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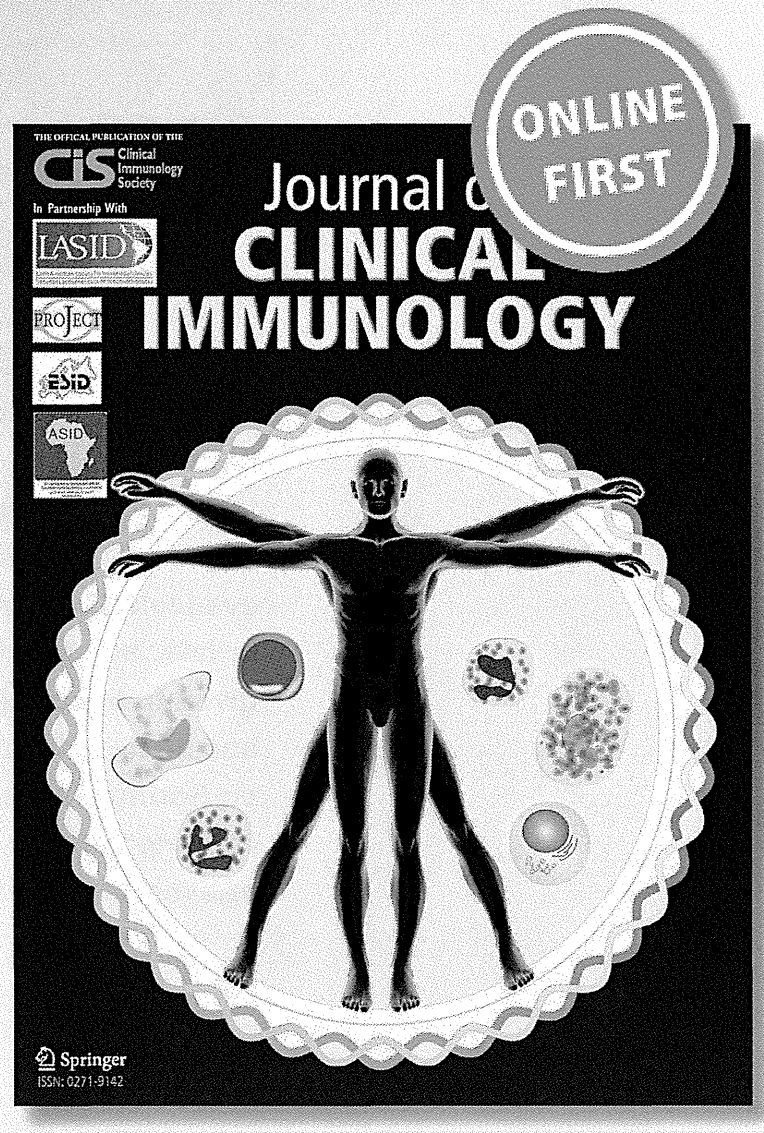

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ORIGINAL RESEARCH

Clinical and Genetic Characterization of Japanese Sporadic Cases of Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis Syndrome from a Single Medical Center in Japan

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Abstract
Purpose To investigate clinical presentation, genetic background and cytokine profile of Japanese sporadic cases of periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome.
Methods Nine PFAPA syndrome patients were recruited. DNA sequence analysis of auto inflammatory disorder susceptibility genes, *MEFV*, *MVK*, *NLRP3*, and *TNFRSF1A*, were performed. Serum cytokine levels and monocyte IL-1β levels were measured by ELISA.
Results The study population consisted of six males and three females (mean age of onset 26.8 months). Febrile episodes lasted 3–6 days with symptom-free intervals ranging from 2 to 12 weeks. Fever was accompanied by pharyngitis (n=8), aphthous stomatitis (n=4), and cervical adenitis (n=5). White blood cells and C-reactive protein were increased during the attack phase. Mean IgD serum levels were 7.32±9.51 mg/dl during the attack phase, and were mildly elevated in two patients. Heterozygous *MEFV*, *NLRP3* and *TNFRSF1A* variants were detected in four, one and three cases, respectively. Serum TNF-α and IL-18 levels were elevated during the attack-free and attack periods compared with controls. Other cytokines, IL-1β, IL-1ra, IL-6, and sTNFR1, were only increased during the attack phase. Oral prednisolone was administered to eight patients and immediately reduced fever. Tonsillectomy performed in five patients induced cessation of fever in four patients. One case with repeated fever attacks after tonsillectomy showed increased monocyte IL-1β production, similar to the other active case with genetic variants of auto inflammatory disorder-associated genes.
Conclusions Japanese PFAPA syndrome patients may have cytokine regulation dysfunction as a result of genetic variants of auto inflammatory disorder-associated genes.

Keywords PFAPA · IgD · *MEFV* · IL-1β · tonsillectomy

Introduction

Periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome is the most common auto inflammatory disease in childhood [1] and was first described in 1987 by Marshall et al. [2]. It is characterized by periodic episodes of high fever, accompanied by at least one of three clinical signs, including aphthous stomatitis, pharyngitis and cervical adenitis [2, 3]. Disease onset is generally before the age of 5 years, with attacks lasting 3–6 days and recurring every 3–8 weeks. Patients are asymptomatic between attacks and show normal growth and development [3]. PFAPA syndrome responds well to corticosteroid therapy, and usually resolves in adolescence [4].

PFAPA syndrome is diagnosed based on the exclusion of other diseases, such as infections, immunodeficiencies, malignant diseases and autoimmune diseases. In addition, diagnosis of PFAPA syndrome must exclude cyclic neutropenia and hereditary periodic fever syndromes (HPFs), which include familial Mediterranean fever (FMF), TNF receptor-associated periodic syndrome (TRAPS), mevalonate kinase deficiency (MKD), cryopyrin-associated periodic syndromes

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(CAPS) and others. HPFs are characteristically monogenic disorders of the innate immune system [5]. PFAPA syndrome is understood to be a non-hereditary disease of unidentified etiology, although infection, abnormal host immune responses, or combinations of both are related to its pathogenesis [6, 7]. Both HPFs and PFAPA syndrome sometimes overlap clinically [8–10]. During the attack phase of HPFs and PFAPA syndrome, C-reactive protein (CRP), serum amyloid A (SAA), erythrocyte sedimentation rate (ESR) and white blood cell (WBC) counts are elevated, indicating acute inflammatory reactions [8]. These indicators return to normal levels between episodes in PFAPA syndrome patients and possibly in HPF patients. Since these laboratory findings are non-specific, they cannot discriminate between PFAPA syndrome and HPFs. Therefore, it is important to differentiate PFAPA syndrome from other HPFs.

Here, we studied the clinical characteristics and serum inflammatory cytokine levels and performed genetic analysis of Japanese children with PFAPA syndrome during attack-free and attack phases, since few studies have characterized Japanese PFAPA syndrome patients [11]. Our findings suggest that the dysfunction of cytokine regulation as a result of genetic variants of auto inflammatory disorder-associated genes may contribute to the inflammatory phenotype of PFAPA syndrome in Japanese patients.

Methods

Patients

Nine PFAPA syndrome patients ranging in age from 1 to 10 years were selected to participate in this study. A total of 13 patients with PFAPA syndrome visited our hospital between April 2009 and March 2013. Of these, we only selected sporadic cases of PFAPA for further analysis. We excluded four patients from this study: three patients had a family history of periodic fevers in childhood and thus HPFs could not be excluded, and the fourth was because of different ethnicity (non-Japanese). The diagnosis of PFAPA syndrome was according to accepted criteria as follows: (1) consistent recurring fevers (>38.5 °C) from an early age (<5 years); (2) symptoms in the absence of upper respiratory tract infection with at least one of the following clinical signs: aphthous stomatitis, pharyngitis or cervical lymphadenitis; (3) exclusion of cyclic neutropenia; (4) completely asymptomatic intervals between episodes; and (5) suitable height and weight as their age in Japanese children with no signs of delayed development [3]. The clinical manifestations associated with periodic fevers; pharyngitis, aphthous stomatitis, cervical adenitis, thoracic pain, abdominal pain, diarrhea, skin rash, arthralgia and headache were determined (Table I). The diagnostic score was calculated according to criteria reported previously suggested by the Gaslini Institute [12]. A score of 1.32 or higher determined a case with PFAPA-like febrile attacks to be at high risk of carrying relevant mutations in genes associated with periodic fevers. An attack-free phase was defined as PFAPA patients with no symptoms of PFAPA attack, such as fever, aphthous stomatitis, pharyngitis or cervical lymphadenitis, and with a normal range of WBC and CRP in blood. Blood was collected from febrile patients within 96 h from fever onset. Patients had not received oral prednisolone treatment when blood samples were obtained during the attack phase. WBC and CRP were measured during the attack phase and attack-free phase. IgD levels were measured during the attack phase. Drug treatments for patients were chosen from a single dose of oral prednisolone 1.0 mg/kg, cimetidine at a dose of 30 mg/kg/day, colchicine at a dose of 0.5 mg/day and/or, montelukast sodium at a dose of 5 mg/day.

This study was performed according to the Helsinki Declaration. All subjects provided informed consent to participate in the study.

Specific PFAPA Syndrome Case

Case 6 was a 13-year-old girl with no history of growth or developmental problems. Since 20 months of age, she had recurrent fever episodes with an interval of four to eight weeks. The fever was accompanied by pharyngitis and cervical adenitis. Tonsillectomy was performed as she was diagnosed with habitual tonsillitis. However, recurrent fever episodes continued. Although she received a second tonsillectomy at the age of 9, recurrent fever episodes continued at intervals of 2 to 3 weeks. Because she carried the gene variation of *MEFV* (described later), colchicine treatment was started at the age of 9 years and 7 months. Attacks of PFAPA syndrome continued for 5 months and then spontaneously resolved at the age of ten years. Regrettably, eight months later, she relapsed with recurrent fever at intervals of 2–4 weeks. It should be emphasized that a single dose of oral prednisolone was immediately effective against each attack of periodic fever.

DNA Sequencing

Genomic DNA was extracted from leukocytes using SepaGene (EIDIA, Tokyo, Japan). DNA fragments included the coding regions of Mediterranean fever (*MEFV*), mevalonate kinase (*MVK*), Nod-like receptors family, pyrin domain containing 3 (*NLRP3*) and TNF receptor superfamily, and member 1A (*TNFRSF1A*) genes were amplified by polymerase chain reaction (PCR), and analyzed using big dye terminator bidirectional sequencing (Applied Biosystems, Foster City, CA, USA).

Table I The clinical profiles and genotypes of PFAPA patients

Case	1	2	3	4	5	6	7	8	9
Gender	M	M	M	F	F	F	M	M	M
Age at onset (month)	35	20	43	12	24	20	24	52	11
Duration of attacks (days)	4–5	5–6	3–4	3	3–5	5–6	3	3	5
Duration of symptom free intervals (weeks)	4	4	4	2–4	4	4–8	3	3–12	4–10
Fever	+	+	+	+	+	+	+	+	+
Pharyngitis	+	+	+	−	+	+	+	+	+
Aphthous stomatitis	+	−	+	+	−	−	+	−	−
Cervical adenitis	+	−	+	−	−	+	+	+	−
Thoracic pain	−	−	−	−	−	−	−	−	−
Abdominal pain	−	−	−	−	−	−	−	−	−
Diarrhea	−	−	−	−	−	−	−	−	−
Skin rash	−	−	−	−	−	−	−	−	−
Arthralgia	−	−	−	−	−	−	−	−	−
Headache	−	−	−	−	−	−	−	−	−
Laboratory findings									
Attack-free phase									
WBC (/μl)	5,490	6,480	7,840	4,780	8,700	6,920	5,900	7,300	10,950
CRP (mg/ml)	0.06	<0.02	0.03	0.07	0.20	0.15	0.04	0.02	0.02
Attack phase									
WBC (/μl)	7,770	11,750	10,300	18,690	8,570	10,220	10,390	10,120	13,400
CRP (mg/ml)	6.05	1.96	5.54	2.95	1.47	0.33	3.00	7.64	10.60
IgD (mg/dl)	25.4	<0.6	2.0	23.7	2.2	7.2	5.4	<0.6	<0.6
Treatment									
Corticosteroid	response	response	response	not done	response	response	response	response	response
Cimetidine	partial response	not done	not done	not done	poor response	poor response	not done	not done	not done
Colchicine	not done	not done	not done	not done	not done	poor response	not done	not done	not done
Montelukast sodium	not done	not done	not done	not done	not done	not done	not done	partial response	not done
Tonsillectomy	response	not done	response	not done	response	poor response	response	not done	not done
Present status	remission at the age of seven	spontaneous resolution at the age of four	remission at the age of six	spontaneous resolution at the age of nine	remission at the age of four	active	remission at the age of eight	active	active
Gaslini diagnostic score	−3.849	−1.340	−5.889	−2.308	−1.608	−1.340	−3.112	−3.484	−0.737
MEFV Genotype	N.D.	N.D.	N.D.	I591M	P369S-R408Q	R202Q	N.D.	N.D.	L110P-E148Q

Table I (continued)

Case	1	2	3	4	5	6	7	8	9
MVK Genotype	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NLRP3 Genotype	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	G809S
TNFRSF1A Genotype	N.D.	N.D.	N.D.	c.218-9 T>C	N.D.	N.D.	N.D.	c.625+10A>G	c.625+10A>G

M male; *F* female; *WBC* white blood cells; *CRP* the serum C-reactive protein level; *N.D.* not detected. Normal IgD levels were less than 9.0 mg/dl

Analysis of Serum Cytokine Levels by ELISA

Serum samples of patients and controls were stored at −80 °C until assayed. Tumor necrosis factor (TNF)-α concentrations were measured by Immunoassay Kit (Biosource International, Carlsbad, CA, USA) with a detection limit of 1.7 pg/ml. Similarly, interleukin (IL)-6 and IL-1β concentrations were measured by Immunoassay Kit (Biosource) with detection limits of 1.7 and 1.0 pg/ml, respectively. IL-1ra and sTNFR1 concentrations were measured by ELISA (R&D Systems, Minneapolis, MN, USA) with detection limits of 6.26 and 0.77 pg/ml, respectively. IL-18 was assayed by ELISA (MBL, Nagoya, Japan), with a detection limit of 25.6 pg/ml. When cytokine levels were below the detection limit, cytokines were given the lowest detectable value. Cytokine levels are described as the mean value ± SD throughout the study.

IL-1β Production from Monocytes

We selected control subjects ranging in age between 5 and 15 years with no history of recurrent fevers including PFAPA syndrome, tonsillectomy, serious infections and other serious diseases. In addition, three HPF child patients were recruited for comparison with PFAPA patients. One patient aged 4 years was affected by familial cold auto inflammatory syndrome (FCAS), as described in our previous report [13]. The other two patients were 13 years old and fulfilled the Tel Hashomer criteria [14] for the diagnosis of FMF. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of control donors and from patients by gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). CD14-positive cells were purified from PBMCs using CD14 MACS MicroBeads and MACS magnetic columns according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). The purity of CD14-positive cells was verified by fluorescence activated cell sorting analysis involving PE-conjugated anti-CD14 staining (BD Biosciences, San Jose, CA). CD14-positive cells were cultured in medium consisting of RPMI 1,640 supplemented with 10 % heat-inactivated fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 μg/ml). CD14 positive cells were seeded to a density of 3.0×10^5/ml and cultured with the addition of 1.0 μg/ml LPS-EB Ultrapure (Invivogen, San Diego, CA, USA) and 20 μg/ml IFN-γ (R&D Systems, Minneapolis, MN, USA) for 24 h at 37 °C in a humidified atmosphere of 5 % CO_2 and pulsed with 5 mM ATP (Sigma-Aldrich, St. Louis, MO, USA) for 30 min before harvesting. The cell-culture supernatants were harvested, and stored at −80 °C until assayed. IL-1β levels were measured by ELISA.

Statistical Analyses

The statistical significance of serum cytokine levels between the attack-free phase and attack phase was analyzed using the Wilcoxon signed-rank test. The statistical significance of serum cytokine levels between control and each phase was analyzed using Mann–Whitney *U*-test. *P*-values<0.05 were considered statistically significant. For the production levels of IL-1β, the statistical significance between controls and each case was analyzed using Dunnett's multiple comparison test. *P*-values<0.05 were considered statistically significant.

Results

Patient Characteristics and Genetic Analysis

The clinical profiles and genotypes of these cases are summarized in Table I. The study population consisted of six males and three females. Normal growth was observed in all patients. The mean age of onset was 26.8 months. Febrile episodes lasted 3–6 days with symptom-free intervals ranging from 2 to 12 weeks. Fever attacks were accompanied by pharyngitis in eight cases, aphthous stomatitis in four cases, and cervical adenitis in five cases. None of the patients showed atypical symptoms, such as abdominal pain, arthralgia, skin rash or headache. All patients were asymptomatic between their fever episodes. The Gaslini diagnostic score [12] was calculated to differentiate PFAPA syndrome from monogenic periodic fevers and showed a low risk of carrying mutations of auto inflammatory disorder-related responsible genes in all patients. Mean WBC and CRP values of patients during the attack phase were 11,246±3,046/μl and 4.39± 3.13 mg/ml, respectively. WBC and CRP values during attack-free periods decreased to 7,150±1,760/μl and 0.07± 0.06 mg/ml, respectively. Mean IgD levels were 7.32± 9.51 mg/dl during the attack phase. IgD levels in Cases 1 and 4 were mildly elevated to 25.4 and 23.7 mg/dl, respectively. Oral prednisolone was administered to eight patients and immediately reduced fever in all patients. Case 4 was not treated with any medication including oral prednisolone because she had already resolved spontaneously prior to the diagnosis of PFAPA syndrome. Tonsillectomy was performed in five patients, inducing remission of fever episodes in four patients. However, Case 6 had relapsing episodes of recurrent fever after tonsillectomy. Genetic analysis identified heterozygous R202Q (c. 605G > A) in exon 2, P369S (c. 1105C > T)-R408Q (c. 1223G > A) in exon 3, I591M (c. 1,773 T > G) in exon 9 and L110P (c. 329 T > C)-E148Q (c.442G > C) in exon 2 of *MEFV* in Cases 4, 5, 6, and 9, respectively. Case 4 carried an intronic polymorphism of c.218-9 T > C and Cases 8 and 9 carried intronic polymorphisms of c.625+10A > G of *TNFRSF1A*. Moreover, in Case 9, heterozygous G809S (c.2425 G > A) in *NLRP3* was identified. No gene substitutions of *MVK* were detected for all cases.

Cytokine Profiles of Patients

Serum IL-1β, IL-6 and TNF-α levels were not detected in the sera of healthy control subjects. The mean concentration ± SD of serum IL-18 and IL-1ra in healthy control subjects was 169.2±85.7 pg/ml and 213.4±87.1 pg/ml, respectively [13]. The mean serum sTNFR1 level in healthy control subjects was 1,009.0±276.4 pg/ml [15]. Fig. 1 shows the serum cytokine profiles from patients during the attack-free phase and attack phase. Serum IL-1β, IL-1ra, IL-6 and sTNFR1 levels were not elevated during the attack-free phase. However, during attacks, serum IL-1β, IL-1ra, IL-6, IL-18 and sTNFR1 levels were increased. IL-1ra showed a marked increase during the attack phase compared with controls and during the attack-free phase. Interestingly, TNF-α and IL-18 were increased during the attack-free phase.

Figure 2a shows the production levels of IL-1β from monocytes treated with lipopolysaccharide (LPS), IFN-γ and/or ATP stimulation in Cases 8 and 9 and in Case 6 after tonsillectomy. IL-1β was 12.7±16.9 pg/ml in the monocytes of healthy control subjects (n=5) without stimulation. The mean concentration of IL-1β from monocytes of healthy control subjects stimulated with 20 ng/ml IFN-γ or 1 μg/ml LPS was 14.2±15.3 pg/ml and 120.0±70.6 pg/ml, respectively. The mean concentration of IL-1β from monocytes of healthy control subjects stimulated with 1 μg/ml LPS and 5 mM ATP was 181±183 pg/ml. The mean concentration of IL-1β from monocytes of healthy control subjects stimulated with both 20 ng/ml IFN-γ and 1 μg/ml LPS was 215± 118 pg/ml. The mean concentration of IL-1β from monocytes of healthy control subjects stimulated with 20 ng/ml IFN-γ, 1 μg/ml LPS and 5 mM ATP was 367±188 pg/ml. IL-1β secretion from monocytes of Case 6 was significantly increased compared with healthy controls when the monocytes were co-stimulated with LPS and IFN-γ with added ATP. In Case 9, IL-1β secretion was significantly increased compared with healthy controls when the monocytes were co-stimulated with LPS and IFN-γ or LPS and IFN-γ with added ATP. Additionally, patients with HPFs showed increased IL-1β secretion compared with PFAPA patients (Fig. 2b). It should be noted that the purity of CD14-positive cells was 95.50±2.71 %.

Discussion

HPFs that cause recurrent fevers demonstrate several clinical overlapping phenotypes with PFAPA syndrome [8]. In particular, the clinical phenotype of MKD, with findings such as fever duration, aphthous stomatitis and cervical adenitis, is

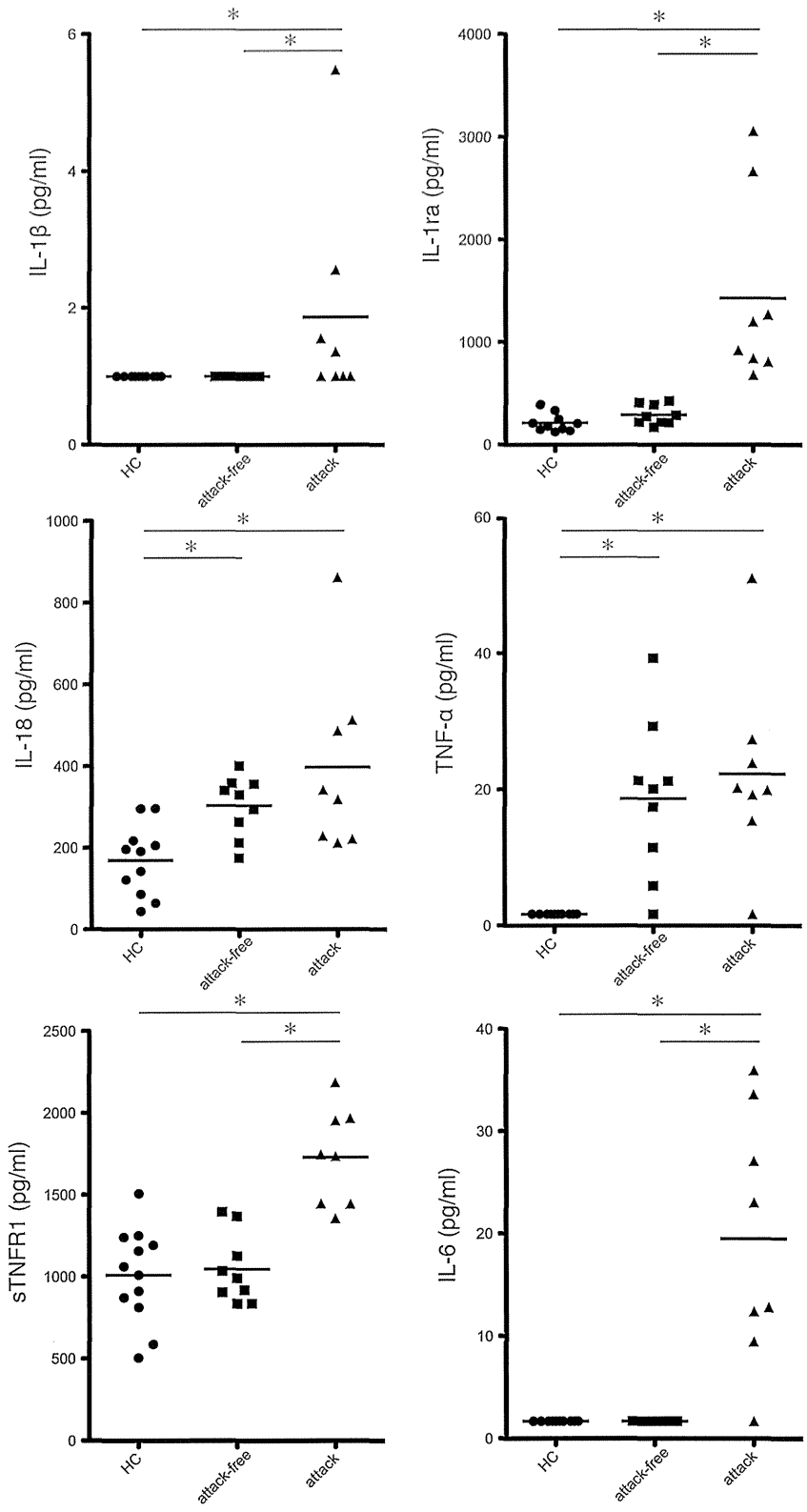

Fig. 1 Inflammatory cytokine levels from eight patients of PFAPA syndrome during the attack-free phase and attack phase. *$P<0.05$

quite similar to PFAPA syndrome [16]. The diagnosis of PFAPA syndrome is made in accordance with clinical criteria after the exclusion of other diseases including HPFs. However, the attack phase of PFAPA patients, including patients in the current study, showed increased WBC and CRP similar to that observed in HPFs. In contrast, these markers returned to their normal range during the attack-free phase. Moreover, IgD serum levels (normal value<9.0 mg/dl) were mildly

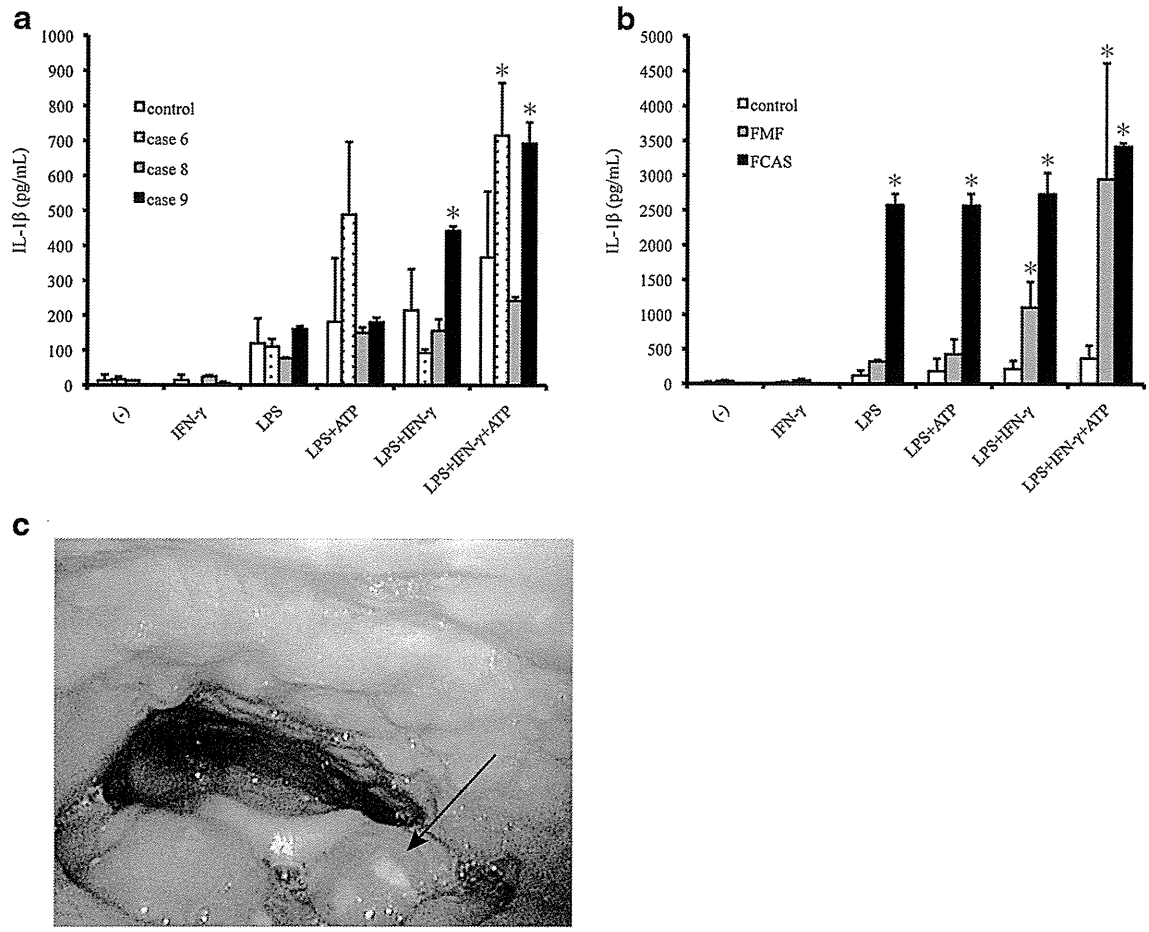

Fig. 2 **a** IL-1β levels from monocytes in Cases 6, 8 and 9. *White* bars = controls, *dotted* bars = Case 6, *grey* bars = Case 8, and *black* bars = Case 9. *$P<0.05$. **b** IL-1β levels from monocytes in HPF patients. *White* bars = controls, *grey* bars = FMF patients, and *black* bars = FCAS patients. *$P<0.05$. **c** Clinical presentation of pharyngitis in Case 6 after tonsillectomy. The *white* exudates are present in the remaining tonsillar tissue after tonsillectomy (*arrow*)

elevated in two patients (22 %) in this study, similar to MKD patients with elevated serum IgD levels [1]. Previous studies reported elevated serum IgD levels in PFAPA patients [4, 17], although it is known that such elevation is not unique to MKD and can be seen in other conditions such as lymphoma and tuberculosis. As slightly elevated IgD levels may not be helpful as a diagnostic test for MKD or PFAPA syndrome, there are currently no specific laboratory tests that can be used to perform a diagnosis of PFAPA syndrome. In our patients, mutations of *MVK* gene were not detected, even in patients with infancy-onset (Cases 4 and 9). It should be noted that a patient with suspected PFAPA syndrome with periodic fever, especially at the beginning of infancy, should be tested for mevalonic acid excretion in the urine, activity of the MVK enzyme or undergo *MVK* gene analysis to differentiate it from MKD.

In general, most cases of PFAPA syndrome are sporadic, as observed in the present study. However, PFAPA syndrome patients with a family history have been reported [11, 18–20]. These reports suggested a possible role of the genetic background in PFAPA syndrome. However, to date no genome-wide association study of PFAPA has been performed to help classify the genetic etiology of each case. In this study, we found that five of nine patients with PFAPA syndrome had heterozygous variants in the *MEFV* and/or *NLRP3* genes, or intronic polymorphisms of the *TNFRSF1A* gene, which are associated with FMF, CAPS and TRAPS, respectively. The simple scoring system suggested by Gattorno indicated that patients showing early onset, family history, thoracic pain and abdominal pain, well known symptoms of FMF and TRAPS, and diarrhea, a symptom of MKD, have a higher risk of HPFs [12]. Interestingly, although all enrolled PFAPA patients in this study had negative scores, the above-mentioned gene variants were identified in approximately 56 % of PFAPA patients. Previous reports described the involvement of *MEFV* in PFAPA syndrome [21] and demonstrated that 27 % of cases diagnosed as PFAPA syndrome exhibited *MEFV* gene variants [22]. A recent study indicated the involvement of the *MEFV* gene in FMF and some clinical overlap with PFAPA syndrome [23]. Several reports also demonstrated that PFAPA

syndrome could be associated with *TNFRSF1A* gene variants [8, 22, 24]. In particular, children with the R92Q variant displayed a higher degree of similarity with the phenotype of PFAPA syndrome than with TRAPS patients carrying structural mutations [25]. In this study, two intronic polymorphisms of *TNFRSF1A*, c.218-9 T > C and c.625+10A > G were identified. To date, there have been no reports of PFAPA syndrome with these *TNFRSF1A* polymorphisms. The allele frequency of c.625+10A > G polymorphisms in *TNFRSF1A* is 20 % in Japanese, according to the international HapMap Project (http://hapmap.org/). This intronic polymorphism was identified in Cases 8 and 9, which demonstrated a frequency similar to that of the Japanese population. This therefore increases the insignificance of these variants. There were no obvious differences in symptoms accompanied by fever and cytokine profiles between these two cases and others, except that their symptom-free intervals were longer than those of other cases. However, the allele frequency of c.218–9 T > C polymorphisms in *TNFRSF1A* has not been reported in Japanese and the functional consequence of this variant is unknown. Although it is unclear whether these *MEFV* and *TNFRSF1A* polymorphisms are related to the PFAPA phenotype, they may contribute to the presentation of PFAPA.

Interestingly, in the current study, only one patient carried *NLRP3* variants. The patient was heterozygous for the *NLRP3* G809S and *MEFV* L110P-E148Q variants. We previously reported that the G809S variant is functional and that synergistic effects with *NLRP3* G809S and *MEFV* haplotype variants could modify the phenotype of atypical auto inflammatory syndrome [15]. The phenotype of this patient, Case 9, may also be associated with these two gene variants. In contrast, a recent report demonstrated that 12 of 57 patients with PFAPA syndrome had heterozygous variants of *NLRP3* including V198M, R488K and Q703K [24]. Furthermore, their PFAPA syndrome patients were sometimes accompanied by arthralgia or cutaneous rash such as CAPS [26], although these complications were not observed in our patients. These differences suggest that allele frequencies vary widely in humans. For instance, the allele frequency of Q703K variant in *NLRP3* is 1 % in Japanese and 5.8 % in Europeans, according to the international HapMap Project. The *NLRP3* nonsynonymous variant, except G809S, is rare in Japanese but common in European subjects. As *MEFV* variants were identified in our patients, this might be a notable feature of Japanese PFAPA syndrome patients. Consequently, the high frequency of auto inflammatory disease-associated variants in patients with PFAPA syndrome suggests that the dysregulation of inflammasome activation may be involved in the pathogenesis of PFAPA syndrome.

Inflammatory cytokines are typically associated with inflammatory responses and play important pathogenic roles in monogenic HPFs [27]. In particular, dysfunctional IL-1β processing is often involved in HPF pathogenesis [28–30]. In an attempt to reveal the pathophysiology of PFAPA syndrome, we examined the concentration of serum cytokines in children with PFAPA syndrome during the attack and attack-free phases. Patients with PFAPA syndrome had a marked increase in pro-inflammatory cytokines, especially IL-1ra, during the attack phases, which may indicate a role for IL-1β in the pathogenesis of PFAPA syndrome and activation of the inflammasome as seen in CAPS [13]. IL-1β from monocytes of two active patients with PFAPA syndrome was increased compared with control subjects in this study. Recent studies demonstrated that environmental factors such as LPS could trigger the activation of complement, IL-1β and IL-18 during PFAPA syndrome flares, with the induction of Th1 chemokines and subsequent reduction of Th2 anti-inflammatory responses [31, 32]. In addition, Stojanov et al. reported that a recombinant IL-1R antagonist (anakinra) was useful for the treatment of PFAPA attacks. This indicates that IL-1β plays a central role of the pathophysiology of PFAPA syndrome. PFAPA syndrome resembles other HPFs regarding the importance of IL-1β in its pathogenesis. However, the expression of genes encoding complement factors, IL1-related molecules, and IFN-induced molecules was previously shown to be overexpressed during the attack phase of PFAPA syndrome compared with other HPFs [31]. This study reported that IFN-γ-induced chemokines, G-CSF, and other proinflammatory cytokines such as IL-1β, IL-6, and IL-18 were elevated in the serum at fever onset. Our study found that production levels of IL-1β from stimulated monocytes were up-regulated in two PFAPA syndrome patients, in accord with the study by Kolly et al. [24]. Interestingly, they had missense variants in the genes responsible for HPFs. Future work involving a larger sample size could help identify differences in cytokine profiles between patients with different genetic variants. However, it was noticeable that concentrations of TNF-α and IL-18 were even higher during the symptom-free period. This may be caused by lasting inflammation during attack-free periods. In accordance with our findings, Stojanov et al. described significantly elevated serum concentrations of TNF-α compared with controls during symptom-free periods [7]. Interestingly, serum IL-18 levels were elevated during both flare and non-flare states in FMF patients as well as in our PFAPA patients [33]. Since inflammasome activation leads to the direct release of IL-1β, and the counter regulators IL-1ra, and IL-18 [34], we suggest that dysregulation caused by the gene variants of auto inflammatory disease-associated genes in patients with PFAPA syndrome may have already induced potential activation of the inflammasome during the attack-free period, and subsequently one more "hit" because of environmental factors could induce fever episodes.

Tonsillectomy is the most effective surgical intervention for the long-term resolution of PFAPA syndrome symptoms [35]. The effectiveness of tonsillectomy for PFAPA syndrome suggests the presentation of microbial agents in tonsillar tissue,

which could trigger auto inflammatory reactions [36]. A systemic review of tonsillectomy for PFAPA syndrome showed the rate of complete resolution emerging from the combined analysis of all treated children was 83 % (95 % confidence interval, 77–89 %) [37]. This supports a view of PFAPA syndrome in which environmental exposure and possible immunologic variants cause recurrent febrile episodes [6, 7]. In our study, tonsillectomy was successful in four of five PFAPA patients who received tonsillectomy is done. Several previous studies reported tonsillectomy was successful in all PFAPA patients [38, 39]. However, in Case 6 of the current study, relapsing episodes of recurrent fever were observed after tonsillectomy, suggesting the patient's tonsillar tissue was unable to be completely removed by surgery (Fig. 2c). In addition, monocytes from Case 6 after tonsillectomy secreted high levels of IL-1β associated with inflammasome activation, as recently described [24]. Additionally, a recent case of PFAPA syndrome relapse in an adult after tonsillectomy during childhood was described [40]. There is a consensus on the efficacy of tonsillectomy for PFAPA syndrome, but its role may be limited in some cases. Therefore, patients with PFAPA syndrome should be carefully observed after tonsillectomy.

Conclusions

PFAPA patients showed a non-specific pattern of inflammatory reactants including serum IgD and inflammatory cytokines compared with HPFs. Furthermore, variants of responsible genes of HPFs were often identified in PFAPA patients. Therefore, we cannot discriminate between PFAPA and HPFs using only these parameters. This study also suggests that PFAPA syndrome could be caused by a dysfunction of cytokine regulation linked to genetic variants of auto inflammatory disease-associated genes in Japanese patients. Our findings may help to clarify the diagnosis and pathophysiology of PFAPA syndrome.

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Conflict of Interest The authors declare no conflicts of interest.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Compound Heterozygous Deletions in Pseudoautosomal Region 1 in an Infant With Mild Manifestations of Langer Mesomelic Dysplasia

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Haploinsufficiency of *SHOX* on the short arm pseudoautosomal region (PAR1) leads to Leri–Weill dyschondrosteosis (LWD), and nullizygosity of *SHOX* results in Langer mesomelic dysplasia (LMD). Molecular defects of LWD/LMD include various microdeletions in PAR1 that involve exons and/or the putative upstream or downstream enhancer regions of *SHOX*, as well as several intragenic mutations. Here, we report on a Japanese male infant with mild manifestations of LMD and hitherto unreported microdeletions in PAR1. Clinical analysis revealed mesomelic short stature with various radiological findings indicative of LMD. Molecular analyses identified compound heterozygous deletions, that is, a maternally inherited ~46 kb deletion involving the upstream region and exons 1–5 of *SHOX*, and a paternally inherited ~500 kb deletion started from a position ~300 kb downstream from *SHOX*. In silico analysis revealed that the downstream deletion did not affect the known putative enhancer regions of *SHOX*, although it encompassed several non-coding elements which were well conserved among various species with *SHOX* orthologs. These results provide the possibility of the presence of a novel enhancer for *SHOX* in the genomic region ~300 to ~800 kb downstream of the start codon.

Key words: *SHOX*; langer mesomelic dysplasia; deletion; enhancer

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Am J Med Genet Part A 9999:1–6.

INTRODUCTION

SHOX on the short arm pseudoautosomal region (PAR1) is a transcription factor gene exclusively expressed in the developing limbs and pharyngeal arches [Rao et al., 1997; Clement-Jones et al., 2000]. Haploinsufficiency of *SHOX* leads to idiopathic short stature and Leri–Weill dyschondrosteosis (LWD; OMIM #127300), and nullizygosity of *SHOX* results in Langer mesomelic dysplasia (LMD; OMIM #249700) [Rao et al., 1997; Belin et al., 1998; Shears et al., 1998; Zinn et al., 2002]. Previous studies in patients with LWD and LMD have identified several copy-number abnormalities in PAR1 that involved coding exons and/or the putative upstream or downstream enhancer regions, together with multiple point

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mutations in the coding region [Rao et al., 1997; Belin et al., 1998; Shears et al., 1998; Benito-Sanz et al., 2005, 2011, 2012b; Fukami et al., 2005, 2006; Bertorelli et al., 2007; Sabherwal et al., 2007; Chen et al., 2009; Durand et al., 2010]. The putative enhancers of *SHOX* have been mapped to a ~300 kb region ~95 kb upstream, and to a ~30 kb region ~250 kb downstream from the start codon [Benito-Sanz et al., 2005, 2012a,b; Fukami et al., 2006]. The putative downstream enhancer region contains several conserved non-coding elements (CNEs) that exert enhancer activity in the developing chicken limb bud [Sabherwal et al., 2007] and in human osteosarcoma cells [Fukami et al., 2006; Benito-Sanz et al., 2012b]. Since molecular abnormalities have not been detected in a substantial portion of patients with LWD/LMD [Zinn et al., 2002; Fukami et al., 2008; Rosilio et al., 2012], it appears that unknown genetic or environmental factors are also involved in the development of these conditions.

The clinical severity of SHOX abnormalities is highly variable [Binder, 2011]. Relatively severe phenotypes in adult female patients indicate that gonadal estrogens enhance skeletal abnormalities in SHOX abnormalities [Ogata et al., 2001; Binder, 2011]. However, there may be other phenotypic modulators for this condition [Binder et al., 2004]. Here, we report on a male infant with mild LMD phenotype and compound heterozygosity of hitherto unreported microdeletions in PAR1.

CLINICAL REPORT

This Japanese male infant was a dizygotic twin conceived by in vitro fertilization. At 24 weeks gestation, an ultrasound examination delineated short extremities in one fetus and a normal skeletal appearance in the other. At 27 weeks gestation, caesarean section was performed because of bradycardia in both fetuses. At birth, the patient presented with a mesomelic appearance with a body length of 33.5 cm (−1.1 SD), weight of 1,130 g (+0.4 SD), and head circumference of 26.5 cm (+1.0 SD). His twin brother was normally proportioned with body length 35.5 cm (−0.1 SD), weight 854 g (−1.5 SD), and head circumference 25.0 cm (±0 SD). Bone survey of the patient at 2 months of age showed markedly curved radii, hypoplastic ulnas and fibulas, and metaphyseal splaying (Fig. 1A). The patient and his brother received standard medical interventions for prematurity, and were discharged from hospital at 3 months of age.

On his latest visit at 21 months of age, the infant manifested obvious mesomelic short stature with a height of 69.3 cm (−3.9 SD), weight of 8.0 kg (−2.7 SD), and head circumference of 46.6 cm (−1.0 SD; Fig. 1B). His developmental milestones were almost normal. His brother had a proportionate short stature with a height of 74.1 cm (−2.6 SD), weight of 8.5 kg (−2.3 SD), and head circumference of 46.2 cm (−1.3 SD). The mother showed limited movement of the wrist and mesomelic short stature (140 cm, −3.6 SD), while the father was clinically normal and had a normal height (165 cm, −1.0 SD). Radiological examinations were not performed for the parents or brother.

MOLECULAR ANALYSES

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development.

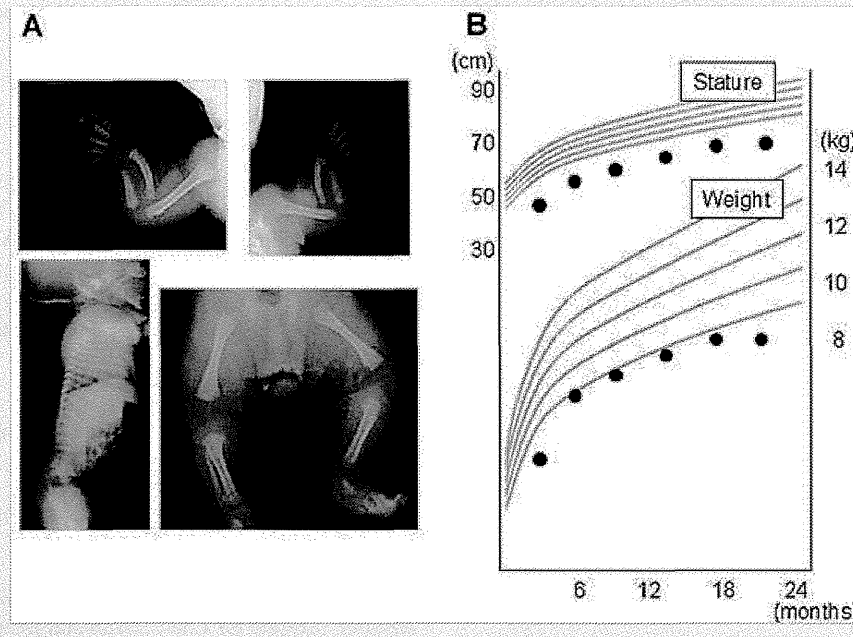

FIG. 1. Clinical findings of the infant. A: X-Ray at 10 weeks of age. Markedly curved radii, hypoplastic ulnas and fibulas, and metaphyseal splaying are shown. B: Growth pattern. Height and weight of the infant are plotted on the longitudinal height and weight standards for Japanese boys (the mean, ±1.0 SD and ±2.0 SD, respectively).

After taking written informed consent from the parents, leukocyte genomic DNA samples were obtained from the infant and parents. First, we performed direct sequencing analysis for *SHOX* by a previously described method [Shears et al., 1998]. No nucleotide alterations in the coding exons were identified in the infant. Next, we performed multiplex ligation-dependent probe amplification (MLPA) using a SALSA MLPA Kit (P018 SHOX-F1, MRC-Holland, Amsterdam, the Netherlands). Two heterozygous deletions in PAR1 were identified in the infant; a deletion involving exons 1–5 of *SHOX*, and a deletion affecting the downstream region of *SHOX* that corresponds to four MLPA probes from 05649-L20176 to 13911-L19678 (Fig. 2). Then, we examined the extent of the deletions by comparative genomic hybridization (CGH) using a custom-built oligo-microarray harboring 26,274 probes for PAR1 and several reference probes for other chromosomal regions (4 × 180 K format, Agilent Technologies, Palo Alto, CA; Fig. 3A). The telomeric deletion included a ~46 kb genomic interval (ChrX: 556,720–603,222; hg 19, Build 37) affecting *SHOX* exons 1–5 and a ~28 kb region at the 5′ side of exon 1. The centromeric deletion encompassed a ~500 kb interval (ChrX: 881,006–1,387,599) and started at a point ~300 kb from the start codon of *SHOX*. In silico analysis using UCSC Genome Browser (http://genome.ucsc.edu/) and rVista 2.0 (http://rvista.dcode.org/) revealed that the deletions did not affect the putative upstream or downstream enhancer regions of *SHOX*, and that the downstream deletion encompassed a gene for cytokine receptor-like factor 2 (*CRLF2*) and several CNEs (Fig. 4). Most of these CNEs were well conserved in fugu, frog, chicken, dog and monkey which preserve orthologs of *SHOX* (*Shox*), as well as in opossum which is likely to preserve *Shox*, and were absent in mouse which lack *Shox* (Ensemble Genome Browser, http://ensembl.org/; Fig. 4). Next, MLPA and CGH were performed on the parental samples. The ~46 and ~500 kb deletions were detected in the mother and father, respectively (Figs. 2 and 3A). Thus, the infant was diagnosed as being compound heterozygous for a maternally inherited ~46 kb deletion on the X chromosome and a paternally inherited ~500 kb deletion on the Y chromosome. The results of MLPA and CGH were confirmed by fluorescence in situ hybridization (FISH). FISH probes for *SHOX* exons 3–5 (probe A) and for a region ~320 kb downstream of *SHOX* (probe B) were generated by PCR using primers 5′-CAGCTCTTCCTCAAATTCTTTCC-3′ and 5′-GTGTCTGTCCCATCTCTGGTATC-3′, and 5′-ATAGTG-CATGGGTATCAGAGGTC-3′ and 5′-GGAAAAAGAGTGGGT-

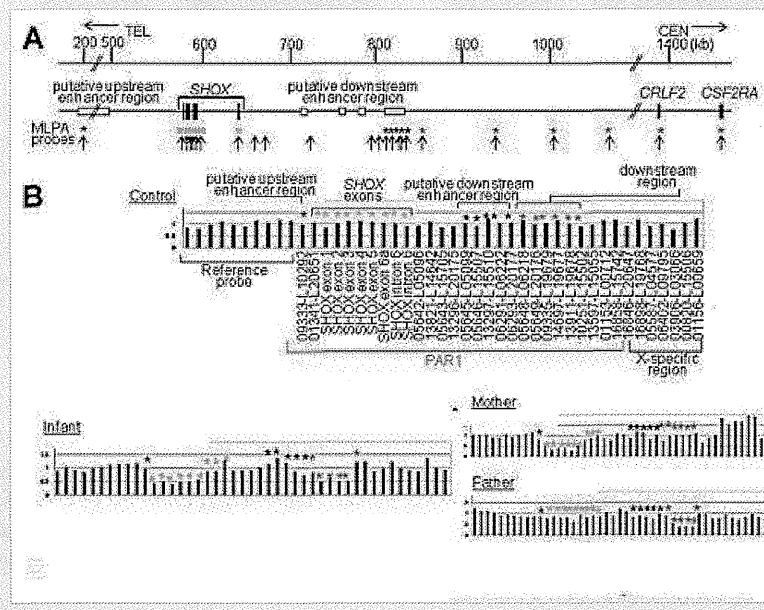

FIG. 2. Multiplex ligation-dependent probe amplification (MLPA) analysis. A: Schematic representation of the short arm pseudoautosomal region (PAR1). The upper horizontal line indicates the physical distance from the Xp/Yp telomere. Genomic positions refer to the Human Genome (hg 19; NCBI Build 37). The black boxes indicate exons of *SHOX*, *CRLF2*, and *CSF2RA* (not all exons are shown). The white box denotes the putative upstream and downstream enhancer regions (elements) of *SHOX* identified in previous studies [Sabherwal et al., 2007; Benito-Sanz et al., 2012a,b]. The gray box indicates putative downstream enhancer elements that exert enhancer activities in the developing chicken limb [Sabherwal et al., 2007]. Vertical arrows indicate genomic positions of MLPA probes; green asterisks indicate probes for *SHOX* exons, blue and black asterisks indicate probes for the putative upstream and downstream enhancer regions respectively, and red asterisks depict probes for the genomic region between the putative downstream enhancer region and *CSF2RA*. TEL, telomere; CEN, centromere. B: Representative results of MLPA. The asterisks indicate the same probes shown in Fig. 2A. The sample data were normalized to a male sample. Decreased peak heights suggest heterozygous deletions. The infant has two deletions; one involving the upstream region and exons 1–5 of *SHOX*, and the other in the downstream region. The mother and father are heterozygous for the deletions.

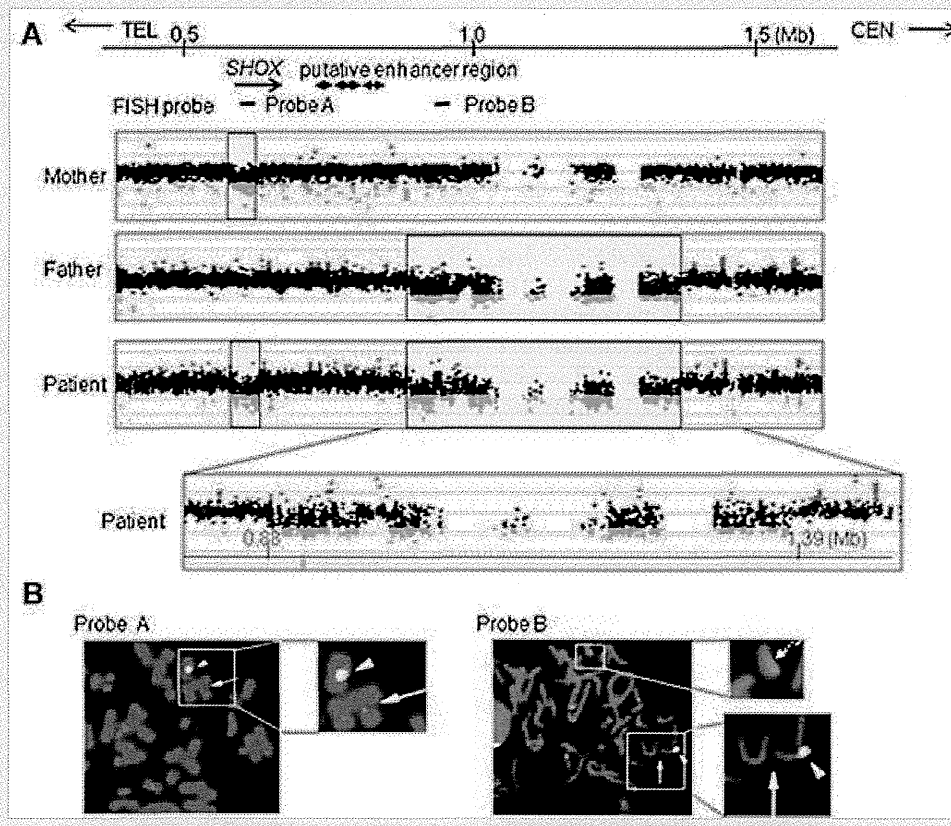

FIG. 3. Comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) analyses. A: CGH analysis in the infant and his parents. The upper horizontal line indicates physical distance from the Xp/Yp telomere. The genomic position of *SHOX*, the putative downstream enhancer elements, and FISH probes are shown. The black, red, and green dots denote signals indicative of the normal, increased (>+0.5), and decreased (<−0.8) copy numbers, respectively. The blue-shaded boxes indicate deleted regions. B: FISH analyses in the infant. Red signals (arrows) indicate probe A for *SHOX* exons 1–3 and probe B for the *SHOX* downstream region, and green signals (arrow heads) depict *DXZ1* control probes on the X chromosome. Probe A detects a signal on the Y chromosome but not on the X chromosome. Probe B detects a signal on the X chromosome but not on the Y chromosome (a dotted arrow).

CAGAACTT-3′, respectively. In the infant, probe A detected only one signal on the Y chromosome and probe B detected only one signal on the X chromosome (Fig. 3B).

We attempted to obtain a DNA sample from the twin brother, because he was predicted to carry the same Y chromosomal deletion as the infant. However, the sample was not available for genetic analysis.

DISCUSSION

We identified compound heterozygous deletions in PAR1 in a male infant. These deletions have not been described previously. The deletion on the X chromosome included most of the coding exons of *SHOX*, and therefore appears to result in complete loss-of-function of the *SHOX* allele. Consistent with this, the mother heterozygous for the same deletion manifested typical clinical features of LWD. In contrast, the deletion on the Y chromosome did not affect exons or the known putative enhancer regions of *SHOX*. Furthermore, this deletion included no genes except *CRLF2*, which has not been implicated in skeletal development [Siracusa et al., 2011]. Clinical examinations of the infant revealed mesomelic short stature and obvious skeletal changes that are more consistent with LMD than with LWD [Fukami et al., 2005; Binder, 2011; Ambrosetti et al., 2013].

Two possible explanations can be made for these results. First, the Y chromosomal deletion in the infant may encompass a hitherto unidentified *cis*-regulatory element of *SHOX*. Recent studies have indicated that several genes such as *SOX9* and *LHX3* have multiple *cis*-acting modules widely distributed around the coding exons [Gordon et al., 2009; Mullen et al., 2012]. Thus, it is possible that coordinated action of multiple regulatory elements is required for adequate *SHOX* expression in the developing limb bud, and that one of such elements is located within the ~500 kb region >300 kb apart from the coding region. Indeed, the Y chromosomal deletion in the infant harbored several CNEs that are well conserved among various species with *SHOX* orthologs.

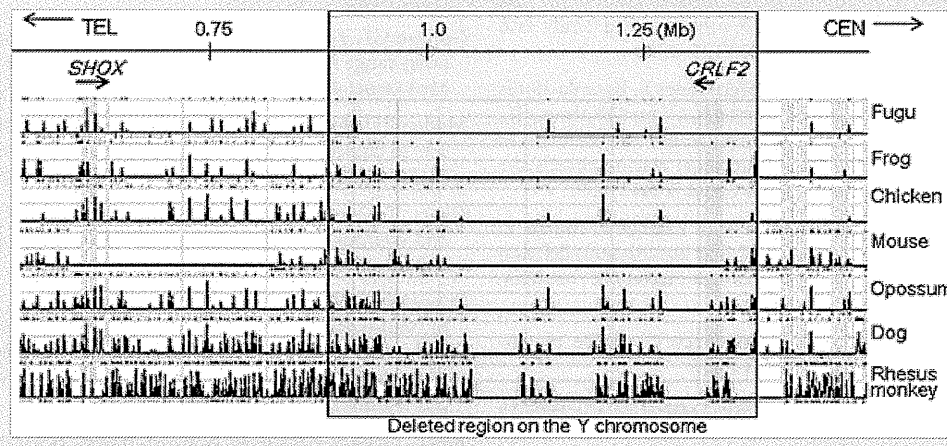

FIG. 4. Conservation analysis in the short arm pseudoautosomal region of the sex chromosomes (PAR1). The deleted region on the Y chromosome (shaded in gray) contains several non-coding elements that are well conserved in species which preserve *SHOX* orthologs (fugu, frog, chicken, dog, and monkey, and possibly opossum as well) and absent in mouse which lacks an *SHOX* ortholog.

One of the CNEs in the deletion may be a distal enhancer of *SHOX/Shox*, because CNEs in the human genome frequently exert enhancer activity [Pennacchio et al., 2006]. In this regard, it is noteworthy that the skeletal features of this infant are milder than those of previously reported patients with *SHOX* nullizygosity [Zinn et al., 2002; Fukami et al., 2005; Ambrosetti et al., 2013]; the infant showed no short stature or rhizomelia at birth. Furthermore, the father with the same Y chromosomal deletion manifested no skeletal abnormalities, and the twin brother with possible Y chromosomal deletion showed only mild proportionate short stature. These results can be explained by assuming that deletions involving only the putative enhancer regions exert a relatively mild effect on skeletal growth compared to mutations/deletions affecting the coding exons. Consistent with this, relatively mild LWD phenotypes have been observed in patients with heterozygous downstream deletions [Rosilio et al., 2012], and mild LMD phenotype has been described in a patient with compound heterozygous deletions for *SHOX* exons and the putative downstream enhancer region [Fukami et al., 2005]. Alternatively, *cis*-regulatory deletions may be associated with broad phenotypic variation compared to exonic deletions/mutations, because Chen et al. [2009] have described profound phenotypes in patients with enhancer deletions.

Second, the phenotype of the infant may be explained as an extremely severe manifestation of LWD. If this is the case, the Y chromosomal deletion would be a functionally neutral copy-number variation. The absence of skeletal changes in the father with the Y chromosomal deletion supports this notion. In this regard, previous studies have indicated that phenotypic severities of *SHOX* haploinsufficiency are variable and likely to be affected by multiple factors [Binder et al., 2004; Binder, 2011]. Thus, some genetic or environmental factors may have enhanced the abnormal skeletal formation of this infant with a maternally derived *SHOX* intragenic deletion.

In summary, we identified hitherto unreported deletions in PAR1 in a Japanese infant with a mild LMD phenotype. Further studies will clarify the presence or absence of a novel downstream enhancer of *SHOX* in the genomic region ~300 to ~800 kb from the start codon.

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RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Prenatal Genetic Testing for a Microdeletion at Chromosome 14q32.2 Imprinted Region Leading to UPD(14)pat-like Phenotype

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TO THE EDITOR:

Human chromosome 14q32.2 imprinted region carries several paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* and maternally expressed genes (*MEGs*) such as *MEG3* (alias, *GTL2*) and *RTL1as* (*RTL1 antisense*), together with the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR (Fig. 1) [da Rocha et al., 2008; Kagami et al., 2008a]. Consistent with this, paternal uniparental disomy 14 (UPD(14) results in a unique phenotype characterized by facial abnormality, small bell-shaped thorax, abdominal wall defects, placentomegaly, and polyhydramnios [Kagami et al., 2005, 2008a,b]. In this regard, we have recently reported that heterozygous microdeletions and epimutations (hypermethylations) affecting unmethylated DMR (s) of maternal origin also lead to UPD(14)pat-like phenotype [Kagami et al., 2008a, 2010, 2012]. Indeed, after studying 26 patients with UPD(14)pat-like phenotype, we identified UPD (14)pat in 17 patients (65.4%), microdeletions in 5 patients (19.2%), and epimutations in 4 patients (15.4%) [Kagami et al., 2012]. Importantly, although there is no report describing recurrence of UPD(14)pat and epimutation in familial members with a normal karyotype, microdeletions can be transmitted recurrently from mothers with the same heterozygous microdeletions to offsprings [Kagami et al., 2008a]. Here, we report on our experience of a prenatal genetic testing for a microdeletion at the chromosome 14q32.2 imprinted region.

A 33-year-old Japanese woman came to us with her husband seeking for prenatal diagnosis of a fetus at 9 weeks of gestation. The first child and the mother have been reported previously as cases 3 and 11 of Family B in Kagami et al. [2008a]. In brief, the child had upd(14)pat-like phenotype and a maternally derived 411,354 bp microdeletion involving *WDR25*, *BEGAIN*, *DLK1*, *MEG3*, *RTL1/RTL1as*, and *MEG8* (Fig. 1). The mother had UPD(14)mat-like phenotype and the same microdeletion on the paternally derived chromosome 14. The parents hoped to deliver the fetus at a local hospital if there is no microdeletion or at our hospital with a neonatal intensive care unit if a microdeletion is identified.

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After thorough consultation, we performed trans-abdominal chorionic villus sampling (CVS) at 12 weeks of gestation. Immediately after the sampling, fluorescence in situ hybridization was carried out with an RP11-566J3 probe detecting a segment within

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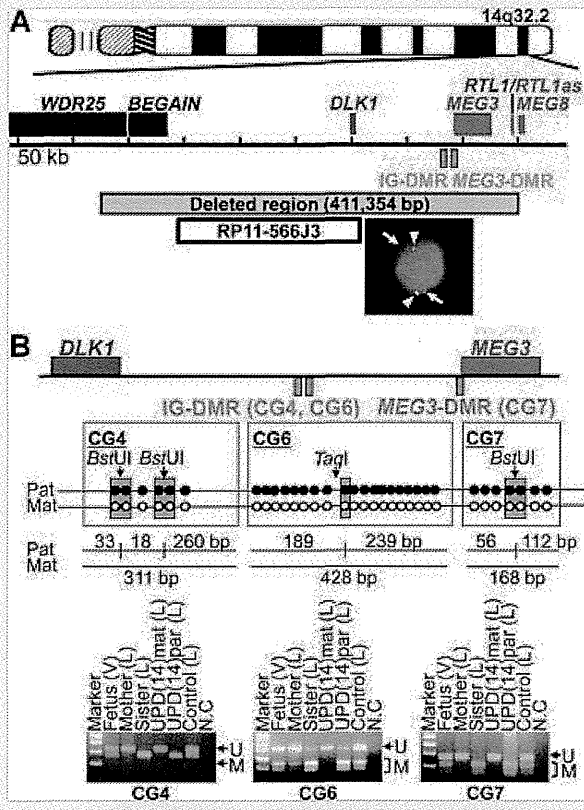

FIG. 1. Summary of the molecular studies. A: The physical map of the 14q32.2 imprinted region and the FISH finding of the fetus. *PEGs* are shown in blue, *MEGs* in red, and the IG-DMR and the *MEG3*-DMR in green. The gray rectangle indicates the 411,354 bp microdeletion identified in the first child and the mother, and the white rectangle denotes the region detected by the RP11-566J3 BAC probe. The FISH analysis reveals two red signals (arrows) identified by the RP11-566J3 BAC probe and two green signals (arrowheads) detected by the RP11-566I2 BAC probe for 14q12 utilized as an internal control. B: The methylation analysis for the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) by COBRA. The black and white circles indicate methylated and unmethylated cytosines at the CpG dinucleotides, respectively. Pat: paternally derived chromosome; and Mat: maternally derived chromosome. PCR products for CG4 (311 bp) are digested with *Bst*UI into three fragments (33, 18, and 260 bp) when cytosines at the first and the second CpG dinucleotides and the fourth and fifth CpG dinucleotides (indicated with orange rectangles) are methylated. The PCR products for CG6 (428 bp) are digested with *Taq*I into two fragments (189 and 239 bp) when the cytosine at the 9th CpG dinucleotide (indicated with an orange rectangle) is methylated. The PCR products of CG7 (168 bp) are digested with *Bst*UI into two fragments (56 and 112 bp) when the cytosines at the fourth and fifth CpG dinucleotides (indicated with orange rectangle) are methylated. Both methylated (M)- and unmethylated (U)-specific bands are identified in the chorionic villus sample. V, villi; L, leukocytes; and N.C, negative control.

the deleted region of the first child and the mother, delineating two signals on villus cell interphase spreads (Fig. 1). Next combined bisulfite restriction analysis (COBRA) was performed for the IG-DMR and the *MEG3*-DMR using villus cell genomic DNA, identifying both methylated- and unmethylated allele-specific bands (Fig. 1B). These findings clearly excluded the presence of a microdeletion in the fetus by 14 weeks of gestation. Subsequent pregnant course was uneventful, and a phenotypically normal infant was delivered at term by a caesarean section.

To our knowledge, this is the first report describing a prenatal genetic testing for a familial microdeletion affecting the chromosome 14q32.2 imprinted region. Although such a genetic testing is possible only when an accurate genetic diagnosis has been made for the proband, it permitted the precise diagnosis before the second to the third trimester when characteristic UPD(14)pat-like features such as bell-shaped small thorax with coat hanger appearance and polyhydroamnios become detectable by ultrasonographic studies [Suzumori et al., 2010; Yamanaka et al., 2010]. Such an early prenatal diagnosis, though it is associated with a certain risk such as CVS-induced abortion, provides critical information for the clinical management. When a microdeletion is excluded as shown in this case, this releases the parents from the anxiety of having an affected fetus and allows for a standard follow-up during pregnancy. By contrast, when a microdeletion is identified, this will allow for appropriate management during pregnancy (e.g., amnioreduction to mitigate the risk of threatened premature delivery) and pertinent therapeutic interventions for the infant (e.g., respiratory management). Thus, prenatal genetic diagnosis appears to be beneficial for the fetus and the parents, when it is performed at appropriate institutes where a multidisciplinary team including a genetic counselor is available.

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