both cases had obviously periodic fever episodes. These suggest the presence of oligogenicity and that variants in *NLRP3* and *MEFV* synergistically modify the symptoms of the atypical autoinflammatory diseases.

There are two important limitations in this study when discussing the pathogenicity of low penetrance rare variants. The first limitation is the limited number of patients in the study. Further study using a large number of patients is necessary to confirm our results. Secondly, we only analyzed a limited number of genes. In this study, we concluded that the presence of an *NLRP3* variant with the co-existence of *MEFV* variants contributed to atypical autoinflammatory disease. However, the patients may have had alternative genetic mutations or other rare variants of inflammasome related genes such as *CARD8* [27] elsewhere in the genome, which are truly disease causing, and the two variants described in these patients may be unrelated.

Conclusions

This study describes the molecular analysis of two cases with heterozygous low penetrance variants in exon5 of *NLRP3* and exon3 of *MEFV*. The findings provide in vivo and in vitro evidence for the effect of an *NLRP3* missense variant. Importantly the mutations are within the same signaling pathway and are associated with inflammasome activation. Our observations suggest that oligogenic inheritance may occur in patients with atypical autoinflammatory syndrome. It is therefore important to consider that the phenotypes could be modified by synergistic effects with plural autoinflammatory-associated gene mutations when the patients have atypical autoinflammatory disease.

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Conflict of Interest The authors have declared no conflicts of interest.

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Characterization of *NLRP3* Variants in Japanese Cryopyrin-Associated Periodic Syndrome Patients

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Abstract The etiology of cryopyrin-associated periodic syndrome (CAPS) is caused by germline gene mutations in NOD-like receptor family, pryin domain containing 3 (*NLRP3*)/cold-induced autoinflammatory syndrome 1 (*CIAS1*). CAPS includes diseases with various severities. The aim of this study was to characterize patients according to the disease severity of CAPS. Five Japanese patients with four kinds of gene variations in *NLRP3* were found and diagnosed as CAPS or juvenile idiopathic arthritis. Two mutations in *NLRP3*, Y563N and E688K, found in CAPS patients exhibit significant positive activities in the nuclear factor-κB reporter gene assay. Increased serum interleukin (IL)-18 levels were only observed in severe cases of CAPS. In mild cases of CAPS, the serum IL-18 levels were not increased, although lipopolysaccharide- or hypothermia-enhanced IL-1β and IL-18 production levels by their peripheral blood mononuclear cells were detectable. This series of case reports suggests that a combination of in vitro assays could be a useful tool for the diagnosis and characterization of the disease severity of CAPS.

Keywords Autoinflammatory disease · cryopyrin · familial cold autoinflammatory syndrome · interleukin-18 · *NLRP3*

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Abbreviations

CAPS	Cryopyrin-associated periodic syndrome
CIAS1	Cold-induced autoinflammatory syndrome 1
CINCA	Chronic infantile neurologic cutaneous and articular
CRP	C-reactive protein
FCAS	Familial cold autoinflammatory syndrome
HEK	Human embryonic kidney
IL	Interleukin
JIA	Juvenile idiopathic arthritis
LPS	Lipopolysaccharide
MWS	Muckle–Wells syndrome
NLRP3	NOD-like receptor family, pryin domain containing 3
NF-κB	Nuclear factor-κB
NOMID	Neonatal-onset multisystem inflammatory disease
PBMCs	Peripheral blood mononuclear cells
TNF	Tumor necrosis factor

Introduction

Cryopyrin-associated periodic syndrome (CAPS) is an autoinflammatory syndrome [1] caused by germline gene mutations in NOD-like receptor family, pryin domain containing 3 (*NLRP3*)/cold-induced autoinflammatory syndrome 1 (*CIAS1*) [2–4]. The diagnosis of CAPS is based on its

characteristic clinical phenotypes and examination of gene mutations in *NLRP3*. A hotspot of gene mutations in *NLRP3* is located on exon 3. On the other hand, approximately 40% of cases with the clinically diagnosed severe form of CAPS, chronic infantile neurologic cutaneous and articular (CINCA)/neonatal-onset multisystem inflammatory disease (NOMID) syndrome, have no detectable germline gene mutations in *NLRP3* [5, 6]. Some of these patients have gene mutations in *NLRP3* outside of exon 3, *NLRP12*, or somatic mosaicism of *NLRP3* [5, 7–10]. In some of the remaining typical CAPS patients, the disease-causing mutations cannot be confirmed. Thus, the clinical phenotypes are very important for diagnosing CAPS patients.

Familial cold autoinflammatory syndrome (FCAS) shows the mildest clinical phenotypes in the spectrum of CAPS, such as cold-induced urticaria-like skin rash, while CINCA/NOMID syndrome shows additional severe phenotypes, such as severe arthritis, patella overgrowth, aseptic meningitis, mental retardation, and progressive sensory neural hearing loss [1]. The diagnosis of FCAS is relatively difficult owing to its mild phenotypes compared with the more severe phenotypes of CAPS (CINCA/NOMID syndrome or Muckle–Wells syndrome (MWS)). On the other hand, and similar to other autoinflammatory syndromes such as familial Mediterranean fever, it is important for CAPS treatment to prevent the onset of renal amyloidosis for consideration of the prognosis. Interleukin (IL)-1β inhibitory drugs, such as anakinra, rilonacept, and canakinumab, can prevent the clinical phenotypes of CAPS including renal amyloidosis [11]. However, the usage of IL-1 blockade for the severe form of CAPS may sometimes be an overtreatment for FCAS because the clinical symptoms are relatively mild and the frequency of onset of renal amyloidosis was reported to be low in FCAS patients [11]. Therefore, precise evaluation of the disease severity of CAPS may contribute to a reduction in the usage of IL-1 blockade. Consequently, a convenient objective standard is anticipated for discrimination between the mild and severe forms of CAPS.

In this study, to diagnose CAPS and characterize the differences between the mild and severe forms of CAPS, we evaluated the serum inflammatory cytokine levels, cytokine production levels by peripheral blood mononuclear cells (PBMCs), and cell-based nuclear factor (NF)-κB reporter gene activities of *NLRP3* variants in patients. Our results provide new insights into the characterization of the severity of CAPS.

Methods

Case Reports

The five clinical cases evaluated in this study are described below, and their characteristics are summarized in Table I.

Table I Genotypes and clinical profiles of the patients

	Analyzed age	Onset age	Gender	Diagnosis	Genotype (*NLRP3*)	CNS		Skin	Joint	Others		The inflammatory markers	
						Meningitis	Mental retardation	Urticaria like rash	Arthritis	Hearing loss	Renal amyloidosis	WBC (/μl)	CRP (mg/dl)
Case 1	3 months	3 months	Female	FCAS	Y563N	–	–	+	–	–	–	14,890	0.48
Case 2	34 years	Unknown	Male	FCAS	Y563N	–	–	+	+	–	–	13,120	3.05
Case 3	14 years	11 months	Male	CINCA/NOMID	E688K, G809S	+	+	+	+	–	–	22,500	12.30
Case 4	45 years	Unknown	Female	MWS	E688K	–	–	+	+	+	–	13,640	4.17
Case 5	3 years	3 years	Female	JIA	E378K	–	–	+	+	–	–	15,200	11.69

CNS central nervous system, *FCAS* familiar cold inflammatory syndrome, *CINCA* chronic infantile neurologic cutaneous and articular syndrome, *MWS* Muckle–Wells syndrome, *JIA* juvenile idiopathic arthritis, *WBC* the count of white blood cells, *CRP* the serum C-reactive protein level

All of the patients' family members and healthy control subjects provided informed consent to participate in the study, and the ethical principles of the Declaration of Helsinki were followed.

Case 1 The onset of disease (FCAS) in this patient occurred at 3 months of age. She exhibited a recurrent generalized urticaria-like skin rash upon exposure to cold temperatures (Fig. 1a). Progressive sensory neural hearing loss and renal amyloidosis were not seen. Her serum C-reactive protein (CRP) levels were continuously and slightly increased (0.24–2.1 mg/dl).

Case 2 Case 2 was the father of case 1. He was a 34-year-old male with a recurrent urticaria-like skin rash, fever, conjunctivitis, and arthralgia that developed following fatigue or exposure to cold temperatures. The precise time of his disease onset was unknown. Progressive sensory neural hearing loss and renal amyloidosis were not seen [12]. His CRP levels were continuously increased (1.52–3.98 mg/dl).

Case 3 The onset of disease (CINCA/NOMID) in this patient occurred at 11 months of age. Continuous aseptic meningitis, urticaria-like skin rash, arthritis at the end of the fingers, and Raynaud's symptoms were observed. Arteriosclerosis of the ophthalmic artery was found. However, severe patella overgrowth was not seen. At 14 years of age, he had heart failure with myocarditis, which was considered to be a rheumatic characteristic. The patient died suddenly at 19 years of age (the detailed clinical case will be described elsewhere by Teramoto et al.).

Case 4 Case 4 was the mother of case 3. The precise time of her disease (MWS) onset was unknown. Initially, she was diagnosed with rheumatic arthritis and received oral prednisolone therapy. She suffered progressive sensory neural hearing loss at 30 years of age and underwent artificial cochlea replacement therapy at 48 years of age. This was greatly effective in improving her hearing ability. Meningitis and renal amyloidosis were not seen.

Case 5 The onset of disease in this patient occurred at 3 years of age. Fever that continued for more than 2 weeks, severe polyarthritis (serum matrix metalloproteinase-3 of >800 ng/ml), and recurrent urticaria-like non-itchy skin rash (Fig. 1b) were observed. Lymphadenopathy, hepatosplenomegaly, and serositis were not seen. Patella overgrowth, aseptic meningitis, progressive sensory neural hearing loss, and renal amyloidosis were not seen. Rheumatoid factor was negative. Other autoantibodies, including anticyclic citrullinated peptide antibody, were not detected. Her serum CRP and ferritin levels were increased (11.69 mg/dl and 255.1 ng/ml, respectively). Based on the below-mentioned hereditary traits and the results of in vitro functional assays, we diagnosed this patient as juvenile idiopathic arthritis (JIA), according to the criteria for JIA from the International League of Associations for Rheumatology [13]. A combination therapy with steroid and tocilizumab was effective.

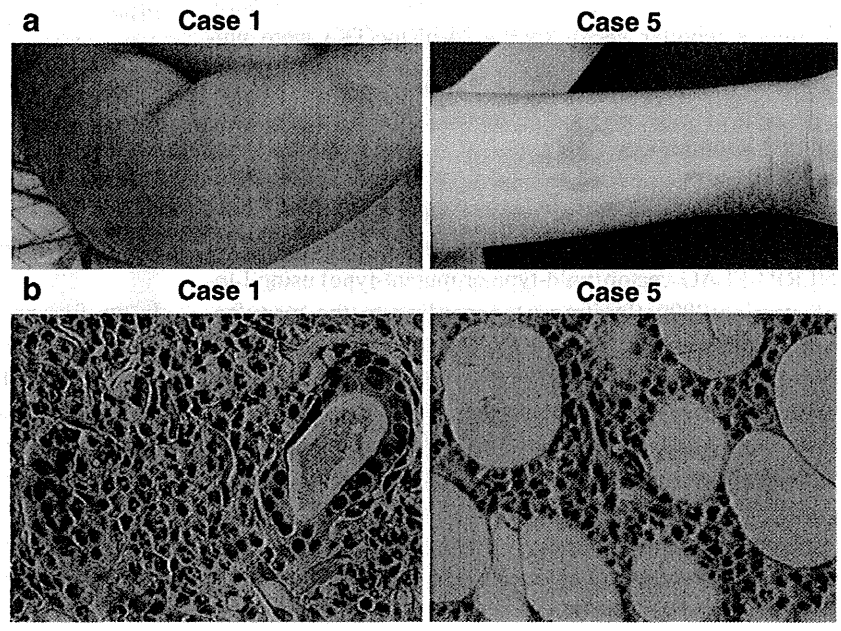

Fig. 1 Urticaria-like skin rash of cases 1 and 5. **a** Clinical appearances of the urticaria-like rash of cases 1 and 5. **b** Histopathological examinations of biopsy specimens from the skin rash of cases 1 and 5. Both skin biopsies show a recurrent cold-induced non-itchy urticaria-like skin rash and also show neutrophil infiltration

DNA Sequencing

Genomic DNA was extracted from leukocytes using Sepa-Gene (Eidia, Tokyo, Japan). A DNA fragment of the *NLRP3* gene was amplified by PCR and analyzed using Big Dye Terminator Bidirectional Sequencing (Applied Biosystems, Foster City, CA, USA).

Cell Culture

PBMCs were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (GE Healthcare, Uppsala, Sweden). The PBMCs were cultured in medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 pg/ml). Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (high glucose-containing DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Vector Preparations

A cDNA encoding *NLRP3* tagged at the C terminus with a FLAG epitope (NLRP3-FLAG) was cloned into the plasmid vector pcDNA3.1+ (Invitrogen). Mutants of *NLRP3* (E378K, Y563N, E688K, and G809S) were generated using a GeneEditor In Vitro Site-Directed Mutagenesis System (Promega, Madison, WI, USA). An ASC variant 1 tagged at the C terminus with a myc epitope (ASC1-myc) was also cloned into pcDNA3.1+. An NF-κB luciferase reporter vector (pGL4.32-luc2P/NF-kappaB-RE/Hygro) and a *Renilla* luciferase reporter vector (pGL4.74-hRluc/TK) were purchased from Promega.

NF-κB Reporter Gene Activity

HEK293T cells in 96-well plates were transfected with 16 ng/well of pcDNA3.1+ control vector or pcDNA3.1+ NLRP3-FLAG vector (wild-type or mutant-type) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The pcDNA3.1+ ASC1-myc vector, NF-κB luciferase reporter vector, and *Renilla* luciferase reporter vector were cotransfected. After transfection, the cells were cultured for 24 h. The luciferase reporter gene activities were analyzed using a Dual-Luciferase Reporter Assay System (Promega). The statistical significance of differences in the luciferase activities between the wild-type and mutant genes in the NF-κB gene reporter assays was analyzed by the Kruskal–Wallis test, and further analysis was performed by the Bonferroni/Dunn test. Statistical significance was assumed for values of $P<0.05$.

Lipopolysaccharide- or Hypothermia-Induced Assays

PBMCs were suspended at 1×10^6 cells/ml in culture medium and cultured in the presence or absence of 10 or 100 ng/ml of LPSO127 (Sigma) for 24 h in six-well plates at 30°C or 37°C in a humidified atmosphere containing 5% CO_2.

Measurements of Tumor Necrosis Factor-α, IL-6, IL-1β, IL-1ra, and IL-18

Sera from the patients and healthy control subjects ($n=10$; age range, 1–35 years) were stored at −80°C until analysis. The sera of cases 1 and 2 were collected when they had the cold-induced rash, but not fever. The sera of cases 3, 4, and 5 were collected during a fever episode as an autoinflammatory symptom. Culture supernatants in test tubes or microtest plates were centrifuged to remove the cells and then stored at −80°C until analysis. The tumor necrosis factor (TNF)-α, IL-6, IL-1β, IL-1ra, and IL-18 concentrations were measured using a Human TNF-α Immunoassay Kit (BioSource, Camarillo, CA, USA), Human IL-6 Immunoassay Kit (BioSource), Human IL-1β Immunoassay Kit (BioSource), Quantikine Human IL-1ra/IL-1F3 ELISA Kit (R&D Systems, Minneapolis, MN, USA), and Human IL-18 ELISA Kit (MBL, Nagoya, Japan), respectively. The detection limits of the cytokine measurement kits were as follows: TNF-α, 1.7 pg/ml; IL-6, 2.0 pg/ml; IL-1β, 1.0 pg/ml; IL-1ra, 6.26 pg/ml; IL-18, 12.5 pg/ml. Values under the detection limits were shown as not detected. The serum cytokine levels were measured at two points at least, and the average values were calculated. The cytokine production levels by PBMCs were measured in duplicate and the average values were calculated. We defined cytokine levels of more than the mean+2 SD as increasing.

Results

Detection of Gene Variations in *NLRP3*

In the five patients, four heterozygous missense variations (E378K, Y563N, E688K, and G809S) of the *NLRP3* gene were identified (Table I). Interestingly, case 3 showed compound heterozygous gene variations, E688K and G809S, while his mother (case 4) had only one mutation, E688K, of *NLRP3*. The G809S allele was inherited from his asymptomatic father. In case 5, a novel missense variation, E378K, in *NLRP3* was identified. In addition, a heterozygous mutation, E148Q, in *MEFV* was identified. Gene mutations in *TNFRSF1A*, *MVK*, *NLRP12*, and *NOD2* were not found.

The genotypes of *NLRP3* and *MEFV* in her asymptomatic mother were the same. It should be noted that E378K and G809S were not present in the *INFEVERS* database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) [14] and were confirmed as rare variants that were not identified in the 100 ethnically matched control subjects.

NF-κB Reporter Gene Activities of the *NLRP3* Variants

Figure 2 shows the ASC-dependent NF-κB activities of the *NLRP3* variants in vitro. The NF-κB reporter gene activities were increased by the Y563N and E688K mutations in *NLRP3*. The activities were higher for D303N (as a positive control *NLRP3* mutation that was previously identified in a CINCA/NOMID patient [5]) and E688K than for the FCAS mutation, Y563N. E378K and G809S did not cause any significant increases in the activities. Initially, we suspected that case 5 had CAPS. However, based on these results, we were able to confirm the diagnosis of case 5 as JIA, rather than CAPS.

Cytokine Profiles of the Patients

The serum IL-1β, IL-6, and TNF-α levels were not detected in the sera of the healthy control subjects. Although we were unable to detect IL-1β in the patients' sera, we clearly detected the serum IL-18 and IL-1ra levels in all cases (Fig. 3a, b). The serum IL-18 levels were extremely high in the CINCA/NOMID (case 3), MWS (case 4), and JIA (case 5) patients compared with the control subjects. The serum IL-1ra and IL-6 levels were increased in cases 2, 3, 4, and 5 (Fig. 3b, c). The serum TNF-α levels were increased in cases 1, 2, and 3 (Fig. 3d).

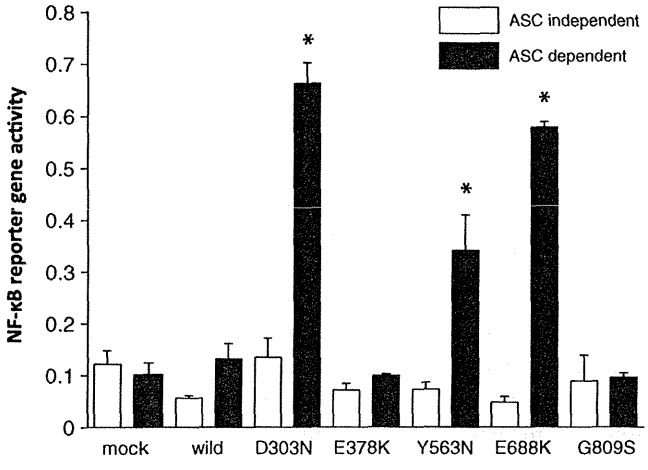

Fig. 2 NF-κB reporter gene activities of the *NLRP3* variants. The *white bars* indicate the NF-κB reporter gene activities of the *NLRP3* variants without cotransfection of ASC, while the *black bars* indicate these activities with cotransfection of ASC. The data shown are the means±SD of triplicate assays. The ASC-dependent NF-κB reporter gene activities are increased for the variants with D303N, Y563N, and E688K. The activities for the CINCA/NOMID mutations, D303N and E688K, are higher than those for the FCAS mutation, Y563N. The variants with E378K and G809S do not show any significant increases in the activities. *$P<0.05$

Interestingly, the serum IL-18 levels in the FCAS patients (cases 1 and 2) did not show any increases compared with the control subjects (Fig. 3a). Furthermore, the levels of spontaneous IL-1β production by PBMCs from the CINCA/NOMID (case 3) and MWS (case 4) patients were increased, whereas those of the control subjects, FCAS patients, and JIA patient (cases 1, 2, and 5) did not show any increases (Fig. 4a).

The lipopolysaccharide (LPS)-induced cytokine production levels by PBMCs from the FCAS and JIA patients are shown in Fig. 4b–d. The IL-1β and IL-18 production levels were increased in the FCAS patients compared with the control subjects. However, TNF-α did not show any significant changes. Comparisons of the cytokine production levels by the PBMCs cultured at 30°C and 37°C are shown in Fig. 5. The PBMCs from the FCAS patients showed obvious increases in the IL-1β and IL-18 production levels after culture at the lower temperature with no stimulation.

Discussion

The diagnosis of CAPS is still based on the clinical symptoms and recognition of a syndrome. Detection of a pathogenic *NLRP3* mutation can confirm the CAPS diagnosis. However, to confirm the diagnosis of CAPS patients with novel identified *NLRP3* variations, some functional experiments regarding the effects of the *NLRP3* mutations, such as the NF-κB luciferase reporter gene assay used in this study, are necessary because of the existence of nonfunctional missense variations of *NLRP3* [7]. Furthermore, although there are many previously reported missense mutations of *NLRP3* associated with CAPS in the *INFEVERS* database [14], the mutations with confirmed functional evidence are limited. In this study, we identified *NLRP3* gene mutations in five patients who were suspected of having autoinflammatory syndromes. Two mutations of *NLRP3*, Y563N and E688K, were previously reported to be disease-causing mutations [15, 16], although in vitro functional assays were not performed. Y563N was first identified in FCAS patients who were diagnosed based on the clinical criteria of FCAS [16, 17]. Our FCAS patients (cases 1 and 2) showed a skin rash, occasional fever, and mild arthritis and did not show any severe symptoms, such as neurological disorders, hearing loss, and renal amyloidosis. On the other hand, E688K was first identified in an Italian male CINCA/NOMID patient [15] who was described as having a skin rash, hearing loss, fever, and transient arthritis without persistent deformities of the involved joints. Our patients with E688K

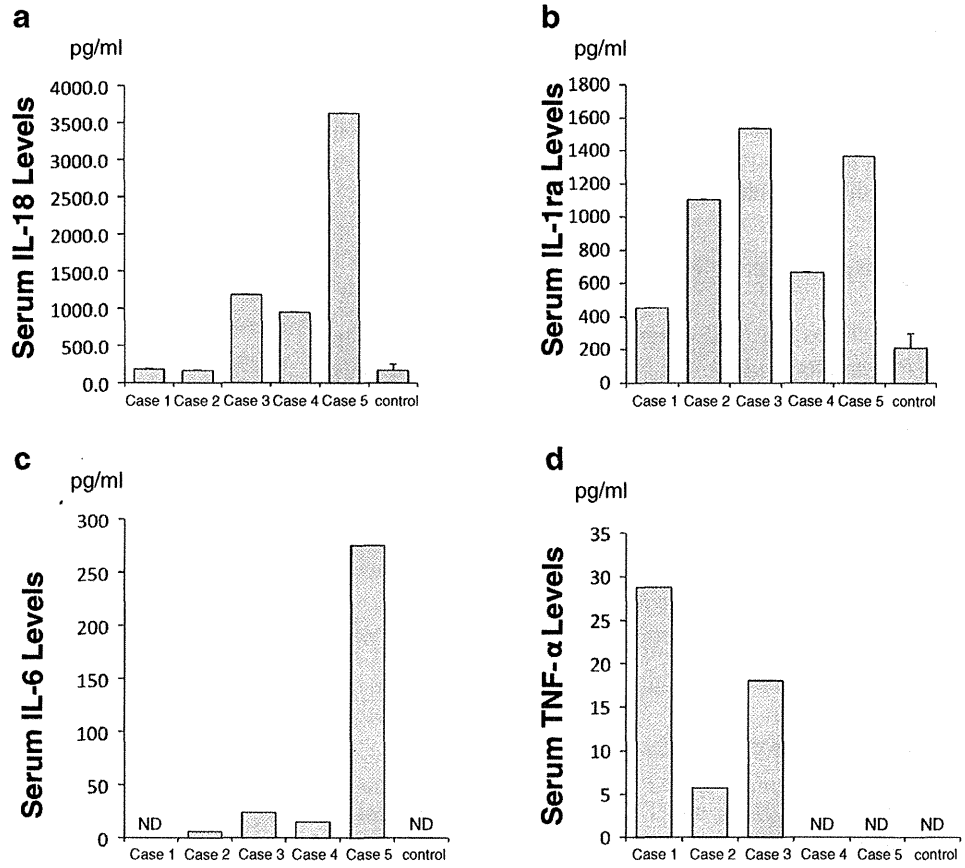

Fig. 3 Serum inflammatory cytokines in the four CAPS cases. IL-1β, IL-6, and TNF-α were not detected in the sera of the control subjects. The means ±D of the serum IL-18 and IL-1ra levels of the healthy control subjects were 169.2±85.7 and 213.4±87.1 pg/ml, respectively (n=10)

(cases 3 and 4) also had no strong deformities of the joints, but had obviously more severe phenotypes than FCAS, such as aseptic meningitis and hearing loss. In the present study, the E688K mutation in the MWS and CINCA/NOMID syndrome patients showed significantly stronger NF-κB activities than the Y563N mutation identified in the FCAS patients. Our findings indicate that the clinical phenotypes and values of the ASC-dependent NF-κB activity assay are well correlated with the genetic mutations, consistent with a previous report [18]. However, the artificial reporter gene assay system used may have little to do with the function of the CAPS pathophysiology, and limited numbers of *NLRP3* variants have been assessed using the assay in the present and previous studies, thereby making it difficult to prove this hypothesis at the present time. Consequently, further experiments including large amounts of pathogenic mutations and accumulation of detailed clinical information about the disease severity of CAPS are necessary to confirm this hypothesis. It should be noted that low-penetrance mutation, G809S, did not show positive activity with this in vitro assay system. But the clinical phenotype of case 3 was obviously more severe than case 4, although the father of case 3, who also was found to have G809S, was as symptomatic. Because of the discrepancy between the patient and the father, it remains unclear whether G809S is a pathogenic mutation or, alternatively, if there is an alternative genetic explanation for disease in the patient not detected by genomic DNA sequencing.

On the other hand, it requires time to build the above-mentioned in vitro experimental system. For the rapid diagnosis and characterization of CAPS, a simple screening system is necessary. In this study, we measured several serum inflammatory cytokine levels in our patients (Fig. 3). The serum IL-6 level is usually used for evaluating the disease severity of rheumatoid arthritis [19]. Moreover, the serum IL-18 level was recently reported to reflect the disease severity of not only JIA but also other diseases such as allergic diseases [20, 21]. In our CAPS patients, the serum levels of IL-18, but not IL-1β, seemed to be correlated with the disease phenotypes. Although the precise reason for this dissociation between the IL-18 and IL-1β levels in the sera is unknown, IL-1β may be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. In fact, serum IL-1ra, which is the counter-regulator of IL-1, was increased in our CAPS patients. Thus, the serum IL-18 levels may be used as an appropriate marker for the evaluation of treatments, although it is unlikely that serum IL-18 can contribute to the differential diagnosis between CAPS and other diseases.

The diagnosis of FCAS seems to be relatively difficult because of its mild phenotypes compared with the other more severe phenotypes of CAPS. The serum inflammatory

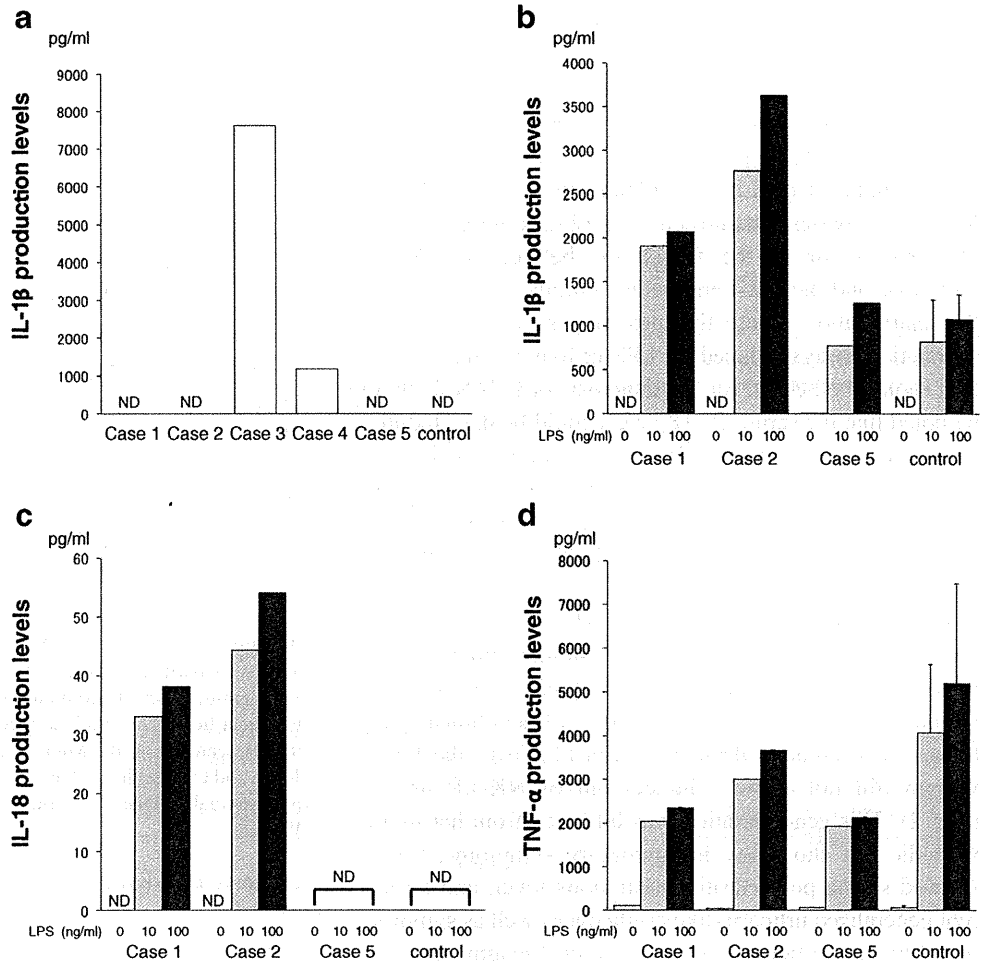

Fig. 4 LPS-induced cytokine production levels in the patients. **a** The *white bars* indicate the spontaneous IL-1β production levels by PBMCs. Increased IL-1β production by PBMCs from case 3 (CINCA/NOMID syndrome) and case 4 (MWS) is detected, whereas no increases are observed for the PBMCs from the control subjects and cases 1, 2 (FCAS), and 5 (JIA). **b, c** The LPS-induced IL-1β and IL-18 production levels by PBMCs from the FCAS patients are increased compared with PBMCs from the control subjects. **d** The TNF-α production levels by PBMCs from the FCAS and JIA patients do not show any significant changes. In **b–d**, the *white bars* indicate the cytokine production levels without stimulation and the *gray* and *black bars* indicate the cytokine production levels after stimulation by 10 and 100 ng/ml LPS, respectively

cytokine levels in our FCAS patients did not show any typical increases, unlike the case for the CINCA/NOMID patient (Fig. 3), indicating that the establishment of an effective and easy screening method is important for the diagnosis of FCAS. Therefore, we focused on the cytokine production levels in these patients' blood cells. First, IL-1β production by nonstimulated PBMCs was observed in our CINCA/NOMID and MWS patients (cases 3 and 4, respectively), as reported previously [5]. However, no enhancement of spontaneous IL-1β production was observed in our FCAS patients (cases 1 and 2) (Fig. 4a), suggesting that this method may not be suitable for screening of FCAS.

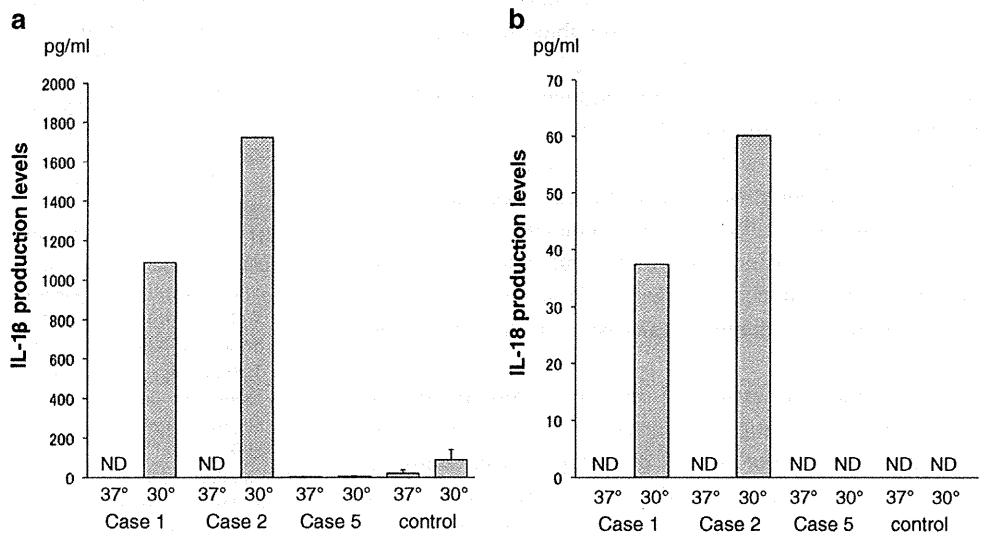

Fig. 5 Hypothermia-induced cytokine production levels by PBMCs from the FCAS and JIA patients. **a, b** Comparisons of the cytokine production levels by PBMCs cultured at 30°C and 37°C. The PBMCs from the FCAS patients (cases 1 and 2) show obvious increases in the IL-1β and IL-18 production levels after culture at lower temperature with no stimulation

Furthermore, the LPS- or hypothermia-induced cytokine production levels by the PBMCs showed marked elevation of IL-1β or IL-18 (Figs. 4a–c and 5b), as reported previously [16, 22]. The phenomena for hypothermic culture were similar to the findings in our recent report that NF-κB activity induced by LPS stimulation through TLR4 is enhanced in low-temperature cultures [23], although the precise mechanism of the association between the *NLRP3* variations and the low-temperature stimulation requires further clarification. These findings suggest that the cytokine production assays induced by LPS or hypothermia stimulation should be helpful for the diagnosis of FCAS. It should be noted that the serum IL-18 levels could be detected in all of the non-CAPS subjects, although the production levels of IL-18 from their PBMCs were lower than the detection limit. This might be dependent on the long half-life of IL-18 in human blood compared with the above-mentioned half-life of IL-1β.

The discrimination between CAPS and JIA cases is sometimes difficult because of their similar clinical characteristics. Interestingly, although case 5 had a rare missense variation in *NLRP3* (E378K) and some of her clinical symptoms were similar to those of CAPS (Table I), the E378K variant did not show enhancement of NK-κB activity (Fig. 2). This gene variation was inherited from her mother who did not show any inflammatory symptoms. Case 5 showed strong polyarthritis, continuous fever, and a recurrent generalized urticaria-like erythema as well as symptoms of CAPS. In particular, histopathological examination of a biopsy specimen from her skin rash revealed infiltration of neutrophils and mononuclear cells, representing similar findings to case 1 (Fig. 1). Thus, it was difficult to discriminate CAPS by the clinical symptoms alone in this case.

Therefore, to discriminate between CAPS and JIA in this case, we focused on her cytokine profiles. Her serum IL-6 and IL-18 levels were extremely high compared with not only the healthy controls but also the other CAPS patients (Fig. 3a, c). These observations resembled the serum cytokine pattern of systemic-onset JIA [21, 24]. Furthermore, the LPS-induced and hypothermia-induced IL-1β and IL-18 production levels by PBMCs from case 5 showed no increases compared with the control subjects (Figs. 4b, c and 5a, b). Recently, Saito et al. [5] reported that another screening method, LPS-induced monocyte cell death, was effective for diagnosing CAPS. The monocytes in case 5 did not show LPS-induced cell death. These objective results also supported the diagnosis of case 5 as JIA, rather than CAPS.

In this study, we evaluated several methods for the limited genotypes of patients with *NLRP3* variants. According to comparisons of the clinical phenotypes of previous case reports and our cases, the disease severity seems to be correlated with the serum cytokine levels and the ex vivo and in vitro responses and is almost completely determined by the specific mutations, which appear to suggest that other genetic or epigenetic determinants or environmental factors do not play a significant role.

Conclusions

A precise and easy method for the diagnosis of CAPS has not yet been established. The characteristics of the clinical phenotypes and the identification of proven gene variations of *NLRP3*, as the etiology of CAPS, are very important for diagnosing CAPS. In addition, the serum IL-18 levels and NF-κB activities of patients with the *NLRP3* variants reflect the phenotypes of disease severity. Evaluation of the cytokine profile is also a useful tool for diagnosing and discriminating the severity of CAPS.

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Conflicts of Interest The authors have declared no conflicts of interest.

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Refractory Chronic Pleurisy Caused by *Helicobacter equorum*-Like Bacterium in a Patient with X-Linked Agammaglobulinemia▽

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We describe a 35-year-old man with X-linked agammaglobulinemia who had refractory chronic pleurisy caused by a *Helicobacter equorum*-like bacterium. Broad-range bacterial PCR targeting the 16S and 23S rRNA genes and *in situ* hybridization targeting the 16S rRNA gene of *H. equorum* confirmed the presence of this pathogen in a human for the first time.

CASE REPORT

A 35-year-old man was referred to us for a low-grade fever, fatigue, and discomfort in the right thorax. He had been diagnosed with X-linked agammaglobulinemia (XLA) during the first year of life (5). Upon diagnosis, he showed extremely low serum immunoglobulin G, A, and M levels (650 mg/liter, under 80 mg/liter, and under 60 mg/liter, respectively) and had a missense mutation, L111R (464T>G), in Bruton's tyrosine kinase (BTK) gene (Fig. 1A). His family pedigree with respect to XLA is shown in Fig. 1B. Substitution therapy with intravenous immunoglobulin was administered every 4 weeks from his childhood. During his high school years, he had acute right pleurisy and a pleural puncture, but the details are unclear. In 2006, he suffered from right pleurisy again and then was repeatedly admitted to our hospital for 2 years. No pathogens causing his chronic pleurisy have ever been detected, even though some conventional cultures of blood and sputum have been performed. However, administration of panipenem/betamipron (PAPM/BP) had been the only way to improve his chest discomfort and transiently reduce C-reactive protein (CRP) levels. Administration of other antimicrobial treatments, such as the use of macrolides, cephems, newquinolones, glycopeptides, and carbapenems other than PAPM/BP, has resulted in no improvement.

On admission in 2008, his laboratory findings showed a normal white blood cell count (7.76×10^9/liter), a high CRP level (50.3 mg/liter), and a very high endotoxin level (131 ng/liter). A chest radiograph showed a thickened right pleura and pneumonia in the right inferior lung (Fig. 2A), and a computed tomography (CT) scan of the chest also showed the thickened right pleura with calcification and an alveolar opacity in the right inferior lung (Fig. 2B). We suspected a Gram-negative bacterial infection due to the transient PAPM/BP effectiveness and the high endotoxin level or *Mycobacterium tuberculosis* complex (MTC) infection due to the CT calcification finding. However, conventional cultures of blood, urine, sputum, and feces were all negative, as they had often been in the past.

A transbronchial lung biopsy and a transcutaneous pleural biopsy were performed for definite diagnosis. Histological examination of the alveolar spaces in the right lung showed intraluminal fibrosis of distal airspaces with foamy alveolar macrophages, suggesting secondary organizing pneumonia (OP) (Fig. 2C). In addition, examination of the right pleura showed chronic inflammation (Fig. 2D). Despite these findings, conventional bacterial cultures of biopsy samples from the right pleura grown in sheep blood agar (Nissui Pharmaceutical, Tokyo, Japan) and chocolate agar (Eiken Kagaku, Tokyo, Japan) plates showed no evidence of infection, and MTC cultures grown in an egg-based solid medium (Ogawa medium) (Kyokuto Pharmaceutical, Tokyo, Japan) was also negative.

To determine the pathogen of this refractory pleurisy, we performed broad-range bacterial PCR and mycobacterial PCR using the pleural samples. The PCR products targeting the bacterial 16S and 23S rRNA genes revealed a 1,473-bp band and a 563-bp band, respectively (Fig. 3A). Sequencing analysis was carried out using a GenBank BLAST search (National Center for Biotechnology, Bethesda, MD). Sequence editing and phylogenetic analyses were performed with ClustalW. The sequence of a 1,473-bp fragment of 16S rRNA gene confirmed the presence of *Helicobacter equorum*-like (99.8% identical) bacterium DNA (GenBank accession no. AB571486). Moreover, the sequence of a 563-bp fragment targeting the bacterial 23S rRNA gene was 98.9% similar to that of *H. equorum* (GenBank accession no. AB571487). On the other hand, PCR amplification of the 16S rRNA gene for MTC determinations was negative.

Next, to further demonstrate that the pleural infection involved an *H. equorum*-like bacterium, we performed *in situ* hybridization

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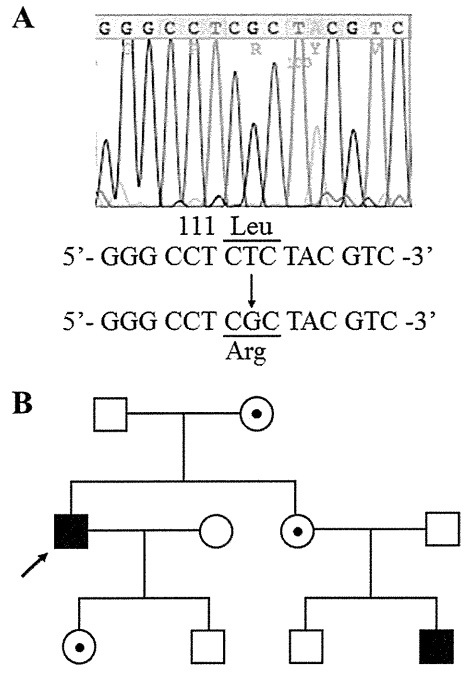

FIG. 1. Molecular analysis of the BTK gene and pedigree of our patient. (A) A missense mutation, L111R in BTK gene, identified in our patient. (B) The family tree for our patient. An arrow indicates the proband with XLA. The solid squares denote patients with XLA; circles with black dots denote mutation carriers.

using a digoxigenin-labeled single-strand RNA probe. The probe for positions 999 to 1118 in the 16S rRNA gene of *H. equorum* (GenBank accession no. AM998804) was designed as follows: 5′-UCCUCACCUUCCUCCUCCUUACGAAGGCAGUCUC CUUAGAGUGCUCAGCCAAACUGCUAGCAACUAAGG ACGAGGGUUGCGCUCGUUGCGGGACUUAACCCAAC AUCUCACGACACGAGC-3′. Positive signals in the pleural sample were confirmed with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) system (Roche Diagnostics, Tokyo, Japan) (Fig. 3B) but not in the control, which included the sense probe (Fig. 3C). These results indicated that the refractory chronic pleurisy in our patient was caused by an *H. equorum*-like bacterium, which in turn caused the development of secondary OP.

We began administration of PAPM/BP at a high dose of 8 g/day and of clarithromycin orally for 2 months. Since then, the patient has had no symptoms, and tests have shown negative CRP results and an endotoxin level of less than 10 ng/liter.

Since the discovery of *Helicobacter pylori* in 1984 (7), various *Helicobacter* species have been described in a wide variety of animal hosts, and transmission to humans has been suggested (3, 10, 14). In general, *Helicobacter pylori* is associated with gastritis, peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (3). Also, non-*H. pylori Helicobacter* species are associated with gastric, intesti-

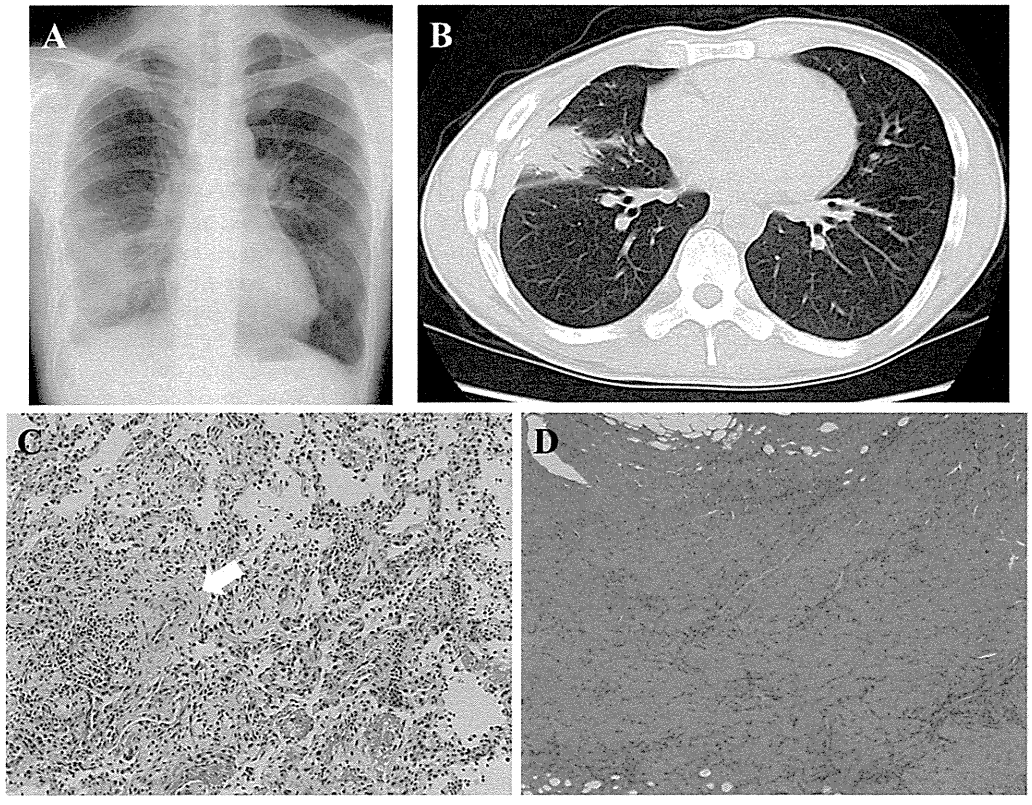

FIG. 2. Imaging and histological findings of refractory chronic pleurisy and secondary OP. (A and B) Results of a chest radiograph (A) and a chest CT scan (B) upon the latest admission of the patient to the hospital. (C) Histological finding in alveolar spaces, showing intraluminal fibrosis (arrow) (hematoxylin and eosin). (D) Histological finding in the right pleura, showing chronic inflammation (hematoxylin and eosin).

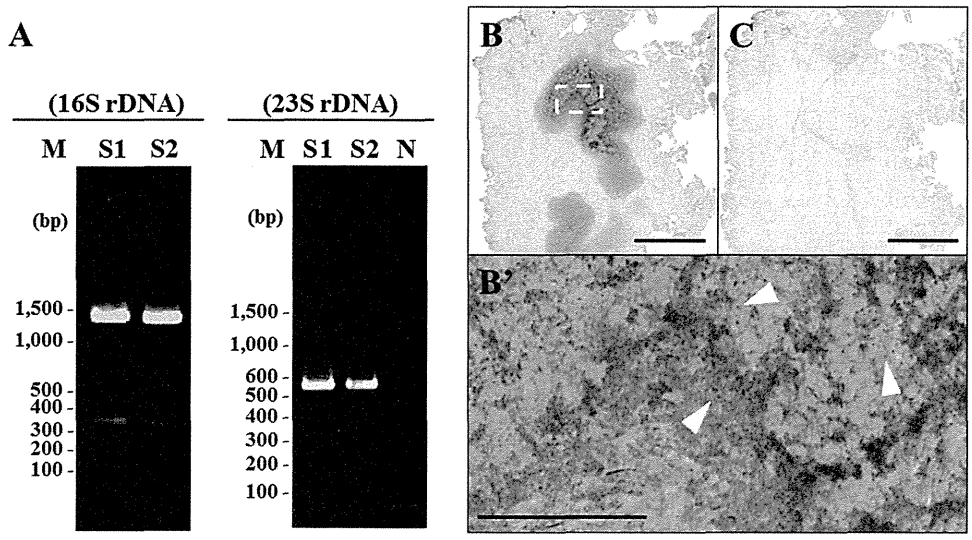

FIG. 3. Detection of *H. equorum*-like bacterium DNA in samples from the right pleura. (A) Broad-range bacterial products from a PCR targeting the 16S rRNA gene (left) and the 23S rRNA gene (right) determined using biopsy samples from the right pleura. S1 and S2 denote DNA samples from our patient. One-tenth the amount used in S1 was used in S2. N, negative control; M, marker. (B) Result of *in situ* hybridization of the pleural samples performed using the probe for the 16S rRNA gene of *H. equorum*. Scale bar, 500 μm. (B') Higher magnification of the bracketed areas shown in panel B. The signals of *H. equorum* were detected (arrowheads). Scale bar, 100 μm. (C) Negative control. Scale bar, 500 μm.

nal, and hepatobiliary diseases in humans (3, 10, 14). This understanding is attributed to molecular diagnosis based on the sequencing of bacterial 16S and 23S rRNA genes, an analytical technique that has already proved useful for various bacterial infections during antimicrobial treatment (11), for rare or unexpected pathogens (11), and particularly for difficult-to-culture bacteria such as non-*H. pylori Helicobacter* species (3). Herein, we have also described a case of refractory chronic pleurisy caused by an *H. equorum*-like bacterium that was subjected to molecular analysis.

Our patient had XLA, which is a rare genetic disorder of B-cell maturation characterized by the absence of mature B cells, very low serum levels of all immunoglobulin isotypes, and a lack of specific antibody production (6). He suffered for 2 years from right chronic pleurisy due to an unknown pathogen. We treated him with PAPM/BP on the basis of the clinical findings, but we were confused because the efficacy was transitory. Molecular diagnosis targeting bacterial 16S and 23S rRNA genes revealed that only DNA of an *H. equorum*-like bacterium that has not previously been reported to have been found in samples from humans was isolated from biopsy samples of our patient. Unfortunately, a culture for the *Helicobacter* species could not be performed for our patient because of the unexpected bacterium, but such culture is also difficult to perform in general, particularly for non-*H. pylori Helicobacter* species (3). We therefore performed *in situ* hybridization using the probe for the 16S rRNA gene of *H. equorum* and thereby confirmed that the infection had been caused by an *H. equorum*-like bacterium.

H. equorum, which is a Gram-negative, curved, and motile bacterium, was recently isolated from horse feces by molecular diagnosis (8). Additional investigation revealed that the prevalence of *H. equorum* was significantly higher in horses under veterinary care than in healthy horses, and *H. equorum* DNA has never been detected in human samples (9). To the best of our knowledge, this is the first case of infection with *H. equorum*-like bacterium in a human with XLA and in the respiratory system. So far, *Helicobacter* infections in patients with XLA have rarely been reported (2, 4, 12, 13), and none of those reported have been due to the presence of *Helicobacter* species in the respiratory system. Freeman and Holland illustrated the importance of humoral immunity in *Helicobacter* infections involving mucosal surfaces, because patients with XLA have been prone to chronic bacteremia, skin infections, and bone infections by the *Helicobacter* species (1). Our patient with XLA showed no evidence of bacteremia or other infections due to the presence of an *H. equorum*-like bacterium. In addition, the studied patient had not had any contact with horse feces, which is a possible vector of *H. equorum*, for the previous 2 years, though he had a history of right pleurisy. Finally, the source of the infection in our patient could not be identified, but we think it would be accurate to say that this infection, which exhibited abnormal humoral immunity, may have been associated with XLA.

Our patient with XLA has been treated with PAPM/BP, but we are unsure as to which antimicrobial treatment to use in a case like this. Because of the difficulty of performing culture, *in vitro* susceptibility testing has scarcely been evaluated or standardized for *H. equorum*. Moyaert et al. reported resistance to cephalotin and nalidixic acid and sensitivity to metronidazole for *H. equorum* (8). We also noted evidence of multiple drug resistance of this organism clinically, as our patient improved only after treatment with PAPM/BP; administration of many other antimicrobial treatments resulted in no improvement. Further investigation is needed, because antimicrobial treatment for *H. equorum* may be difficult.

In conclusion, we have described a case of chronic pleurisy associated with the presence of an *H. equorum*-like bacterium.

All of the clinical findings for our patient—transient PAPM/BP effectiveness, a high serum endotoxin level, and imaging-histological findings of chronic inflammation—were consistent with infections by this organism. This case illustrates both the usefulness of molecular diagnosis of infections with unknown organisms and the pathogenicity of the *H. equorum*-like bacterium in immunocompromised humans. In the future, the issues of whether *H. equorum* is associated with diseases in immunocompetent humans or not and of how patients infected with *H. equorum* are to be treated need to be investigated.

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Multiple Reversions of an IL2RG Mutation Restore T cell Function in an X-linked Severe Combined Immunodeficiency Patient

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Abstract Reversion mosaicism is increasingly being reported in primary immunodeficiency diseases, but there have been few cases with clinically improved immune function. Here, a case is reported of X-linked severe combined immunodeficiency (SCID-X1) with multiple somatic reversions in T cells, which restored sufficient cell-mediated immunity to overcome viral infection. Lineage-specific analysis revealed multiple reversions in T cell receptor (TCR) $\alpha\beta$+ and TCR$\gamma\delta$+ T cells. Diversity of the TCRVβ repertoire was comparable to normal and, furthermore, mitogen-induced

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proliferation of the patient's T cells was minimally impaired compared to healthy controls. *In vivo* and *in vitro* varicella antigen-specific T cell responses were comparable to those of healthy controls, although a reduced level of T cell receptor excision circles suggested that recent thymic output was low. During long-term evaluation of the patient's immunologic status, both the number of CD4+ and CD8+ T cells and T cell proliferation responses were stable and the patient remained healthy. This case demonstrates that multiple but restricted somatic reversions in T cell progenitors can improve the clinical phenotype of SCID-X1.

Keywords Severe combined immunodeficiency · reversion · multiple

Introduction

X-linked severe combined immunodeficiency (SCID-X1) is a recessive hereditary disease characterized by a lack of T cells and natural killer (NK) cells. Without stem cell transplantation, persistent infections with opportunistic organisms uniformly lead to death in the first 2 years of life, except in those with atypically attenuated phenotypes [1–3]. Recently, spontaneous genetic reversion has been reported in primary immunodeficiency disorders. Somatic reversion mosaicism is considered to be 'natural gene therapy'; however, few cases are reported with reversions that restore functional immunity [4–9]. Here, an atypical case of SCID-X1 with somatic mosaicism due to multiple reversions in T cells, which restored sufficient T cell immunity, is described.

Materials and Methods

Patient

A male infant was born prematurely at 34 weeks and 4 days of gestation with a birth weight of 1,660 g to healthy parents. There was no family history of consanguinity or immunodeficiency. He was well until 14 months of age, when he started to have recurrent bacterial respiratory tract infections. At the age of 21 months, laboratory tests were performed. Patient results were compared to age-matched normal controls (controls). Examination of serum Ig revealed a decreased level of IgG (IgG, 1.93 g/L [range of controls: 7.15–9.07 g/L]), and normal levels of IgA (IgA, 0.33 g/L [range of controls: 0.22–1.44 g/L]) and IgM (IgM, 0.72 g/L [range of controls: 0.34–1.28 g/L]). His serum IgG was constantly under 2.0 g/L. In addition, he had a reduced number of CD4+ cells (358/μl, [mean of controls: 1,683± 874]) and CD56+ cells (39/μl [mean of controls: 306± 207]), while CD3+ cells (1,803/μl [mean of controls: 2,997±1,751]), CD8+ cells (1,067/μl [mean of controls: 1,683±874]) and CD19+ cells (1,850/μl [mean of controls: 1,114±976]) were within the normal limits. The patient's T cell proliferative response to phytohemagglutinin (PHA) (stimulation index (S.I.) of 172 [range of controls: 105–225]) and to concanavalin-A (Con-A) (S.I. of 140 [range of controls: 68–154]) was within the normal ranges for his age. From these data, he was diagnosed with common variable immunodeficiency (CVID) at that time. Intravenous immunoglobulin therapy was started and he remained in good health thereafter. Without receiving vaccination, varicella infection at 5 years of age did not cause fever, and he was successfully treated with oral acyclovir at an outpatient clinic. At 9 years of age, warts developed and spread over his body, and he was referred to our hospital for assessment of his immunological status. Physical examination revealed neither detectable lymph nodes nor tonsils, and his thymus appeared hypoplastic on CT scan. Before the laboratory studies were performed, informed consent was obtained from the patient and his parents, in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols with a FACSCalibur flow cytometer (Becton Dickinson, USA). The following fluorochrome-conjugated antibodies (Abs) were used for flow cytometric analysis: CD3 (clone SK7), CD4 (clone CK3), CD8 (clone SK1), CD14 (clone M5E2), CD19 (clone SJ25C1), CD56 (clone B159), CD45RA (clone HI100), CD45RO (clone UCHL1) (BD Biosciences Pharmingen, USA), TCRαβ (clone IP26A), TCRγδ (clone IMMU 510) (Beckman Coulter, Inc., USA), CCR7 (clone 150503, R&D Systems Inc., USA), CD27 (clone O323, eBioscience, Inc., USA), CD132 (clone TUGh4, BD Biosciences Pharmingen), and rabbit anti-Human IgD polyclonal Ab (DAKO Japan Co., Japan).

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Sequencing of Genomic DNA and cDNA, and Subcloning Analysis

Peripheral blood mononuclear cells (PBMCs) were obtained from the patient and his parents and various cell lineages were sorted using a FACSVantage (Becton Dickinson). The genomic DNA was isolated from the sorted samples and the cDNA was obtained using reverse transcriptase Super Script II (Invitrogen, USA) with Oligo $(dT)_{20}$ primer. Genomic DNA and cDNA were amplified with the proofreading PCR enzyme, KOD –Plus– (Toyobo, Japan). Direct sequencing analysis of all exons of the *IL2RG* gene, including introns at least 50 bases adjacent, were performed on an ABI 3700 (Applied Biosystems, USA). For analysing revertant subclones in each PBMC lineage, the genomic DNA and the cDNA isolated from sorted cell fractions were amplified by PCR with primer pairs 5′-TCCCAGAGGTT CAGTGTTTTG-3′ and 5′-TTGCAACTGACAGCCA GAAG-3′, and 5′-CGCCATGTTGAAGCCATC-3′ and 5′-TTGCAACTGACAGCCAGAAG-3′, for the region spanning exons 2 and 3 of *IL2RG,* respectively. These PCR products were subcloned using a TOPO TA Cloning Kit (Invitrogen) and sequenced.

T cell Functional Assays

To obtain PHA-induced T cell blasts, PBMCs were stimulated with PHA (Invitrogen) at 1:100 dilution and cultured in RPMI 1640 (RPMI) supplemented with 5 % fetal calf serum (FCS) with recombinant human IL-2 (50 IU/ml, kindly provided by Takeda Pharmaceutical Company, Japan) at 37 °C for 7 days. After being rested in RPMI with 5 % FCS overnight, the T cell blasts were stimulated with various concentrations of IL-2 for 48 h, and $[^3H]$-thymidine uptake assays were performed as previously described [8]. T cell receptor (TCR) Vβ repertoire analysis and CDR3 spectratyping were performed as described [10, 11]. *In vitro* cytokine production against varicella zoster virus (VZV) antigen was performed as previously described [12]. Spots were enumerated automatically using the KS ELISPOT system (Carl Zeiss). The *in vivo* delayed-type hypersensitivity (DTH) reaction to subcutaneous purified VZV antigen (BIKEN, Japan) was performed as previously described [13]. The T cell receptor excision circles (TRECs) from the patient PBMCs were measured as previously described [14].

Tyrosine Phosphorylation of STAT5 by IL-2

PBMCs (1×10^6) were cultured in RPMI with 5 % FCS at 37 °C for 2 h and then treated with or without IL-2 (10,000 U/ml) for 10 min. The cells were fixed and permeabilized with BD Cytofix Buffer and Phosflow Perm Buffer III (BD Biosciences Pharmingen) according to the manufacturer's instructions. After washing with PBS containing 1 % FCS, the cells were stained with mouse anti-pSTAT5 (pY694) (clone 47, BD biosciences), anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry.

Results and Discussion

At the age of 9 years, the patient presented with generalized warts and no detectable lymph nodes and tonsils. This, coupled with his prior hypogammaglobulinemia, prompted a re-evaluation of his immunological status. He showed a decreased level of IgA and a normal level of IgM but no isohemagglutinin. Mitogen-induced proliferation assays showed a slightly reduced response to PHA and Con A (Table I). Surface marker analysis of PBMCs revealed slightly decreased levels of CD3+ and CD4+ T cells, and a normal level of CD8+ T cells (Table II). Naïve CD4+ T cells

Table I Laboratory investigations (patient aged 9 years)

		Patient (IVIG)	Healthy controls
Blood counts			
White blood cells	(count/μl)	7,400	3,600–9,800
Neutrophil	(count/μl)	4,773	3,000–5,000
Lymphocyte	(count/μl)	2,028	2,500–4,500
Monocyte	(count/μl)	340	200–950
Eosinophil	(count/μl)	252	0–700
Basophil	(count/μl)	7	0–150
Red blood cells	($\times10^6$ count/μl)	5.15	4.08–5.07
Hemoglobin	(g/dl)	12.5	11.6–14.1
Platelet	($\times10^3$ count/μl)	275	201–409
Serum Immunoglobulin levels			
IgG	(g/L)	7.69	10.79±2.63
IgA	(g/L)	0.26	2.46±0.91
IgM	(g/L)	1.08	0.83±0.21
IgD	(mg/L)	<6	55±16
IgE	(IU/mL)	<5	<170
isohemagluttinin		Undetectable	
T cell proliferation			
None	(cpm)	163	127–456
Phytohemagglutinin	(cpm)	16,800	20,500–56,800
Concanavalin A	(cpm)	16,600	20,300–65,700
DTH reaction to subcutaneous varicella virus antigen			
Erythematous change (mm in diameter)		18	≧5.0

Control values of blood counts are shown as the range from 95 % of healthy children aged 9 to 12 years. Control values of serum immunoglobulin levels are based on children aged 8 to 10 years and are shown as the mean ± SD. IVIG indicates monthly intravenous infusion of 2.5 g immunoglobulin

Table II Surface marker analysis of peripheral blood mononuclear cells (patient aged 9 years)

	Patient (count/μl)	Healthy controls (count/μl)
CD3+	1,080	2,813±1,197
CD4+	357	1,699±850
CD8+	582	972±457
TCRαβ+	890	2,154±1,004
TCRγδ+	190	324±182
CD4+CD45RA+CCR7+	8	1,290±756
CD8+CD45RA+CCR7+	25	655±503
CD8+CD45RA+CCR7-	114	221±95.3
CD8+CD45RA-CCR7+	33	30.1±27.6
CD8+CD45RA-CCR7-	410	132±87.4
CD19+	894	1,238±605
CD19+CD27+smIgD-	0.4	86.6±61.3
CD19+CD27+smIgD+	14.3	172±123
CD3-CD56+	Undetectable	271±186

Absolute numbers of cells expressing surface markers are shown. Healthy control values are from children aged 2 to 9 years and are shown as mean ± SD

(CD4+/CD45RA+/CCR7+), naïve CD8+ T cells (CD8+/CD45RA+/CCR7+), and both switched memory B cells (CD19+/CD27+/smIgD-) and unswitched memory B cells (CD19+/CD27+/cmIgD-) were scarce. In addition, natural killer (NK) cells (CD3-/CD56+) were absent. This suggested the existence of a genetic defect causing lack of NK cells, such as an *IL2RG* deficiency and *JAK3* deficiency, and therefore the expression of IL2RG (also known as the common gamma chain or CD132) was examined by flow cytometry. Reduced expression was found on B cells and monocytes, although T cells expressed normal levels of CD132 (Fig. 1a). To determine whether CD132-dependent signal transduction was functioning, STAT5 phosphorylation was analyzed on patient CD4+ and CD8+ T cells in response to IL-2. It was found to be comparable with that of normal controls (Fig. 1b). In addition, a proliferation assay of PHA-induced T cell blasts in response to exogenous IL-2 was performed (Fig. 1c). This confirmed that the patient T cells, which were expressing normal levels of CD132, also had intact IL-2 signaling.

To elucidate the genetic cause of the lineage-dependent CD132 expression abnormalities, *IL2RG* genomic sequencing was performed in various cell lineages. Genomic sequencing of *IL2RG* in B cells, monocytes and buccal mucosa revealed a point mutation, c.284-15A>G, in intron 2 of *IL2RG*. This has been reported as a causative mutation of SCID-X1 [15], producing aberrant mRNA with an insertion of 14 bases spanning nucleotide −14 to −1 of exon 3 (Fig. 2a, b). Genomic sequencing of *IL2RG* in T cells showed overlapping bases at and around the mutation sites, while the cDNA of *IL2RG* from

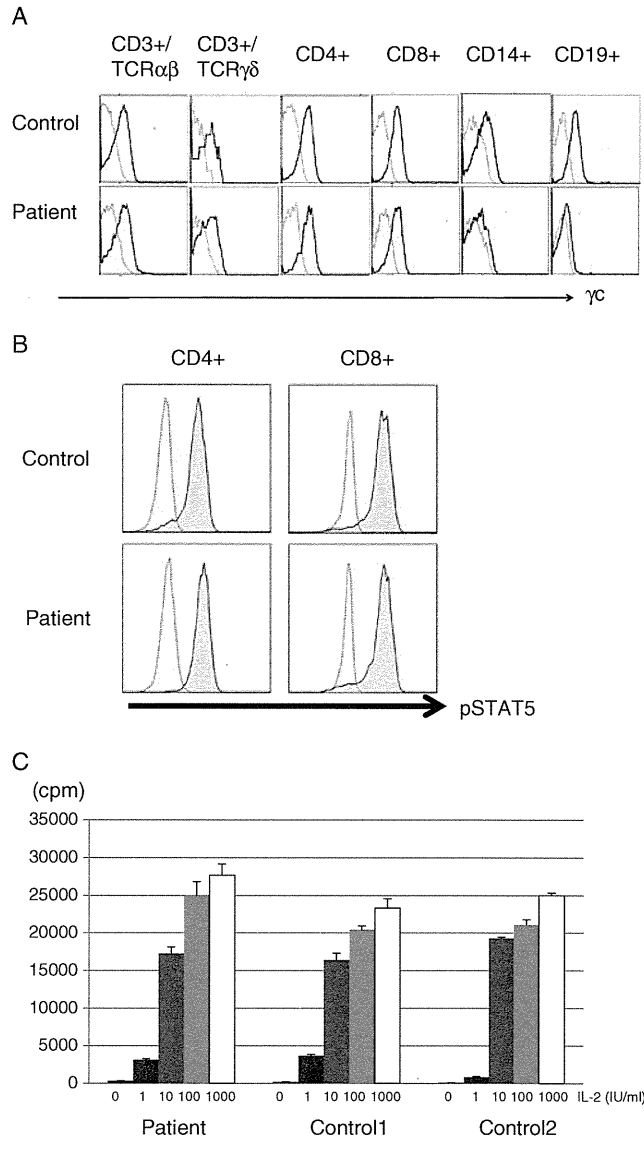

Fig. 1 IL2RG expression and T cell function at 9 years old. **a** Surface expression of IL2RG on PBMCs from the patient and healthy control gated according to the expression of the indicated lineage surface markers. *Black lines* indicate staining for IL2RG (with anti-CD132 Ab) and *gray lines* indicate staining with the isotype control. Data represent one of three independent experiments. **b** STAT5 tyrosine phosphorylation in patient and control CD4+ and CD8+ cells after incubation with (*shaded histograms*) or without IL-2 (*open histograms*). **c** Proliferation of PHA-induced T cell blasts in response to IL-2 stimulation from the patient and two controls. Data are shown as means ± SD

the T cells was normal (Fig. 2a, b). Genomic sequencing of PBMCs from the patient's mother confirmed her as a carrier of the mutation. The possibility of maternal engraftment was excluded by FISH analysis of sex chromosomes (data not shown), and it was concluded that the patient inherited the mutation from his mother and that reversion occurred in the patient's T cells, which led to somatic mosaicism.

To explore the reversions that could have occurred to restore normal *IL2RG* expression in the patient's T cells,

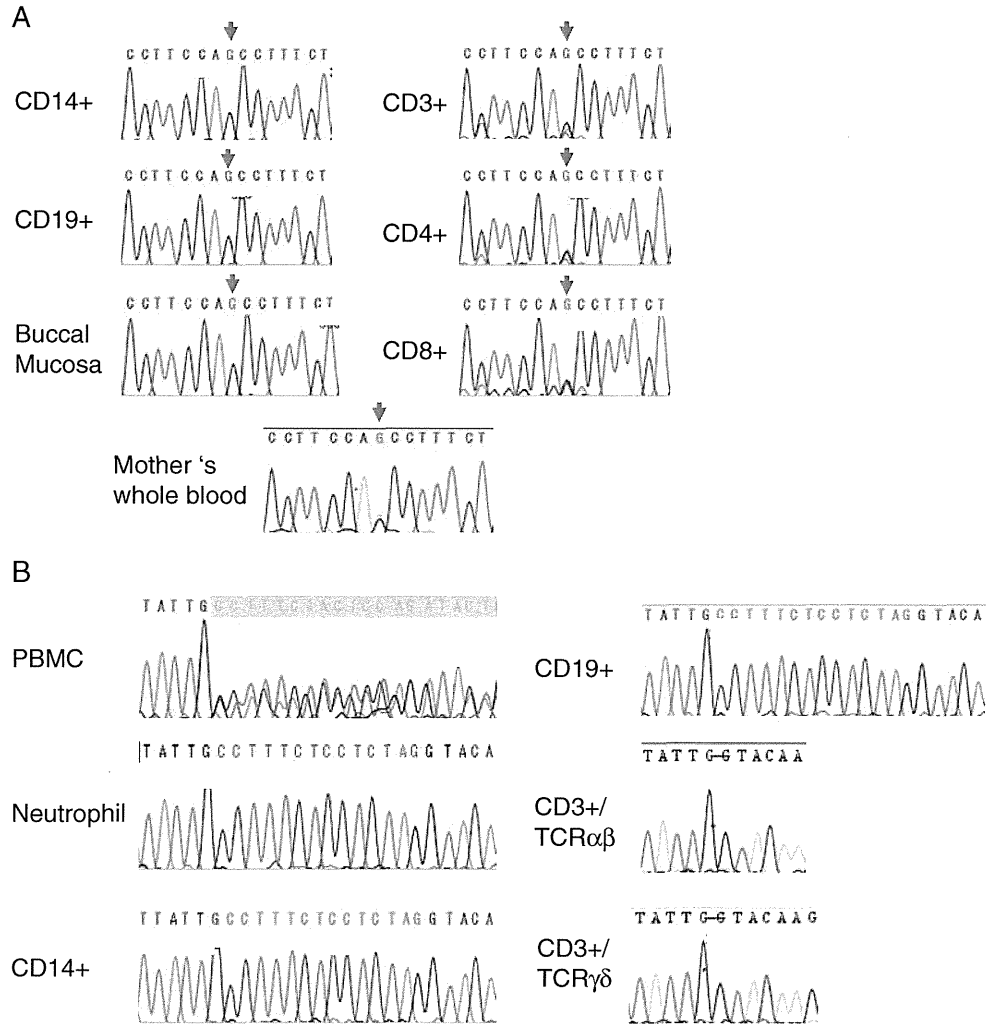

Fig. 2 Genetic analysis of various cell lineages at 9 years old. **a** Sequencing chromatograms of the patient's DNA from various immune cell lineages and buccal mucosa. *Red arrows* indicate the mutated base position c. 284–15. PBMCs from the patient's mother carried the same mutation. The patient's T cells show overlapping base changes at or around the mutated site. Data represent one of three independent experiments. **b** Sequencing chromatograms of the patient's cDNA from various cell types. Re characters indicate the inserted 14 bases spanning nucleotide −14 to nucleotide −1 of exon 3

subcloning and sequencing analysis of genomic DNA and cDNA was performed in various cell lineages. In B cells and monocytes, no reversion was detected and all of the cDNA clones had aberrant splicing (Table III). Analysis of TCRαβ+ cells revealed seven reversions, a true-back reversion, two fully compensating same-site reversions and four second-site reversions, all of which favored a functional reversion according to the splicing analysis software NNSPLICE0.9 [16] (Table IV). None of these base changes were detected in 200 clones from four healthy controls, indicating that the identified intron changes were unlikely to be due to PCR errors. The multiple reversions seen in this case differed from the single reversions seen in other reported cases of reversion mosaicism of SCID-X1 [2, 3]. One possible reason for this is that, compared with the previously reported exonic mutations, an intronic mutation is more likely to acquire additional reversions on top of a true-back mutation. Additionally, the nine-year lifespan of the patient may have provided increased opportunities for extra reversions to occur. TCRVβ V-to-DJ rearrangement is reported to be impaired in some SCID-X1 patients, suggesting that differentiation arrest occurs at the CD4 immature single positive (ISP) stage at which TCRVβ V-to-DJ recombination is completed in normal T cells [17]. Therefore, the

Table III Clonal analysis of *IL2RG* cDNA in various cell lineages

	CD3+	CD4+	CD8+	CD14+	CD19+
Wild-type cDNA	100 % (25/25)	100 % (31/31)	100 % (30/30)	0 % (0/45)	0 % (0/34)
Aberrant cDNA	0 % (0/25)	0 % (0/31)	0 % (0/30)	100 % (45/45)	100 % (34/34)

Data represent the percentages of wild-type or aberrant spliced cDNA subclones in each lineage. The ratio indicates the number of each clone as compared to the total number of clones analyzed, based on subcloning and sequencing analysis