Table 1 Primers utelized in the present study

	Forward primer	Reverse primer
FGFR1 sequence analysis		
Exon 2	CCTTCTGTTTTTCCTTTCCCC	GAGCCTTCCCTGTTGACCA
Exon 3	TCTTCCTCTCTCGCCCCTT	AAGCAGAGTGGGGGCAGAT
Exon 4 + 5	AAATCTAGGGGTCCCTAGGAG	TCCCCTGTTCCCATTACTCTA
Exon 6	GTCATGGGGCCTGCATTTT	AAGTGCCAATCGCTATCCTGA
Exon 7	ATGGCAAGGTCCCCATGA	AAGCGTGAGGAATGATCCCAT
Exon 8	AGGTCTCATGTCCTGTGCTTG	AGGATTCAGCCCTCAAAGCT
Exon 9	CAGGAGACAGGTGTTGCTTTT	AGCCAGAAAATAAGGCCCAA
Exon 10 + 11	TTGGGAAGCCCTGACTAAGAA	TGTTTGCTTGGAATGGGACA
Exon 12	AAAGCAGCCCCTCGACACATA	ACCCCAGCTCAGATCTTCTCC
Exon 13	AACCTGCTCACCTGCTGCT	CACAGGCTGGAAGACTAGGG
Exon 14	CAGAGCAGTGTGGCAGAAGTT	ATGAGGGCACAGGTGGGAA
Exon 15	TGAGAACCCACCCCCCAGT	GCATGGAGAACAGATCAGCCT
Exon 16	TGGGTAGAGGATTTGTGCTGG	TCAGGGACCGTCTCCTGGAGAT
Exon 17	TAAGCCCGAGGAGATGTCG	AAAGCTGGGGGAGTACTGGT
Exon 18	CCATCGCTGCTTCCTCTC	TTACAGCTGACGGTGGAGTCT
FISH probe		
LA-PCR *FGFR1*	AGTAAACAATAGGGTGAGAACTTTTGCT	CCATGTTATAGCATAGAATCAGCACTTT
Reporter vector constuction		
Osteocalcin promoter	ACGCGTAGGTCTGGGGCCAT	AGATCTGGTCTGCACCGAGT
Mutagenesis		
V102I	TATGCTTGCATAACCAGCAGCCCCTC	GGTTATGCAAGCATAGAGGCCGGAGT
S107L	CCCTTGGGCAGTGACACCACCTACTT	GTCACTGCCCAAGGGGCTGCTGGTTA

cells were cultured for an additional 18 hours and then subjected to luciferase analysis using the luciferase reporter assay system (Promega). Relative luciferase activity was measured with Lumat LB9507 (Berthold, Oak Ridge, TN, USA). These experiments were carried out in triplicate within a single experiment and the experiment was repeated three times. The results are expressed as the mean ± SEM, and statistical significance was determined by the *t*-tests. Values of $p \leq 0.05$ were considered significant.

Results

Identification of a submicroscopic deletion involving FGFR1

A heterozygous deletion involving *FGFR1* was identified in one female patient. This deletion was indicated by MLPA and confirmed by FISH (Fig. 1A). CGH revealed that the deletion was ~8.5 Mb in size and included all exons of *FGFR1* (Fig. 1B). In addition to *FGFR1*, this deletion affected 55 genes and pseudogenes (Table 2). Parental samples of the patient were not available for genetic analysis. This deletion was not detected in the control group.

Identification of two FGFR1 missense variants

Direct sequence analysis identified two heterozygous variations, p.V102I (c.304 G>A) and p.S107L (c.320 C>T), in two female patients (Fig. 2A). The father of the p.V102I-positive patient also carried this variation, while parental samples of the p.S107L-positive patient were not available for genetic analysis. The p.V102I and p.S107L variants were found in two and one of the 100 control individuals, respectively.

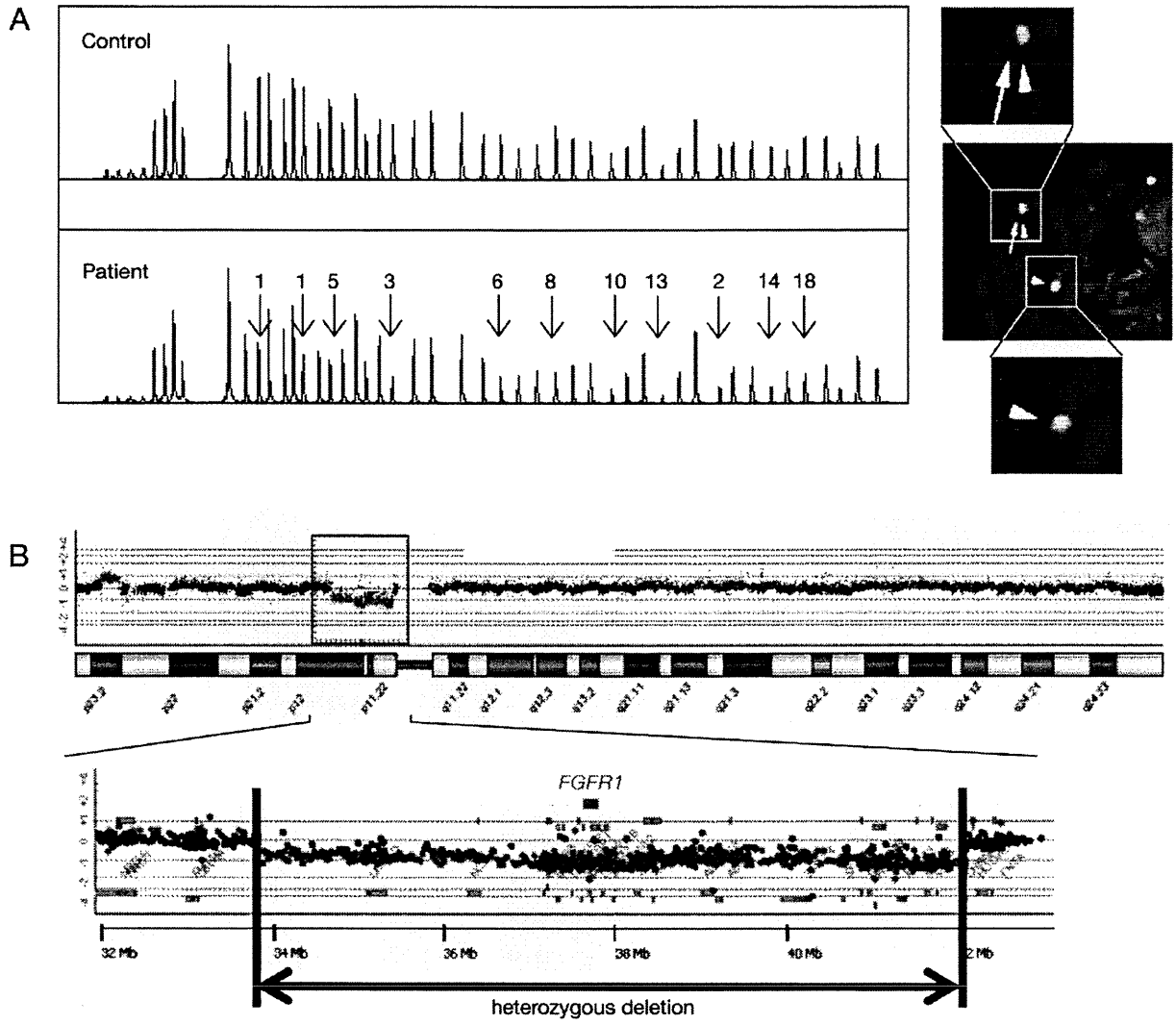

Fig. 1 Submicroscopic deletion involving *FGFR1*
A Deletion analyses. Left: MLPA analysis. The arrows indicate the peaks that correspond to *FGFR1* exons. Reduced peak heights of MLPA probes suggest a heterozygous deletion. Right: FISH analyses on lymphocyte metaphase spreads. The *FGFR1* probe detects only a single red signal (an arrow), whereas the chromosome 8 centromere probe (*D8Z2*) identifies two green signals (arrowheads). **B** Array-based CGH analysis. CGH indicated an ~8.5 Mb deletion at 8p11.1-p12 involving *FGFR1*, together with a small duplication at 8p23.2 that is known as a benign polymorphism (Database of Genomic Variants; http://projects.tcag.ca/variation/).

Functional analysis of missense variants

In vitro functional assays were carried out for the p.V102I and p.S107L variants (Fig. 2B and C). The relative luciferase activity of p.S107L did not significantly differ from that of WT ($p = 0.0552$). Furthermore, although p.V102I showed a slightly lower activity compared to WT ($p = 0.0413$), there was no significant difference in activity between WT and p.V102I plus WT (1:1) ($p = 0.0872$).

Clinical features of the deletion-positive patient

The deletion-positive patient presented with primary amenorrhea at 20 years of age. She had no family history of pituitary dysfunction; her non-consanguineous parents were clinically normal.

Clinical examinations revealed delayed pubertal development (breast, Tanner stage 3; pubic hair, stage 2-3; axillar hair, stage 1), in addition to short stature (-2.7 SD) and learning disability. She had several episodes of convulsion from eight months of age and was

Table 2 Genes and pseudogenes involved in the deletion

Gene symbol (Refseq)	Accession number	Description
UNC5D	NM_080872	unc-5 homolog D (C. elegans)
KCNU1	NM_001031836	potassium channel, subfamily U, member 1
ZNF703	NM_025069	zinc finger protein 703
ERLIN2	NM_007175	ER lipid raft associated 2
LOC728024	NR_003671	chromosome X open reading frame 56 pseudogene
PROSC	NM_007198	proline synthetase co-transcribed homolog (bacterial)
GPR124	NM_032777	G protein-coupled receptor 124
BRF2	NM_018310	subunit of RNA polymerase III transcription initiation factor, BRF1-like
RAB11FIP1	NM_025151	RAB11 family interacting protein 1 (class I)
GOT1L1	NM_152413	glutamic-oxaloacetic transaminase 1-like 1
ADRB3	NM_000025	adrenergic, beta-3-, receptor
EIF4EBP1	NM_004095	eukaryotic translation initiation factor 4E binding protein 1
ASH2L	NM_004674	ash2 (absent, small, or homeotic)-like (Drosophila)
STAR	NM_000349	steroidogenic acute regulatory protein
LSM1	NM_014462	LSM1 homolog, U6 small nuclear RNA associated (S. cerevisiae)
BAG4	M_004874.3	BCL2-associated athanogene 4
DDHD2	NM_001164234	DDHD domain containing 2
PPAPDC1B	NM_001102559	phosphatidic acid phosphatase type 2 domain containing 1B
WHSC1L1	NM_017778	Wolf-Hirschhorn syndrome candidate 1-like 1
LETM2	NM_144652	leucine zipper-EF-hand containing transmembrane protein 2
FGFR1	NM_023105	fibroblast growth factor receptor 1
C8orf86	NM_207412	chromosome 8 open reading frame 86
RNF5P1	NR_003129	ring finger protein 5, E3 ubiquitin protein ligase pseudogene 1
TACC1	NM_001122824	transforming, acidic coiled-coil containing protein 1
PLEKHA2	NM_021623	pleckstrin homology domain containing, family A member 2
HTRA4	NM_153692	HtrA serine peptidase 4
TM2D2	NM_001024381	TM2 domain containing 2
ADAM9	NR_027639	ADAM metallopeptidase domain 9
ADAM32	NM_145004	ADAM metallopeptidase domain 32
ADAM5P	NR_001448	ADAM metallopeptidase domain 5, pesudogene
ADAM3A	NR_001569	ADAM metallopeptidase domain 3A
LOC100130964	NR_046245	ADAM metallopeptidase domain 3A-like
ADAM18	NM_014237	ADAM metallopeptidase domain 18
ADAM2	NM_001464	ADAM metallopeptidase domain 2
IDO1	NM_002164	indoleamine 2,3-dioxygenase 1
IDO2	NM_194294	indoleamine 2,3-dioxygenase 2
C8orf4	NM_020130	chromosome 8 open reading frame 4
ZMAT4	NM_001135731	zinc finger, matrin-type 4
SFRP1	NM_003012	secreted frizzled-related protein 1
GOLGA7	NM_001174124	golgin A7
GINS4	NM_032336	GINS complex subunit 4
AGPAT6	NM_178819	1-acylglycerol-3-phosphate O-acyltransferase 6
NKX6-3	NM_152568	NK6 homeobox 3
ANK1	NM_000037	ankyrin 1, erythrocytic
MIR486	NR_030161	microRNA 486
KAT6A	NM_001099412	K(lysine) acetyltransferase 6A
AP3M2	NM_006803	adaptor-related protein complex 3, mu 2 subunit
PLAT	NM_033011	plasminogen activator, tissue
IKBKB	NM_001556	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
POLB	NM_002690	polymerase (DNA directed), beta
DKK4	NM_014420	dickkopf homolog 4 (Xenopus laevis)
VDAC3	NM_005662	voltage-dependent anion channel 3
SLC20A2	NM_001257180	solute carrier family 20 (phosphate transporter), member 2
C8orf40	NM_138436	chromosome 8 open reading frame 40
CHRNB3	NM_000749	cholinergic receptor, nicotinic, beta 3
CHRNA6	NM_001199279	cholinergic receptor, nicotinic, alpha 6

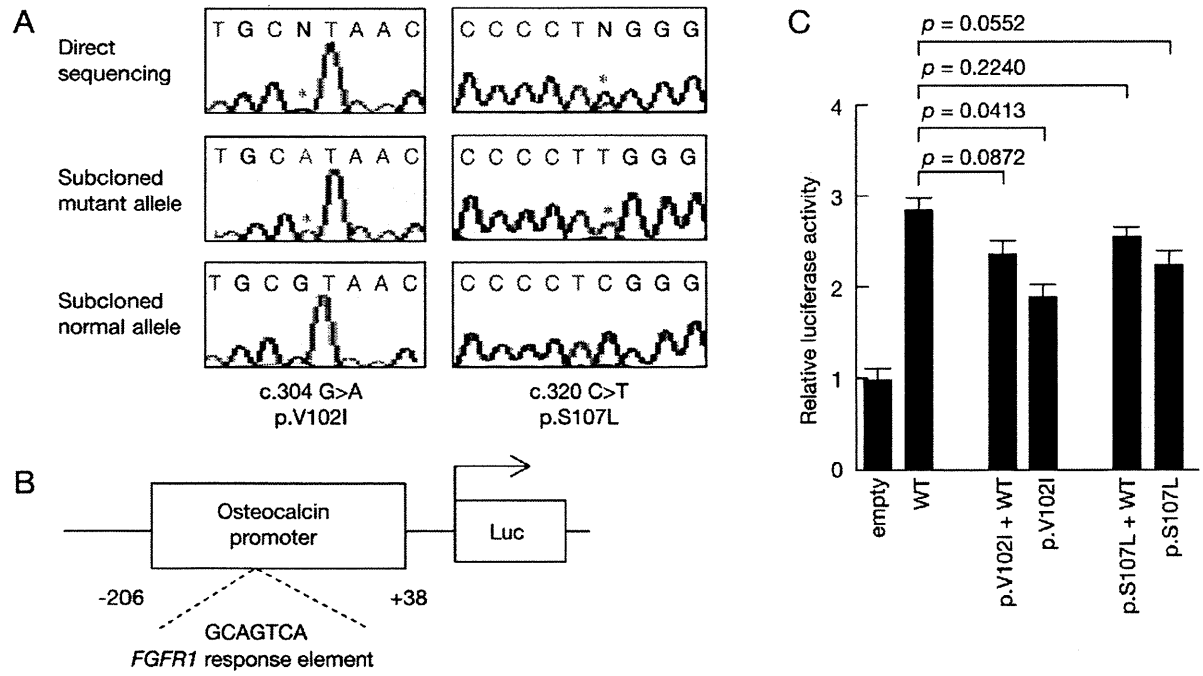

Fig. 2 Missense variations of *FGFR1* identified in the present study
A Electrochromatograms delineating p.V102I and p.S107L variations. Asterisks indicate mutated nucleotides. **B** Luciferase reporter construct utilized in the present study. **C** Representative results of luciferase assays. Relative luciferase activities of the empty expression vector (empty), the wildtype vector (WT), the variant *FGFR1* vectors (p.V102I and p. S107L), and WT plus the variant vectors (1:1) are shown.

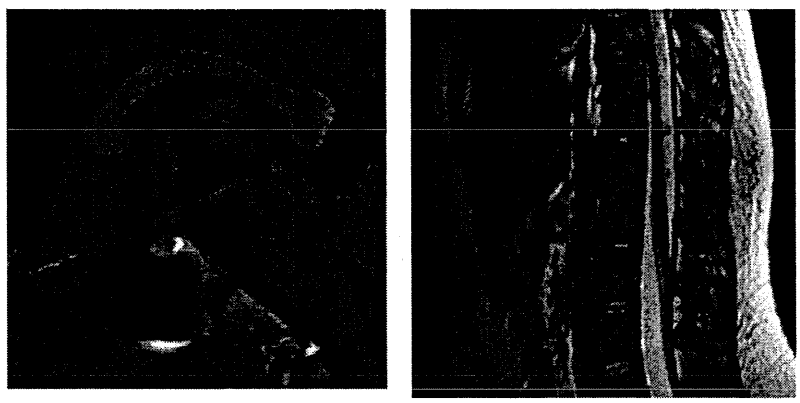

Fig. 3 Brain magnetic resonance imaging of the deletion-positive patient
Chiari type I malformation, syringomyelia, and apparently normal pituitary and stalk are shown.

diagnosed as having epilepsy. Brain magnetic resonance imaging delineated Chiari type I malformation and syringomyelia, together with an apparently normal pituitary and stalk (Fig. 3). She had a normal sense of smell, although we did not perform a quantitative examination of olfactory function.

Endocrine evaluation delineated a combined deficiency of pituitary hormones (Table 3). Blood GH levels were normal at the baseline, but did not increase after insulin stimulation. Gonadotropin levels were low at the baseline, and did not respond to GnRH stimulation. Basal and TRH-stimulated values of TSH remained within the reference ranges, while the basal level of free T4 was low. Basal and TRH-stimulated levels of prolactin were normal.

Table 3 Endocrine data of the deletion-positive patient

Hormone	Stimulus (dosage)	Basal value	Peak value	Peak response[a]
GH (ng/mL)[b]	Insulin (0.1 U/ kg)[c]	4.3 (0.2-23.0)	**4.4** (> 6.0)	at 90 min.
LH (mIU/mL)	GnRH (100 μg/m^2)	**0.1** (0.4-4.1)	**0.7** (8.5-15.5)	no peak
FSH (mIU/mL)	GnRH (100 μg/m^2)	**0.4** (4.8-10.4)	**1.6** (8.3-20.2)	no peak
TSH (μU/mL)	TRH (10 μg/kg)	2.4 (0.9-5.0)	10.8 (5.0-26.5)	at 30 min.
Prolactin (ng/mL)	TRH (10 μg/kg)	7.2 (1.4-14.6)	**18.9** (> 13)	at 30 min.
ACTH (pg/mL)	Insulin (0.1 U/ kg)[c]	54.4 (7.4-55.7)	**89.0** (> 30)	at 60 min.
Cortisol (μg/dL)	Insulin (0.1 U/ kg)[c]	13.4 (3.9-21.3)	**18.9** (> 18.0)	at 60 min.
Estradiol (pg/mL)		**14** (36-113)		
Free T4 (μg/dL)		**0.4** (0.9-1.7)		
IGF1 (ng/mL)		200 (119-389)		

The conversion factor to the SI unit: GH, 1.0 (μg/L); LH 1.0 (IU/L), FSH 1.0 (IU/L), TSH, 1.0 (mIU/L); ACTH, 0.22 (pmol/L); cortisol, 27.59 (nmol/L); estradiol, 3.671 (pmol/L); free T4, 12.87 (pmol/L) and IGF1, 0.131 (nmol/L). Reference range in age-matched individuals are shown in parenthesis. Hormone values below the reference range are boldfaced.

[a] Blood sampling during the provocation tests: 0, 30, 60, 90, and 120 minutes. [b] GH was measured using the recombinant GH standard, and the peak GH value of 6.0 ng/mL are used as the cutoff value for GH deficiency. [c] Sufficient hypoglycemic stimulations were obtained during all the insulin provocation tests.

Discussion

Copy-number analyses identified a submicroscopic deletion involving *FGFR1* in one of 69 patients with CPHD. The phenotype of the deletion-positive patient, such as brain anomalies and CPHD, is likely be associated with *FGFR1* haploinsufficiency, because none of 55 other genes involved in the deletion are known to play a role in the brain development (NCBI database, http://www.ncbi.nlm.nih.gov/; UCSC Genome Browser, http://genome.ucsc.edu/). However, we could not exclude the possibility that this deletion is a non-pathogenic variant, because parental samples of the patient were not available for genetic analysis. In this regard, it is worth mentioning that previous studies have shown that *FGFR1* is clearly expressed throughout the neuroepithelium of the developing brain including Rathke's pouch [6], and FGF signaling regulates the proliferation and differentiation of cultured pituitary progenitor cells [9, 10]. In addition, the CPHD phenotype has also been described in a patient with a large interstitial deletion on 8p11.2 affecting *FGFR1* [8]. These results, in conjunction with previous findings that *FGFR1* mutations and deletions are present in a certain part of patients with IHH, KS, and SOD [3, 7], suggest that *FGFR1* abnormalities can be associated with various developmental defects of the anterior midline in the forebrain. Nevertheless, CPHD seems to be a rare manifestation among patients with *FGFR1* abnormalities, because the majority of previously reported patients with mutations or deletions of *FGFR1* had no hormonal abnormalities with the exception of gonadotropin deficiency [3, 13]. These results can be explained by assuming that during development, GnRH neurons are more vulnerable to *FGFR1* abnormalities than the other parts of the anterior midline in the forebrain. Furthermore, the high frequency of gonadotropin deficiency and the rarity of other pituitary hormone deficiencies in patients with *FGFR1* abnormalities are consistent with the results of *in vitro* assays; Norlin *et al.* have suggested that in the developing pituitary, FGFs play a particularly important role in the specification of the *POU1F1*-independent progenitor cells including gonadotrophs [9].

The deletion-positive patient manifested a combined deficiency of GH, LH and FSH. In addition, normal TSH values along with a low free T4 level are indicative of central hypothyroidism [14]. Since endocrine evaluation of this patient was fragmentary, it remains unknown whether the primary lesion of her hormonal defects resides in the hypothalamus or in the pituitary. Further studies, such as GHRH stimulation tests or GnRH stimulation tests after GnRH priming, will clarify the origin of CPHD in patients with *FGFR1* abnormalities.

Mutation analysis identified two heterozygous *FGFR1* variations (p.V102I and p.S107L) in two of 69 patients with CPHD. *In vitro* assays indicated that heterozygosity of the p.V102I and p.S107L vari-

ants resulted in nearly normal transactivating function. Furthermore, p.V102I and p.S107L were found in a small subset of unaffected individuals. Thus, these two substitutions are more likely to be benign polymorphisms rather than disease-causing mutations. These results imply that *FGFR1* intragenic mutations account for only a minor part of the etiology of CPHD, if any. However, given the relatively small number of patients analyzed in the present study, further studies are necessary to clarify the contribution of *FGFR1* mutations to the development of CPHD.

In summary, the present study indicates a possible role of *FGFR1* in anterior pituitary function and the rarity of *FGFR1* abnormalities in patients with CPHD.

Acknowledgements

This work was supported by grants from the Ministry of Health, Labor and Welfare and from Takeda Science Foundation, by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, by Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology and by the Grant of National Center for Child Health and Development.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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

EXTENDED REPORT

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

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Handling editor Tore K Kvien

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

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

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

ABSTRACT

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

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

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

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

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

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

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

PATIENTS AND METHODS

Patients

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

NLRP3 analyses

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

Bioinformatics analyses

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

Functional studies

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

Statistical analyses

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

RESULTS

Genetic analyses

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

Bioinformatics and functional analyses

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

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

Clinical features of patients with somatic *NLRP3* mosaicism

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

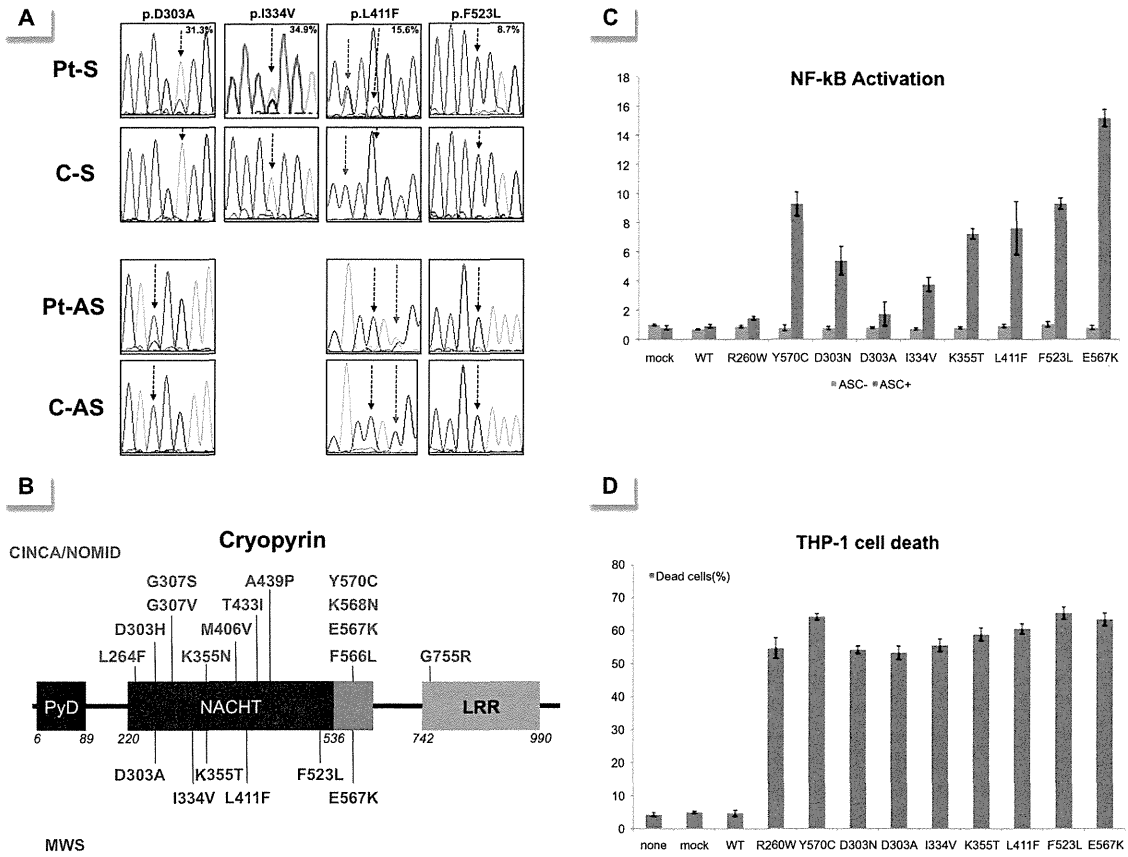

Figure 1 (A) Sense (upper rows) and antisense (bottom rows) chromatograms from four patients with somatic *NLRP3* mosaicism and controls obtained by Sanger sequencing using genomic DNA extracted from whole blood. The black arrows show the *NLRP3* positions where the somatic mutations were detected. The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

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

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

Outcome of anti-IL-1 blockade

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

Comparative phenotype analyses

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

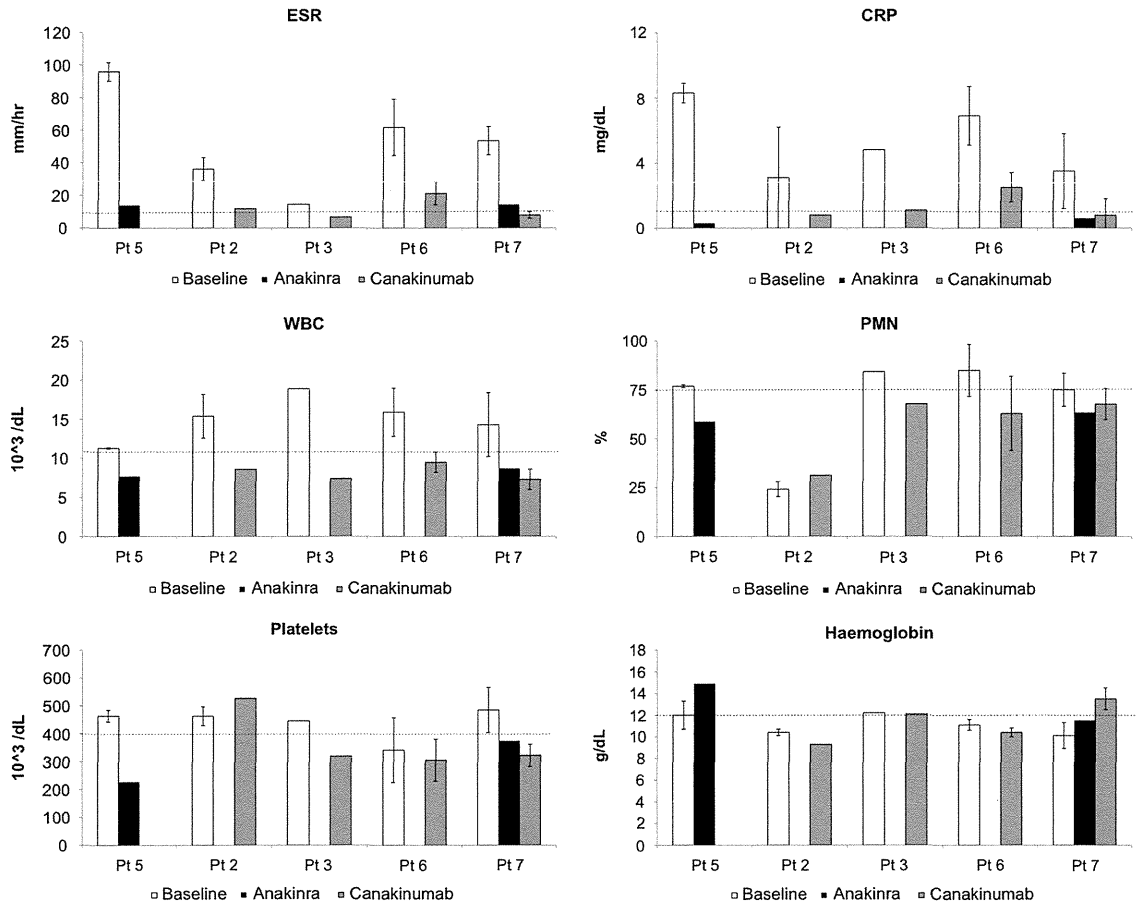

Figure 2 Laboratory values obtained in the five patients treated with different anti-interleukin 1 drugs. Patient's graphics were ordered as follows: First, those graphics from the patient who only received treatment with anakinra (Pt 5), followed by those from patients who only received treatment with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

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

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

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

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

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

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

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

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

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

Competing interests None.

Patient consent Obtained.

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

Provenance and peer review Not commissioned; externally peer reviewed.

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

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

Notes

Human Immunology 74 (2013) 1579–1585

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Down-regulation of CD5 expression on activated CD8$^+$ T cells in familial hemophagocytic lymphohistiocytosis with perforin gene mutations

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ARTICLE INFO

Article history:
Received 18 June 2013
Available online 16 September 2013

ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is characterized by uncontrolled activation of T cells and macrophages with overproduction of cytokines. Familial HLH type 2 (FHL2) is the most common form of primary HLH and is caused by mutations in *PRF1*. We have recently described a significant increase in the subpopulation of CD8$^+$ T cells with clonal expansion and CD5 down-regulation in Epstein-Barr virus associated-HLH, which represented a valuable tool for its diagnosis. However, this unusual phenotype of CD8$^+$ T cells has not been investigated fully in patients with FHL2. We performed immunophenotypic analysis of peripheral blood and measured serum pro-inflammatory cytokines in five patients with FHL2. All patients showed significantly increased subpopulations of activated CD8$^+$ T cells with down-regulation of CD5, which were negligible among normal controls. Analysis of T-cell receptor Vβ repertoire suggested the reactive and oligoclonal expansion of these cells. The proportion of the subset declined after successful treatment concomitant with reduction in the serum levels of cytokines in all patients except one who continued to have a high proportion of the subset and died. These findings suggest that down-regulation of CD5 on activated CD8$^+$ T cells may serve as a useful marker of dysregulated T cell activation and proliferation in FHL2.

1. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal disease that is characterized by marked systemic inflammation and unregulated activation of macrophages and T cells [1,2]. Patients with HLH may present with fever, cytopenia, hepatosplenomegaly, liver dysfunction, coagulation abnormalities, and hemophagocytosis [1,2]. HLH is comprised of primary and secondary forms. Primary HLH includes familial HLH (FHL), which is caused by genetic defects related to granule-dependent cytotoxicity, and immunodeficiencies, such as X-linked lymphoproliferative syndrome. Mutations in the *PRF1*, *UNC13D*, *STX11*, and *STXBP2* genes cause FHL type 2 (FHL2), FHL3, FHL4, and FHL5, respectively. Perforin is a crucial effector molecule for cytotoxicity that is present in the granules of cytotoxic T lymphocytes and natural killer (NK) cells. FHL2 (perforin deficiency) accounts for more than half of the FHL cases in Japan [3]. Secondary HLH is associated with a variety of infections, autoimmune diseases and malignancies. Epstein-Barr virus (EBV)-associated HLH (EBV–HLH) is the most frequent subtype of HLH in Japan [4]. Establishing a diagnosis of HLH may be difficult when based solely on clinical and laboratory findings, because those findings are often present in severely ill patients. It is also difficult to differentiate between primary and secondary HLH and diagnose a specific subtype of HLH during the acute phase of HLH.

Abbreviations: EBV, Epstein-Barr virus; FHL, familial hemophagocytic lymphohistiocytosis; HLA, human leukocyte antigen; HLH, hemophagocytic lymphohistiocytosis; IM, infectious mononucleosis; mAb, monoclonal antibody; NK, natural killer; PE, phycoerythrin; PBMCs, peripheral blood mononuclear cells; TCR, T-cell receptor.

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0198-8859/$36.00 - see front matter © 2013 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.humimm.2013.09.001

We have recently reported the clonal proliferation of activated $CD8^+$ T cells with down-regulation of CD5 in patients with EBV–HLH [5]. This unique immunophenotype of $CD8^+$ T cells could be a valuable tool for the diagnosis of EBV–HLH [5]. However, the immunophenotypic features of T cells in other subtypes of HLH have not been fully characterized. Human CD5 is a membrane glycoprotein that belongs to the scavenger receptor cysteine-rich family of receptors [6–9]. It is expressed on thymocytes, mature peripheral T cells and a small population of B cells, and is involved in the modulation of antigen-specific receptor-mediated activation and differentiation signals [6–9]. It has recently been reported that CD5 is recruited and colocalized with CD3 at the immunological synapse and inhibits T-cell receptor (TCR) signaling in T cells without interfering with immunological synapse formation [10]. Although an expanded subpopulation of $CD8^+$ T cells lacking expression of CD5 has been reported in a single case of FHL2 [11], the nature of $CD8^+$ T cells with down-regulation of CD5 in FLH remains to be elucidated. In this report, we describe the down-regulation of CD5 on activated $CD8^+$ T cells in patients with FHL2 and discuss the relationship between down-regulation of CD5 and systemic inflammation.

2. Materials and methods

2.1. Patients

We studied five patients with FHL2 from five families, all of whom were born to non-consanguineous Japanese parents. Table 1 presents the clinical and laboratory data of the patients. All patients showed typical features of HLH, such as persistent fever, hepatosplenomegaly, cytopenia, liver dysfunction, and hypercytokinemia, i.e., neopterin, interferon-γ, and interleukin-6 at the onset of FHL2. Defective NK cell activity was a universal feature. In patient P3, HLH was triggered by a primary EBV infection, in which the major cellular target of EBV infection, as assessed by *in situ* hybridization for EBV-encoded small RNA1 was B cells (data not shown) but not $CD8^+$ T cells, resulting in marked lymphocytosis with atypical lymphocytes. The disease onset was during early infancy in all patients except for patient P4. Patient P2 did not respond to the HLH-2004 treatment protocol [12], and died at 12 days of age. Patients P1, P3, P4 and P5 underwent stem cell transplantation at the ages of 5 months, 2 years, 5 months and 5 months, respectively [13]. Patients P1 and P3 are alive with no

Table 1
Patient characteristics.

	P1	P2	P3	P4	P5	Normal range
Onset age	1 month	1 day	2 yr	3 months	1 month	
Fever	+	+	+	+	+	
Hepatosplenomegaly	+	+	+	+	–	
Hemophagocytosis*	+	NA	+	+	NA	
Stem cell transplantation	+	–	+	+	+	
Outcome	Alive	Deceased	Alive	Deceased	Alive	
Laboratory findings						
NK cell activity (%)	3.8	2.0	0.0	0.0	0.0	18–40
WBC (/μL)	3100	16,600	51,000	3330	5700	5000–19,500
Neutrophils (/μL)	580	13,600	4290	830	470	3150–6200
Lymphocytes (/μL)	2290	1660	40,800	2060	4380	1500–3000
Hemoglobin (g/dL)	9.2	18.6	8.0	7.8	6.4	9.0–14.0
Platelets (×103/μL)	86	75	50	18	81	150–350
Triglycerides (mg/dL)	356	105	600	129	158	30–149
Fibrinogen (mg/dL)	147	117	NA	59	NA	183–381
Ferritin (ng/mL)	9983	532	2400	1038	427	4.6–204.0
sIL-2R (IU/mL)	3306	11,209	31,000	18,355	4180	220–530
Lymphocyte subsets						
$CD3^+$ (%)	69.2	87.6	92.6	77.0	66.4	64.4–80.2
$CD4^+$ (%)	34.4	40.2	33.5	20.8	29.5	47.3–58.9
$CD8^+$ (%)	31.0	42.1	56.8	53.7	25.1	10.3–24.3
Serum cytokines						
Neopterin (nmol/L)	60	90	125	120	78	2–8
IFN-γ (pg/mL)	37	510	57	1200	205	<5
IL-6 (pg/mL)	122	18	<5	52	<5	<5

NK, natural killer; WBC, white blood cells; sIL-2R, soluble interleukin-2 receptor; IFN-γ, interferon-γ; IL-6, interleukin-6; NA, not available.
* Hemophagocytosis in bone marrow.

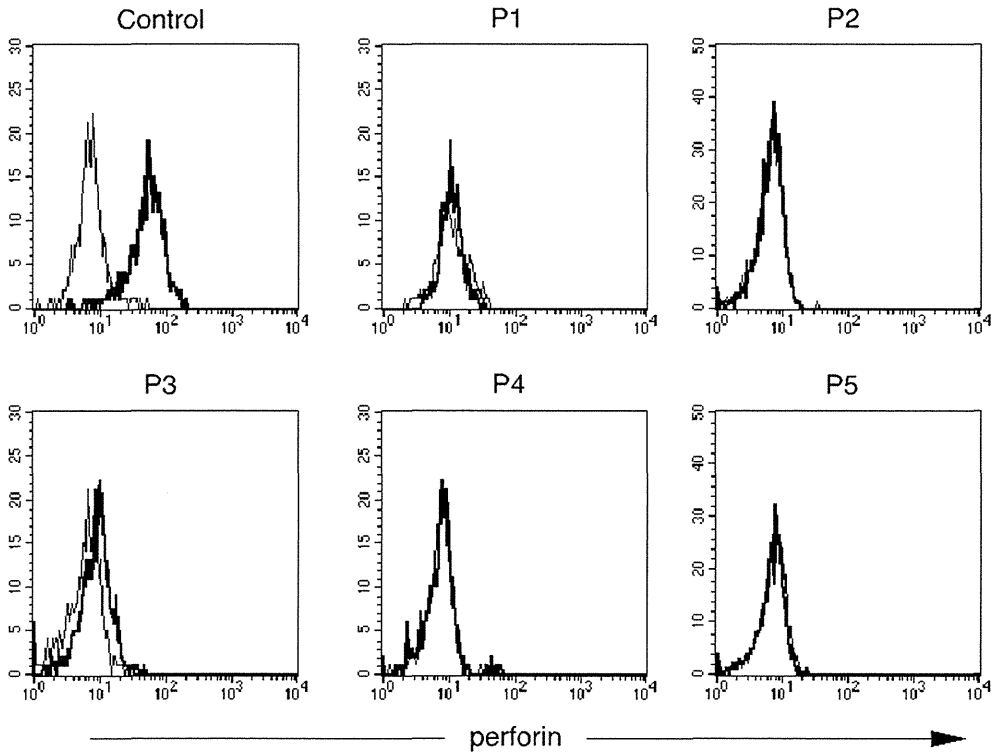

Fig. 1. Perforin expression. Intracellular expression of perforin in $CD3^-$ $CD56^+$ NK cells. Thin lines indicate control antibody; thick lines represent monoclonal antibody specific for perforin.

Table 2
PRF1 mutations.

	Nucleotide mutation	Predicted effect
P1	c.1090_1091delCT	p.Leu364fs
	c.1562A>G	p.Tyr521Cys
P2	c.996_1000delGCCCG	p.Pro333fs
	c.1090_1091delCT	p.Leu364fs
P3	c.1246C>T	p.Gly416X
	c.1349C>T	p.Thr450Met
P4	c.1090_1091delCT*	p.Leu364fs
P5	c.1090_1091delCT*	p.Leu364fs

* Homozygous mutation.

evidence of disease, but patient P4 died of acute graft-versus-host disease 5 months later. In patient P5, the transplantation was performed recently. We also investigated 10 cases of EBV–HLH and 13 cases of infectious mononucleosis (IM) as disease controls [5,14]. All patients with EBV–HLH showed typical clinical features of HLH and exhibited high viral copy numbers. It was noted that $CD8^+$ T cells were the major cellular targets of EBV in all EBV–HLH patients by *in situ* hybridization for EBV-encoded small RNA1 in the available samples. No detectable mutations within the *SH2D1A* or *XIAP/BIRC4* genes were observed in the five male patients with EBV–HLH [5]. The diagnosis of IM was clinically determined. All patients with IM exhibited self-limited disease. Primary EBV infection was serologically confirmed for all cases of EBV–HLH and IM. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

2.2. Flow cytometry

For the analysis of perforin expression, peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls by Ficoll-Hypaque gradient centrifugation. PBMCs were stained with fluorescein isothiocyanate-conjugated anti-CD56 and peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibodies (mAbs). After washing, cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit and incubated with phycoerythrin (PE)-conjugated anti-perforin mAb or control Ab (BD, San Diego, CA, USA). Perforin expression was evaluated in $CD3^-$ $CD56^+$ NK cells with a FACSCalibur flow cytometer using CellQuest software (BD Bioscience, Tokyo, Japan). For the analysis of CD5 expression, whole blood was stained with fluorescein isothiocyanate-conjugated anti-CD5, PE-conjugated anti-human leukocyte antigen (HLA)-DR (both from BD), and R-PE-Cy5-conjugated anti-CD4 or anti-CD8 (Dako, Glostrup, Denmark) mAbs. CD5 expression on $CD8^+$ T cells was considered negative if it was similar to levels of the NK cells and most of the B cells that do not express CD5 [5]. Analysis of differences among the data groups was performed using Student's unpaired *t*-test. *p*-Values less than 0.05 were considered significant. Flow cytometric analysis of the TCR Vβ repertoire was performed as previously described [15].

2.3. Cytokine determination

Serum or plasma concentrations of cytokines were determined using the following enzyme-linked immunosorbent assay kits: interferon-γ and interleukin-6 (R&D systems, Minneapolis, MN, USA); and neopterin (IBL, Hamburg, Germany) [14].

2.4. Mutation analysis

DNA was extracted from blood samples using standard methods. The *PRF1* gene was amplified from genomic DNA using specific primers [16]. Sequencing was performed on purified polymerase chain reaction products using the ABI Prism BigDye Terminator

Cycle sequencing kit on an ABI 3130 automated sequencer (Applied Biosystems, Foster, CA, USA).

2.5. Cell cultures

To stimulate T cells, PBMCs were incubated for 72 h with 5 μg/mL phytohemagglutinin, with 1 μg/mL anti-CD3 (OKT3) plus 5 μg/mL anti-CD28 mAb, or with 20 ng/mL phorbol myristate acetate plus 250 ng/mL ionomycin in RPMI 1640 medium containing 10% fetal calf serum and antibiotics [17].

3. Results

3.1. Perforin expression and PRF1 mutations

We first analyzed perforin expression by flow cytometry. As shown in Fig. 1, perforin was not detectable in NK cells from patients P1, P2, P4 or P5. NK cells from patient P3 showed residual expression of the mutated perforin, which might be associated with the later age of onset, compared with the other patients. Table 2 presents the sequencing data for each patient. Patient P1 was a compound heterozygote bearing Leu364fs and Tyr521Cys mutations in *PRF1*. The effect of the novel missense mutation Tyr521Cys was evaluated using a web-based analysis tool, and was found to be deleterious on the basis of the SFIT program [18]. Two distinct frameshift mutations, Pro333fs and Leu364fs, were demonstrated in patient P2. The former is a novel deletion mutation. The 1090_1091delCT (Leu364fs) mutation which was found in patients P1, P2, P4 and P5 has been repeatedly reported in Japanese patients with FHL2 [3]. All patients had at least one nonsense or frameshift mutation.

3.2. Increased subpopulation of activated CD8⁺ T cells with down-regulation of CD5

Immunophenotypic analysis of the lymphocytes demonstrated an increased percentage of CD8⁺ T cells expressing the activation marker HLA-DR in the acute phase of the disease (Table 1, Fig. 2A). Compared with CD8⁺ T cells, CD4⁺ T cells were less activated, except for patient P3 who had primary EBV infection. More importantly, the activated CD8⁺ T cells exhibited down-regulation of CD5, which was normally expressed on both CD4⁺ and CD8⁺ T cells from normal individuals (Fig. 2A, B). In contrast to the CD8⁺ T cells, CD4⁺ T cells from the patients with FHL2 exhibited normal expression of CD5. Although patients with IM have been reported to exhibit marked immune responses to regulate EBV-infected B cells and an increased subpopulation of highly activated CD8⁺ T cells, down-regulation of CD5 was not detected in these CD8⁺ T cells (Fig. 2B) [5].

The percentage of this unique subset (CD5⁻ HLA-DR⁺ CD8⁺ T cells) decreased after treatment with the HLH-2004 protocol in patient P1 (22.1–3.9%), P4 (35.2–0.4%) and P5 (20.3–0.8%) concomitant with the relief of clinical symptoms and serum levels of neopterin and IFN-γ (Fig. 2C). We did not perform serial analysis in patient P3 due to the availability of sample. In contrast, patient P2 did not respond well to the treatment and continued to exhibit a high percentage of the subset (58.3–63.0%) and died at 12 days of age. CD107 cytotoxicity assay was not performed in our patients.

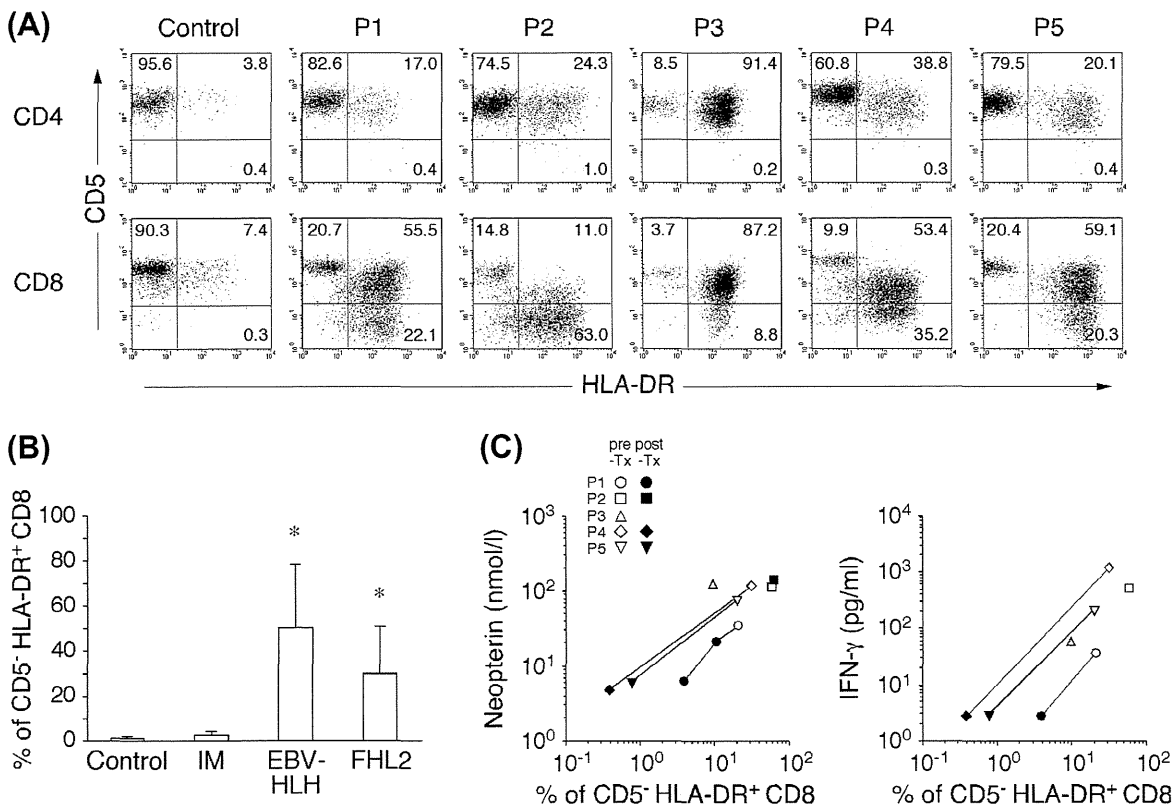

Fig. 2. Expression of CD5 by CD8⁺ T cells (A) Expression of CD5 and HLA-DR on the CD4⁺ and CD8⁺ T cells. The percentage of cells gated in each region is shown. (B) The frequency of CD5⁻ HLA-DR⁺ CD8⁺ T cells. Shown are the percentages of CD5⁻ HLA-DR⁺ cells among CD8⁺ T cells in controls ($n = 10$), IM ($n = 13$), EBV–HLH ($n = 10$), and FHL2 ($n = 5$). Bars represent the standard deviation. *$p < 0.01$. (C) Correlation between the percentages of CD5⁻ HLA-DR⁺ CD8⁺ T cells and serum levels of neopterin and IFN-γ. Open symbols indicate data of pre-treatment (Tx) time points; solid symbols represent those of post-Tx time points. EBV, Epstein-Barr virus; FHL2, familial hemophagocytic lymphohistiocytosis type 2; IM, infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis; IFN-γ, interferon-γ.