cells. From these findings, a diagnosis of MCC was made. MCPyV large T-antigens (3) were detected in the tumour by immunostaining (Fig. 1e) and MCPyV DNA had 0.281 copies per cell, as determined by real-time PCR (4). However, HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59, and 68 types and also HPV types 5, 8, and 17 types which are EV-specific HPVs, were not detected using PCR.

Mutation analysis by PCR amplification using genomic DNA derived from his blood sample revealed a homozygous mutation, c.1824-1G>A, in the *EVER2/TMC8* gene. This mutation can cause an aberrant splicing such as exon 15 skipping or activation of cryptic splice sites and therefore is a highly possible pathogenic mutation. No pathogenic mutation in the *EVER1/TMC6* gene was found. His son and daughter did not present EV symptoms. Unfortunately, we did not have an opportunity to test his family for the *EVER2/TMC8* gene mutation. We established the diagnosis of MCPyV$^+$ MCC in the patient with EV on the basis of clinical features, histopathological findings, and gene mutation analysis. The patient underwent radiation therapy for MCC using 45.6 Gy to treat the tumour and 33 Gy to treat the left cervical lymph nodes, respectively. During treatment, the tumour and lymph node swelling disappeared. However, the patient decided to discontinue treatment on his own judgement. The tumour and lymph node swelling reoccurred after 5 months, and an additional round of radiation therapy was performed. It was effective for at least 4 months, but he died of unknown causes one month later.

DISCUSSION

Recently, the potential causative role of MCPyV has been suggested in the pathogenesis of MCC, because this virus is identified in approximately 90% of MCC cases (2). On the other hand, MCPyV is usually not detected in other skin tumours such as SCC (5). Patients with EV are incapable of clearing HPVs, therefore, they continue to have persistent infections that ultimately result in malignant transformation. To the best of our knowledge, only 2 EV cases with MCC and MCPyV infection have been reported (6, 7), and in one of these cases, the patient was also positive for HPVs in MCC (7). Although we did not detect the 3 types of EV-specific HPVs (as well as the mucosal HPVs that are indicative of high risk for cervical carcinoma), we cannot neglect a possibility that other HPVs are involved in MCC development. Patients with EV are susceptible to disease-specific HPVs and MCPyV infections. Thus, when physicians encounter patients with EV, they should pay attention to the possibility of SCC induced by EV-specific HPVs, as well as to MCC induced by MCPyV. In addition, we strongly recommend regular check-ups for reoccurrence and/or appearance of skin neoplasms.

Immunosuppressed patients with MCPyV infections cannot eliminate viruses after persistent infection and may eventually develop MCC (6, 8, 9). MCPyV has been detected in non-MCC skin lesions, SCCs and common warts in EV patients (8, 9). However, HPV 5 and/or 8 (8) and HPV17 (9) have also been detected in these skin lesions. In addition, MCPyV$^+$ SCC alone (10) and MCPyV$^+$ SCC and MCC (11) have been reported in immunosuppressed patients. However, the association of MCPyV with SCC onset remains unclear as most SCC cases also show positivity for HPVs.

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Aicardi-Goutières Syndrome Is Caused by *IFIH1* Mutations

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Aicardi-Goutières syndrome (AGS) is a rare, genetically determined early-onset progressive encephalopathy. To date, mutations in six genes have been identified as etiologic for AGS. Our Japanese nationwide AGS survey identified six AGS-affected individuals without a molecular diagnosis; we performed whole-exome sequencing on three of these individuals. After removal of the common polymorphisms found in SNP databases, we were able to identify *IFIH1* heterozygous missense mutations in all three. In vitro functional analysis revealed that *IFIH1* mutations increased type I interferon production, and the transcription of interferon-stimulated genes were elevated. *IFIH1* encodes MDA5, and mutant MDA5 lacked ligand-specific responsiveness, similarly to the dominant *Ifih1* mutation responsible for the SLE mouse model that results in type I interferon overproduction. This study suggests that the *IFIH1* mutations are responsible for the AGS phenotype due to an excessive production of type I interferon.

Aicardi-Goutières syndrome (AGS [MIM 225750]) is a rare, genetically determined early-onset progressive encephalopathy.[1] Individuals affected with AGS typically suffer from 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 basal ganglia calcification, white matter abnormalities, and cerebral atrophy.[3,4] Cerebrospinal fluid (CSF) analyses show chronic lymphocytosis and elevated levels of IFN-α and neopterin.[3–5] AGS-affected individuals are often misdiagnosed as having intrauterine infections, such as TORCH syndrome, because of the similarities of these disorders, particularly the intracranial calcifications.[1] In AGS, etiological mutations have been reported in the following six genes: *TREX1* (MIM 606609), which encodes a DNA exonuclease; *RNASEH2A* (MIM 606034), *RNASEH2B* (MIM 610326), and *RNASEH2C* (MIM 610330), which together comprise the RNase H2 endonuclease complex; *SAMHD1* (MIM 606754), which encodes a deoxynucleotide triphosphohydrolase; and *ADAR1* (MIM 146920), which encodes an adenosine deaminase.[6–9] Although more than 90% of AGS-affected individuals harbor etiological mutations in one of these six genes, some AGS-affected individuals presenting with the clinical characteristics of AGS still lack a genetic diagnosis, suggesting the existence of additional AGS-associated genes.[1]

We recently conducted a nationwide survey of AGS in Japan and reported 14 AGS-affected individuals.[10] We have since recruited three other Japanese AGS-affected individuals, and among these 17 individuals, we have identified 11 individuals with etiologic mutations; namely, *TREX1* mutations in six, *SAMHD1* mutations in three, and *RNASEH2A* and *RNASEH2B* mutations in one each. Of the remaining six individuals without a molecular diagnosis, trio-based whole-exome sequencing was performed in three whose parents also agreed to participate in further genome-wide analyses (Figure 1A). Genomic DNA from each individual and the parents was enriched for protein-coding sequences, followed by massively parallel sequencing. The extracted nonsynonymous or splice-site variants were filtered to remove those with minor allele frequencies (MAF) > 0.01 in dbSNP137. To detect de novo variants, any variants observed in family members, listed in Human Genetic Variation Database (HGVD), or with MAF > 0.02 in our in-house exome database were removed. To detect autosomal-recessive (AR), compound heterozygous (CH), or X-linked (XL) variants, those with MAF > 0.05 in our in-house database were removed (Figure S1 available online). All samples were collected with the written informed consents by parents, and the study protocol was approved by the ethical committee of Kyoto University Hospital in accordance with the Declaration of Helsinki.

After common polymorphisms were removed, we identified a total of 40, 18, 89, and 22 candidate variants under the de novo, AR, CH, and XL inheritance models, respectively, that were present in at least one of the three individuals (Table S1). Among them, missense mutations were identified in *IFIH1* (MIM 606951, RefSeq accession

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http://dx.doi.org/10.1016/j.ajhg.2014.06.007.

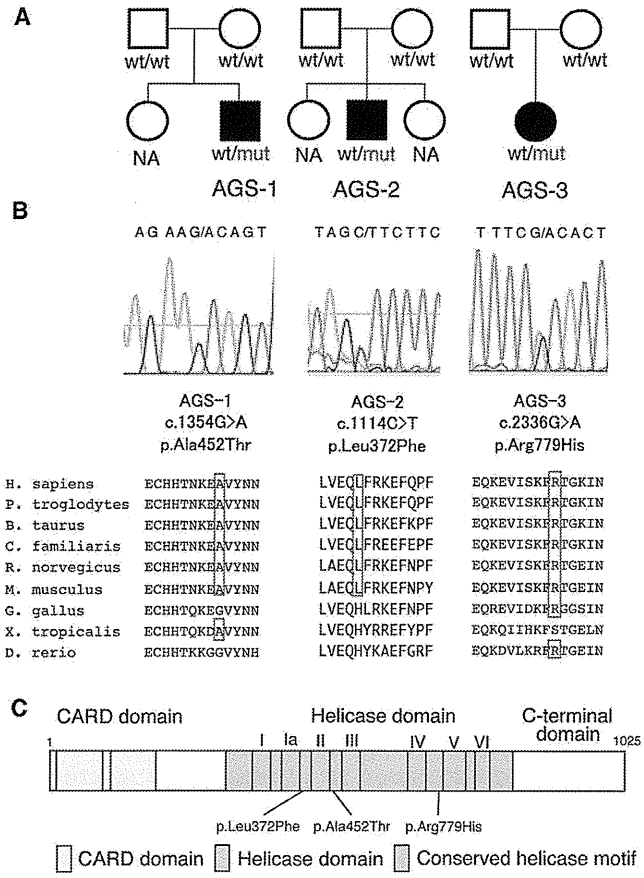

Figure 1. Pedigree Information for the AGS-Affected Individuals and Details of the *IFIH1* Mutations Identified

(A) The pedigrees of the three families indicating the AGS probands.

(B) Sanger sequencing chromatograms of the three *IFIH1* mutations found in the AGS-affected individuals. The locations of these mutations in the amino acid sequence of the MDA5 protein are shown in alignment with the conserved amino acid sequences from several species. This alignment was obtained via ClustalW2. The amino acids that are conserved with human are circled in red.

(C) The MDA5 protein domain structure with the amino acid substitutions observed in these AGS-affected individuals.

number NM_022168.2), which encodes MDA5 (RefSeq NP_071451.2). These missense mutations are c.1354G>A (p.Ala452Thr) in AGS-1; c.1114C>T (p.Leu372Phe) in AGS-2; and c.2336G>A (p.Arg779His) in AGS-3 (Figure 1B). None of the mutations are found in HGVD, including the 1,208 Japanese samples, or our in-house exome database of 312 Japanese individuals. Multiple-sequence alignment by ClustalW2 revealed that each of the amino acids affected by these mutations are conserved among mammals (Figure 1B). The subsequent amino acid alterations were all suggested to be disease causing in at least one of the four function-prediction programs used (Table 1). None of the other genes identified in the de novo inheritance model, or any of the genes identified in the other three inheritance models, were mutated in all three individuals. The *IFIH1* mutations identified were validated by Sanger sequencing. The other coding exons of *IFIH1* were also examined by Sanger sequencing, and no other mutations were found.

MDA5 is one of the cytosolic pattern recognition receptors that recognizes double-stranded RNA (dsRNA).[11] MDA5 consists of N-terminal tandem CARD domains, a central helicase domain, and a C-terminal domain (Figure 1C). When bound to dsRNA, MDA5 forms a closed, C-shaped ring structure around the dsRNA stem and excludes the tandem CARD as well as creates filamentous oligomer on dsRNA.[12] It is hypothesized that the tandem CARD interacts each other and activates MAVS on the mitochondrial outer membrane. Oligomerization of MAVS induces TBK1 activation, IRF3 phosphorylation, and induction of type I interferon transcription, resulting in the activation of a large number of interferon-stimulated genes (ISGs).

The neurological findings of the individuals with these *IFIH1* mutations are typical of AGS (Table S2). They were born with appropriate weights for their gestational ages without any signs of intrauterine infection. However, they all demonstrated severe developmental delay in early infancy associated with progressive microcephaly. No arthropathy, hearing loss, or ophthalmological problems were observed. As for extraneural features, all three individuals had at least one of the following autoimmune features: positivity for autoantibodies, hyperimmunoglobulinemia, hypocomplementemia, and thrombocytopenia. Notably, none of the individuals with *IFIH1* mutations had chilblain lesions, although all the five individuals with *TREX1* mutations and two of the three individuals with *SAMHD1* mutations in the Japanese AGS cohort showed chilblain lesions.[10] Individuals with *SAMHD1* mutations and *IFIH1* mutations both show autoimmune features; however, chilblain lesions have been observed only in individuals with *SAMHD1* mutations.[10]

To predict the effects of the identified amino acid substitutions on MDA5, three-dimensional model structures of MDA5 mutants were generated from the crystal structure of human MDA5-dsRNA complex[12] (Protein Data Bank [PDB] code 4gl2), using PyMOL (Schroedinger) and MOE (Chemical Computing Group) (Figure S2A). The oligomeric model of MDA5 was generated using the electron microscopy imaging data of MDA5 filament lacking CARD domain[13] (Electron Microscopic Data Bank [EMDB] code 5444) (Figure S2B). The three amino acid substitutions in the AGS-affected individuals are all located within the helicase domain (Figures 1C and S2A). Because Ala452 directly contacts the dsRNA ribose O2′ atom, the p.Ala452Thr substitution probably affects the binding affinity to dsRNA due to an atomic repulsion between the side chain of Thr452 and the dsRNA O2′ atom (Figures S2C and S2D). Leu372 is located adjacent to the ATP binding pocket, and the p.Leu372Phe substitution could increase the side chain volume of the binding pocket, affecting its ATP hydrolysis activity (Figures S2E and S2F). In our oligomeric model, Arg779 is located at the interface between the two monomers, which is consistent with the

Table 1. Functional Predictions of the *IFIH1* Variants

Individuals	Nucleotide Change	Amino Acid Change	SIFT	PolyPhen2	Mutation Taster	PROVEAN
AGS-1	c.1354G>A	p.Ala452Thr	tolerated	benign	disease causing	neutral
AGS-2	c.1114C>T	p.Leu372Phe	tolerated	probably damaging	disease causing	neutral
AGS-3	c.2336G>A	p.Arg779His	tolerated	probably damaging	disease causing	deleterious

The potential functional effects of the *IFIH1* variants identified in the AGS-affected individuals were predicted via SIFT, PolyPhen2, Mutation Taster, and PROVEAN.

recent report showing that Lys777, close to Arg779, is in close proximity to the adjacent monomer.[12] Furthermore, in our model, Arg779 is in close to Asp572 on the surface of the adjacent monomer. We speculate that losing the positive charge due to the p.Arg779His substitution would possibly affect the electrostatic interaction between the MDA5 monomers (Figures S2G and S2H).

To connect the identified *IFIH1* mutations with the AGS phenotype, we examined the type I interferon signature in the individuals by performing quantitative RT-PCR (qRT-PCR) of seven ISGs.[14] Peripheral blood mononuclear cells (PBMCs) from the three AGS-affected individuals showed upregulation of ISG transcription (Figure 2), confirming the type I interferon signature in the individuals with *IFIH1* mutations.

To elucidate the disease-causing capability of the identified *IFIH1* mutations, three FLAG-tagged *IFIH1* mutant plasmids containing these mutations were constructed via site-directed mutagenesis. These plasmids were transiently expressed on human hepatoma cell line Huh7 and the *IFNB1* promoter activity as well as endogenous expression of *IFIT1* (MIM 147690) was measured 48 hr after transfection.[15] The three mutant plasmids activated the *IFNB1* promoter in Huh7 cells more strongly than the wild MDA5 and nearby missense variants reported in dbSNP (Figures 3 and S3). The upregulation of endogenous *IFIT1* was also observed in the transfected cells (Figure S4), suggesting that these AGS mutations enhance the intrinsic activation function of MDA5. Recent genome-wide association studies (GWASs) showed association of the *IFIH1* with various autoimmune diseases, such as systemic lupus erythematosus (SLE), type I diabetes, psoriasis, and vitiligo.[16–19] We examined *IFNB1* promoter activity induced by the c.2836G>A (p.Ala946Thr) polymorphism (rs1990760) identified in the GWASs. Although the c.2836G>A polymorphism partially activated the promoter activity, the induced activity was lower than those of the AGS-derived mutants. In addition, the dominantly inherited SLE mouse model in the ENU-treated mouse colony is reported to have the *Ifih1* mutation, c.2461G>A (p.Gly821Ser).[15] These observations suggest that *IFIH1* has strong association with various autoimmune diseases, especially SLE, which also has a type I interferon signature.[20] Because alteration of *TREX1* has been reported to cause AGS as well as SLE,[21] it seems quite plausible for *IFIH1* to also be involved in both AGS and SLE. Interestingly, all the individuals identified with *IFIH1* mutations had autoantibodies, suggesting the contribution of *IFIH1* mutations to autoimmune phenotypes.

To further delineate the functional consequences of the three *IFH1* mutations, we measured the ligand-specific *Ifnb* mRNA induction by stimulating *Ifih1*^{null} mouse embryonic fibroblasts (MEFs) reconstituted with retrovirus expressing the *IFIH1* mutants by an MDA5-specific ligand, encephalomyocarditis virus (EMCV).[22] None of the MEF cells expressing the three mutant *IFIH1* responded to the EMCV, which suggested that the MDA5 variants lacked the ligand-specific responsiveness. The response of the three AGS mutants against the MDA5-specific EMCV was similar to that of the p.Gly821Ser variant reported in the dominantly inherited SLE mouse model with type I interferon overproduction[15] (Figures 4 and S5).

During the revision of this manuscript, Rice et al. identified nine individuals with *IFIH1* mutations, including the c.2336G>A mutation we identified, in a spectrum of neuroimmunological features consistently associated with enhanced type I interferon states including AGS.[23] Although we agree that the *IFIH1* mutations cause constitutive type I interferon activation, Rice et al. show that the mutated MDA5 proteins maintain ligand-induced responsiveness, which was not the case in our study. Because we measured the ligand-specific responsiveness of MDA5 in different experimental conditions, further analysis remains to be performed to reveal the biochemical mechanism of interferon overproduction by the mutated MDA5.

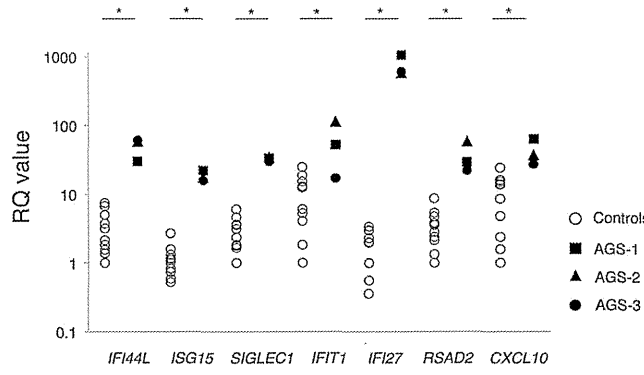

Figure 2. Quantitative RT-PCR of a Panel of Seven ISGs in PBMCs Obtained from the *IFIH1*-Mutated Individuals and Healthy Control Subject

qRT-PCR was performed as previously described.[15] The relative abundance of each transcript was normalized to the expression level of β-actin. Taqman probes used were the same as previous report,[14] except for *ACTB* (MIM 102630). Individual data were shown relative to a single calibrator (control 1). The experiment was performed in triplicate. Statistical significance was determined by Mann-Whitney U test, *$p < 0.05$.

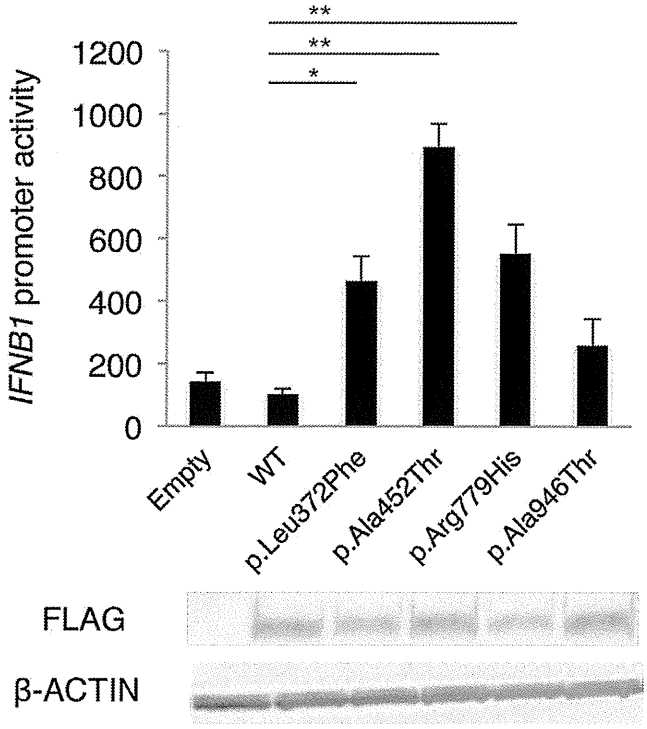

Figure 3. The Effects of the Three MDA5 Variants on *IFNB1* Expression

Huh7 cells were transfected with a reporter gene containing *IFNB1* promoter (p-55C1B Luc), an empty vector (BOS), and expression vectors for FLAG-tagged human wild-type *IFIH1*, c.2836G>A polymorphism (p.Ala946Thr) in the GWASs, and the identified *IFIH1* mutants. Luciferase activity was measured 48 hr after transfection, and the MDA5 protein accumulation was examined by immunoblotting as previously described.[15] FLAG indicates the accumulation of FLAG-tagged MDA5. Each experiment was performed in triplicate and data are mean ± SEM. Shown is a representative of two with consistent results. Statistical significance was determined by Student's t test. *$p < 0.05$, **$p < 0.01$.

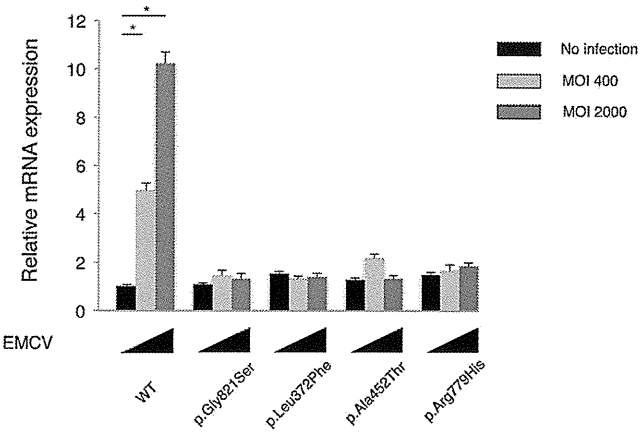

Figure 4. *Ifnb* mRNA Levels in *Ifih1*-Deficient MEFs Expressing *IFIH1* Mutants

The MEFs were infected with retroviruses encoding mouse wild-type *Ifih1*, mouse *Ifih1* with c.2461G>A (p.Gly821Ser) (RefSeq NM_027835.3) mutation, or the three AGS mutants of human *IFIH1*. At 48 hr after the retroviral infection, these MEFs were infected with indicated multiplicity of infection (MOI) of EMCV for 6 hr, and *Ifnb* mRNA levels were measured by qRT-PCR. The relative abundance of each transcript was normalized to the expression level of 18S ribosomal RNA. Data are shown as mean ± SEM of triplicate samples. Shown is a representative of two independent experiments. Statistical significance was determined by Student's t test, *$p < 0.001$. The expression of the retrovirally transduced FLAG-tagged constructs was confirmed by immunoblotting (Figure S5).

In conclusion, we identified mutations in *IFIH1* as a cause of AGS. The individuals with the *IFIH1* mutations showed encephalopathy typical of AGS as well as the type I interferon signature with autoimmune phenotypes, but lacked the chilblains. Further analysis remains to elucidate the mechanism of how the *IFIH1* mutations identified in AGS cause the type I interferon overproduction.

Supplemental Data

Supplemental Data include five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.06.007.

Acknowledgments

We are very grateful to Y. Takaoka (Kyoto University) and E. Abe (RIKEN Center for Integrative Medical Sciences) for their technical assistance on Sanger sequencing, to E. Hirano (Kyoto University) for her technical assistance on functional analyses of the AGS mutants, to M. Takazawa (Kazusa DNA Research Institute) for his contribution to exome data analysis, and to T. Taylor for his critical reading of the manuscript. This work was supported by the Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was supported by Grants-in-aid for Scientific Research from the Japanese Ministry of Health, Labor and Welfare and the Japanese Ministry of Education, Culture, Sports, Science, Technology (MEXT).

Received: March 1, 2014
Accepted: June 11, 2014
Published: July 3, 2014

Web Resources

The URLs for the data presented herein are as follows:

Burrows-Wheeler Aligner, http://bio-bwa.sourceforge.net/
ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
EMDataBank, http://www.emdatabank.org/index.html
GATK, http://www.broadinstitute.org/gatk/
Human Genetic Variation Database (HGVD), http://www.genome.med.kyoto-u.ac.jp/SnpDB/
MutationTaster, http://www.mutationtaster.org/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
PolyPhen-2, http://www.genetics.bwh.harvard.edu/pph2/
PROVEAN, http://provean.jcvi.org/index.php
RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
SIFT, http://sift.bii.a-star.edu.sg/

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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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Handling editor Tore K Kvien

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

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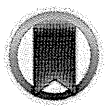
Received 27 July 2013
Revised 16 October 2013
Accepted 24 November 2013
Published Online First 10 December 2013



To cite: Nakagawa K, Gonzalez-Roca E, Souto A, *et al. Ann Rheum Dis* 2015;**74**:603–610.

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

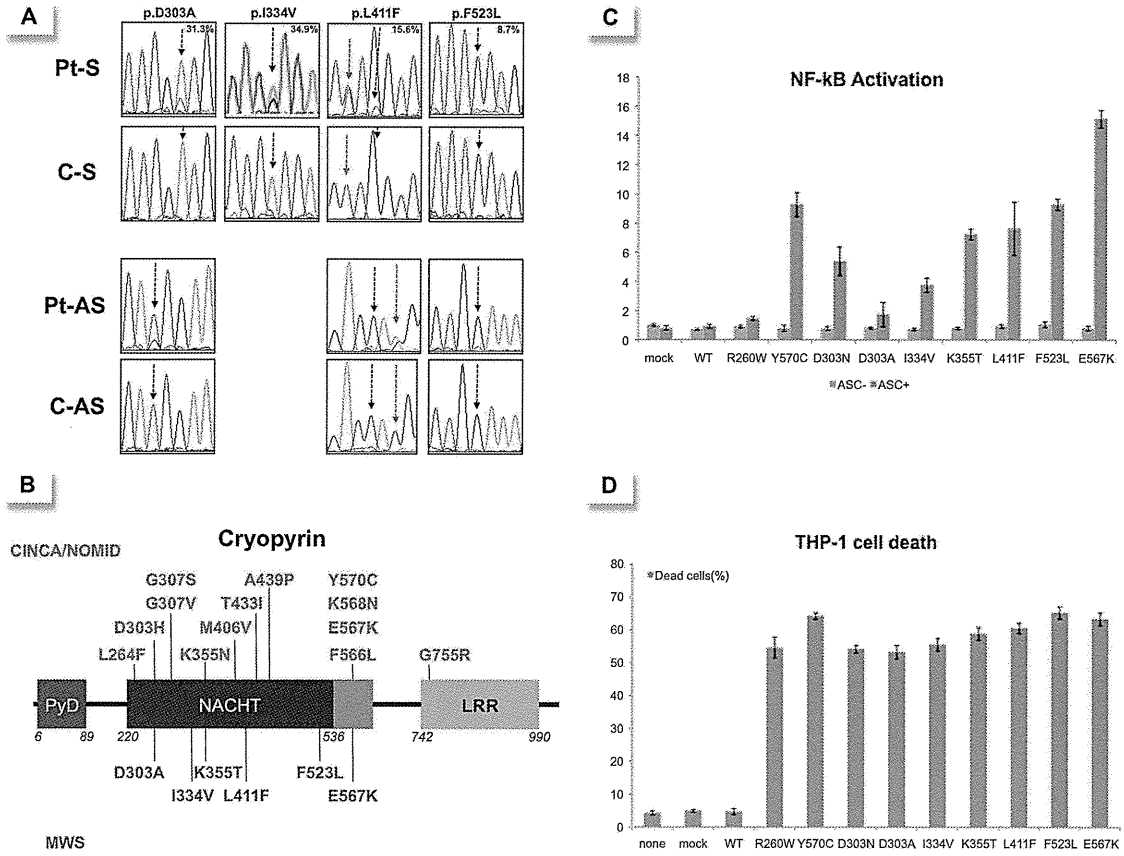


Figure 1 (A) Sense (upper rows) and antisense (bottom rows) chromatograms from four patients with somatic *NLRP3* mosaicism and controls obtained by Sanger sequencing using genomic DNA extracted from whole blood. The black arrows show the *NLRP3* positions where the somatic mutations were detected. The 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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

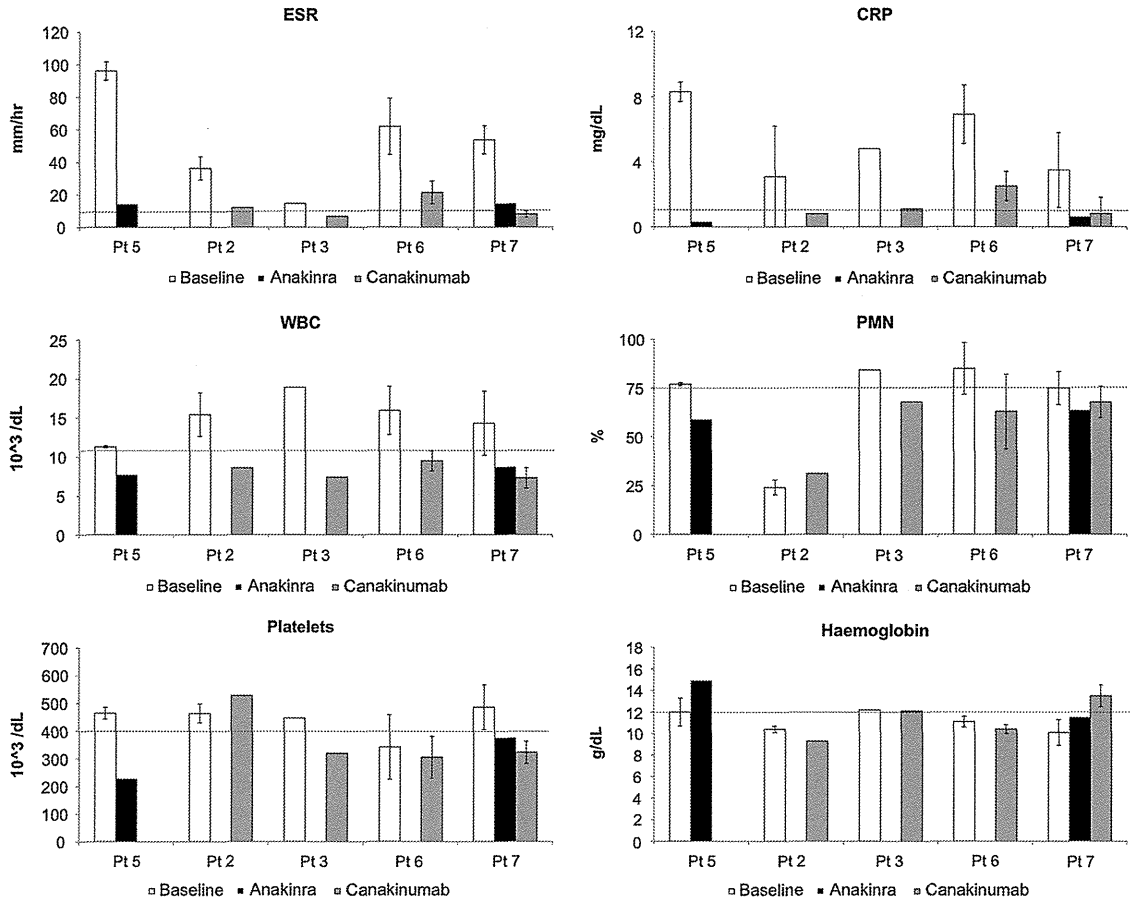


Figure 2 Laboratory values obtained in the five patients treated with different anti-interleukin 1 drugs. Patient's graphics were ordered as follows: First, those graphics from the patient who only received treatment with anakinra (Pt 5), followed by those from patients who only received treatment with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

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

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

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

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

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

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

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

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

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

Competing interests None.

Patient consent Obtained.

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

Provenance and peer review Not commissioned; externally peer reviewed.

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

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Ann Rheum Dis 2015 74: 603-610 originally published online December 10, 2013
doi: 10.1136/annrheumdis-2013-204361

J Clin Immunol
DOI 10.1007/s10875-014-0058-8

BRIEF COMMUNICATION

A Complement Factor B Mutation in a Large Kindred with Atypical Hemolytic Uremic Syndrome

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Received: 9 July 2013 / Accepted: 15 May 2014

Abstract
Purpose Gain-of-function mutations in complement factor B (*CFB*) were recently identified in patients with atypical hemolytic uremic syndrome (aHUS), but are extremely rare. Our purpose is to describe a large kindred with aHUS associated with a *CFB* mutation and to further understand *CFB*-mutated aHUS patients.
Methods and Results We report a large kindred in which 3 members had aHUS. This kindred revealed that 9 of 12 members, including 2 affected patients, had persistent activation of the alternative pathway with low complement component 3 and that those 9 members showed a *CFB* mutation (c.1050G>C, p.Lys350Asn) in exon 8. This missense mutation was heterozygous in 8 of them and homozygous in only one. From structural studies, this mutation is shown to be located in close proximity to the Mg^2-binding site within a von Willebrand factor type A domain of CFB, resulting in a gain-of-function effect of CFB and predisposition to aHUS. At present, 2 of the 3 members with aHUS have maintained normal renal function for a long-term period.
Conclusions This kindred illustrates that a *CFB* mutation (c.1050G>C, p.Lys350Asn) can result in aHUS. In the future, phenotype-genotype correlations and outcome in *CFB*-mutated aHUS patients need to be further investigated by accumulation of a number of cases.

Keywords Atypical hemolytic uremic syndrome · complement alternative pathway · complement factor B · gain-of-function · large kindred

Abbreviations

aHUS	Atypical hemolytic uremic syndrome
CFH	Complement factor H
CFI	Complement factor I
MCP	Membrane cofactor protein
CFB	Complement factor B
C3	Complement component 3
THBD	Thrombomodulin
VWA	von Willebrand factor type A
SP	Serine protease

Introduction

Atypical hemolytic uremic syndrome (aHUS) is known to be a disorder of the regulation of the complement alternative pathway, and is also broadly interpreted as a primary immunodeficiency

Electronic supplementary material The online version of this article (doi:10.1007/s10875-014-0058-8) contains supplementary material, which is available to authorized users.

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Published online: 08 June 2014

disease or an autoinflammatory disease [1–3]. Approximately 50 % of aHUS patients have been reported to have mutations in the genes coding for regulators or components of the complement alternative pathway, complement factor H (*CFH*), complement factor H-related 5, complement factor I (*CFI*), membrane cofactor protein (*MCP*), complement factor B (*CFB*), complement component 3 (*C3*) and thrombomodulin (*THBD*) [1, 4, 5]. In particular, *CFB* mutations in aHUS patients are extremely rare [1, 6–10], accounting for 0 to 3 % of aHUS patients [1, 4], and are only noted marginally in updated classifications of primary immunodeficiency diseases [2].

To further understand aHUS associated with a *CFB* mutation, we analyzed a large kindred with aHUS associated with a *CFB* missense mutation (c.1050G>C, p.Lys350Asn) in exon 8.

Methods

Patients

The pedigree of a nonconsanguineous Japanese kindred that we studied is depicted in Fig. 1a, and a summary of the clinical and laboratory data of 3 members with aHUS is given in Table I.

DNA Sequencing

Genomic DNA was extracted from leukocytes using SepaGene (Eidia, Tokyo, Japan). DNA fragments of the *CFH*, *CFI*, *MCP*, *CFB*, *C3*, and *THBD* were amplified by PCR and analyzed using Big Dye Terminator Bidirectional Sequencing (Applied

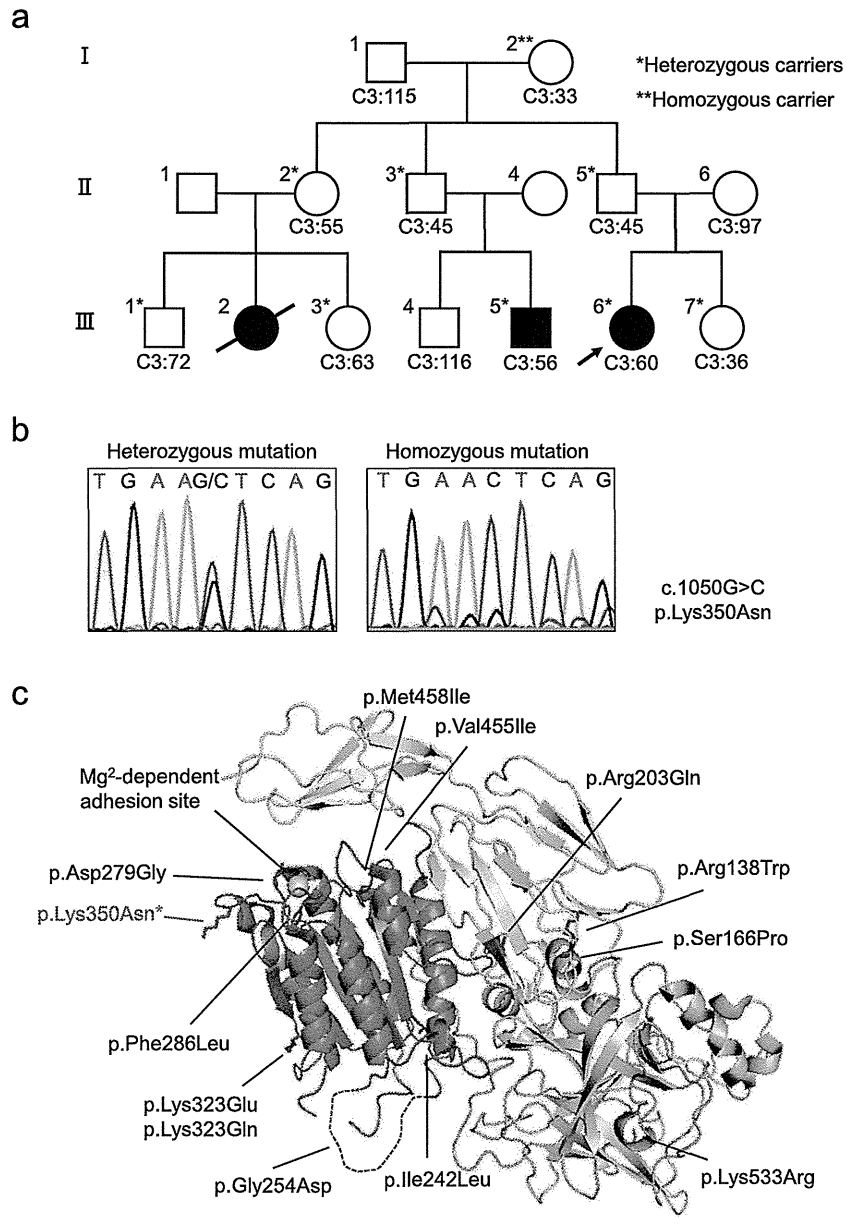

Fig. 1 **a**, Pedigree of kindred we studied. Individuals are identified by numbers within each generation. Arrow indicates the proband. Black and white symbols indicate, respectively, affected and unaffected individuals. One deceased individual is crossed. Heterozygous mutation carriers are indicated by asterisks, and one homozygous mutation carrier by double asterisks. Serum C3 levels (mg/dl) in a normal situation are shown below symbols **b**, Genetic analysis of the *CFB* **c**, Structures of the von Willebrand type A (VWA) domain (blue), the serine protease (SP) domain (green), and three complement-control protein (CCP) domains (yellow) in CFB. The location of the mutation we identified (*) and previously reported mutations are shown

Table I Clinical characteristics and laboratory data on admission of aHUS patients

	Patient III-2	Patient III-5	Patient III-6	Normal values
Onset (year)	1998	2010	2007	
Onset age (months)	8	6	20	
Gender	Female	Male	Female	
Extrarenal manifestations	No	No	No	
Chief complaint	Vomiting	Rhinorrhea	Cough	
	Fever	Fever	Fever	
	Paleness	Paleness	Paleness	
Diarrhea	Negative	Negative	Negative	
Hemoglobin (g/dl)	4.7	5.9	6.9	9.0–13.0
Platelet (/μl)	99,000	54,000	71,000	200,000–250,000
LDH (IU/l)	1,121	2,285	2,359	235–335
BUN (mg/dl)	72.6	53.0	96.6	10.0–23.0
Creatinine (mg/dl)	0.7	0.72	1.03	0.03–0.50
Direct antiglobulin test	Negative	Negative	Negative	Negative
C3 (mg/dl)	28	48	33	80–165
C4 (mg/dl)	33	33	15	12–30
ADAMTS13 activity (%)	N/A	44	59	>50
Anti-FH antibody	N/A	N/A	Negative	Negative
Hematuria	3+	3+	3+	–
Proteinuria	3+	3+	3+	–
Stool culture	Negative	Negative	Negative	
Main treatment for uremia	SP inhibitor	PE, HF	PE, HF	
Outcome	Deceased	Alive (>3 years) with 2 recurrences	Alive (>6 years)	

LDH, lactate dehydrogenase; BUN, blood urea nitrogen; C3, complement component 3; C4, complement component 4; ADAMTS, a disintegrin and metalloproteinase with thrombospondi motifs; FH, factor H; N/A, not available; SP, Serine protease; PE, plasma exchange; HF, hemofiltration

Biosystems, Foster City, CA, USA). Primer sequences are available upon request. Numbering is made with the first methionine counted as 1.

SIFT and PolyPhen-2 Algorithm Analyses

We used two popular algorithms, SIFT (sift.jcvi.org) and PolyPhen-2 (genetics.bwh.harvard.edu/pph2), for predicting the damaging effects of previously identified mutations.

Structural Studies

PyMOL (www.pymol.org) was used to draw the ribbon diagram of protein structure of a single von Willebrand factor type A (VWA) domain and a carboxyl-terminal serine protease (SP) domain by using PDB file, 2WXB [11].

Results and Discussion

To demonstrate the causal factor of aHUS in this kindred, we studied complement profiles and the genomic analysis of aHUS-associated genes. We first examined the serum C3 level to screen kindred members with a predisposed aHUS condition, because a low serum C3 level that reflects complement activation and consumption is sometimes observed in aHUS patients with *CFH*, *CFI*, *CFB*, and *C3* mutations but not *MCP* mutations [4, 12]. And, we found that 9 of 12 members, including 2 affected patients (III-5 and III-6), except a deceased patient (III-2), presented low serum C3 levels in a normal situation. We also examined the well-known susceptibility genes including *CFH*, *CFI*, *CFB*, and *C3*, and identified a heterozygous *CFB* missense mutation (c.1050G>C, p.Lys350Asn) in 8 of 9 members with low C3, including 2 affected patients (III-5 and III-6), and a homozygous mutation in one unaffected member (I-2) with low C3 (Fig. 1b). No p.Lys350Asn mutations were present in an ethnically matched control population of 100 normal individuals.

So far, some researchers have reported *CFB* mutations in aHUS patients [1, 6–10]. p.Lys350Asn that we identified in this study has also been reported in sporadic case with aHUS by Roumenina et al. [7]. To review the protein function of some *CFB* mutations identified in previous reports [1, 6–10], we analyzed these reported mutations using SIFT and PolyPhen-2 algorithms. The SIFT score of p.Arg203Gln and

p.Phe286Leu was less than 0.05 (indicated to be damaging), whereas the PolyPhen-2 score of p.Arg203Gln, p.Gly254Asp, p.Phe286Leu, and p.Lys350Asn was range from 0.85 to 1.00 (indicated to be probably damaging). To further investigate how these mutations predicted to be damaging can affect the function of the protein, we next analyzed these mutations from a structural point of view. CFB is composed of an amino-terminal region of three complement control protein domains, a 40-amino acid linker region, a single VWA domain and a carboxyl-terminal SP domain [13]. When Bb fragment of CFB binds to C3b, a metal ion-dependent adhesion site is formed at the apex of the VWA domain and mediates Mg^2-dependent C3b binding [11]. Therefore, the mutation in close proximity to the Mg^2-binding site within the VWA domain, causes resistance to decay acceleration and increases C3b-binding affinity and C3bBb stability [14]. In fact, p.Phe286Leu and p.Lys350Asn in the mutations predicted to be damaging are located in close proximity to the Mg^2-binding site within the VWA domain of CFB (Fig. 1c), and these functional expression studies were reported to result in increased formation of the C3bBb complex, indicating a gain-of-function effect of CFB [6, 7]. Moreover, while we were preparing this manuscript, Roumenina and colleagues studied the functional consequences of 10 *CFB* mutations and described that 6 *CFB* mutations including p.Lys350Asn are related to aHUS pathogenesis [15]. These revealed that p.Lys350Asn is the causal factor of aHUS in this kindred from several perspectives.

In this kindred, only 3 members had aHUS, and other heterozygous mutation carriers and a homozygous mutation carrier have been in a healthy state without any diseases despite having low C3. Persistent activation of the alternative pathway by the *CFB* mutation can result in aHUS via triggering events, as reported previously [6], but the mechanism of incomplete penetrance remains unclear. Recent studies have shown that multiple disease-associated polymorphisms or at-risk CFH and MCP haplotypes are strongly associated with aHUS [6, 9, 16, 17]. To investigate the aHUS-onset-associated genetic factor, we further studied aHUS-associated genes including *MCP* and *THBD*. However, no specific mutation, polymorphism or at-risk CFH and MCP haplotypes in 2 affected members (III-5 and III-6) could be found (Supplementary Table 1 and 2). This may relate to other specific mutations, the unique nature of the affected members, or undefined aspects in the environment.

In general, the overall prognosis of patients with mutations in aHUS associated genes is poor [4, 18], but the clinical course and outcome in *CFB*-mutated aHUS patients is not well documented. In our study, although patient III-2 died of aHUS-associated myocardiopathy because of no available specific treatment at that time, patients III-5 and III-6 have had normal renal function for more than 3 and 6 years, respectively. These patients will need to be further followed up over a long period of time to study the renal outcome of aHUS patients with *CFB* mutations.

Conclusions

We described a large kindred in which a *CFB* missense mutation was the causal factor of aHUS. Phenotype-genotype correlations and outcome in *CFB*-mutated aHUS patients need to be further investigated by accumulation of a number of cases, which will lead to better treatment.

Acknowledgments We thank Masanori Matsumoto, Yoko Yoshida, and Yoshihiro Fujimura (Nara Medical University, Kashihara, Japan) for ADAMTS13 activity assay and Nobuaki Takagi and Hiromu Mae (Hyogo college of Medicine, Nishinomiya, Japan) for anti-factor H antibody assay.

This work was supported in part by Health and Labor Sciences Research Grants of Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare.

Declaration of Interest The authors declare that they have no conflict of interest.

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