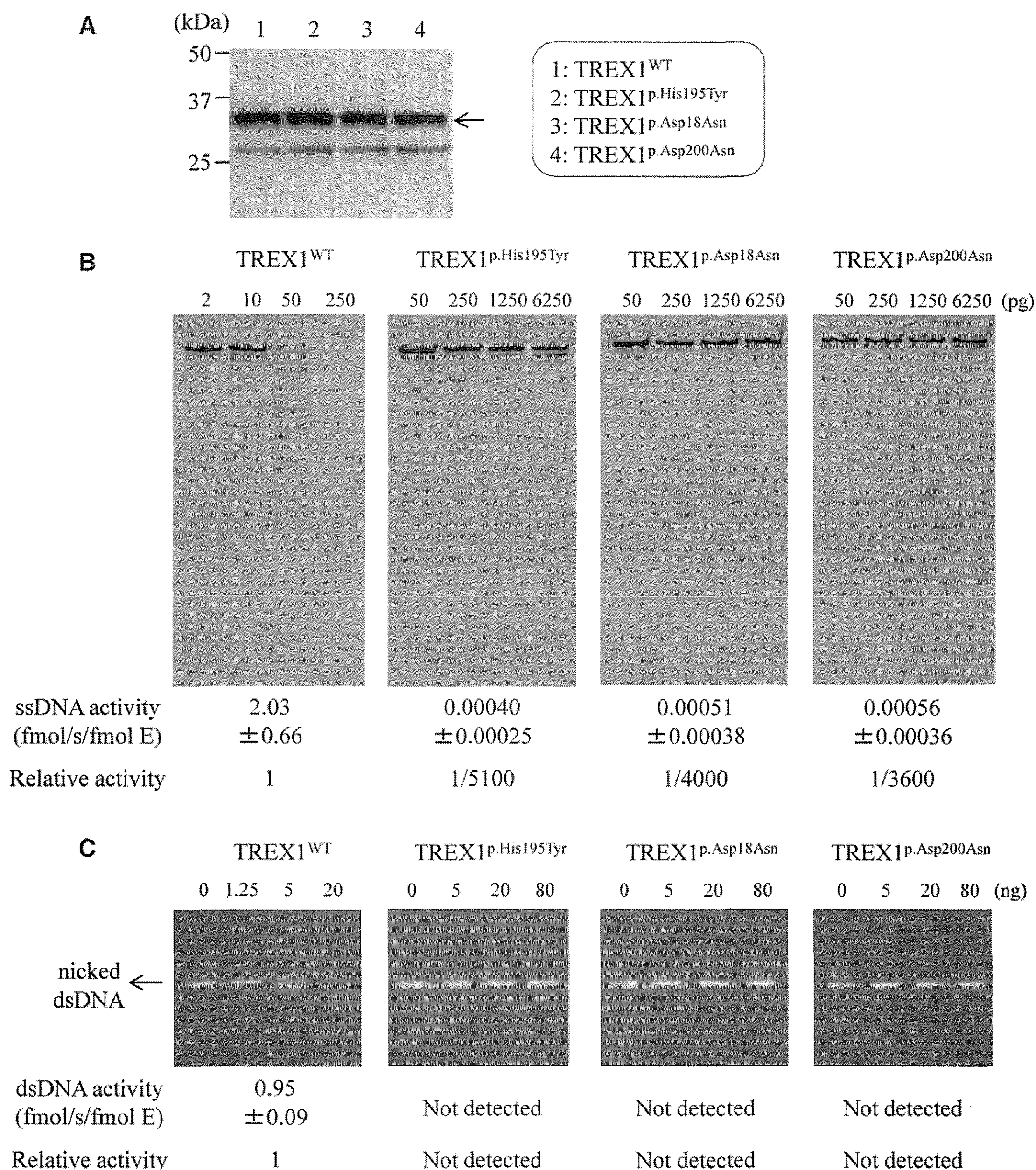
Clinical findings

All of the patients in the present cohort are currently alive (Table 2). Of the 13 patients for whom neonatal information was available, 11 were born at term, but 6 were small for their gestational age. All patients showed the first symptoms of AGS within 6 months of birth, with a median disease onset of 1.5 months. Four patients (patients 9, 11, 13 and 14) were early onset cases, which were affected within the first days of life; patients 9 and 11 had thrombocytopenia at the time of disease onset. Of the other subacute-onset cases, the earliest symptoms were largely related to encephalopathy, including neurological abnormalities and fever; however, patients 1 and 2 presented with rashes on the extremities that were later diagnosed as chilblains.

In the present study, all patients showed developmental delay, which was severe in 13 cases and moderate in 1 (patient 1). Seizures were observed in seven patients (patients 1, 5, 7, 8, 11, 13 and 14), including febrile convulsions in two. We also identified microcephaly in nine patients (patients 4–10, 12 and 14), dystonia in six (patients 5, 6, 8, 9, 12 and 13) and hypotonia in five (patients 2, 5, 7, 9 and 13).

Chilblain lesions were observed in seven patients (patients 1–5, 8 and 10) (Fig. 2A). All five patients harbouring *TREX1* mutations had chilblain lesions, which were absent in patients without gene mutations. The lesions

FIG. 2 Genetic and molecular analysis of the *TREX1* mutations



(**A**) Western blotting of purified recombinant human TREX1 proteins expressed in bacteria. The arrow indicates the position of the TREX1 proteins. The exonuclease activity of the recombinant TREX1 proteins in the (**B**) ssDNA assay and the (**C**) dsDNA assay. The amount of TREX1 protein used in the assays is indicated. The enzymatic activity of wild-type and mutated TREX1 proteins and the relative activity, which is shown as the ratio of the activity of the mutant to that of the wild type, are shown below each figure. Values are expressed as the mean (S.D.) of three independent experiments. The arrow in (C) indicates non-degraded, nicked dsDNA.

appeared on the fingers and toes in all seven cases; four of the seven also had lesions on the ears. In all cases the lesions were worse during the winter.

Recurrent fever was observed in seven patients (patients 1, 2, 4, 5, 8, 9 and 13). Regarding articular diseases, patient 4 suffered from scoliosis and a hip dislocation, and patient 10 had arthritis. Patients 7 and 9 suffered from hearing loss, and four patients (patients 2, 4, 8 and 14) had ophthalmological problems.

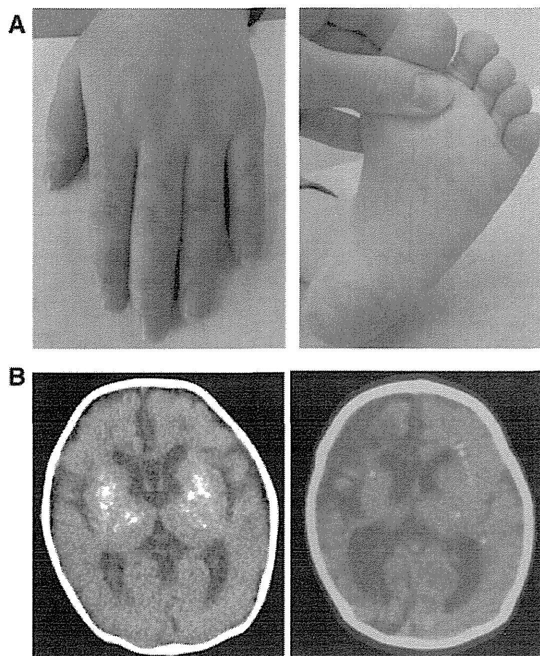
Patients 8 and 9, both harbouring *SAMHD1* mutations, fulfilled the ACR diagnostic criteria for SLE [29]. In addition, patient 10, who also harboured a *SAMHD1* mutation, was diagnosed with SS according to the Japanese criteria for SS [30].

TABLE 2 Clinical findings of Aicardi-Goutières syndrome patients

Patient	Genotype	Age, years	Sex	GA, weeks	BW, g	Age at disease onset	Manifestations at disease onset	Developmental delay	Other neurological manifestations	Chilblain lesions	Extraneural manifestations
1	*TREX1* AD	15	F	38	2840	23 d	Rash (extremities)	Moderate	Febrile convulsion	Yes	Recurrent fever, abdominal pain
2	*TREX1* AD	15	M	39	2792	2 mo	Rash (extremities)	Severe	Hypotonia	Yes	Recurrent fever, conjunctivitis, posterior synechia
3	*TREX1* AD	8	M	39	2828	4 mo	Delayed head control	Severe	None	Yes	None
4	*TREX1* AR	19	F	38	2044	3 mo	Delayed head control	Severe	Microcephaly, spastic quadriplegia	Yes	Recurrent fever, oral ulcer, abdominal pain, visual impairment, scoliosis, hip dislocation, neurogenic bladder dysfunction
5	*TREX1* AR	12	M	38	3000	18 d	Fever	Severe	Dystonia, hypotonia, microcephaly, febrile convulsion, mixed quadriplegia, startle reaction	Yes	Recurrent fever
6	*RNASEH2B* ?	6	F	Unknown	Unknown	3 mo	Eye movement disorder	Severe	Dystonia, microcephaly	No	None
7	*RNASEH2A* AR	2	F	36	1931	3 mo	Involuntary movement	Severe	Hypotonia (trunk), hypertonia (extremities), microcephaly, seizure	No	Hearing loss
8	*SAMHD1* AR	14	F	40	2052	1 mo	Irritability	Severe	Dystonia, microcephaly, seizure	Yes	Recurrent fever, hepatosplenomegaly, glaucoma, corneal perforation
9	*SAMHD1* AR	6	F	38	2534	5 d	Fever and feeding difficulties with thrombocytopenia	Severe	Dystonia, hypotonia, microcephaly	No	Recurrent fever, photosensitization, hearing loss
10	*SAMHD1* AR	16	M	39	2330	<4 mo	Irritability	Severe	Microcephaly	Yes	Arthritis
11	ND	4	M	36	2780	4 d	Omphalitis with thrombocytopenia	Severe	Hypertonia, seizure, spastic quadriplegia	No	Idiopathic interstitial pneumonia
12	ND	6	M	39	3290	6 mo	Developmental delay	Severe	Regression, dystonia, microcephaly	No	Atopic dermatitis
13	ND	7	F	40	2144	0 d	Conjugate deviation	Severe	Dystonia, hypotonia, seizure, spastic paralysis	No	Recurrent fever
14	ND	4	F	37	2056	0 d	Conjugate deviation	Severe	Regression, microcephaly, seizure, hemiplegia	No	Congenital cataract

GA: gestational age; BW: birth weight; AR: autosomal recessive; AD: autosomal dominant; ND: not detected; M: male; F: female; d: day(s); mo: month(s). Patients 1–10 are the same as presented in Table 1. No significant mutations in six genes associated with AGS were detected in patients 11–14. Patients 13 and 14 are siblings.

Fig. 3 Clinical presentations of Aicardi-Goutières syndrome patients



(**A**) Chilblain lesions observed in AGS patients. The left panel shows the hand of patient 1. The right panel shows the foot sole of patient 3. (**B**) Cranial calcifications on head CT scans. The left panel shows the head CT scan of patient 7 at the age of 4 months. The right panel shows head CT scan of patient 8 at the age of 3 months.

Laboratory findings

All of the AGS patients examined showed at least one CSF abnormality, including lymphocytosis, elevated IFN-α levels or elevated neopterin levels (Table 3). All three patients harbouring dominant-type *TREX1* mutations (patients 1-3) showed elevated serum IFN-α levels at the age of $\geqslant$7 years.

Seven patients showed elevated levels of serum autoantibodies: ANA in four cases (patients 8-10 and 12), anti-ssDNA antibodies in four cases (patients 5, 7, 9 and 10), anti-dsDNA antibodies in patients 5 and 9, anti-RNP antibodies in patients 8 and 10, anti-DNA antibody in patient 8, anti-SS-A antibody in patient 10 and anti-LKM1 antibody in patient 11. All three patients harbouring *SAMHD1* mutations showed increases in multiple autoantibody types. Other immunological findings included hypergammaglobulinaemia in patients 9-11 and hypocomplementaemia in patients 9 and 11.

Abnormal liver function was observed in nine cases (patients 4-6, 8-11, 13 and 14). Hematological abnormalities included thrombocytopenia in four cases (patients 8, 9, 11, and 13) and anaemia in patients 8 and 14. Regarding endocrine complications, we found moderate hypothyroidism in patients 1 and 9 and diabetes mellitus in patients 8 and 10.

Imaging findings

CT scans revealed cranial calcifications in all 14 patients (Fig. 2B). Eleven patients (patients 1-7 and 9-12) had calcifications in the bilateral basal ganglia, and some had calcifications in the thalamus, periventricular lesions or cerebellar hemispheres. Eleven patients (patients 3-9 and 11-14) had white matter abnormalities. High-intensity signals were observed mainly in periventricular lesions, frontal lobes or temporal lobes on T2-weighted MRI scans. Brain atrophy was evident in all but patient 1; this patient showed only moderate developmental delay.

Discussion

Here we present the results of the first nationwide survey of AGS patients conducted to examine the clinical manifestations and genotype-phenotype correlations of this disease. *RNASEH2B* is reported to be the most common gene associated with AGS (50 of 127 pedigrees) [3]; however, this mutation was identified at a significantly lower rate in the Japanese cohort ($P < 0.05$). The majority of the AGS patients in the present study harboured mutations in *TREX1* and *SAMHD1*. Although our cohort was small, the results suggest that the frequency of AGS gene mutations is variable in different ethnic populations.

AGS is usually inherited in an autosomal recessive manner, and previous studies have shown that consanguineous pedigrees are often involved [2, 3]. However, there was no consanguinity among our patients. Interestingly, we identified two patients harbouring *de novo* dominant-type *TREX1* mutations; this is a higher proportion than previously reported [3], since such patients are reported very rarely [2, 10-12]. AGS patients usually show severe neurological disabilities and rarely have children. Thus pedigrees are unhelpful when identifying *de novo* dominant-type AGS; however, the presence of parental consanguinity would facilitate the diagnosis of the more common recessive-type AGS. Since similar *de novo* mutations are expected to occur among different ethnic origins, we speculate that there might be more AGS patients harbouring *de novo* dominant-type mutations in other countries, regardless of the presence or absence of consanguinity.

The neurological symptoms shown by our AGS cohort were similar to those reported in previous studies, and included developmental delay, seizures, microcephaly, dystonia and hypotonia [2, 3]. The cohort also comprised a high percentage of patients with severe developmental delay, although AGS cases that are neurologically milder were recently described [2]. All of the patients in the present cohort presented with the first symptoms of the disease by the age of 6 months. This could be because the cohort harboured few *RNASEH2B* mutations, which are associated with less severe neurological findings and later-onset presentation [2, 3].

AGS is highly associated with type I IFN-related autoimmunity [2, 16]. SLE shows many similarities to AGS, and previous reports describe two patients with