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ORIGINAL ARTICLE

Characteristics of FDG-PET findings in the diagnosis of systemic juvenile idiopathic arthritis

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Abstract

Objective: To examine and delineate inflammatory focus in patients with juvenile idiopathic arthritis (JIA), ^{18}F-Fluoro-deoxy-glucose (FDG)-positron emission tomography (PET) (^{18}F-FDG-PET) was applied to patients with JIA, and the images of these patients were compared.

Methods: Sixty-eight children (59 with systemic JIA (s-JIA) and 9 with polyarticular JIA) were included. The diagnosis of JIA was done to meet the International League of Associations for Rheumatology (ILAR) criteria. After 6-h fasting, whole-body positron emission tomography (PET) scans were acquired 60 min after intravenous injection of 3–5 MBq/kg ^{18}F-FDG. The interpretation of ^{18}F-FDG uptake was based on visual characteristics.

Results: Two types of PET images were outstanding in s-JIA; one was ^{18}F-FDG uptake in red bone marrow, such as the spine, pelvis, and long bones as well as spleen (12 cases), and other type was the uptake in the major joints, such as hips, elbows, wrists, knees, and ankles (8 cases). The former findings were correlated with elevated levels of inflammatory markers, while the latter were with significantly increased levels of MMP-3 ($p < 0.05$).

Conclusion: There was a noticeable accumulation of ^{18}F-FDG uptake in bone marrow of s-JIA patients which may indicate the inflammatory focus of this disease and play an important role in the pathogenic basis of arthritis and systemic inflammation of s-JIA.

Keywords:

Diagnosis, F-Fluoro-deoxy-glucose-positron emission tomography, Systemic juvenile idiopathic arthritis

History

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Introduction

Systemic juvenile idiopathic arthritis (s-JIA) is a systemic chronic inflammatory disease, the main symptoms of which are remittent fever, rheumatoid rash, and arthritis [1]. During the clinical course, about 7% of patients suffer from macrophage activation syndrome (MAS), which can be life-threatening. Some patients develop MAS as the first symptom of the disease [2,3]. In s-JIA, the whole range of symptoms is rarely observed at the onset, with remitting fever being an early symptom in most cases, while arthritis tends to appear later. Therefore, early diagnosis is required but no appropriate diagnostic marker has been established to date. However, it has been determined that heme oxygenase-1 (HO-1) and interleukin-18 (IL-18) are markedly increased at the active phase of s-JIA, unlike in polyarticular and oligoarticular juvenile idiopathic arthritis, and these two factors have been reported to be useful serological diagnostic markers [4,5]. In addition, matrix metalloproteinase 3 (MMP-3), which is produced by inflamed synovial cells and fibroblasts, is a useful activity marker for cartilage destruction. It has become possible to diagnose the disease and evaluate its activity relatively easily. Together with a marked elevation in inflammatory markers, such as C-reactive protein (CRP) and blood sedimentation rate, the diagnosis of s-JIA can become more accurate.

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Although s-JIA is currently classified as one subtype of JIA, there is a school of thought considering the disease as an autoinflammatory syndrome [6]. Affected joints are markedly different from polyarticular and oligoarticular JIA, and the process of cartilage and bone destruction is also different, suggesting that s-JIA is a different disease. Marked osteoporosis and poor development of epiphyseal nuclei are observed in the systemic type, whereas narrowing of the joint space is characteristic of the articular type [7,8], suggesting different mechanisms responsible for disease onset of systemic versus articular type.

In the present study, the primary inflammatory lesion was investigated by ^{18}F-FDG-PET (FDG-PET) in >400 cases with remittent fever. The FDG-PET findings were compared in 59 cases of s-JIA diagnosed based on serological evaluation and clinical course, so that characteristic FDG-PET findings for the disease could be determined. In cases that do not receive a firm diagnosis, these characteristic FDG-PET findings may be used as potent diagnostic tools.

Patients and methods

A total of 68 cases with JIA examined by FDG-PET between January 2002 and December 2011 at our hospital were retrospectively investigated. There were 59 cases of s-JIA (31 boys and 28 girls; average age, 9.1 ± 3.9 years) and nine polyarticular JIA (p-JIA) (5 boys and 4 girls; average age, 11.6 ± 5.2 years). Joints at the bilateral shoulders, elbows, hands, hips, knees, and ankles were evaluated. Articular symptoms of tenderness and swelling of each joint were recorded. White blood cell count (WBC), CRP,

serum amyloid A (SAA), erythrocyte sedimentation rate (ESR), ferritin, and FDP-E were assessed as inflammatory markers, while MMP-3 was used as a marker of articular destruction; plasma G-CSF, IL-6, and IL-18 were measured at the same time.

For FDG-PET, ^{18}F-FDG was intravenously infused after 6 h of fasting, and images were scanned 1 h later. SUVmax $\geq$0.5 was considered positive for defining ^{18}F-FDG accumulation. Next, clinical symptoms and laboratory data at the time of FDG-PET and accumulation patterns of ^{18}F-FDG were compared among the 12 joints listed above. In addition, cases with characteristic ^{18}F-FDG accumulation findings were extracted and their images were examined.

This study was carried out as part of advanced medical research of the hospital (Registration No. 158-1) and all patients participated in the study only after they gave informed consent.

Results

^{18}F-FDG accumulation in children without inflammation

After exploration of a remittent fever, a case without inflammation is presented here, as an example (Figure 1). In children without inflammation, ^{18}F-FDG accumulates in the brain, heart, bladder, and joints during growth. This patient is an 8-year-old girl with low-grade fever of unknown origin and pain in the extremities. Her complaints were severe and her parents wanted to have her examined as extensively as possible. We undertook FDG-PET after obtaining informed consent from her and her parents. It was confirmed that she had no inflammation and she was diagnosed as suffering from fibromyalgia.

Characteristics of ^{18}F-FDG accumulation in cases with p-JIA

In 11 cases with p-JIA, ^{18}F-FDG accumulation showed a diffuse distribution pattern in inflamed joints. It was often observed that ^{18}F-FDG accumulated in almost all large joints as in (Figure 2a) and (Figure 2b) or in those joints with severe inflammation as in (Figure 2c). There was no accumulation in the bone marrow and no significant difference in accumulation in the liver or spleen.

Relationship between arthritis and ^{18}F-FDG accumulation in cases with p-JIA

Relationships between SUVmax and other laboratory data such as CRP, ESR, WBC, and MMP-3 were examined, but no significant correlations were found (Figure 2).

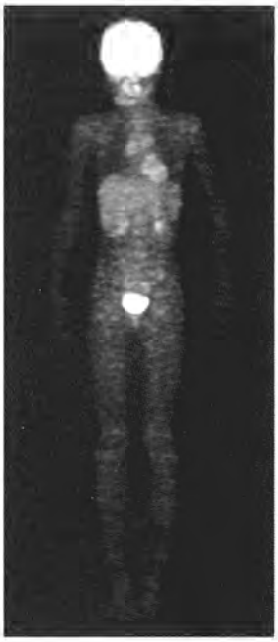

Figure 1. FDG-PET findings in a healthy child. Despite no abnormal inflammation, accumulation in the brain, heart, bladder, and joints at the growth stage is observed.

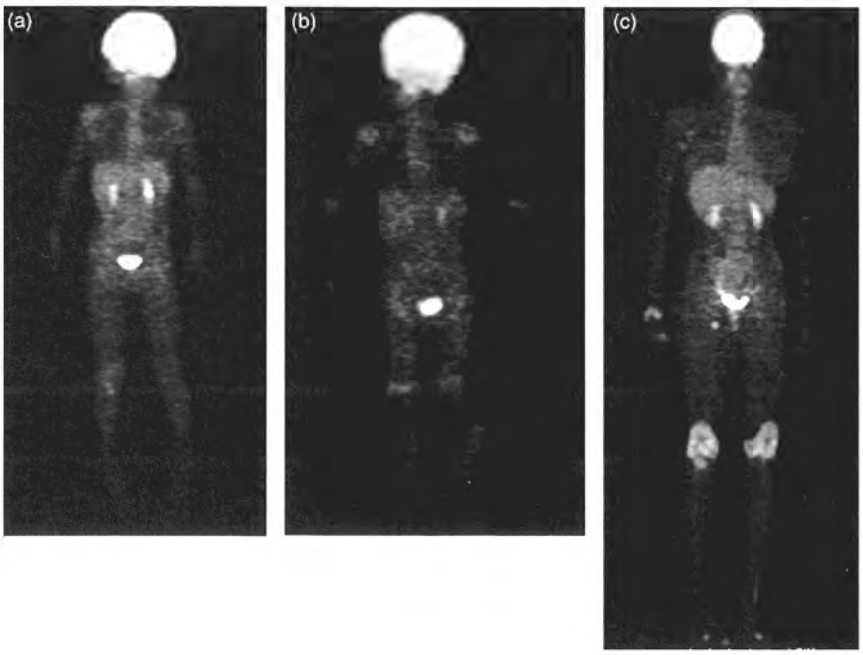

Figure 2. FDG accumulation in cases with p-JIA. (a) Accumulation is observed at the bilateral shoulders, elbows, wrists, and in the knees. In particular, marked accumulation was observed at the bilateral shoulders, the left elbow, and in the right knee. (b) Accumulation is observed at the bilateral shoulders, in the elbows, wrists, hips, knees, and ankles. (c) Marked accumulation is observed in the bilateral knees and the right wrist.

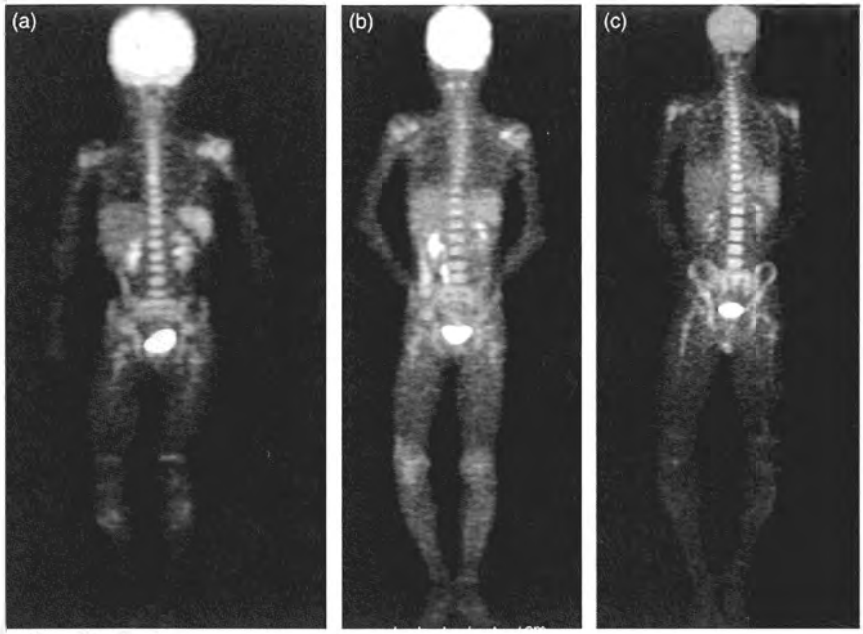

Figure 3. FDG accumulation in s-JIA (type I). (a) Accumulation is observed at the bilateral shoulders, in the vertebrae, pelvis, spleen, and bilateral knees. Accumulation is centered on the head of the humerus at the shoulder and the proximal tibial bone at the knee. (b) Accumulation is observed at the bilateral shoulders, in the vertebrae, pelvis, spleen, and bilateral knees. (c) Marked accumulation at the bilateral shoulders, in the vertebrae, pelvis, and spleen. Accumulation is also observed in the knee. At the shoulder and in the knee, accumulation is observed not in the joint but at the epiphysis (bone marrow tissue). In the pelvis, accumulation is marked at the ala of the ilium that contained red bone marrow.

Characteristics of ^{18}F-FDG accumulation in cases with s-JIA

There were two characteristic patterns in ^{18}F-FDG accumulation in the 59 cases with s-JIA. They were designated type I and type II as follows:

(1) Characteristic accumulation was found in all vertebral bodies and pelvis and around large joints, such as shoulders and knees. The accumulation was not in the joint synovia but the bone itself or at the end of the long bones. It was considered that accumulation was not in the joints but in the bone marrow. In addition, compared with the liver, greater accumulation in the spleen was characteristic (Figure 3) (type I, 12 cases).

(2) As in cases with p-JIA, diffuse accumulation in inflamed joints was recognized. There was no accumulation in the bone marrow and no significant difference between the liver and spleen (Figure 4) (type II, 8 cases).

Relationship between arthritis and ^{18}F-FDG accumulation in cases with s-JIA

Relationships between SUVmax and other laboratory data such as CRP, ESR, WBC, and MMP-3 were examined, but no significant correlations were found.

Comparison of different accumulation patterns in cases with s-JIA

Age, gender, duration from onset, treatment intervention, transition to MAS, reduction in the steroid dose 1 year later, cases treated with biological agents, and number of refractory cases were compared between type I and type II. With regard to laboratory data, WBC, CRP, SAA, ESR, ferritin, FDP-E, MMP-3, IL-6, IL-18, and G-CSF were compared (Tables 1 and 2, and Figure 5).

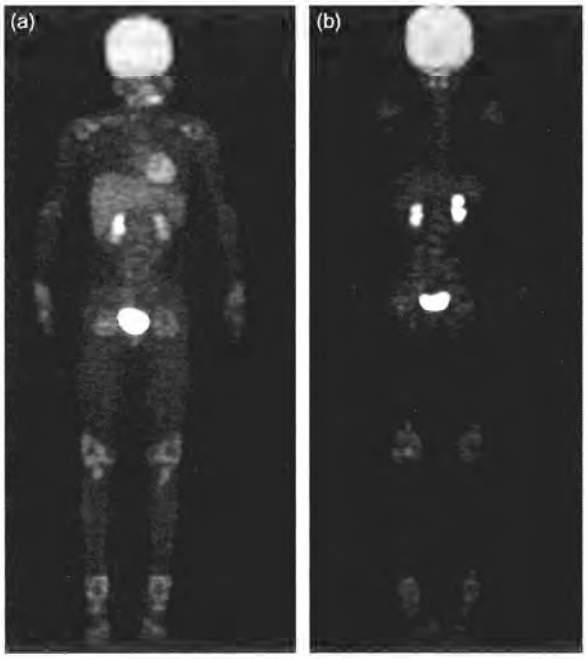

Figure 4. FDG accumulation in s-JIA (type II). (a) Accumulation is observed at the bilateral shoulders, in the elbows, hands, hips, knees, and ankles. In particular, it is marked in the hands, knees, and ankles. (b) Accumulation is observed at the bilateral shoulders, in the hands, knees, and ankles. It is marked in the knees and ankles.

Type I accumulation in FDG-PET was observed in boys, in particular, immediately after onset and before treatment; the risk of developing MAS seemed high. There was no significant difference in treatment resistance.

The test results (Figure 5) showed that inflammation markers such as WBC, CRP, SAA, ESR, ferritin, IL-6, IL-18, and G-CSF

were significantly higher in type I, whereas the synovitis marker MMP-3 was significantly higher in type II.

Discussion

FDG-PET results for the diagnosis of remittent fever and reference findings of JIA were collected for retrospective comparison over a 10-year period at our department. Of these, 68 cases with JIA were analyzed. In cases with p-JIA, ^{18}F-FDG accumulated in the joints with synovitis, as is seen in adult rheumatoid arthritis. These findings were almost identical to the physical, ultrasonographic, and magnetic resonance imaging (MRI) findings for arthritis. Although it was reported that SUVmax correlates with the severity of arthritis, there was no such significant correlation in the present study. The degree of accumulation was influenced by the interval from radioisotope injection to scanning, a meal and exercise taken before the test, and age and body build of the subjects, and there was a large interindividual variation in measurements.

Table 1. Comparison of type-I and type-II s-JIA (at scanning).

Background of s-JIA subtypes	s-JIA type I	SJIA type II
Number of cases	12	8
Age (years)	8.8±2.8	7.9±4.1
(range)	(3~14)	(3~18)
Boy:girl ratio	8:4	3:5
Duration from	1.3±1.7	23.4±43
onset (months)	(0~10)	(0~66)
No treatment	7 cases	1 case
when PET taken	(58%)	(13%)

Table 2. Comparison of type-I and type-II s-JIA (all clinical courses).

Course of s-JIA subtypes	s-JIA type I	s-JIA type II
Number of cases	12	8
Macrophage activation syndrome	3 cases (25%)	0 cases
Reduction rate of PSL dosage (after 1 year)	−63.1%	−54.9%
Treatment with biologics	11 cases (91.7%)	6 cases (85.7%)
Refractory cases	3 cases (1 articular 2 systemic course)	5 cases (all cases had articular courses)

Characteristic images of ^{18}F-FDG accumulation in the red bone marrow tissue of the whole body were obtained in 12 cases, as shown in Figure 3. Among cases diagnosed with s-JIA, findings similar to those of p-JIA were sometimes obtained. Cases with accumulation in the bone marrow tissue had a shorter period from onset, had received less intensive treatment, and showed an increase in serological markers for systemic inflammatory status (WBC, CRP, SAA, ESR, ferritin, FDP-E, IL-6, IL-18, and G-CSF). Diagnosis of s-JIA at an early stage after onset is a critical issue for treatment selection; these findings will be useful for diagnosis in this regard.

For reference, in this study, FDG-PET images from 23 juvenile systemic lupus erythematosus patients, 20 juvenile dermatomyositis, 10 mixed connective tissue disease, 8 systemic sclerosis, and 10 Kawasaki disease patients were examined and no characteristic findings in these diseases were observed.

It has been demonstrated that imaging modalities are useful for the diagnosis and evaluation of arthritis in JIA, and recent advances in joint ultrasonography, in particular, for p-JIA, have been remarkable [9]. It is highly significant that this modality enables real-time evaluation of inflammation based on the presence of synovitis, retention of synovial fluid, stratification of synovial membrane, and increased blood flow by power Doppler imaging. Additionally, joint ultrasonography is useful for the evaluation of arthritis in cases with s-JIA, but differential diagnosis is very difficult when the patient presents with a remittent fever with unclear arthritis. However, an elevation in HO-1 [4] and IL-18 [5] is disease-specific and these can be useful serological markers for diagnosis. Therefore, it is expected that they will be widely used as serological markers.

FDG-PET showed characteristic findings in this study. In type I s-JIA, ^{18}F-FDG accumulation was observed in the bone marrow, in particular the red bone marrow, reflecting systemic inflammation; accumulation was also more marked in the spleen than in the liver. These findings are otherwise seen only in some diseases

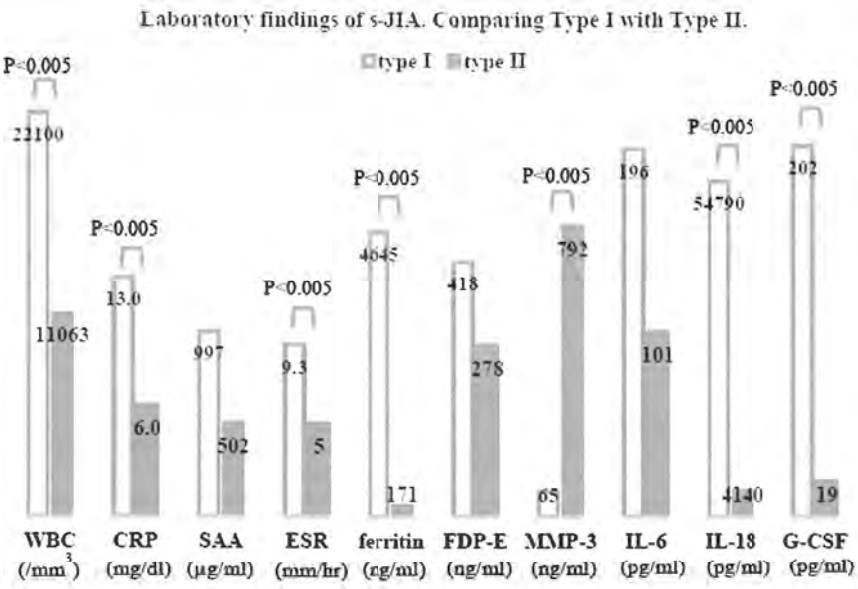

Figure 5. Laboratory findings of s-JIA: comparing type I with type II. Inflammation markers, such as WBC, CRP, SAA, ESR, ferritin, IL-6, IL-18, and G-CSF, were significantly higher in type I, whereas the synovitis marker MMP-3 was significantly higher in type II.

such as sepsis and are thus considered relatively specific for the diagnosis of s-JIA in combination with serological tests. FDG also accumulates in the bone marrow of patients with leukemia. Nevertheless, FDG-PET in s-JIA is different in that there is relatively homogeneous accumulation in the bone marrow, whereas in leukemia it has a speckled distribution.

It should be noted that adult-onset Still's disease, a disease similar to s-JIA, also exhibits characteristic accumulation of ^{18}F-FDG in the bone marrow, spleen, and lymph nodes, and FDG-PET is considered an effective modality for its diagnosis at the stage of exploration of remittent fever in adults [10]. In addition, FDG accumulation was observed in inflamed joints as a characteristic finding in type-II s-JIA, suggesting synovitis. Furthermore, there was no significant difference in the amount of FDG accumulation in the liver relative to the spleen, and there was no accumulation in the bone marrow. In type-II s-JIA, some cases manifest arthritis as the main symptom after a long clinical course, while others have a tendency to improve after anti-inflammatory treatment such as with steroids. The clinical course of type-I and type-II s-JIA is different, as reflected in differences at the sites of ^{18}F-FDG accumulation. In p-JIA, ^{18}F-FDG accumulated only at the joints with inflammation and there was no significant difference in the accumulation in the bone marrow and spleen. These findings are consistent with the FDG-PET findings observed in adult rheumatoid arthritis [11] and are interpreted as ^{18}F-FDG accumulation at the joint synovial membrane and synovial fluid in the joint capsule.s-JIA rarely develops a remittent fever, rash, and arthritis at the same time during disease progression. It starts with a remittent fever and rash, following which arthritis develops, and in the long-term eventually causes problems in daily life activities due to polyarthritis [2]. It is an inflammatory disease in which systemic inflammation precedes the appearance of arthritis that eventually becomes the main symptom.

Exploration by FDG-PET in this study showed two patterns of type-I and type-II s-JIA ^{18}F-FDG accumulation, with type-I revealing a pattern at the early stage after onset based on clinical findings and laboratory data, and showing inflammation localized to the bone marrow and spleen. In contrast, type II is an advanced inflammatory disease and progression to arthritis is expressed similarly to p-JIA and rheumatoid arthritis.

According to the national survey on exploration of "remittent fever," s-JIA is the most frequent outcome with a definite diagnosis [12]. In general, it takes a long time before a definite diagnosis can be made. In addition, 6.8 to 13% of cases develop MAS and their prognosis is often poor [13]. Therefore, early diagnosis is desirable for s-JIA to initiate appropriate mitigating treatment. Our results that FDG-PET showed a characteristic accumulation pattern for early systemic inflammation indicate that this imaging modality is useful for early diagnosis of s-JIA.

Accumulation of ^{18}F-FDG in the bone marrow suggests that these cells are proliferating, and differentiating [14]. G-CSF and GM-CSF administered to counter the adverse effects of chemotherapy is associated with accumulation of ^{18}F-FDG in the bone marrow and spleen [15]. In that study, plasma G-CSF levels were markedly increased at the time of FDG-PET in cases with s-JIA [15], most of whom also showed an increase in peripheral blood granulocytes. Therefore, it was suggested that excessive G-CSF was involved in the accumulation of ^{18}F-FDG in systemic inflammation at the early stage of s-JIA, and thus not only IL-6 but also G-CSF was potentially involved in disease pathogenesis [16].

Because HO-1 and IL-18 are increased in the serum [4,5] and amyloidosis is an important factor influencing the development of joint destruction, marked osteoporosis [6], and the prognosis of s-JIA, it is clear that this disease exhibits different characteristics relative to p-JIA. The systemic type has the characteristics of an "autoinflammatory syndrome" as a systemic inflammatory disease lacking associations with external factors, and its tentative assignment into this disease category is currently under consideration [17,18].

Characteristics of FDG-PET findings were elucidated in the present study. After infectious disease and malignancy were ruled out based on a blood culture test and bone marrow testing, the findings of FDG accumulation in the bone marrow (red bone marrow) and spleen are consistent with the proposal that s-JIA should be classified as an "autoinflammatory disease". Investigations of larger numbers of similar cases to support the utility of FDG-PEG for disease diagnosis is now required.

Conflict of interest

Masaaki Mori has received lecture fees from MSD, Sumitomo Dainippon Pharma, and Phizer Japan Inc, and has served as a consultant adviser to Bristol-Myers Squbb and Astellas Pharm. Shumpei Yokota hold a patent for tocilizumab and receives royalties for Actemra. All other authors have declared no conflicts of interest.

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RESEARCH ARTICLE

PADI4 and the HLA-DRB1 shared epitope in juvenile idiopathic arthritis

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Abstract

Objective

Both genetic and environmental factors are associated with susceptibility to juvenile idiopathic arthritis (JIA). Many studies have reported that both a 'shared epitope' (SE) encoded by several HLA-DRB1 alleles and the peptidyl arginine deiminase type 4 (PADI4) gene polymorphisms are associated with susceptibility to rheumatoid arthritis (RA). However, it is uncertain whether JIA and RA share the latter genetic risk factor. Therefore, here we investigated relationships between HLA-SE and PADI4 polymorphisms with clinical subtypes of JIA.

Methods

JIA patients (39 oligoarthritis, 48 RF-positive polyarthritis, 19 RF-negative polyarthritis and 82 systemic) and 188 healthy controls were genotyped for HLA-DRB1 by PCR-sequence-specific oligonucleotide probe methodology. Three PADI4 gene single nucleotide polymorphisms (SNPs), rs2240340, rs2240337 and rs1748033, were genotyped using TaqMan SNP Genotyping Assays.

Results

Frequencies of the HLA-SE were higher in RF-positive polyarticular JIA than in healthy controls. RF-positive polyarticular JIA was associated with HLA-SE (OR = 5.3, 95% CI = 2.5–11.9, pc < 0.001). No associations were found between clinical subtypes of JIA and PADI4 allele frequency. Nonetheless, rs2240337 in the PADI4 gene was significantly associated with anti-cyclic citrullinated peptide antibody (ACPA)-positivity in JIA. The A allele at rs2240337 was a significant risk factor for ACPA positivity in JIA (OR = 5.6, 95% CI = 1.71–23.7 pc = 0.03).

Ltd., Mitsubishi Tanabe Pharma, AbbVie LLC, UCB Japan Co. Ltd., Astellas Pharma Inc., and Eisai Co., Ltd. Dr. Mori has also received lecture fees from MSD K.K and AbbVie LLC, and consulting fees from Daiichi Sankyo Co., Ltd. and Taisho Pharmaceutical Co., Ltd. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Conclusion

PADI4 gene polymorphism is associated with ACPA-positivity in JIA. The association of HLA-SE with RF-positive polyarticular JIA as well as RA is confirmed in Japanese. Thus, HLA-SE and PADI4 status both influence JIA clinical manifestations.

Introduction

Juvenile idiopathic arthritis (JIA) is defined as a chronic arthritis developing in children <16 years of age and persisting for ≥6 weeks. According to the International League of Associations for Rheumatology (ILAR) classification criteria for JIA, it has 7 subtypes [1]. The 4 major subtypes are oligoarthritis, rheumatoid factor (RF)-positive polyarthritis, RF-negative polyarthritis and systemic arthritis. The major pathology of oligoarthritis and polyarthritis is articular inflammation and joint destruction. RF-positive polyarthritis is considered to be a counterpart of adult rheumatoid arthritis (RA) [2]. In contrast to the above forms of JIA, the major pathology of systemic JIA is systemic inflammation, which is considered similar to adult Still's disease [3,4].

In RA and JIA, both genetic and environmental factors are associated with disease susceptibility [5]. HLA class II gene polymorphisms are considered the most influential for RA susceptibility [6]. Many studies have reported the association of a 'shared epitope' (SE) encoded by several HLA-DRB1 alleles with RA susceptibility in adults [7]. Similarly, an association between HLA-SE and susceptibility to JIA has been reported in Caucasians [8]. We have previously reported that HLA-DRB1*04:05, a major SE-containing allele, is associated with polyarticular JIA also in the Japanese population [9].

More recently, a number of RA susceptibility genes outside of the HLA region have been identified by genome-wide association studies (GWAS) [10,11]. One of these, peptidyl arginine deiminase type 4 (PADI4) was first reported in Japanese RA patients [12,13], and subsequently confirmed in several Asian groups and subgroups of Europeans [14–17]. PADI4 is one member of PADI gene family. It codes for enzymes responsible for the posttranslational conversion of arginine residues into citrulline. It was indicated that an RA susceptibility haplotype in PADI4 was associated with increased stability of PADI4 mRNA [13]. And it could lead to accumulation of PADI4 protein, with subsequent increases in citrullinated proteins and enhanced production of autoantibodies against these citrullinated peptides [18].

PADI4 mRNA is detected in hematological cells and pathological synovial tissues [19,20]. And it was reported that PADI4 significantly overexpressed in the blood cells of RA patients [21]. Moreover, PADI4 have a nuclear localization signal, which affects the expression control of various genes [22]. PADI4 may have various role in the immune system and associated with development of autoimmune disease.

In each of the JIA subtypes, age of onset, clinical course and serological findings are different, which may be accounted for by different influences of the genetic background. However, it is uncertain whether JIA (particularly the RF-positive polyarthritic form) and RA share any genetic risk factors other than HLA-SE. There are no reports that PADI4 risk alleles are involved in JIA disease susceptibility. In the present study, which includes our previous cohort [9], we investigated relationships between HLA-SE and PADI4 polymorphisms, and clinical subtypes of JIA in the Japanese population.

Materials and methods

Study population

Patients were eligible if they met the ILAR classification criteria for JIA. A total of 188 JIA patients (39 oligoarthritis, 48 RF-positive polyarthritis, 19 RF-negative polyarthritis and 82 systemic), comprising 59 boys and 129 girls, was enrolled in this study and followed at the Yokohama City University Hospital between December 2006 and December 2009. This cohort included the 106 oligo- and poly-articular JIA patients who were described in our previous study [9]. Clinical data including age at onset, gender, RF and anti-cyclic citrullinated peptide antibody (ACPA) status were reviewed.

We conducted this study in accordance with the Declaration of Helsinki and with the approval of the Ethics Committee of the Yokohama City University School of Medicine. Written informed consent was obtained from each patient and/or their guardian. (Approval number: A090528002)

HLA genotyping

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen K.K., Tokyo, Japan). JIA patients and healthy adult controls were genotyped for HLA-DRB1 using PCR sequence-specific oligonucleotide probes (SSOP) by the Luminex method with Genosearch HLA-A, -B and -DRB1 Ver. 2 (Medical & Biological Laboratories Co., Ltd. Nagoya, Japan), as described previously [9]. HLA-DRB1*01:01, *04:01, *04:04, *04:05, *04:10, *10:01, *14:02 and *14:06 were regarded as HLA-SE alleles [23].

PADI4 genotyping

Three single nucleotide polymorphisms (SNPs), rs2240340, rs2240337 and rs1748033 in the PADI4 gene were selected based on previous research [12,13]. Genotyping for these in 188 JIA patients and 188 healthy adult controls was performed using TaqMan SNP Genotyping Assays (AB assay ID: C__16176717_10 for rs2240340, C___3123009_1 for rs2240337 and C___7541083_1 for rs1748033). These SNPs were analyzed by real-time PCR using the AB7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) under the conditions recommended by the manufacturer. Allele discrimination was accomplished using SDS software version 1.4 (Applied Biosystems).

Statistical analysis

The statistical significance of the differences in the frequencies of HLA-DRB1 alleles or PADI4 gene polymorphisms between JIA subtypes was evaluated by Fishers exact test. A corrected P-value (Pc) was calculated by multiplying the P-value by the number of HLA-DRB1 alleles tested at each locus. For the PADI4 gene polymorphisms, we examined 3 SNPs and used a total of 5 independent tests.

Results

Patients' characteristics

Characteristics of the patients studied are shown in Table 1. Patients comprised 39 children with oligoarthritis, 48 with RF-positive polyarthritis, 19 with RF-negative polyarthritis and 82 with systemic arthritis. The mean age at onset of oligoarthritis was 5.6 years, RF-positive polyarthritis was 8.2 years, RF-negative polyarthritis was 7.1 years and systemic arthritis 5.0 years.

Table 1. Clinical characteristics of JIA patients.

	Oligo articular JIA (n = 39)	RF positive, polyarticular JIA (n = 48)	RF negative, polyarticular JIA (n = 19)	Systemic JIA (n = 82)
Age at JIA onset (years, mean)	5.6	8.2	7.1	5
Gender (female,%)	35 (90%)	40(83%)	10(53%)	44 (53%)
ANA (>1:160,%)	16 (41%)	19(40%)	3(16%)	3/78 (4%)
RF (>14.0 (IU ml-1),%)	9 (23%)	48(100%)	0(0%)	-
Anti-CCP (>4.5(U ml-1),%)	8 (21%)	40(83%)	0(0%)	0/43 (0%)

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HLA-DRB1 and JIA subtypes

188 healthy controls was genotyped for HLA-DRB1 to determine associations of HLA-DRB1 and HLA-SE with JIA subtype susceptibility. According to ILAR classification criteria for JIA, RF-positive oligoarticular JIA is classified as "undifferentiated". Thus, such cases were excluded from the oligoarthritis group in HLA association studies. RF-positive polyarticular JIA was significantly associated with HLA-DRB1*04:05 and HLA-SE (OR = 5.1, 95% CI = 2.5–11, pc < 0.001; OR = 5.3, 95% CI = 2.5–11, Pc < 0.001, respectively) (Table 2). In contrast, frequencies of HLA-DRB1*04:05 and HLA-SE were not higher in the other types of JIA patients.

PADI4 polymorphisms and JIA subtypes

Frequencies of PADI4 gene polymorphisms studied in JIA patients and controls are shown in Table 3. There were no associations between clinical subtypes of JIA and PADI4 gene polymorphisms. Nonetheless, the PADI4 SNPs were significantly associated with ACPA positivity in JIA (Table 4). Because the ACPA status of all systemic JIA patients measured in this study was negative (0/43), systemic JIA was excluded from the data in Table 4. Hence, the A allele at rs2240337 is a significant risk factor for ACPA positivity in oligo- and poly-articular JIA (OR = 5.6, 95% CI = 1.7–24 Pc = 0.03). Finally, there were no associations between HLA-SE and PADI4 gene polymorphisms in oligo- and poly-articular JIA (Table 5).

Table 2. Association of HLA-DRB1 *04:05 and HLA-SE with susceptibility to JIA subtypes.

HLA-DRB1*0405	Genotype (*0405/any)	OR	95% CI	P-value	Pc
control (n = 188)	40 (21.3%)	-	-	-	-
Oligoarticular JIA (n = 30)	1(3.3%)	0.1	0.01–0.82	0.02	NS
RF positive, polyarticular JIA (n = 48)	28 (58.3%)	5.1	2.50–10.7	<0.001	<0.001
RF negative,polyarticular(n = 19)	4(21.1%)	1	0.30–4.42	0.98	NS
RF negative(oligo+poly)(n = 49)	5(10.2%)	0.4	0.86–8.17	0.078	NS
Systemic JIA (n = 82)	21 (25.6%)	1.3	0.66–2.42	0.43	NS
HLA-SE	Genotype (SE/any)	OR	95% CI	P-value	Pc
control (n = 188)	68 (36.2%)	-	-	-	-
Oligoarticular JIA (n = 30)	6(20.0%)	0.4	0.14–1.18	0.082	NS
RF positive polyarticular JIA (n = 48)	36 (75.0%)	5.3	2.47–11.9	<0.001	<0.001
RF negative,polyarticular(n = 19)	5(26.3%)	0.6	0.17–1.96	0.39	NS
RF negative(oligo+poly)(n = 49)	15(30.6%)	0.8	0.37–1.60	0.47	NS
Systemic JIA (n = 82)	33 (40.2%)	1.8	0.67–2.09	0.59	NS

SE, shared epitope: HLA-DRB1*04:05,01:01,04:01,04:10,10:01,14:02,14:06

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Table 3. Association between PADI4 gene polymorphisms and susceptibility to JIA subtypes.

rs2240340	G allele	A allele	MAF	OR	95% CI	P	Pc
Control (n = 188)	223	153	0.41	-	-	-	-
Oligoarticular JIA (n = 30)	37	23	0.38	0.9	0.49–1.64	0.73	NS
RF positive, polyarticular JIA (n = 48)	49	47	0.49	1.4	0.87–2.25	0.17	NS
RF negative,polyarticular(n = 19)	24	14	0.37	0.9	0.39–1.78	0.64	NS
RF negative,oligo+poly articular(n = 49)	61	37	0.38	0.9	0.54–1.43	0.6	NS
Systemic JIA (n = 82)	92	72	0.44	1.1	0.77–1.68	0.51	NS
rs2240337	G allele	A allele	MAF	OR	95% CI	P	Pc
Control (n = 188)	350	26	0.07	-	-	-	-
Oligoarticular JIA (n = 30)	57	3	0.05	0.7	0.13–2.43	0.45	NS
RF positive, polyarticular JIA (n = 48)	85	11	0.12	1.7	0.75–3.82	0.14	NS
RF negative,polyarticular(n = 19)	37	1	0.03	0.4	0.01–2.36	0.25	NS
RF negative,oligo+poly articular(n = 49)	94	4	0.04	0.6	0.14–1.71	0.21	NS
Systemic JIA (n = 82)	149	15	0.18	1.4	0.65–2.74	0.38	NS
rs1748033	G allele	A allele	MAF	OR	95% CI	P	Pc
Control (n = 188)	239	137	0.36	-	-	-	-
Oligoarticular JIA (n = 30)	42	18	0.30	0,7	0.39–1.39	0.33	NS
RF positive, polyarticular JIA (n = 48)	55	41	0.43	1.3	0.80–2.10	0.29	NS
RF negative,polyarticular(n = 19)	26	12	0.32	0.8	0.36–1.72	0.55	NS
RF negative,oligo+poly articular(n = 49)	68	30	0.31	0.8	0.46–1.27	0.28	NS
Systemic JIA (n = 82)	120	44	0.27	0.6	0.42–0.97	0.03	NS

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Table 4. Association between PADI4 gene polymorphisms and ACPA positivity in oligo- and poly- articular JIA patients (n = 106).

		Anti-CCP(-) (<4.5U ml-1) (n = 58)	Anti-CCP (+) (>4.5U ml-1) (n = 48)	OR	95% CI	P	Pc
rs2240340	allele	75	46	2	1.1–3.6	0.018	NS
	recessive	26	11	2.7	1.1–7.1	0.024	NS
	dominant	49	35	2	0.70–6.0	0.158	NS
rs2240337	allele	112	80	5.6	1.7–24	0.002	0.03
	recessive	54	32	6.6	1.9–30	<0.001	<0.001
	dominant	-	-	-	-	-	-
rs1748033	allele	80	52	1.9	1.0–3.4	0.03	NS
	recessive	30	14	2.6	1.1–6.4	0.029	NS
	dominant	53	38	2.8	0.78–11	0.095	NS

Recessive: GG versus (GA/AA), dominant: (GG/GA) versus AA

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Table 5. Association between PADI4 gene polymorphisms and SE positivity in oligo- and poly- articular JIA (n = 106).

		GG	GA/AA	OR	95% CI	P-value
rs2240340	SE-	20	31	1.4	0.60–3.5	0.42
	SE+	17	38	-	-	-
rs2240337	SE-	42	9	1.2	0.39–3.5	0.81
	SE+	44	11	-	-	-
rs1748033	SE-	23	28	1.3	0.57–3.1	0.56
	SE+	21	34	-	-	-

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Discussion

Susceptibility to RA is influenced by both genetic and environmental factors such as smoking. Many studies have determined that the major RA disease susceptibility genes are the HLA class II alleles. The shared epitope (SE) hypothesis for risk of RA is well-established [7], indicating that multiple HLA-DRB1 alleles are the strongest known genetic risk factors for RA by virtue of encoding a shared amino acid sequence, known as a shared epitope, SE [6]. Several studies have also reported associations between the genetic background and JIA susceptibility [5], including associations with HLA alleles [24–29]. An association between HLA-SE and susceptibility to JIA has been confirmed in 204 RF- or ACPA-positive Caucasian JIA patients [8].

The contribution of HLA to RA susceptibility, however, accounts for only about 30% of incidence, implying that genes other than those in the HLA region are involved; some estimates suggest as many as 100. Other genes influencing RA susceptibility have now been identified, such as PADI4, PTPN22 and CTLA4. Numerous non-HLA JIA susceptibility genes have also been imputed using GWAS [11]. Variants at the PTPN22, STAT4, TNF-α, TNFAIP3, MIF, WISP3, SLC11A1 and IL2-Ra loci have been reported as risk factors for JIA by several investigators [5], although it was also reported that several of these are not necessarily shared between different ethnic groups [10,30]. Thus, there are likely to be different genetic risk factors for JIA in different ethnic groups. Therefore, here we sought an influence of HLA-SE and PADI4 on JIA susceptibility in Japanese, because both HLA-SE and PADI4 were reported as significant genetic risk factors for RA independent of ethnicity [14,15,31].

We previously reported an association of HLA-A*02:06 with JIA accompanied by uveitis and of HLA-DRB1*04:05 with polyarticular JIA [9]. In the present study, we confirmed the association between HLA-SE and RF-positive polyarticular JIA in Japanese. However, we found that HLA-SE was not associated with oligoarticular or systemic JIA in our cohort. Recently, it was reported that five amino acids in three HLA molecules, including three amino acid positions (11, 71 and 74) in HLA-DRB1, were associated with RF-seropositive RA by the HLA-imputation method [32]. It should therefore be evaluated whether these HLA amino acids are also associated with JIA susceptibility in future.

In addition to RF, ACPA is the most specific serologic marker in adult RA with a specificity of 95% and a sensitivity of 80%, similar to RF [33,34]. Considering all JIA subtypes together, ACPA was detected in 1.8–28.6% of patients, a low frequency compared to RA. However, ACPA was present in 70–90% of RF-positive polyarticular JIA patients [35]. Bone destruction is more severe in these ACPA-positive patients [36]. These results suggest that ACPA-positive polyarticular JIA may be similar to RA with regard to pathogenetic processes.

PADI4, a member of the PADI family, was first reported to be associated with RA in a Japanese population [12,13]. It encodes a peptidyl arginine deiminase responsible for the post-translational conversion of arginine residues into citrulline. We investigated associations between PADI4 gene polymorphisms and ACPA positivity in JIA in our Japanese population. The stability of PADI4 mRNA differs according to these gene polymorphisms, which may represent the mechanism by which it influences the production of ACPA [13]. To the best of our knowledge, there are no reports that PADI4 risk alleles are involved in JIA disease susceptibility. It is likely that PADI4 is also a JIA susceptibility gene in ethnic groups other than Japanese, especially in ACPA-positive JIA. This hypothesis needs further exploration.

We found no association between HLA-SE and PADI4 in JIA patients, implying that HLA-SE and PADI4 are independent JIA susceptibility genes. However, an association between HLA-SE and citrullination in the pathogenesis of RA has been noted [37]. The electropositive P4 pocket of HLA-DRB1*04:01/04 can accommodate citrulline-containing epitopes, and the $CD4^+$ T cell repertoire for citrullinated antigens is increased in RA patients

harboring HLA-DRB1*04:01/04. These potential pathogenetic mechanisms may also contribute to JIA. Further study is needed to determine whether this is the case.

In conclusion, we found an association of PADI4 gene polymorphisms with ACPA-positivity in JIA, as was already known for RA. We also confirmed the influence of HLA-SE on RF-positive polyarticular JIA in the Japanese population. Thus, JIA may be classified into clinical and genetic background-based subtypes using HLA-SE and PADI4 genotyping.

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BRIEF REPORT

Treatable renal disease in children with silent lupus nephritis detected by baseline biopsy: association with serum C3 levels

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Abstract Lupus nephritis is identified in up to 75% of patients with juvenile systemic lupus erythematosus and may present with abnormal urinary findings (overt lupus nephritis) or be apparent only upon renal biopsy (silent lupus nephritis). We investigated whether serum complement levels correlate with renal pathology in pediatric patients with silent lupus nephritis. We performed baseline renal biopsy in 45 children diagnosed with juvenile systemic lupus erythematosus who were admitted to Kagoshima University Hospital between January 2000 and June 2015. Patients were classified as having overt or silent lupus nephritis based on urinary findings at renal biopsy. *Silent lupus nephritis was identified in 55.5% (25/45) of cases. Of these, 6 (13.3%) were classified as class III nephritis, according to the International Society of Nephrology/Renal Pathology Society criteria.* Decreased serum C3 levels were associated with the renal pathology classification for patients with silent but not with overt lupus nephritis. No differences in serum C4 levels were identified between cases of silent and overt lupus nephritis. Baseline renal biopsy is a critical component of the work-up of juvenile systemic lupus erythematosus as treatable renal pathology may be present in the absence of urinary signs. Serum C3 may be an important marker of the progression of silent lupus nephritis.

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Keywords Biomarker · Children · Complement · Renal biopsy · Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect multiple organ systems, including the kidneys, central nervous system, hematopoietic system, and skin [1]. Juvenile SLE (JSLE) accounts for 15–20% of all cases of SLE, with lupus nephritis (LN) being a serious complication of JSLE. Identified in 20–75% of patients with JSLE, LN is a predictor of poor disease prognosis [2, 3].

LN-associated activation of an immune response in renal tissue involves multiple factors, including activation of T- and B-lymphocytes, and macrophages and dendritic cells; production of pro-inflammatory cytokines and type I interferons; and deposition of immune complexes in the mesangium and other renal tissues [4]. Notably, serum complement components, such as C1q and C3, are associated with disease activity in JSLE and could potentially be used as biomarkers of LN disease activity in patients with JSLE [5–7]. However, the relationship between serum levels of complement components and renal pathology in LN remains unclear, due in part to an incomplete understanding of the mechanisms underlying renal pathology in LN. Moreover, pediatric patients can present, clinically, with either overt LN (oLN), defined by abnormal urinalysis findings of proteinuria and hematuria, or silent LN (sLN), characterized by normal urinalysis findings.

Decreased serum levels of C3 and C4 complement components have been reported to correlate with disease activity in adults with LN [8–12]. Specifically, C3 deposition in renal tissues correlates with morphological disease classification [13] and is likely to contribute to renal pathology in sLN. Therefore, the goals of our study were to evaluate the

importance of baseline renal biopsy in pediatric patients with sLN and to evaluate the association between serum C3 and C4 levels and the renal pathology classification.

Materials and methods

Statement of ethics and description of the study group

Our study conformed to ethical principles of the Declaration of Helsinki and was approved by the institutional review board at Kagoshima University Hospital, with parents or legal guardians providing informed consent for minors.

The study group consisted of 45 children admitted to the Department of Pediatrics at Kagoshima University Hospital, between January 2000 and June 2015, with a diagnosis of JSLE. All patients fulfilled the revised criteria of the American College of Rheumatology for SLE [14], presenting with at least four of the 11 criteria. As standard of care, all patients underwent renal biopsy, independent of urinalysis findings, at the time of JSLE diagnosis. Patients who had received immunosuppressive therapy prior to renal biopsy were excluded from this study. All the children in the study group were from a Japanese population.

JSLE patients with LN were enrolled and classified into two groups: 20 patients with persistent proteinuria (>0.5 g/day) at the time of renal biopsy were classified as having oLN (1 male, 19 females; 12.4 ± 2.2 years old; median, 12.6 years), with the remaining 25 patients, having no proteinuria and no hematuria, were classified as having sLN (6 males, 19 females; 12.5 ± 2.3 years old; median, 12.0 years).

Laboratory and pathology analyses

Laboratory data, including antibody titers and proteinuria levels, were obtained using the standard methods of our hospital's clinical laboratory. LN diagnosis was confirmed by a single pathologist using hematoxylin–eosin (HE), periodic acid–Schiff (PAS), periodic acid methenamine (PAM), and Masson's trichrome staining, and electron microscopy images. LN class was determined using the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification [15].

Complement components

Blood serum was collected prior to renal biopsy and treatment. Blood samples were maintained at room temperature and tested within 30 min of collection. Serum C3 and C4 levels were obtained using commercially available immunity turbidimetric assay kits (C3, catalog# TBA-84A5; C4, catalog# RM73-852TK). All reagents and equipment used for complement component measurement were purchased from LSI Medience (Tokyo, Japan).

Statistical analysis

Between-group differences were evaluated using the Mann-Whitney *U* test for continuous data and Fisher's exact test for qualitative and ordinal data (SPSS, version 12.0; Chicago, IL). Differences across groups were analyzed using the Steel–Dwass test (R-Statistical Package, R Foundation for Statistical Computing, Vienna, Austria). *P* values <0.05 were considered to be statistically significant.

Results

Clinical features and laboratory data for both groups are listed in Table 1. All patients who underwent biopsy had some evidence of LN pathology. The distribution of ISN/RPS classes of LN in the oLN group was as follows: class I, 0%; class II, 25% (5 patients); class III, 55% (11 patients); and class IV, 20% (4 patients). The distribution in the sLN group was as follows: class I, 12% (3 patients); class II, 64% (16 patients); class III, 24% (6 patients); and class IV, 0% (Fig. 1).

Serum levels of C3 and C4 were comparable between oLN and sLN groups, when LN class was not considered (Table 1). Serum C3 and C4 levels are reported by LN class in Fig. 2a–d. For the sLN group, serum C3 levels varied between LN classes, with significantly lower levels for patients in class III compared to class II and for class II compared to class I (Fig. 2a). In contrast, in the oLN group, serum C3 levels were comparable between patients in classes II, III, and IV (Fig. 2b). There was no significant difference in serum C3 levels between the oLN and sLN groups for a given class: class II, $P = 0.592$, and class III, $P = 0.615$. Serum C4 levels were comparable across the four classes of LN in both the sLN (Fig. 2c) and oLN (Fig. 2d) groups.

Discussion

LN is an immune complex-mediated glomerulonephritis [2]. Despite progress in understanding the pathogenesis of LN, our understanding of the pathophysiology of sLN, in which SLE-associated glomerulonephritis exists in the absence of abnormal urinalysis, is limited, especially in pediatric patients. Our study underscores the importance of baseline renal biopsy, with 6/25 patients (24%) diagnosed with a class III LN in the absence of significant proteinuria or casts on light microscopy. Baseline biopsy in patients with JSLE without urinary symptoms, therefore, may alter treatment decisions. Patients with a LN class

Table 1 Clinical features and laboratory data of the study group

	oLN ($n = 20$)	sLN ($n = 25$)	P value oLN versus sLN
Male, n (%)	1 (5.0)	6 (24.0)	0.112#
Median age, years (range)	12.6 (7.9–15.9)	12.0 (8.0–15.9)	0.811***
Proteinuria, n (%)	20 (100.0)	0 (0.0)	<0.001#*
C3, mg/dL**	50.6 ± 34.6	56.3 ± 27.6	0.299***
C4, mg/dL**	6.2 ± 5.2	5.1 ± 3.7	0.591***
Anti-dsDNA antibody, IU/mL**	166.5 ± 166.8	153.2 ± 152.6	0.875***
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Anti-SSB antibody, U/mL**	21.0 ± 43.3	61.8 ± 221.5	0.391***
ISN/RPS classification			
Class I, n (%)	0 (0.0)	3 (12.0)	0.242#
Class II, n (%)	5 (25.0)	16 (64.0)	0.016#*
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Class IV, n (%)	4 (20.0)	0 (0.0)	0.033#*
Class I and II, n (%)	5 (25.0)	19 (76.0)	0.001#*
Class III and IV, n (%)	15 (75.0)	6 (24.0)	0.001#*

LN lupus nephritis, *oLN* overt LN, *sLN* silent LN

*Significant at $P < 0.05$

**Data are presented as mean ± standard deviation

***Mann-Whitney U test

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≥III would be eligible for immunosuppressive therapy, whereas patients with a LN class <III would not be.

Serum C3 as a marker of silent LN

Prognostic biomarkers of SLE and LN have primarily been investigated in adults, in whom active nephritis and levels of certain autoantibodies, such as anti-C1q, are clearly correlated [8, 10, 16, 17], with a further association between low C3 and C4 levels and LN disease activity [8–12]. However, a specific correlation between low complement component levels and the development of other organ involvement has been less clear. Serum complement components, such as C1q and C3, have been associated with overall SLE disease activity in children [7, 18, 19], with a decrease in serum C3 being a risk factor for LN [20]. The potential role of complement component levels as biomarkers in pediatric sLN has not been fully elucidated.

We analyzed serum C3 and C4 levels in children with oLN and sLN, reporting, for the first time, a significant decrease in serum levels of C3, but not C4, which was associated with the ISN/RPS class in patients with sLN. Our findings agree with those of Klein et al. [21] who identified a correlation between serum levels of C3, but not C4, and renal pathology in

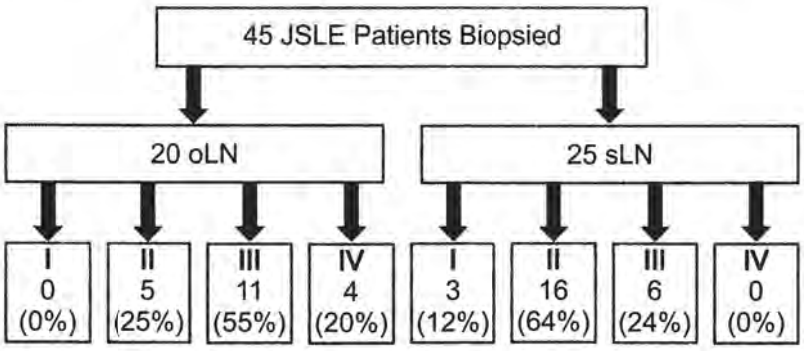

Fig. 1 Flow chart showing the distribution of ISN/RPS classes of LN in all patients who underwent biopsy

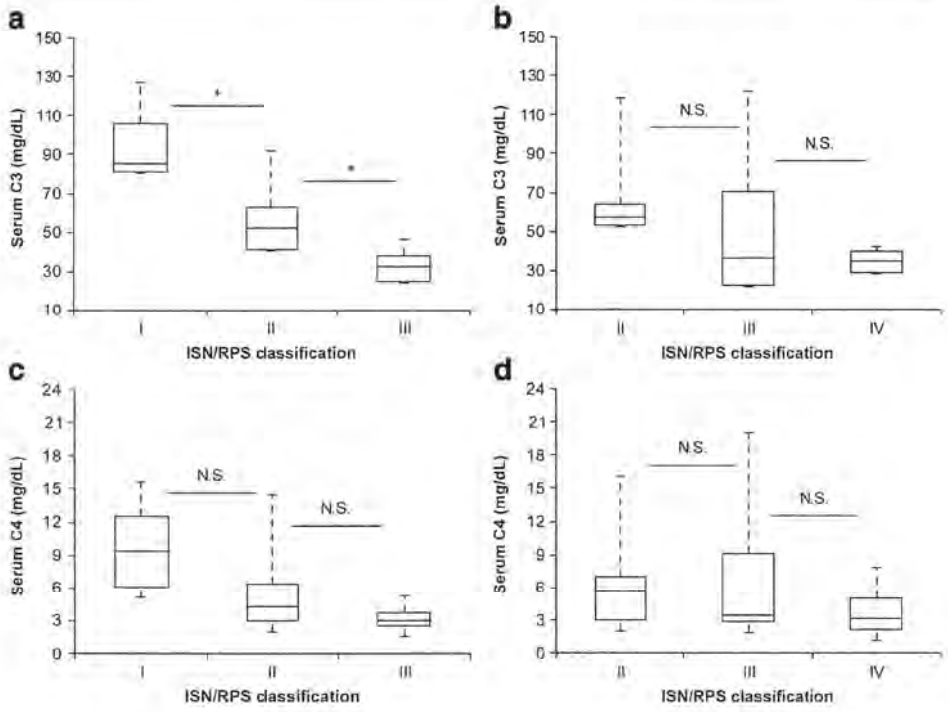

Fig. 2 Relationship between serum complement levels and ISN/RPS classification. Serum levels of C3 (**a**, **b**) and C4 (**c**, **d**) were measured prior to treatment in patients in both the sLN (**a**, **c**) and oLN (**b**, **d**) groups using an immunity turbidimetric assay and analyzed according to LN class as determined by renal biopsy. Data are presented as the first to third quartile (*box*) and median (*horizontal line*), with *whiskers* indicating minimum and maximum values; *asterisk* indicates significant at $P < 0.05$ by the Steel-Dwass test. *N.S.* not significant

pediatric patients with LN. Marks et al. [3] also reported that patients with JSLE and class IV LN tended to have lower serum C3, but not C4, levels. However, we did not identify differences in either C3 or C4 levels by ISN/RPS class in patients with oLN. It is possible that the relatively small number of patients in each group and/or the difference in classification system used (i.e., World Health Organization, WHO versus ISN/RPS) contributed to this difference between studies. We also did not identify a between-group difference in C3 levels when ISN/RPS class was not considered, or by LN class between oLN and sLN groups. These findings are in contrast with the previous research by Zabaleta-Lanz et al. who reported significant differences in C3 levels in adult patients with oLN versus sLN [22]. Additional research is required to clarify whether contrasting findings reflect small sample sizes in both studies or fundamental differences in the pathogenesis of LN between pediatric and adult forms of SLE.

Our study provides level IV evidence of an association between decreased serum C3 levels and renal pathology in pediatric patients with sLN. The role of renal complement component deposition in LN pathogenesis has been established in animal models and human disease [3, 12]. This study provides further evidence that decreased serum C3 levels in patients with sLN may be due to consumption of C3 by activated autoimmune processes, rather than to an underlying deficiency in C3. We propose that serum C3 levels may be useful as an indicator of poor outcome in children with sLN.

Since class II/III oLN C3 levels were similar to those in class II/III sLN, C3 consumption is likely present in our oLN patients as well. However, this does not explain our finding that serum C3 levels did not differ among ISN/RPS classification in the oLN group. In a longitudinal study of adults with LN, Wada et al. reported that decreased serum C3 levels in patients with sLN, together with elevated levels of anti-dsDNA autoantibodies, preceded the progression from sLN to oLN by approximately 24 months [23]. Additional studies are needed to clarify the protective versus destructive roles of C3, both in sLN pathology and in the development of oLN from previously silent disease in pediatric patients with LN. Our study was a small, single-center study, investigating an ethnically homogeneous group of patients. Our findings should be validated in larger, multi-center studies with an ethnically diverse cohort.

In conclusion, we report that substantial numbers of pediatric patients diagnosed with JSLE without urinary signs nevertheless have treatable renal disease. In addition, serum C3 levels are significantly associated with the ISN/RPS classification in pediatric patients with sLN. Serum C3 levels, in combination with other biomarkers, could be useful to identify the need for renal biopsy and to predict risk of disease progression from sLN to oLN in pediatric patients. Further studies are warranted to confirm our findings in a larger clinical population and to determine appropriate testing guidelines and treatment strategies for pediatric patients with sLN.

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Compliance with ethical standards

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Disclosures None.

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BRIEF REPORT

Treatable renal disease in children with silent lupus nephritis detected by baseline biopsy: association with serum C3 levels

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Serum C3 as a marker of silent LN

Prognostic biomarkers of SLE and LN have primarily been investigated in adults, in whom active nephritis and levels of certain autoantibodies, such as anti-C1q, are clearly correlated [8, 10, 16, 17], with a further association between low C3 and C4 levels and LN disease activity [8–12]. However, a specific correlation between low complement component levels and the development of other organ involvement has been less clear. Serum complement components, such as C1q and C3, have been associated with overall SLE disease activity in children [7, 18, 19], with a decrease in serum C3 being a risk factor for LN [20]. The potential role of complement component levels as biomarkers in pediatric sLN has not been fully elucidated.

We analyzed serum C3 and C4 levels in children with oLN and sLN, reporting, for the first time, a significant decrease in serum levels of C3, but not C4, which was associated with the ISN/RPS class in patients with sLN. Our findings agree with those of Klein et al. [21] who identified a correlation between serum levels of C3, but not C4, and renal pathology in

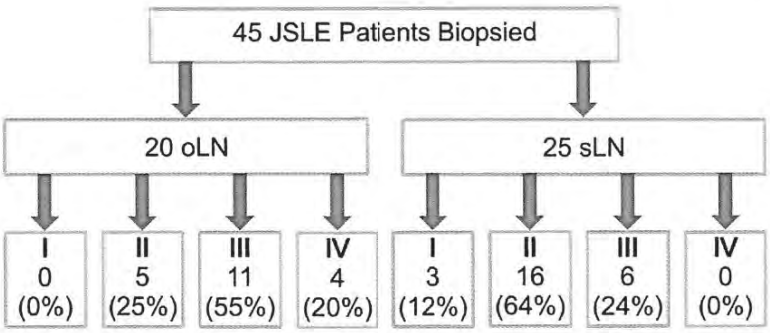

Fig. 1 Flow chart showing the distribution of ISN/RPS classes of LN in all patients who underwent biopsy

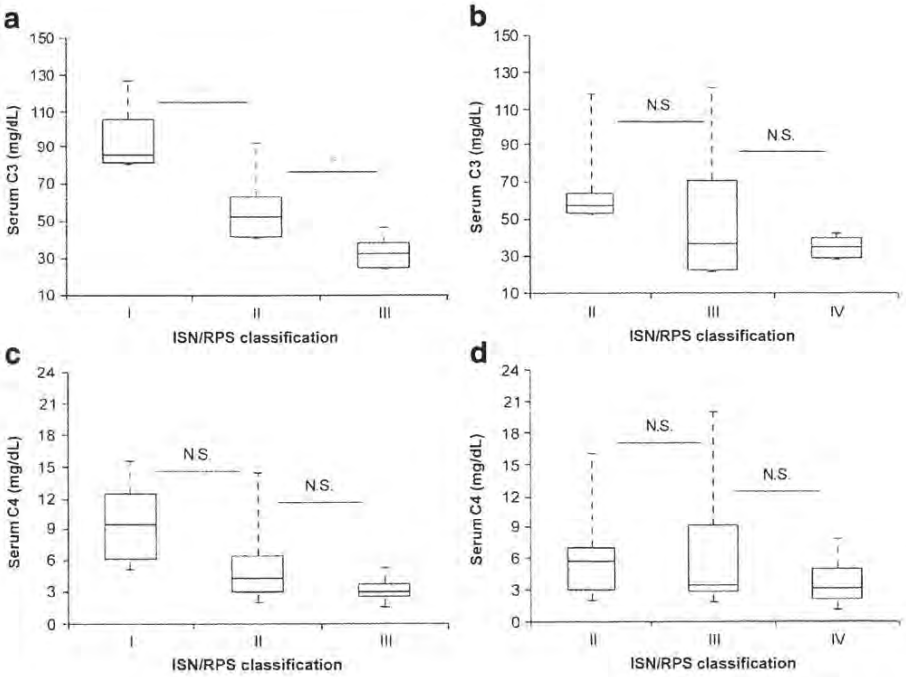

Fig. 2 Relationship between serum complement levels and ISN/RPS classification. Serum levels of C3 (**a**, **b**) and C4 (**c**, **d**) were measured prior to treatment in patients in both the sLN (**a**, **c**) and oLN (**b**, **d**) groups using an immunity turbidimetric assay and analyzed according to LN class as determined by renal biopsy. Data are presented as the first to third quartile (*box*) and median (*horizontal line*), with *whiskers* indicating minimum and maximum values; *asterisk* indicates significant at $P < 0.05$ by the Steel-Dwass test. *N.S.* not significant

pediatric patients with LN. Marks et al. [3] also reported that patients with JSLE and class IV LN tended to have lower serum C3, but not C4, levels. However, we did not identify differences in either C3 or C4 levels by ISN/RPS class in patients with oLN. It is possible that the relatively small number of patients in each group and/or the difference in classification system used (i.e., World Health Organization, WHO versus ISN/RPS) contributed to this difference between studies. We also did not identify a between-group difference in C3 levels when ISN/RPS class was not considered, or by LN class between oLN and sLN groups. These findings are in contrast with the previous research by Zabaleta-Lanz et al. who reported significant differences in C3 levels in adult patients with oLN versus sLN [22]. Additional research is required to clarify whether contrasting findings reflect small sample sizes in both studies or fundamental differences in the pathogenesis of LN between pediatric and adult forms of SLE.

Our study provides level IV evidence of an association between decreased serum C3 levels and renal pathology in pediatric patients with sLN. The role of renal complement component deposition in LN pathogenesis has been established in animal models and human disease [3, 12]. This study provides further evidence that decreased serum C3 levels in patients with sLN may be due to consumption of C3 by activated autoimmune processes, rather than to an underlying deficiency in C3. We propose that serum C3 levels may be useful as an indicator of poor outcome in children with sLN.

Since class II/III oLN C3 levels were similar to those in class II/III sLN, C3 consumption is likely present in our oLN patients as well. However, this does not explain our finding that serum C3 levels did not differ among ISN/RPS classification in the oLN group. In a longitudinal study of adults with LN, Wada et al. reported that decreased serum C3 levels in patients with sLN, together with elevated levels of anti-dsDNA autoantibodies, preceded the progression from sLN to oLN by approximately 24 months [23]. Additional studies are needed to clarify the protective versus destructive roles of C3, both in sLN pathology and in the development of oLN from previously silent disease in pediatric patients with LN. Our study was a small, single-center study, investigating an ethnically homogeneous group of patients. Our findings should be validated in larger, multi-center studies with an ethnically diverse cohort.

In conclusion, we report that substantial numbers of pediatric patients diagnosed with JSLE without urinary signs nevertheless have treatable renal disease. In addition, serum C3 levels are significantly associated with the ISN/RPS classification in pediatric patients with sLN. Serum C3 levels, in combination with other biomarkers, could be useful to identify the need for renal biopsy and to predict risk of disease progression from sLN to oLN in pediatric patients. Further studies are warranted to confirm our findings in a larger clinical population and to determine appropriate testing guidelines and treatment strategies for pediatric patients with sLN.

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Compliance with ethical standards

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Disclosures None.

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CASE REPORT

Anasarca as the initial symptom in a Japanese girl with Sjögren's syndrome

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ABSTRACT

Generalised oedema, or anasarca, is a rare complication of systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) and usually results from nephrotic syndrome or protein-losing enteropathy. We report a 14-year-old girl presented with anasarca and persistent fever. Despite hypoalbuminemia, no or little protein loss was observed in her urine or stool. She was diagnosed as having SS by positive anti-SSA antibodies and ductal dilation and glandular destruction of her parotid gland on magnetic resonance sialography. Elevated levels of serum C-reactive protein, ferritin and plasma D-dimer suggested that systemic inflammation caused anasarca by both decreased production of albumin and hyperpermeability associated with vascular endothelial damage similar to systemic capillary leak syndrome. Methylprednisolone pulse therapy and low-dose intravenous cyclophosphamide therapy followed by oral prednisolone and azathioprine were effective.

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Anasarca; systemic lupus erythematosus; Sjögren's syndrome; systemic inflammation; systemic capillary leak syndrome

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterised by skin erythema and multiple organ involvement associated with an array of autoantibodies. Systemic manifestations of the disease such as fever, generalised lymphadenopathy and renal involvement are more common in childhood SLE than in adult cases [1,2].

Sjögren's syndrome (SS) is an autoimmune disease predominantly affecting glandular tissues. SS develops in association with or without other connective tissue diseases, designated as secondary or primary SS, respectively. Although both dry eyes and dry mouth are characteristics of SS, some patients demonstrate extraglandular manifestations such as fever, fatigue, myalgia and arthralgia [3,4]. Children with SS often lack glandular symptoms except parotid swelling and are commonly presented with the extraglandular manifestations associated with positive anti-SS-A and/or anti-SS-B antibody [5,6].

Generalised oedema, or anasarca, is a rare complication of SLE and SS, which usually results from complicating nephrotic lupus nephritis or protein-losing enteropathy [1–4]. We report a 14-year-old Japanese girl with SS possibly associated with SLE who presented with anasarca despite lack of nephrotic syndrome or protein-losing enteropathy.

The patient and her parents gave informed consent prior to submission of this article.

Patient presentation

A 14-year-old girl was referred to our hospital because of fever persisting for 12 days, lymphocytopenia, thrombocytopenia, pleural effusion, ascites and anasarca. She was 156.0 cm in height and weighed 65.0 kg, gaining 2.0 kg after the onset. On admission, she demonstrated mild tachypnoea, hepatosplenomegaly and erythema on her right cheek. There were no gastrointestinal manifestations such as diarrhoea or vomiting. Laboratory examination demonstrated erythrocyte sedimentation rate 69 mm/h, white blood cell counts 5400/μl with 73% of neutrophils, haemoglobin 115 g/l, haematocrit 33.6%, platelet count 46×10^3/μl. Biochemical findings were as follows; total protein 47 g/l, albumin 15 g/l, aspartate aminotransferase (AST) 200 IU/l, alanine aminotransferase (ALT) 130 IU/l, lactate dehydrogenase 483 IU/l, creatine phosphokinase 26 IU/l, urea nitrogen 130 mg/l, creatinine 9.3 mg/l, C-reactive protein (CRP) 195.5 mg/l, C3 10.8 g/l, C4 1.3 g/l, CH50 54.8 U/ml, ferritin 967 ng/ml, and soluble IL-2-R 1326 U/ml. Coagulation studies showed prothrombin time 16.8 s, activated partial

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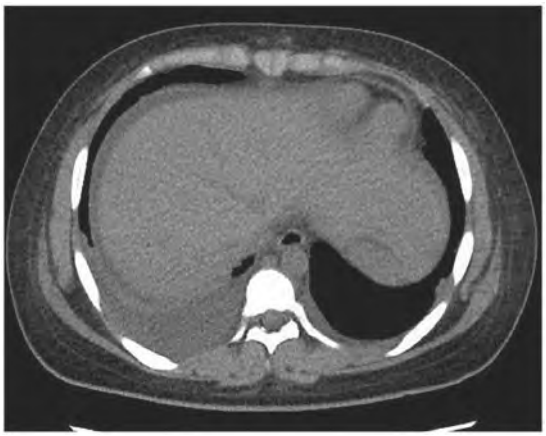

Figure 1. Chest CT scan. Chest CT scan demonstrated bilateral pleural effusion and perihepatic ascites without enhancement.

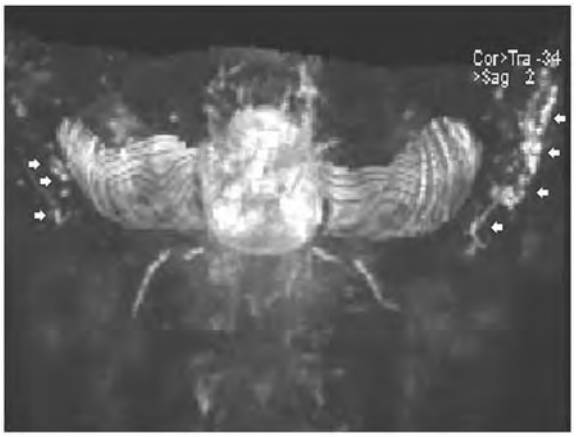

Figure 2. MR-sialography showed ductal dilation and mild glandular destruction of parotid gland with left side dominance (arrows).

thromboplastin time 33.1 s, fibrinogen 5.73 g/l, D-dimer 7.03 μg/ml. Immunological tests demonstrated positive anti-nuclear (1:320) and anti-SS-A antibody (125.5 index) but negative for other autoantibodies including platelet-associated IgG, anti-SS-B, anti-double-strand DNA, anti-Sm, anti-phospholipid and anti-neutrophil cytoplasmic antibodies. No myositis-specific autoantibodies were detected by RNA-immunoprecipitation or immunoprecipitation-Western blot methods. Urinalysis demonstrated specific gravity over 1.040, but no proteinuria, haematuria or glycosuria. Bone marrow examination demonstrated no apparent abnormalities such as malignant cells or hemophagocytosis. Alpha1-antitrypsin clearance test demonstrated no excretion of the protein in the stool. Computed tomography scans of her chest and abdomen demonstrated hepatosplenomegaly, pleural effusion and ascites (Figure 1). There was no pericardial effusion or cardiac dysfunction on echocardiography. Magnetic resonance sialography (MR-sialography) demonstrated ductal dilation and glandular destruction of her parotid gland equivalent to Rubin-Holt's Grade 3 (Figure 2). Histopathological test from her cheek showed perivascular dermatitis which is consistent with SLE or SS. She was diagnosed as having SS and possibly SLE. She was initially treated with prednisolone (PSL, 40 mg/kg/day for 2 days followed by 60 mg/kg/day for 8 days) and infusion of albumin to restore circulation volume and reduce systemic oedema. Lip biopsy was carried out after stabilisation of her general condition and showed mononuclear cell infiltration into the minor salivary gland which is classified as Greenspan's Grade 2. As her body weight increased to 69 kg on the 14th hospital day, methylprednisolone (mPSL) pulse therapy (1 g/day for 3 consecutive days) was commenced. Six courses of low dose intravenous cyclophosphamide (IVCY) therapy (500 mg/dose every two weeks) were also adopted which were followed by oral administration of azathioprine (75 mg/day). Her platelet count, D-dimer and CRP levels returned to normal levels associated with defervesce and recovery of body weight to 64 kg within 2 weeks after the commencement of mPSL pulse therapy. Renal biopsy demonstrated endothelial enlargement and focal intratubular proliferation without immune complex deposition (Figure 3). Oral prednisolone therapy (50 mg/day) was started following three courses of mPSL pulse therapy and gradually decreased. She is currently treated with combination of azathioprine and 7.5 mg/day of prednisolone without relapse.

Discussion

Our patient showed globular destruction of the salivary gland on MR-sialography associated with positive ANA and anti-SS-A antibodies. MR-sialography findings correspond well with sialography findings in childhood SS [7], supporting the diagnosis of SS defined by Japanese Diagnostic Criteria for SS [8]. Although the grade of focal lymphoid infiltration in the minor salivary glands was not enough to confirm the diagnosis, focal infiltration of less than 50 mononuclear cells is suggestive of childhood SS [9,10]. SS and SLE share several similarities in both clinical and laboratory findings. A faint facial erythema in our patient was indistinguishable from each other on both clinical and histopathological examinations. Lymphocytopenia, thrombocytopenia and positive anti-nuclear antibody, all of which are included in the classification criteria by both American College of Rheumatology and Systemic Lupus International Collaborating Clinics [11,12], are also observed in primary SS. Although pleural effusion and ascites were detected on CT scan, she complained no chest or abdominal pain, suggesting effusion associated with generalised oedema rather than serositis. Histological

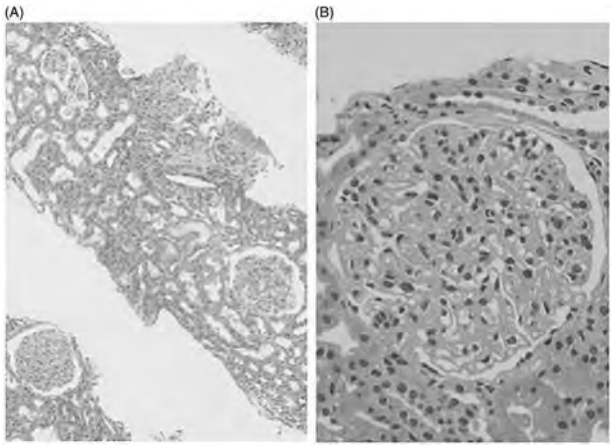

Figure 3. Renal pathology. Histological studies demonstrated endothelial enlargement and focal intratubular proliferation but no apparent interstitial nephritis (haematoxylin–eosin staining, A ×40, B ×100). No deposit of immunoglobulins or complements was observed by immunofluorescent staining (not shown).

showed endothelial enlargement and focal intratubular proliferation of the kidney which are consistent with lupus nephritis on HE staining but lacked immune complex deposition [13]. On the other hand, serum complement levels remained at a lower limit of normal ranges despite extremely high levels of other acute phase reactant, suggesting consumption of complements, a hallmark of SLE. Thus, the diagnosis of SLE is possible but not definitive in our patient.

Anasarca has been reported as a rare complication of juvenile dermatomyositis (JDM) and dermatomyositis [14,15]. However, she demonstrated no muscle weakness, JDM-specific erythema, abnormal signals on muscle MRI, or myositis-specific autoantibodies [16]. Nephrotic syndrome and protein-losing enteropathy are causes of hypoalbuminemia in both SLE and SS [1–4] but were absent in our patient. Systemic capillary leak syndrome (SCLS) is characterised by oedema, hypoalbuminemia and hemoconcentration without nephrosis or other causes of protein loss, and often results in hypovolemic shock [17,18]. SCLS develops alone or in association with malignancies, infectious diseases and medication [18]. Recent reports have demonstrated that SCLS is a rare complication of connective diseases such as SS, polymyositis, antiphospholipid syndrome and Kawasaki disease [19–22]. Interestingly, one report demonstrated that four of the five autoimmune-associated SCLS cases were diagnosed as SS [19], suggesting that patients with SS have a predisposition to SCLS. It is suggested that serum proteins with large molecular weights including albumin leak to interstitial spaces [17]. Although our case lacked several characteristics of SCLS, such as hemoconcentration or hypotension, inexplicable hypoalbuminemia suggests similar mechanism. Given that the severity of SCLS associated with connective tissue diseases varies from self-limiting to fatal [19], our case could be mild and responded to immunosuppressive therapy before the development of irreversible tissue damage. Based on the pathological findings of the skin and renal biopsies and elevated levels of D-dimer, we hypothesise that systemic vasculitis/perivasculitis and endothelial damage increase the vascular permeability. Vasculitis complicates 11–36% of SLE and 10% of SS [23,24]. Vasculitis in SLE tends to associate with high disease activity and/or co-existing diseases causing vascular damage such as SS or anti-phospholipid antibody syndrome [24]. Prolonged systemic inflammation as suggested by fever and elevated levels of ESR, fibrinogen and CRP may be also involved in the decreased production of albumin. Together with thrombocytopenia and the elevated levels of AST and ferritin, these findings suggest hypercytokinaemia as observed in macrophage activating syndrome complicating systemic juvenile idiopathic arthritis or SLE [25,26]. This is consistent with a recent report demonstrating that elevated levels of cytokines, such as vascular endothelial growth factor, interleukin-1β, interleukin-6 and tumour necrosis factor-α, are associated with increased vascular permeability in primary SCLS [27].

She was initially treated with prednisolone and albumin replacement to stabilise her haemodynamics. Although clinical effects of high-dose intravenous immunoglobulin therapy, β2-stimulants and theophylline have been reported in SCLS [18,20,22,28], we chose immunosuppressive therapy to control the possible underlying SS and SLE. Clinical effect of mPSL pulse therapy has also been reported in a case of SCLS (Clarkson's disease) [29]. Recurrence of capillary leak attack is another characteristic of SCLS but has not occurred for 2 years of follow-up on the concomitant administration of azathioprine with corticosteroid.

In conclusion, anasarca is a complication of SS and/or SLE even in the absence of nephrotic syndrome or protein-losing enteropathy. Systemic inflammation-associated hyperpermeability similar to SCLS could be a mechanism of oedema and require intensive immunosuppressive therapy.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Recurrence of juvenile dermatomyositis 8 years after remission

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Key word: juvenile dermatomyositis.

INTRODUCTION

Juvenile dermatomyositis (JDM) is a chronic inflammatory disease characterized by typical skin lesions and muscle weakness, which occurs in children and adolescents younger than 16 years.[1] JDM is classified into 3 clinical types according to the posttreatment course: (1) monocyclic, in which there is one episode with permanent remission within 2 years after diagnosis; (2) polycyclic, with multiple relapses within 2 years; and (3) continuous, with pathologic states persisting for more than 2 years.[2] Early treatment with prednisolone is suggested to limit the disorder to the monocyclic course.[3] Only 2 case reports in which monocyclic JDM recurred more than 3 years after remission have been described in the English-language literature.[4,5] Of these 2 reported cases, 1 patient had no initial treatment and the other had oral prednisolone (PSL) alone.[4,5] Recently a well-designed randomized, controlled trial found that aggressive therapeutic approaches, such as PSL plus methotrexate (MTX) after methylprednisolone (mPSL) pulse therapy, outperform PSL monotherapy after mPSL pulse therapy with respect to clinical remission, treatment failure, and discontinuation of PSL.[6] Here we present a case of monocyclic JDM that recurred 8 years after remission despite initial treatment with PSL plus MTX after mPSL pulse therapy.

Abbreviations used:

CDASI:	Cutaneous Dermatomyositis Area and Severity Index
JDM:	juvenile dermatomyositis
MTX:	methotrexate
mPSL:	methylprednisolone
PSL:	prednisolone

CASE REPORT

A 4-year-old Japanese boy presented with eruptions on the face, ears, elbows, and knees and with muscular weakness. Physical examination found erythema on the cheeks and ears, keratotic papules and purplish erythema on the dorsa of the hands, and scaly erythema on the knees (Fig 1, *A* and *B*). This patient had no symptoms of dysphonia. Cutaneous Dermatomyositis Area and Severity Index (CDASI) was 8. The histopathology of the left knee showed vacuolar changes in the epidermis, deposition of mucin, pigment incontinence, and infiltration of lymphocytes in the papillary dermis (Fig 2, *A*). Biochemical examination found elevated levels of creatine kinase 425 IU/L (normal range, 12−170 IU/L) and aldolase 19.0 IU/L (2.7−7.5 IU/L). Antinuclear antibody and anti-Jo-1 antibody were negative. Magnetic resonance imaging (T2) found diffuse high-intensity areas in the proximal muscles of the extremities, which suggests edema caused by inflammation (Fig 2, *B*). Based on the clinical, histopathologic, and radiologic findings, the diagnosis of JDM was made. According to the recommended regimen at that time,[7] the patient was treated with 2 courses of mPSL pulse therapy (30 mg/kg/d for 3 consecutive days per course) followed by

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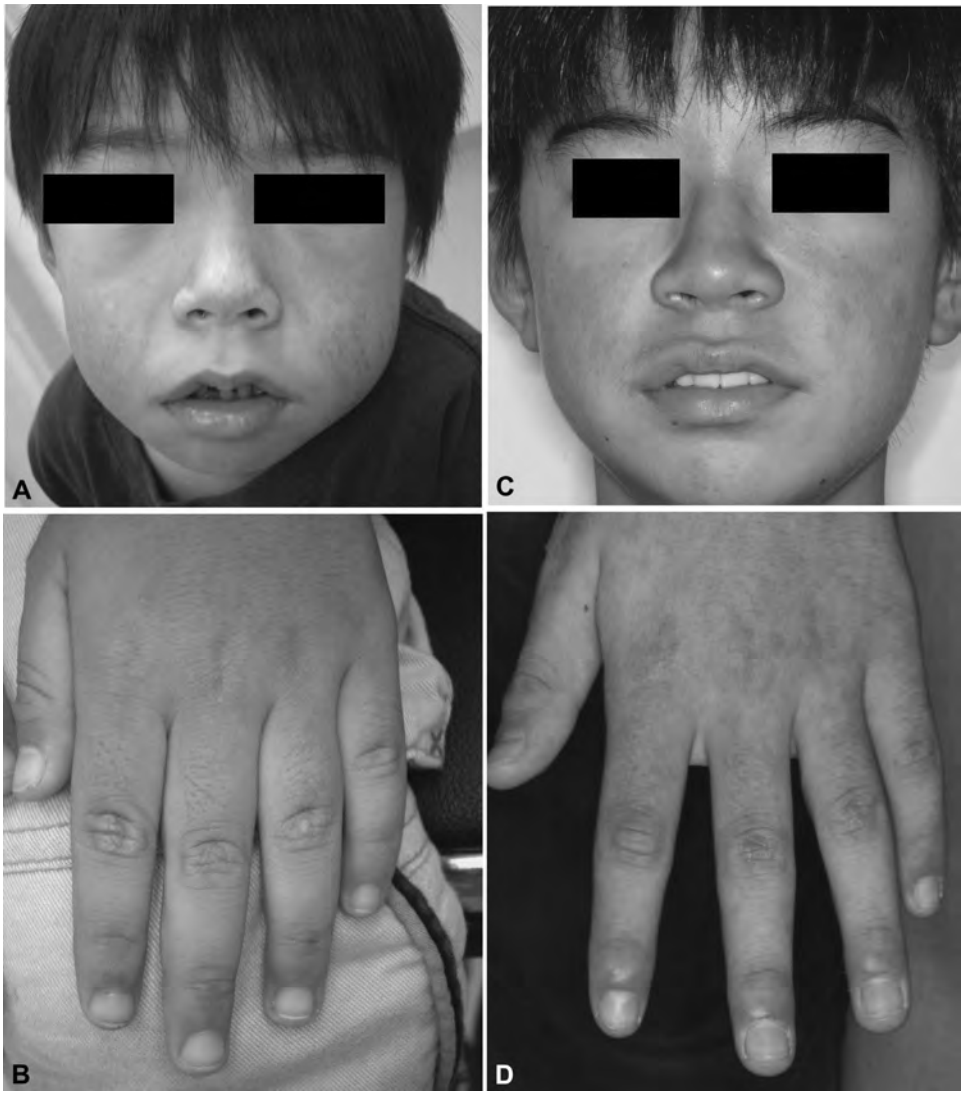

Fig 1. Erythema on the cheeks and ears at initial onset (4 years old) (**A**) and at relapse (12 years old) (**C**). Keratotic papules and purplish erythema on the dorsal of the left hand at initial onset (**B**) and at relapse (**D**).

combination therapy with PSL (1 mg/kg/d) and MTX (0.4 mg/kg/wk), both of which were tapered out in 6 months. Both clinical and biochemical remission was achieved and persisted for 8 years, suggesting a monocyclic course.

At 12 years of age, the patient presented to us with similar symptoms affecting the skin and proximal muscles but without preceding infectious episodes within the previous 3 months (Fig 1, *C* and *D*). Elevated levels of aspartate aminotransferase, 104 IU/L (0−35 IU/L); alanine aminotransferase, 53 IU/L (0−35 IU/L); lactate dehydrogenase, 506 IU/L (80−200 IU/l); creatine kinase, 1930 IU/L (12−170 IU/L), and aldolase 28.6 IU/L (2.7−7.5 IU/L) were observed. Antinuclear, anti-Jo-1, anti-Sm, anti-SS-A, anti-SS-B and anti-RNP antibodies were all negative. The IgM class of antiparvovirus B19 antibodies was not detected. Computed tomography scans showed neither interstitial pneumonia nor visceral malignancy. The clinical, histopathologic, and radiologic findings were virtually identical to those observed 8 years before (Fig 2, *C* and *D*). These findings confirmed the diagnosis of JDM relapse. Both the skin condition and muscle strength improved with 2 courses of mPSL pulse therapy (1 g/d for 3 consecutive days per course) followed by PSL (0.78 mg/kg/d) and MTX (0.20 mg/kg/wk). Serum levels of muscle-derived enzymes also returned to normal ranges. However, when the PSL dose was decreased to 0.29 mg/kg/d, elevation of muscle-derived enzymes and muscle weakness recurred, accompanied by pseudohypertrophy of the gastrocnemius

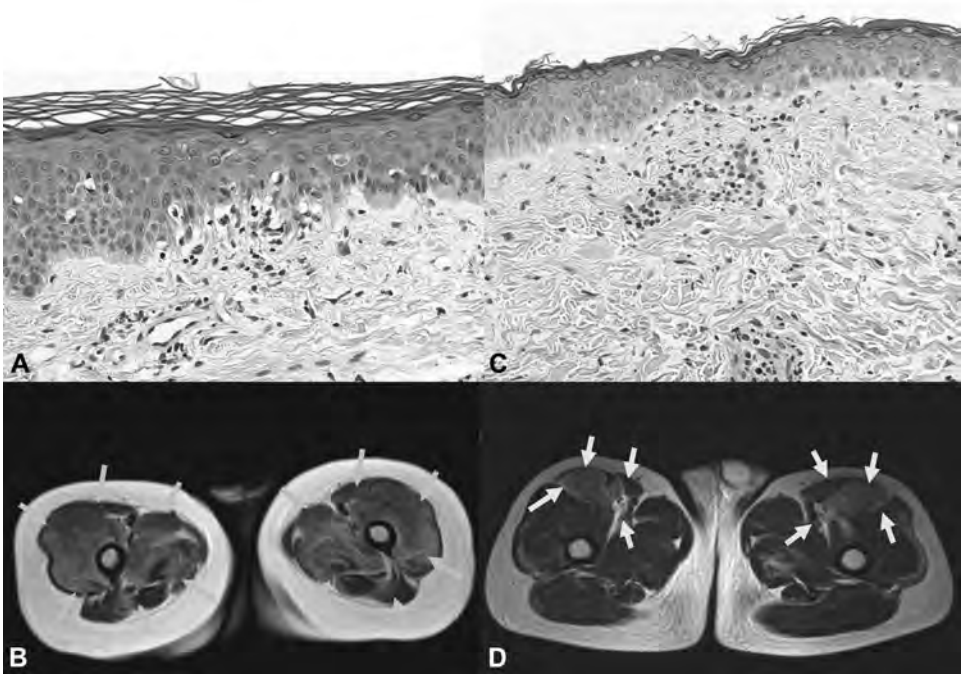

Fig 2. Vacuolar changes at the dermoepidermal junction of the epidermis, and deposition of mucin, pigment incontinence, and infiltration of lymphocytes in the papillary dermis are observed in the biopsy specimen of the left cheek at initial onset (4 years old) (**A**) and of the right knee at relapse (12 years old) (**C**). At the initial onset (T2) (**B**) (*orange arrows*) and at relapse (STIR) (**D**) (*yellow arrows*), magnetic resonance imaging shows high-intensity areas in the proximal muscles of the thighs, which suggests edema caused by inflammation. (**C**, Hematoxylin-eosin stain; original magnification: ×200.)

muscles. Erythema on the cheeks and keratotic papules on the dorsal hands also reappeared. Although his muscle strength and serum levels of muscle-derived enzymes returned to normal levels after the addition of cyclosporine (0.20 mg/kg/d) and an increase of PSL dose (to 0.78 mg/kg/d), the pseudohypertrophy and the eruptions persisted. The change of cyclosporine to tacrolimus (0.04 mg/kg/d) and decrease of MTX (to 0.08 mg/kg/wk) maintained the normal levels of muscle-derived enzymes and muscle strength. There were no sequelae such as calcinosis, muscular contracture, or cutaneous or gastric ulcers during his course. This patient will continue monthly follow-up, with a gradual PSL dose reduction planned for a minimum of 2 years unless a relapse of JDM occurs.

DISCUSSION

There are no established methods for predicting the clinical course of JDM. JDM is usually treated with corticosteroid therapy alone or in combination with immunosuppressive agents such as MTX.[8] It is suggested that early and intensive corticosteroid-based therapy leads to a monocyclic course.[3] Although clinical remission was achieved by early intensive treatment with mPSL pulse therapy followed by oral PSL and weekly MTX in the initial episode of JDM in our case, the maintenance therapy was discontinued at 6 months to prevent adverse events associated with long-term corticosteroid use. Because the treatment for JDM is usually continued for at least 2 years,[6,8] the duration of the initial treatment seems short. However, premature cessation of treatment usually leads to early relapse of JDM. Thus, the short duration of treatment may not have been associated with the relapse 8 years after the initial onset in our patient. Although infections often trigger the onset or relapse of JDM,[9,10] there were no infectious episodes in our patient within 3 months before the relapse of JDM. Recently, 2 possible factors, dysphonia and high CDASI[11] score (CDASI >20), have been associated with relapse in a population of dermatomyositis and JDM.[12] However, this patient did not have dysphonia, and CDASI was less than 20.

The prognosis of late recurrent JDM is not fully understood. Of the 2 previously reported cases, one had been successfully treated with PSL monotherapy until the relapse, whereas the other showed

spontaneous remission.[4,5] Although the initial episode of JDM was completely cured by short-term corticosteroid-based treatment, additional intensive immunosuppressive therapy with tacrolimus was required to control the prolonged skin lesions in the relapse. Thus, the late recurrence of monocyclic JDM could be intractable and require attention.

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LETTER

Tacrolimus in combination with methotrexate and corticosteroid for the treatment of child-onset anti-signal recognition particle antibody-positive necrotizing myopathy

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Anti-signal recognition particle (SRP) antibody-associated immune-mediated necrotizing myopathy (SRP-IMNM) is a rare but the most severe form of both adult and juvenile inflammatory myopathies and often refractory to various therapies (1, 2). We report a boy with SRP-IMNM who was successfully treated with tacrolimus in combination with corticosteroid and weekly methotrexate (MTX).

An 8-year-old Japanese boy was admitted to Shizuoka Children's Hospital because of muscle weakness, muscle pain, and gait disorder which had slowly progressed since he was 6 years old and was suddenly exaggerated after an influenza infection. Initial examination demonstrated generalized muscle atrophy and weakness graded 3 on manual muscle testing (MMT). Gower's sign and symmetrical winged scapula were evident, although his facial muscles, respiration, and swallowing were spared. Laboratory examination demonstrated a white blood cell count of 7×10^9/L with normal differentiation, haemoglobin 119 g/L, platelet count 402×10^9/L, C-reactive protein < 10 mg/L, aspartate aminotransferase 149 IU/L, alanine aminotransferase 131 IU/L, lactate dehydrogenase 924 IU/L, creatine phosphokinase (CK) 5896 IU/L, and aldolase 63.9 IU/L. Serum levels of immunoglobulin and complements were within normal levels. Immunological studies demonstrated positive antinuclear antibodies at 1:160, but tests were negative for anti-Jo-1 and anti-ribonucleoprotein antibodies. T2-weighted magnetic resonance imaging (MRI) demonstrated high-intensity signals in his proximal muscles. Muscle biopsy demonstrated necrosis and regeneration of the muscle fibres with patchy dense infiltration of inflammatory cells (Figure 1). There were no skin, pulmonary, or cardiac lesions. Although limb-girdle muscular dystrophy was initially suspected, progression of muscle weakness and atrophy in association with fever suggested inflammatory myopathy. Two courses of methylprednisolone pulse therapy led to partial improvement of his muscle power and declines in his serum levels of CK (278 IU/L) and aldolase (7.0 IU/L). Prednisolone (PSL) was started at a dose of 40 mg (2 mg/kg) and gradually decreased. When he was referred to Hokkaido University Hospital, he could not raise his head and legs from the prone position, although the muscle power of his both proximal and distal extremities had recovered to grade 4–5 on MMT. As the dose of PSL was gradually decreased to 7.5 mg, serum levels of CK and aldolase elevated to 800 IU/L and 15.5 IU/L, respectively, which required an increase in the dose of PSL. Weekly methotrexate (MTX) 8 mg/week showed no apparent steroid-sparing effect. At the age of 11 years, the disease flared, with elevated CK levels and MRI abnormalities. High-dose intravenous immunoglobulin therapy was not tolerated because of aseptic meningitis soon after the therapy. Following the commencement of tacrolimus (2 mg/day), the muscle power of his body trunk gradually recovered. Currently, he has no weakness on PSL 6 mg/day in combination with tacrolimus and weekly MTX. Anti-SRP antibodies were detected by an RNA-immunoprecipitation assay in the serum obtained at referral to Hokkaido University Hospital. Other myositis-specific or -associated autoantibodies such as anti-aminoacyl tRNA synthetase, anti-CADM-140, anti-MJ, and anti-155/140 antibodies were all negative.

SRP-IMNM accounts for up to 4% of adult idiopathic inflammatory myopathies and only 1–2% of juvenile idiopathic inflammatory myopathies (1, 2). SRP-IMNM consists of two types: chronic and acute/subacute (3). Our case showed insidious onset, and accordingly, was the chronic type. In addition to extremely elevated serum CK levels, necrosis and regeneration of the muscle fibre with no or little cellular infiltration in SRP-IMNM often lead to a misdiagnosis of muscular dystrophy (2). Similarly to our patient, acute exacerbation of SRP-IMNM immediately after an influenza-like illness has been reported in juvenile cases (4).

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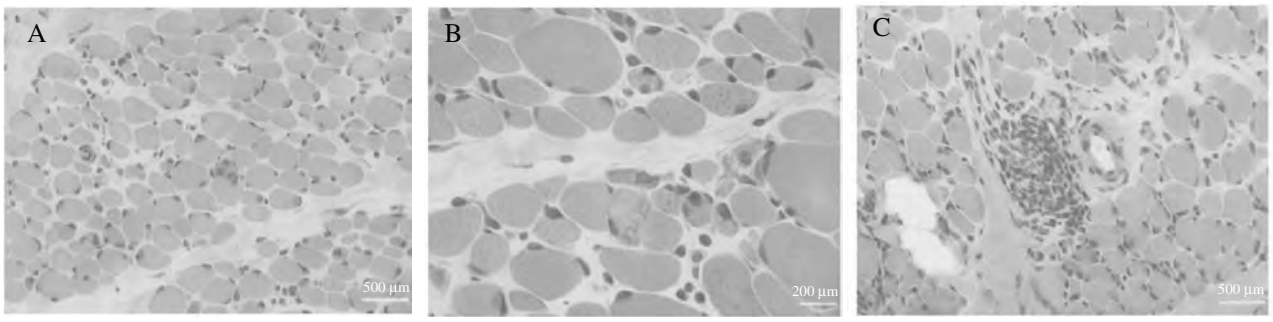

Figure 1. Histological findings of the muscle biopsy (hematoxylin–eosin staining). (A) Regenerated muscle fibres are found in the background of mildly atrophic muscle. (B) The muscle fibres with regeneration and necrosis are occasionally clustered intermingled with small lymphocytes. (C) Patchily distributed dense infiltration of inflammatory cells is seen.

To date, eight paediatric patients with mostly acute/subacute SRP-IMNM have been reported (4–6). Cytotoxic agents in combination with infliximab have failed to suppress the disease progression (4). One case report demonstrated a good response to plasma exchange in combination with steroid pulse and intravenous cyclophosphamide therapies (6). In adult cases, a clinical effect of rituximab has been reported, which supports an antibody-mediated complement-dependent mechanism suggested by *in vitro* studies in SRP-IMNM (7, 8). On the other hand, the involvement of T-helper (Th1) cells expressing interferon-γ, a potential inducer of major histocompatibility complex (MHC) class I molecules, has been reported (9). Indeed, muscle fibres of IMNM patients aberrantly express MHC class I molecules, which may cause muscle damage associated with endoplasmic reticulum stress and unfolded protein responses (2, 10). The clinical effect of tacrolimus in our patient and previously reported adult patients with a chronic course (2) supports a pathological role of T cells, at least in a subset of SRP-IMNM.

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Original article

Clinical and laboratory features of fatal rapidly progressive interstitial lung disease associated with juvenile dermatomyositis

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Abstract

Objective. Rapidly progressive interstitial lung disease (RP-ILD) is a rare but potentially fatal complication of JDM. The aim of this study was to establish markers for the prediction and early diagnosis of RP-ILD associated with JDM.

Methods. The clinical records of 54 patients with JDM were retrospectively reviewed: 10 had RP-ILD (7 died, 3 survived), 19 had chronic ILD and 24 were without ILD. Routine tests included a high-resolution CT (HRCT) scan of the chest and measurement of serum levels of creatine phosphokinase, ferritin and Krebs von den Lungen-6 (KL-6). Anti-melanoma differentiation-associated gene 5 (MDA5) antibodies and IL-18 levels were measured by ELISA.

Results. No differences were found in the ratio of juvenile clinically amyopathic DM between the three groups. Initial chest HRCT scan findings were variable and could not distinguish between RP-ILD and chronic ILD. Anti-MDA5 antibodies were positive in all 8 patients with RP-ILD and 10 of 14 with chronic ILD, but none of the patients without ILD. Serum levels of anti-MDA5 antibody, ferritin, KL-6 and IL-18 were significantly higher in the RP-ILD group than in the chronic ILD and non-ILD groups. Serum levels of IL-18 positively correlated with serum KL-6 ($R = 0.66$, $P < 0.001$).

Conclusion. High serum levels of IL-18, KL-6, ferritin and anti-MDA5 antibodies (e.g. >200 units by ELISA) are associated with RP-ILD. These can be used as an indication for early intensive treatment. Both alveolar macrophages and autoimmunity to MDA5 are possibly involved in the development of RP-ILD associated with JDM.

Key words: juvenile dermatomyositis, interstitial lung disease, interleukin-18, anti-melanoma differentiation-associated gene 5, KL-6, ferritin.

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Introduction

JDM is a rare inflammatory disease characterized by typical skin rashes and muscle weakness. It affects 2-3/million children/yr, however, the frequency differs between ethnic groups [1-3]. Prior to the 1960s, more than one-third of patients died of the disease [4]. Advances in treatment with corticosteroids and immunosuppressants have reduced the mortality rate of JDM to 1–5% [5, 6]. Interstitial lung disease (ILD) is observed in up to 50% of adult DM cases and is a major cause of death when rapidly progressive ILD (RP-ILD) develops in association with clinically amyopathic DM (CADM) [7]. Nevertheless, radiologically confirmed ILD complicates only 2–14% of JDM cases [8, 9]. In a recent Japanese nationwide physician questionnaire-based survey of severe paediatric rheumatic diseases from 2005 to 2009, 13 deaths in patients with JDM were reported; there were >3 deaths in patients with SLE during the same period [10]. Complete clinical records and sera were available in 6 of the 13 JDM patients who died. Surprisingly, all six deaths were attributed to ILD. The Japanese survey demonstrates that RP-ILD is a major cause of death related to JDM in Japan. This prompted this study to establish markers for the prediction and early diagnosis of RP-ILD associated with JDM.

We have reported that the serum Krebs von den Lungen-6 (KL-6) level is a useful marker of ILD associated with JDM [11]. Furthermore, similar to adult cases, anti-CADM-140/melanoma differentiation-associated gene 5 (MDA5) autoantibodies are possible diagnostic markers of JDM-associated ILD [12–14]. Recent studies on adult DM-associated ILD have demonstrated elevated serum levels of both ferritin and IL-18, which is produced by macrophages and dendritic cells (DCs) and activates Th1 response [15–18]. However, cytokine profiles have not been reported in JDM-associated ILD, possibly because of the rarity of the complication. In the present Japanese nationwide collaborative study, we focused on the clinical, radiological and laboratory features of patients with JDM-associated RP-ILD and compared them with those of JDM patients without RP-ILD.

Patients and methods

Definition

Classic JDM was diagnosed according to Bohan and Peter [1]. As this was a retrospective study, muscle weakness was determined by the assessing physician and was not based on validated muscle assessment such as manual muscle test. Juvenile CADM (JCADM) was diagnosed according to modified Gerami *et al.* [19] criteria: hypomyopathic DM was defined as patients with classical cutaneous manifestations of DM and no proximal muscle weakness but with evidence of myositis on laboratory, electrophysiological and/or radiological testing; amyopathic DM patients had no clinical or laboratory evidence of myositis. Gerami *et al.* [19] originally defined JCADM as patients fulfilling the above conditions for ⩾6 months after onset without systemic treatment. However, in the present study we classified all patients without weakness at the commencement of treatment as having CADM, because most of the patients with ILD require early treatment with systemic corticosteroids and immunosuppressants, usually within 6 months after the onset of JDM. The diagnosis of ILD was made by using high-resolution CT (HRCT) scan of the chest and was confirmed by both a radiologist and a paediatric rheumatologist at each institute. RP-ILD was defined as the progression of dyspnoea or HRCT findings within 3 months after the onset of respiratory symptoms or at the time of diagnosis of JDM.

Patients

In the nationwide survey of severe paediatric rheumatic diseases, paediatricians in inpatient health care facilities in Japan were asked about the number of patients with rheumatic disease who had visited the clinic in 2009 and the number of deaths between 2005 and 2009. All the patients were Japanese. Nine institutes reported 13 deaths of children with classic JDM or JCADM. Furthermore, respondents were asked to answer questions about data based on the patients' medical charts. Among the 13 deaths in children with JDM, complete clinical records and sera were available in six patients and were retrospectively analysed. One patient died of *Pneumocystis jirovecii* pneumonia and was excluded. The other five patients died from RP-ILD [10, 21–24]. The number of cases of RP-ILD was increased by including two patients who died of JDM-associated RP-ILD before 2004 and by including three surviving cases with RP-ILD available from the authors' institutes [14, 25]. As a result, 10 patients (7 deceased and 3 survivors) were included in this category. Twenty patients with ILD associated with JDM (followed up by the authors) did not show a rapidly progressive course. One patient was excluded, leaving 19 patients in this chronic ILD group. The excluded patient had chronic ILD and severe myositis complicated by macrophage activation syndrome (MAS); the associated macrophage activation was presumed to be the cause of markedly elevated levels of IL-18 [20]. The clinical features of seven patients with ILD have been previously reported [14, 20–25]. Twenty-four patients with JDM without ILD on chest CT scan from Shinshu University Hospital, Aichi Children's Health and Medical Center and Hokkaido University Hospital were included in the non-ILD group.

Biochemical and serological analyses

Sera were collected from the patients at diagnosis of ILD or, in the case of patients without ILD, at diagnosis of JDM and stored at −20°C until use. Routine laboratory tests included measurement of serum levels of creatine phosphokinase (CK), ferritin and a marker for ILD, KL-6. Anti-MDA5 antibody levels were measured by both ELISA and immunoprecipitation as previously described [13]. Serum IL-18 levels were measured by ELISA according to the manufacturer's protocol (MBL, Nagoya, Japan).

Statistical analyses

The data were analysed by Tukey–Kramer's multiple comparison tests, Fisher's exact tests with Bonferroni adjustment and Pearson's product–moment correlation coefficient using JMP 10.0 for Windows (SAS Institute, Cary, NC, USA)

Ethics

The ethics committee of Shinshu University approved the present study. Written consent was obtained from the parents of the patients according to the Declaration of Helsinki.

Results

Clinical and radiological features of JDM-associated RP-ILD

The clinical and laboratory findings of 10 children with RP-ILD are shown in Table 1. Eight patients showed apparent muscle weakness, although the other two patients were classified as having JCADM. Nine of the 10 patients had characteristic skin findings and high fever (temperature >38°C). Gottron's papules were the most common skin lesion, followed by malar erythema and erythema of the knees and elbows. In contrast, heliotrope rash and ulcerative lesions were rarely observed. Respiratory symptoms such as dry cough, dyspnoea and fine crackles were observed in five, four and six patients, respectively, at diagnosis of ILD. The remaining patients had no respiratory symptoms or signs of ILD at diagnosis. Seven of the 10 patients with RP-ILD died of respiratory failure 1–4 months after the diagnosis of ILD.

HRCT findings of RP-ILD

Lung HRCT scan findings at the time of diagnosis of ILD are summarized in Table 1. Subpleural curvilinear shadow was the most predominant finding. Although localized ground glass opacity (GGO) was observed in six patients, GGOs developed during the disease course in all patients who died of ILD. Three patients (patients 3, 4 and 6) had consolidation around bronchovascular bundles (CABBs) accompanying extensive GGOs. Four patients (patients 1, 4, 6 and 7) developed an air leak such as pneumomediastinum and pneumothorax during the course of the disease. Although patient 1 had only bilateral pleural effusion on the initial HRCT, both elevated serum KL-6 levels and increased gallium-67 uptake on scintigraphy of the lungs were noted. One month later, chest HRCT demonstrated marked consolidation at the base of both lungs [22]. In all cases of death, the final diagnosis leading to death was acute interstitial pneumonia with acute and progressive respiratory failure accompanied by GGOs on HRCT. The clinical diagnosis was consistent with diffuse alveolar damage (DAD) patterns on autopsy or biopsy.

Comparison of the clinical features of the chronic ILD and non-ILD groups

The clinical features of the three groups are summarized in Table 2. The age of onset was significantly higher in the chronic ILD group than in the non-ILD group. No differences were found in the ratio of JCADM among the three groups. Seven of the 10 patients with RP-ILD died despite intensive treatment with methylprednisolone pulse therapy in combination with CSA and/or i.v. CYC, whereas none of the patients in the chronic ILD and non-ILD groups died.

Comparison of the laboratory features of the chronic ILD and non-ILD groups

The laboratory findings of the three groups are summarized in Table 2. Although serum CK levels were significantly higher in the non-ILD group than in the chronic ILD group, there was no significant difference between the RP-ILD and non-ILD groups. Serum KL-6 levels were significantly higher in the RP-ILD group than in either the chronic ILD or the non-ILD group. Serum ferritin levels in both the RP-ILD and chronic ILD groups were higher than those in the non-ILD group.

All 8 patients with RP-ILD and 10 of 14 patients with chronic ILD were positive for anti-MDA5 antibodies, but none of the 22 patients without ILD were positive for the antibodies. Titres of anti-MDA5 antibodies were significantly higher in the RP-ILD group than in either the chronic ILD or the non-ILD group (Table 2 and Fig. 1).

Serum IL-18 levels were significantly higher in the RP-ILD group than in the chronic ILD or non-ILD group (Table 2 and Fig. 2). Serum IL-18 levels correlated well with serum KL-6 levels ($R=0.66$) but not with ferritin levels (Fig. 3 and data not shown). A patient with severe myositis, MAS and chronic ILD, who was excluded from statistical analyses, showed high serum levels of IL-18 (3265 pg/ml), ferritin (2235 ng/ml) and KL-6 (2096 U/ml), but only mild elevation of anti-MDA5 antibody levels (9.5 units) [20].

Sera were serially tested in two patients with RP-ILD who survived and two patients with chronic ILD who were positive for anti-MDA5 antibody. Serum levels of anti-MDA5 antibodies and IL-18 returned to below the cut-off values or to undetectable levels at remission of ILD (data not shown).

Discussion

Although previous studies have reported multiple organ involvement such as muscle weakness, aspiration pneumonia, myocarditis, peptic ulcer and sepsis as causes of death in JDM, the prognosis has improved through recent advances in the management of the disease [5, 26]. The Japanese nationwide survey demonstrated RP-ILD as a remaining major cause of death in JDM. The UK and Ireland national registry from 2000 to 2005 indicated only one death in patients with JDM [27]. A recent study in the USA that enrolled 329 patients with JDM recorded only eight deaths from 1999 to 2011, three of which were due to ILD [28]. Given the higher prevalence of adult CADM-associated RP-ILD in Asian countries, Japanese children with JDM may also be predisposed to RP-ILD compared with other ethnicities [7, 29]. In our previous nationwide survey we analysed the number of patients

Table 1 Clinical course of 10 children with JDM or JCADM-associated rapidly progressive ILD

Sex	Male	Female	Female	Male	Female	Female	Male	Female	Female	Male
Diagnosis	JDM	JCADM	JDM	JDM	JDM	JCADM	JDM	JDM	JDM	JDM
Age of JDM onset, years	9	4	4	10	6	4	7	1	13	7
Fever (>38°C)	+	+	+	+	+	+	+	+	+	−
Gottron's sign	+	+	+	−+	+	+	+	+	+	+
Malar rashes	+	+	+	−	+	+	+	+	+	+
Heliotrope rashes	−	−	−	−	−	+	+	+	+	+
Skin ulceration	−	−	−	−	−	−	+	+	−	−
Dry cough or dyspnoea at diagnosis of ILD	−	+	−	+	+	+	+	−	−	−
Interval between onset of JDM and diagnosis of ILD, months	5	3	1	1	5	6	2	5	1	1
Initial CT scan	PE	SCS	CABB, GGO, TB, SCS	CABB, GGO, SCS	SCS	CABB, GGO, TB, SCS	GGO	CABB, SCS	GGO, SCS	GGO, SCS
Outcome	Died	Died	Died	Died	Died	Died	Died	Alive	Alive	Alive
Autopsy	DAD	DAD	DAD	DAD[a]	DAD	ND	DAD			
Interval between diagnosis of ILD and death, months	4	4	1	3	2	1	2			
Interval between onset of respiratory symptoms and death, months	4	2	1	7 weeks	6.5	5	2			
Reference	[22]		[23]				[20]		[20]	

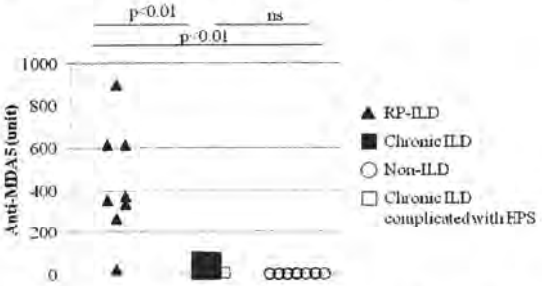
[a]Surgical lung biopsy. CABB: consolidation around bronchovascular bundles; DAD: diffuse alveolar damage; GGO: ground-glass opacity; JCADM: juvenile clinically amyopathic dermatomyositis; ND: not done; PE: pleural effusion; RP-ILD: rapidly progressive interstitial lung disease; SCS: subpleural curvilinear shadow; TB: traction bronchiectasis.

Table 2 Comparison of clinical and laboratory features

				P-value			
	RP-ILD ($n=10$)	Chronic-ILD ($n=19$)	Non-ILD ($n=24$)	*a*	*b*	*c*	Statistical analysis
Age, mean (S.D.), years	6.3 (3.5)	9.0 (3.6)	6.0 (3.3)	0.119	0.073	0.979	Tukey–Kramer test[b]
Sex, male/female	4/6	7/12	11/13	0.466	0.390	0.636	Fisher's exact test[a]
JCADM, *n*	2	2	3	0.337	0.613	0.367	Fisher's exact test[a]
Mortality, % (*n* dead/ *n* alive)	70.0 (7/3)	0.0 (0/19)	0.0 (0/24)	<0.001	0.390	<0.001	Fisher's exact test[a]
CK, mean (S.D.), IU/l	403.1 (604.2)	473.7 (1304.8)	1790.3 (3202.1)	0.999	0.012	0.043	Tukey–Kramer test[b]
KL-6, mean (S.D.), U/ml	2045.6 (881.5)	718.3 (699.0)	283.1 (113.3)	<0.001	0.001	<0.001	Tukey–Kramer test[b]
Anti-MDA5, mean (S.D.), units	387.9 (288.9) ($n=8$)	33.3 (30.1) ($n=14$)	2.4 (1.7) ($n=22$)	<0.001	0.734	<0.001	Tukey–Kramer test
Anti-MDA5 positive cases (cut-off value 8.0 units), %	100 ($n=8$)	71.4 ($n=14$)	0.0 ($n=22$)	0.327	<0.001	<0.001	Fisher's exact test[a]
IL-18, mean (S.D.), pg/ml	1447.0 (941.4) ($n=8$)	470.0 (341.6) ($n=19$)	570.1 (474.0) ($n=24$)	<0.001	0.904	<0.001	Tukey–Kramer test
Ferritin, mean (S.D.), ng/ml	355.4 (136.8) ($n=8$)	222.8 (129.3) ($n=19$)	131.2 (160.4) ($n=24$)	0.119	0.018	<0.001	Tukey–Kramer test[b]

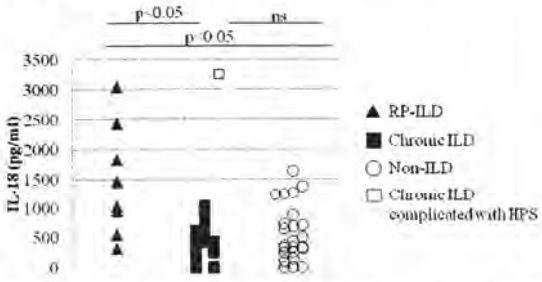
[a]To identify the difference among three groups, Fisher's exact test with Bonferroni adjustment was performed. $P<0.016$ was considered significant. [b]Tukey–Kramer test after log transformation. *a*: RP-ILD *vs* chronic-ILD; *b*: chronic-ILD *vs* non-ILD; *c*: RP-ILD *vs* non-ILD; CK: creatine phosphokinase; ILD: interstitial lung disease; JCADM: juvenile clinically amyopathic DM; KL-6: Krebs von Lungen-6; MDA5: differentiation-associated gene 5; ns: not significant; RP-ILD: rapidly progressive ILD.

Fig. 1 Chart of serum anti-MDA5 concentration



Serum anti-MDA5 antibody titres were significantly higher in RP-ILD than inchronic ILD or non-ILD with $P < 0.05$ (Tukey–Kramer test). One patient with chronic ILD (open square) is excluded from the statistical analysis. ns: not significant; ILD: interstitial lung disease; RP-ILD: rapidly progressive interstitial lung disease; HPS: haemophagocytic syndrome.

Fig. 2 Chart of serum IL-18 concentration

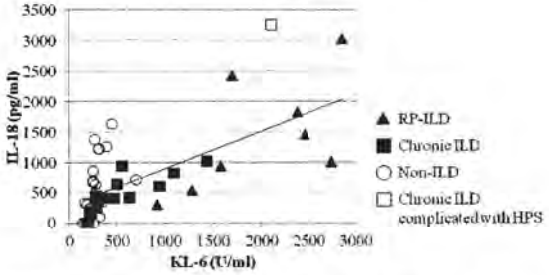


Serum IL-18 levels were significantly higher in RP-ILD than in chronic ILD or non-ILD with $P < 0.05$ (Tukey–Kramer test). One patient with chronic ILD (open square) is excluded from the statistical analysis. ns: not significant; ILD: interstitial lung disease; RP-ILD: rapidly progressive interstitial lung disease; HPS: haemophagocytic syndrome.

with rheumatic diseases who visited hospitals in 2009 and the number and causes of deaths between 2005 and 2009. Furthermore, we included five additional patients with RP-ILD who had presented to our hospital before the study period. A cohort study that captured all JDM cases nationwide is needed to determine the actual prevalence of ILD and the mortality rate of Japanese patients with JDM.

In the present study we could not identify any characteristic clinical features specific to JDM associated with RP-ILD. HRCT showed diffuse GGOs in all the deceased patients, which was consistent with a DAD pattern on autopsy. However, initial HRCT findings were variable and indistinguishable from those of survivors with RP-ILD or chronic ILD. Final radiological and pathological findings may represent merely the end stage of the disease,

Fig. 3 Correlation between KL-6 and IL-18



The closed triangle, closed square and open circle plots show RP-ILD, chronic ILD and non-ILD groups respectively. The coefficient of determination for total data was 0.66 ($P < 0.001$) indicating strong correlation between KL6 and IL18 (Pearson product–moment correlation coefficient). One patient with chronic ILD (open square) is excluded from the statistical analysis. ILD: interstitial lung disease; RP-ILD: rapidly progressive interstitial lung disease; HPS: haemophagocytic syndrome.

regardless of the original clinicopathological entities of ILD. Two of the four patients with pneumomediastinum and pneumothorax (patients 6 and 7, respectively) showed ulcerative lesions of their skin. Vasculopathy associated with JDM could have caused ulceration of both skin and airway walls, as suggested in adult cases [30].

We have reported that anti-MDA5 antibody is a useful disease marker of JDM-associated ILD [14]. The present study also demonstrated that higher levels of the antibody (e.g. >200 units by ELISA) were found in all but one of the RP-ILD group (patient 1), whereas the chronic ILD group had lower titres (<100 units). In addition, the titre of anti-MDA5 antibody declined to below the cut-off value (data not shown). Thus the antibody may help to differentiate RP-ILD from chronic ILD and may help in monitoring the response to treatment in JDM-associated RP-ILD. A similar correlation of the antibody titre with the activity of ILD has recently been reported in adult DM [31]. The antibody was originally reported as a disease marker of CADM-associated RP-ILD but is also detected in some adult cases of DM with chronic ILD on the basis of sensitive immunoblot analyses [12, 13, 32]. Given that anti-MDA5 antibodies were detected regardless of the severity of muscular lesions in our series, the antibodies are possibly related to ILD itself rather than JCADM. On the other hand, anti-MDA5 antibodies are associated with symmetrical arthritis, myositis and ILD, but not with CADM-associated RP-ILD in the USA [33]. Thus the clinical significance of the antibodies may differ between ethnic groups.

In addition to anti-MDA5 antibody, serum levels of ferritin, KL-6 and IL-18 were associated with RP-ILD, although the highest serum level of IL-18 was detected in a patient with severe myositis complicated by MAS and

chronic ILD. IL-18 is produced by both macrophages and DCs in the muscle tissues of patients with DM; IL-18 attracts plasmacytoid DCs that produce type 1 IFNs and correlates with disease activity in adult DM/PM patients without ILD [16, 17]. Extremely high levels of ferritin and IL-18 are reported in systemic JIA-associated MAS [34]. In addition, the association of elevated serum IL-18 levels with RP-ILD, as demonstrated in the present study, has also been reported in adult DM-associated ILD [35–37]. Activated alveolar macrophages are consistently found in bronchoalveolar lavage fluid from patients with DM-associated ILD [15]. Together, macrophages or DCs in the muscle, bone marrow and lungs are likely to play critical roles in myositis, MAS and JDM-associated ILD, respectively. Thus high levels of IL-18 or ferritin may not necessarily reflect the presence of RP-ILD in patients with severe myositis or MAS.

Studies of bronchoalveolar lavage fluid have also demonstrated restricted Vβ gene usage of T cell receptors and the presence of $CD8^+HLA\text{-}DR^+$ T cells in DM-associated ILD [38, 39]. Because IL-18 stimulates Th1 cells [18], T cells activated by alveolar macrophage-derived IL-18 could contribute to tissue destruction of the lung in an antigen-specific manner. Furthermore, since autoantigens identified by reactivity with autoantibodies also stimulate self-reactive T cells [40], MDA5 could be a target antigen of the T cells in DM/JDM-associated RP-ILD.

Of note, there was strong correlation between serum levels of IL-18 and KL-6. KL-6 is produced by type II pneumonocytes and bronchiolar epithelial cells, particularly during their regeneration. Furthermore, KL-6 functions as a chemoattractant for fibroblasts and is generally considered a biomarker of ILD that reflects the severity of the disease [41–43]. Given that the correlation between KL-6 and IL-18 is not initially observed in adult DM-associated ILD [44], remodelling of the lung may occur at an earlier phase of RP-ILD in JDM rather than in adulthood.

All three survivors with RP-ILD were found to have ILD on the basis of elevated KL-6 levels and HRCT findings and were treated with methylprednisolone pulse therapy in combination with CSA and/or monthly i.v. CYC before the development of respiratory symptoms. Thus screening for ILD using chest HRCT scan and routine monitoring of KL-6 levels is recommended for all patients with JDM regardless of respiratory symptoms. We have reported the efficacy of CSA in combination with methylprednisolone pulse therapy for JDM-associated ILD [21]. Early combination therapy with corticosteroid, CSA and i.v. CYC may further reduce the mortality of RP-ILD as reported in adult cases [45].

In conclusion, our results suggest the involvement of both autoimmunity and alveolar macrophages in the development of RP-ILD. Initial CT findings are often indistinguishable between RP-ILD and chronic ILD associated with JDM, however, early development of diffuse GGOs may predict poor outcome. Elevated serum levels of IL-18, KL-6, ferritin and anti-MDA5 antibody (>200 units by ELISA) are associated with RP-ILD in JDM and are indications for early intensive treatment.

Rheumatology key messages

- Rapidly progressive interstitial lung disease is a major cause of death in Japanese patients with JDM and needs early attention.
- Elevated serum IL-18, KL-6, ferritin and MDA5 antibodies are associated with RP-ILD.
- Both autoimmune processes and alveolar macrophages could be involved in the development of JDM-associated RP-ILD.

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ORIGINAL ARTICLE

A CD57$^+$ CTL Degranulation Assay Effectively Identifies Familial Hemophagocytic Lymphohistiocytosis Type 3 Patients

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Abstract
Purpose Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) is a genetic disorder that results in immune dysregulation. It requires prompt and accurate diagnosis. A natural killer (NK) cell degranulation assay is often used to screen for FHL3 patients. However, we recently encountered two cases of late-onset FHL3 carrying novel *UNC13D* missense mutations: in these cases, the degranulation assays using freshly isolated and interleukin (IL)-2-activated NK cells yielded contradictory results. Since the defective degranulation of CD57$^+$ cytotoxic T lymphocytes (CTLs) in these cases was helpful for making the diagnosis, we assessed whether the CD57$^+$ CTL degranulation assay more effectively identified FHL3 patients than the NK cell assays.
Methods Forty additional patients with hemophagocytic lymphohistiocytosis were prospectively screened for FHL3 by measuring the perforin expression in NK cells and the expression of Munc13-4, syntaxin-11, and Munc18-2 in platelets and by performing NK cell and CTL degranulation assays. The results were confirmed by genetic analysis.
Results The freshly isolated NK cell degranulation assay detected FHL3 patients with high sensitivity (100%) but low specificity (71%). The IL-2-stimulated NK cell assay had improved specificity, but 3 out of the 31 non-FHL3 patients still showed degranulation below the threshold level. The CD57$^+$ CTL degranulation assay identified FHL3 patients with high sensitivity and specificity (both 100%).
Conclusions The CD57$^+$ CTL degranulation assay more effectively identified FHL3 patients than the NK cell-based assays.

Keywords Familial hemophagocytic lymphohistiocytosis type 3 · lysosomal degranulation defect · functional screening assay · *UNC13D*

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Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening syndrome that is characterized by immune dysregulation and hyper-inflammation. It is also histologically characterized by the presence of benign hemophagocytic macrophages [1–3]. HLH is classified into primary (genetic) or secondary (acquired) forms, but this distinction is difficult to make in clinical practice [1, 2].

Familial hemophagocytic lymphohistiocytosis (FHL) is the main form of primary HLH. Several FHL mutations have been identified, namely, in the genes encoding perforin (*PRF1*;

FHL2) [4], Munc13-4 (*UNC13D*; FHL3) [5], syntaxin-11 (*STX11*; FHL4) [6], and Munc18-2 (also known as syntaxin-binding protein 2) (*STXBP2*; FHL5) [7, 8]. Perforin is an effector molecule that is contained in the cytolytic granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Munc13-4, syntaxin-11, and Munc18-2 are involved in the intracellular trafficking or the fusion of these granules to the plasma membrane and the delivery of their contents into target cells. Thus, the hallmark finding of FHL is the defective cytotoxic activity of CTLs and NK cells [9].

The early diagnosis of FHL is clinically important because its treatment strategy differs greatly from that of secondary HLH. While aggressive immunosuppressive therapy is required for both the primary and secondary forms of HLH in the initial period, it can be gradually tapered for most secondary HLH cases once the clinical remission is achieved. By contrast, strong maintenance therapy followed by hematopoietic stem cell transplantation (HSCT) is mandatory for patients with FHL [1, 2].

While certain combinations of common laboratory parameters are useful for identifying patients with a high possibility of FHL, functional and molecular analyses are mandatory for confirming the diagnosis [10, 11]. A reliable way to screen for FHL2 is to use flow cytometry to detect perforin expression in NK cells [12, 13]. Functional screening for FHL3-5 involves the detection of CD107a expression on the surface of NK cells; this measures the release of cytolytic granules [7, 8, 14–16]. However, a considerable proportion of secondary HLH patients also exhibits abnormal NK cell degranulation and some patients with FHL3-5 show normal NK cell degranulation after stimulation with interleukin (IL)-2 [16, 17].

Another possibility is to measure CTL degranulation. While such a CTL-based assay has been described, the methodology is not standardized and contradictory results have been reported [16–18]. Nevertheless, numerous studies have shown that the CTL expressing CD57 have high cytotoxic potential [19]. It was also recently reported that CTL expression of CD57 correlates strongly with the intracellular expression of cytolytic molecules such as perforin and granzymes [20] and is a measure of the degranulation capacity of CTLs [21]. Thus, it is proposed that analyzing $CD57^+$ CTL degranulation may be useful for detecting patients with defective cytolytic granules release. However, the usefulness of this method has not been evaluated.

In this report, we describe two late-onset FHL3 patients carrying novel *UNC13D* missense mutations. Notably, these patients exhibited normal IL-2-activated NK cell degranulation but had defective $CD57^+$ CTL degranulation. The latter observation was helpful for diagnosing these patients. Consequently, to test the ability of the $CD57^+$ CTL degranulation assay to detect FHL3 patients, we prospectively screened HLH patients with this assay. We found that this assay distinguished the FHL3 patients from patients with other forms of HLH with high sensitivity and specificity.

Materials and Methods

Patients

Two late-onset FHL3 cases were diagnosed in 2012 (patients 1 and 2). Thereafter, in February 2013–April 2014, prospective FHL screening was performed on 40 additional patients who were suspected by their referring physicians to have FHL (patients 3–42). As a control, blood obtained from healthy adults at the time of patient sampling was shipped for screening along with the patient samples. None of the patients had any sign of oculo-cutaneous albinism or had giant granules in their peripheral blood leucocytes. FHL was screened by measuring perforin expression in NK cells, by measuring Munc13-4, syntaxin-11, and Munc18-2 expression in platelets and by NK cell and CTL degranulation assays. Genetic analysis was performed in all patients to confirm the results. The characteristics of the enrolled patients and the genetic defects detected in the FHL patients are summarized in Tables 1 and 2, respectively. Informed consent was obtained from the patients and their parents in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Protein Expression Assays

All patients were assessed for perforin expression by their NK cells and their platelet expression of Munc13-4, syntaxin-11, and Munc18-2 as previously described, with some modifications [22, 23]. To determine the effect of the *UNC13D* missense mutations observed in the two late-onset FHL3 patients on Munc13-4 protein expression, FLAG-tagged complementary DNA (cDNA) carrying wild-type or missense mutated *UNC13D* sequences were constructed and transiently transfected into HEK293T cells. After overnight culture, the cells were harvested with or without additional incubation in the presence of 0.35 mM cycloheximide. Cell extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the fractionated proteins were electro-transferred onto polyvinylidene fluoride membranes. The membranes were blocked overnight in blocking buffer (5% skim milk) and incubated for 1 h at room temperature with anti-FLAG antibodies, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG

Table 1 Characteristics and assay outcomes of the enrolled patients

Patient no.	Final diagnosis	Age (months) at onset	Gender	Fresh NK cell degranulation (%)	Stimulated NK cell degranulation (%)	$CD57^+$ CTL degranulation (%)
1	FHL3	90	M	***5.1*** (***4.8***)	*38.2* (*40.3*)	***4.7*** (***2.5***)
2	FHL3	190	F	***6.9*** (***5.6***)	*43.0* (*38.6*)	***8.6*** (***9.3***)
3	FHL3	1	M	***8.1*** (***8.5***)	***9.8*** (***21.2***)	***0.5*** (***2.1***)
4	FHL3	0	M	***2.3*** (***5.2***)	***14.1*** (*33.2*)	***4.2*** (***3.7***)
5	FHL3	4	F	***8.3***	*43.0*	***4.5***
6	FHL3	9	M	***3.7***	***20.1***	***0.5***
7	FHL3	1	M	***8.8***	***22.2***	***9.7***
8	FHL3	1	M	***8.8***	***23.2***	***8.0***
9	FHL2	1	F	18.0	62.7	38.2
10	FHL2	0	M	29.4	91.8	45.3
11	XLP2	64	M	10.4	73.8	77.4
12	sJIA-MAS	80	M	***5.3*** (18.2)	46.4	28.7
13	CAEBV	156	M	***8.5***	51.8	28.1
14	EBV-HLH	15	M	19.4	67.9	30.2
15	EBV-HLH	17	M	**9.6** (**9.3**)	66.2 (70.7)	28.1 (57.6)
16	Unknown	61	M	20.2	73.3	56.0
17	EBV-HLH	14	M	11.3	46.6	74.1
18	Unknown	138	F	20.7	86.7	69.5
19	EBV-HLH	23	F	AU	AU	41.7
20	Sepsis	0	F	***6.8***	64.7	AU
21	EBV-HLH	62	F	***4.4*** (32.8)	*31.8*	**13.1** (52.5)
22	Unknown	183	F	13.3	48.8	70.6
23	sJIA MAS	9	F	24.7	73.7	41.8
24	Unknown	20	M	***7.5***	*39.6*	**22.5**
25	Unknown	11	M	29.1	79.9	57.8
26	Unknown	8	F	13.3	55.4	67.0
27	Unknown	12	F	16.2	71.0	77.2
28	EBV-HLH	18	F	AU	AU	98.3
29	Unknown	19	F	16.7	74.9	33.6
30	EBV-HLH	114	F	39.1	AU	76.4
31	HSV-HLH	0	F	***6.9***	57.9	71.5
32	Unknown	13	F	AU	93.8	AU
33	sJIA-MAS	19	M	31.5	78.7	59.7
34	Unknown	39	F	24.0	81.8	83.2
35	EBV-HLH	21	M	23.1	80.0	49.6
36	EBV-HLH	311	F	***6.6***	53.6	40.1
37	Unknown	4	M	***4.2***	66.1	67.8
38	Unknown	14	F	22.7	86.5	56.5
39	Unknown	5	F	14.5	83.5	65.2
40	Unknown	100	F	23.3	68.6	59.3
41	CMV-HLH	0	M	***7.9***	56.1	51.6
42	EBV-HLH	79	F	19.4	*33.0*	45.5

The assay results that were below the laboratory-defined thresholds (10% for the fresh NK, 30% for the IL-2 stimulated NK, and 25% for the $CD57^+$ CTL assays) are shown in bold letters, while those below the ROC-determined optimum thresholds (9.2% for the fresh NK, 44.7% for the IL-2 stimulated NK, and 11.5% for the $CD57^+$ CTL assays) are shown in italics. The numbers in the parentheses indicate the result of the second evaluation and were excluded from the statistical analyses

AU analysis unavailable, *HLH* hemophagocytic lymphohistiocytosis, *FHL* familial HLH, *XLP* X-linked lymphoproliferative syndrome, *EBV* Epstein-Barr virus, *CAEBV* chronic active EBV infection, *MAS* macrophage activation syndrome, *sJIA* systemic-onset juvenile idiopathic arthritis

polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Specific bands were visualized by the standard enhanced chemiluminescence method.

Antibodies

Rabbit polyclonal antibodies specific for human Munc13-4 and syntaxin-11 proteins were described previously [23, 24]. Rabbit polyclonal antibodies against human Munc18-2 protein were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit polyclonal anti-integrin αIIb (Santa Cruz Biotechnology), mouse polyclonal anti-β-actin (Sigma Aldrich, St. Louis, MO), and mouse monoclonal anti-FLAG (Sigma-Aldrich) antibodies served as primary antibodies in Western blotting. The monoclonal antibodies used in the flow cytometric analyses were fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (eBioscience, San Diego, CA), phycoerythrin (PE)-Cy7-conjugated anti-CD3 (Beckman Coulter, Brea, CA), FITC-conjugated anti-CD8 (eBioscience), V500-conjugated anti-CD16 (BD Biosciences), PE-conjugated anti-CD41a (BD Biosciences, San Jose, CA), allophycocyanin (APC)-conjugated anti-CD56 (Beckman Coulter), APC-conjugated anti-CD57 (BD Biosciences, San Jose, CA), PE-conjugated anti-CD107a (eBioscience), and PE-conjugated anti-perforin (eBioscience).

Table 2 Mutations identified in the FHL patients

Patient no.	Diagnosis	Gene mutated	1st allele	2nd allele
1	FHL3	*UNC13D*	c.754-1G>C (S)	c.2759A>G (M)
2	FHL3	*UNC13D*	c.1992+1G>A (S)	c.767G>A (M), c.1240C>T (M)
3	FHL3	*UNC13D*	c.754-1G>C (S)	c.118-308C>T (T)
4	FHL3	*UNC13D*	c.118-308C>T (T)	c.118-308C>T (T)
5	FHL3	*UNC13D*	c.2381delT (F)	c.322-1G>A (S)
6	FHL3	*UNC13D*	c.754-1G>C (S)	c. 1596+1G>C (S)
7	FHL3	*UNC13D*	c.118-308C>T (T)	c. 1596+1G>C (S)
8	FHL3	*UNC13D*	c.118-308C>T (T)	c. 1596+1G>C (S)
9	FHL2	*PRF1*	c. 1090_1091delCT (F)	c. 1288_1289insG (F)
10	FHL2	*PRF1*	c. 1090_1091delCT (F)	c.1A>G (LS)

Predicted mutation effects are shown in parentheses

S splice error, *M* missense, *T* transcriptional dysregulation, *F* frameshift, *LS* loss of start codon

Mutation Analyses

Genomic DNA was isolated from the peripheral blood mononuclear cells (PBMCs) of the patients by standard procedures. Primers were designed to amplify the coding exons and adjacent intronic sequences of the *PRF1*, *UNC13D*, *STX11*, *STXBP2*, *SH2D1A*, *BIRC4*, and *ITK* genes. Primers that would detect the deep intronic mutation in the UNC13D intron 1 were also designed. Primer sequences are available upon request. The amplified products were sequenced with an Applied Biosystems ABI3130 Genetic Analyzer (Life Technologies, Carlsbad, CA) or with a GS Junior System (Roche, Basel, Switzerland).

Lysosomal Degranulation Assays

To quantify lysosome exocytosis by NK cells, 2×10^5 PBMCs that were freshly isolated or stimulated for 36–48 h with IL-2 (50 U/mL) were cultured with or without 2×10^5 K562 cells and incubated in complete medium (RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum) for 2 h at 37 °C in 5% CO_2. For CTL degranulation analyses, 2×10^5 PBMCs stimulated for 36–48 h with IL-2 (50 U/mL) were cultured with 2×10^5 P815 cells with or without 0.5 μg/mL anti-CD3 mAb (OKT3). The cells were resuspended in phosphate-buffered saline supplemented with 2% fetal calf serum and 2 mM EDTA; stained with anti-CD3, anti-CD8, anti-CD16, anti-CD56, anti-CD57, and anti-CD107a monoclonal antibodies; and then analyzed by flow cytometry.

Statistical Analyses

Receiver operating characteristic (ROC) analysis was performed to determine the optimal threshold of each assay that would discriminate FHL3 patients from other HLH patients with the greatest sum of sensitivity and specificity. Laboratory-defined thresholds were also determined (i.e., the lowest degranulation value observed in the control subjects). The sensitivity and specificity of each assay were evaluated on the basis of these thresholds. The controls were excluded from the analyses. In the case of patients who were tested multiple times with particular assays, only the data from the first evaluation were included in the statistical analyses.

Results

Late-Onset FHL3 Patients

Patient 1 was a 90-month-old boy who developed HLH after a *Mycoplasma pneumoniae* infection (Table 1). Oral prednisolone was effective in controlling the disease, but subsequent tapering of the drug resulted in disease recurrence that associated with cerebral symptoms. Genetic analysis identified compound heterozygous *UNC13D* mutations, namely, c.754-1G>C on the paternal allele and c.2759A>G (p.Y920C) on the maternal allele (Table 2). Patient 2 was a girl who developed HLH associated with the abnormal infiltration of non-malignant lymphocytes in a vertebra at the age of 190 months (Table 1). She also developed cerebral symptoms but responded well to oral prednisolone therapy. *UNC13D* sequencing revealed that she carried a c.1992+1G>A mutation on the paternal allele and two missense mutations, namely, c.767G>A (p.R256Q) and c.1240C>T (p.R414C), on the maternal allele (Table 2).

Since the missense mutations found in the patients were novel, the diagnosis of FHL3 could not be made with certainty. Moreover, although the freshly isolated PBMCs of both cases exhibited decreased NK cell degranulation, their IL-2-stimulated NK cells released normal levels of cytolytic granules (Fig. 1a, b). Nevertheless, they both exhibited defective degranulation of their $CD57^+$ CTLs (Fig. 1c) and reduced expression of platelet Munc13-4 protein. These findings strongly supported the diagnosis of FHL3 (Fig. 1d). We

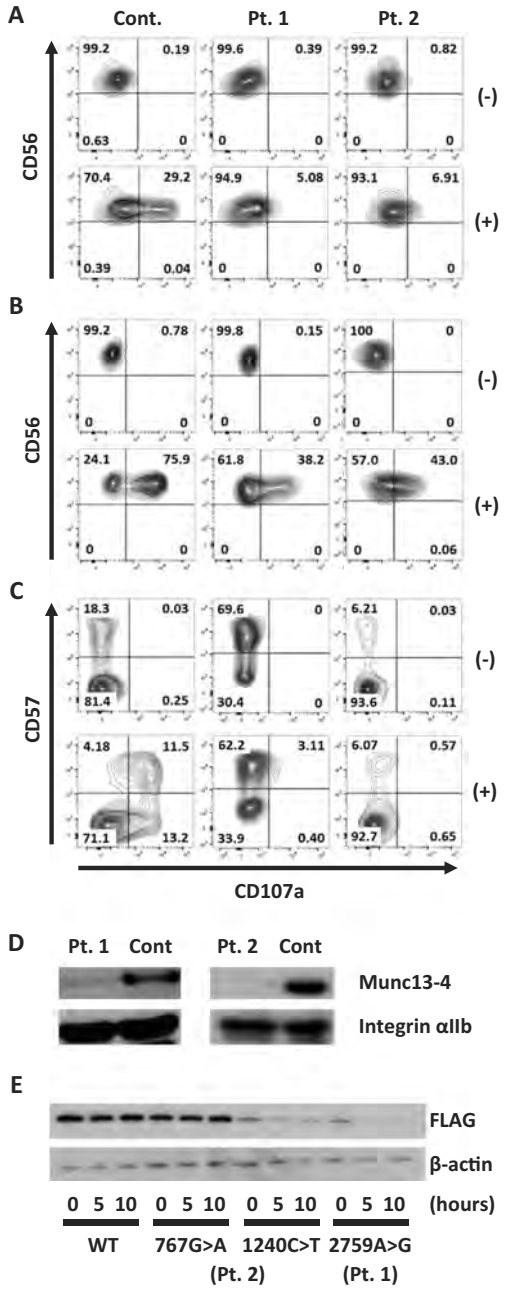

Fig. 1 FHL screening results of late-onset FHL3 patients. Freshly isolated (**a**) or IL-2 stimulated (**b**) PBMCs were co-cultured with (+) or without (−) K562 cells, and the expression of CD107a on the $CD3^{-}CD16^{+}CD56^{+}$ cell population was evaluated. **c** IL-2-stimulated PBMCs were co-cultured with P815 cells in the presence (+) or absence (−) of an anti-CD3 antibody and the expression of CD107a on the $CD3^{+}CD8^{+}$ cell population was evaluated. **d** Munc13-4 protein expression in the platelets of the patients was evaluated by Western blotting. **e** HEK293T cells were transfected with FLAG-tagged cDNA carrying wild-type or missense mutated *UNC13D* sequences. After overnight culture, the cells were harvested with or without additional incubation in the presence of cycloheximide for 0, 5, and 10 h. The Munc13-4 protein expression levels were then analyzed by Western blotting. *WT* wild type. Representative results of two independent experiments are shown

therefore assessed whether the *UNC13D* missense mutations in our patients were responsible for their reduced platelet Munc13-4 expression by transfecting HEK293T cells with FLAG-tagged cDNA carrying wild-type or missense *UNC13D* sequences. Indeed, the c.2759A>G mutation in patient 1 and the c.1240C>T (but not the c.767G>A) mutation in patient 2 were responsible for the reduced Munc13-4 protein expression (Fig. 1e). This confirmed the diagnosis of FHL3 in patients 1 and 2.

Comparison of Degranulation Assays

Given that the $CD57^{+}$ CTL degranulation assay more effectively identified our late-onset FHL3 patients than the NK cell-based assays, we compared these three assays prospectively with 40 additional pediatric HLH patients who were recruited after patients 1 and 2 were diagnosed (Table 1). Six of the 40 patients (patients 3–8) had FHL3. None of these patients carried missense *UNC13D* mutations (Table 2). As shown in Fig. 2a, the freshly stimulated NK cells from the six FHL3 patients exhibited decreased cytolytic granule release. However, the NK cells from the non-FHL3 patients also showed some defects in degranulation. When using the laboratory-defined threshold of 10%, the degranulation assay with freshly stimulated NK cells was found to discriminate FHL3 patients from other HLH patients with a sensitivity of 100% and a specificity of 68%. ROC analysis showed that the optimum threshold value was 9.2%. When this value was used as the discriminatory threshold, the specificity improved to 71% without changing the sensitivity.

While IL-2 stimulation restored the degranulation of NK cells from the non-FHL patients in all cases, the IL-2-stimulated NK cells from a FHL3 patient (patient 5), similar to the IL-2-stimulated NK cells from patients 1 and 2, showed normal degranulation levels. The laboratory-defined threshold of 30% showed that the IL-2-stimulated NK cell degranulation assay discriminated FHL3 patients from other HLH patients with a specificity of 100% but a low sensitivity of 63%. ROC analysis showed that the optimum threshold value was 44.7%. When this threshold was used, the sensitivity improved to 100%; however, the specificity decreased to 90% (Fig. 2b).

By contrast, when the laboratory-defined threshold of 25% was used for $CD57^{+}$ CTL degranulation assay, this assay discriminated FHL3 patients from other HLH patients with a sensitivity of 100% and a specificity of 94%. ROC analysis indicated that the optimum threshold was 11.5%. When this value was used, both the sensitivity and specificity were 100% (Fig. 2c).

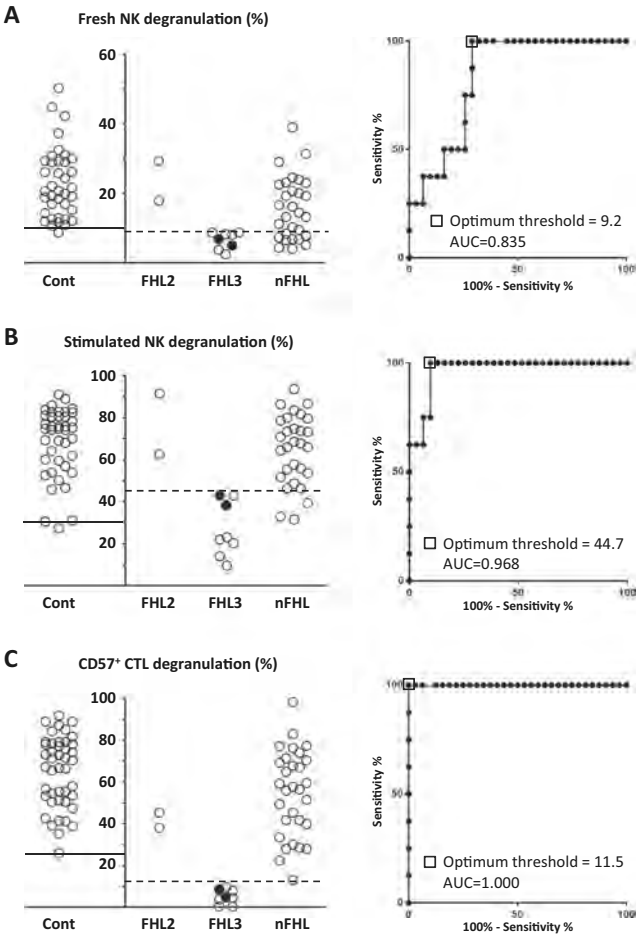

Fig. 2 Sensitivity and specificity with which the NK and CD57$^+$ CTL degranulation assays discriminate FHL3 patients from other HLH patients. The figures show the results of lysosomal degranulation assays and their ROC curves using the freshly isolated NK cells (**a**), IL-2 stimulated NK cells (**b**), and of IL-2 stimulated CD57$^+$ CTLs (**c**) of the enrolled patients. *Solid lines* indicate the laboratory-defined threshold values, while *dashed lines* indicate the optimum threshold values that were determined by ROC analyses to discriminate FHL3 patients from other HLH patients. *Closed circles* indicate patients 1 and 2. *nFHL* non-FHL

Discussion

FHL is a life-threatening inherited immune disorder that is caused by mutations in genes that participate in the cytotoxic activity of lymphocytes. The inability to clear the antigenic stimulus hyper-activates cytolytic lymphocytes. This results in over-production of inflammatory cytokines, which, in turn, leads to sustained inflammation and activation of macrophages [1, 2, 9]. The ability to rapidly screen for FHL rapidly would facilitate the initiation of life-saving immunosuppressive therapy and the preparations for HSCT.

The lysosomal exocytosis assay is a comprehensive method that is used to identify patients with a degranulation defect. It has been used to screen for FHL3-5 and hereditary HLH syndromes associated with oculo-cutaneous albinism [7, 8, 14–16]. In the present study, the FHL3 patients exhibited reduced degranulation of resting NK cells. However, as was shown by a previous study [16], non-FHL3 patients also exhibited some defects in resting NK cell degranulation (Fig. 2a). While IL-2 stimulation restored the degranulation of the NK cells from these non-FHL3 patients, the degranulation of the IL-2-stimulated NK cells from some of the FHL3 patients was comparable to that seen in the control subjects (Fig. 2b). By contrast, the decreased degranulation of CD57$^+$ CTLs was more indicative of FHL3 patients (Fig. 2c). Specifically, when laboratory-defined thresholds were used to discriminate FHL3 patients from the other HLH patients, the fresh NK cell degranulation assay had a sensitivity and specificity of 100 and 68%, respectively, while the IL-2-stimulated NK cell degranulation assay had a sensitivity and specificity of 63 and 100%, respectively. Thus, when using laboratory-defined thresholds, the CD57$^+$ CTL assay was as sensitive as the fresh NK cell assay and a bit less specific than the IL-2-stimulated NK cell assay. However, while 18 patients in total exhibited decreased degranulation below the threshold levels of either NK cell assay, only ten patients exhibited decreased degranulation of CD57$^+$ CTLs (Table 1). This is clinically important because it means that the CD57$^+$ CTL assay essentially made genetic testing unnecessary for eight patients. Moreover, when ROC-determined optimal thresholds were used to discriminate FHL3 patients from the other HLH patients, the fresh NK cell degranulation assay had a sensitivity and specificity of 100 and 71%, respectively, while the IL-2-stimulated NK cell degranulation assay had a sensitivity and specificity of 100 and 90%, respectively. By contrast, the sensitivity and specificity of the CD57$^+$ CTL degranulation assay were both 100%. Notably, one patient with other forms of HLH exhibited CD57$^+$ CTL degranulation levels just above the optimum threshold (patient 21 in Table 1): 1 week later, however, this patient exhibited normal degranulation levels. This was in clear contrast to the reevaluated FHL3 patients (patients 1–4 in Table 1), all of whom showed sustained defects in CD57$^+$ CTL degranulation. Although it is possible that immunosuppressive therapies may affect CTL function, the two FHL2 patients who had been treated with multiple immunosuppressive drugs exhibited normal lysosomal degranulation. Thus, these treatments had minimal effects on the assay. Taken together, we propose that the CD57$^+$ CTL degranulation assay effectively identifies FHL3 patients.

A workflow for the diagnosis of primary HLH has been proposed on the basis of a study by Bryceson et al. on HLH patients in Europe. The assays using NK cells were the mainstay screening methods for identifying patients with a defect in cytolytic granule exocytosis. The study reported that these assays had a higher specificity than the assays reported in the current study [16]. However, in the study by Bryceson et al., secondary HLH patients were defined as patients who developed a single episode that fulfilled the clinical criteria for HLH

and exhibited sustained complete remission for at least 6 months after completing HLH therapy; patients with a refractory HLH course were excluded from the statistical analysis. Examination of these excluded patients showed that many had reduced NK cell degranulation [16]. Since the patient cohort in the present study included many patients with a severe and refractory course of HLH, we believe that the results of our analysis are similar to those reported by Bryceson et al.

The current study suggests that the $CD57^+$ CTL degranulation assay may be more useful for diagnosing FHL3 than the assays employing NK cells. However, several issues must be addressed before it can serve as a standard method. One limitation of this assay is that neonates and young infants have very few numbers of CD57-expressing CTLs in their peripheral blood [25]. Indeed, we could not perform the analysis in two non-FHL patients due to the extreme paucity of this cell population (Table 1). However, all FHL patients in this study had substantial numbers of $CD57^+$ CTLs in their peripheral blood. This probably reflects the pathophysiology of the disease. Another limitation of the current study was the lack of patients with other forms of genetic degranulation defects; this reflects their extreme rarity. While a patient with *BIRC4* deficiency (X-linked lymphoproliferative syndrome type 2) was included in our cohort (Table 1), this hereditary HLH syndrome does not associate directly with cytolytic defects [26, 27]. Further evaluations are required to determine whether the $CD57^+$ CTL degranulation assay is useful for screening FHL4, FHL5, and hereditary HLH syndromes with oculocutaneous albinism. Notably, all FHL3 patients who have been diagnosed at our laboratory (including eight patients presented in this study) lack or have significantly reduced platelet Munc13-4 protein expression (data not shown). This suggests that the detection of Munc13-4 expression is another useful method for screening for FHL3.

The fact that two late-onset FHL3 cases had relatively well-preserved activated NK cell degranulation yet still had poor $CD57^+$ CTL degranulation suggests that Munc13-4 protein may play different roles in the lysosomal degranulation of the two cell subsets. While the molecular mechanism underlying this phenomenon is unclear, we speculate that the preserved degranulation capacity of NK cells may have influenced the clinical picture of the patients. CTLs and NK cells play distinct roles in the pathogenesis of FHL. Analysis of perforin-deficient mice showed that CTLs, but not NK cells, are necessary for the development of FHL symptoms [28]. Moreover, a recent report shows that NK cell cytotoxicity plays an immunoregulatory role and protects against FHL pathology [29]. In addition, FHL3 patients with atypical presentations, like our two late-onset cases, are reported to have relatively preserved NK cell degranulation, especially after culture with IL-2 [17]. Indeed, another FHL3 patient in our cohort who showed high levels of activated NK cell degranulation (patient 5 in Table 1) only developed the symptoms of HLH at 4 months of age: this is relatively late for FHL3. We speculate that the preserved cytolytic capacity of NK cells had contributed to the mild clinical courses of these three patients.

Conclusions

We propose that the $CD57^+$ CTL degranulation assay effectively discriminates FHL3 patients from those with other forms of HLH. Further studies that assess whether these assays are useful for screening patients with FHL4, FHL5, and other forms of degranulation defect-associated hereditary HLH syndromes are warranted.

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Authorship Contributions Contribution: T.Y., R.N., and T.H. designed the research; M.I., N.N., H.T., and E.I. treated patients 1 and 2; M.H., S.S., H.S., E.H., K.I., and T.K. performed the degranulation and protein expression assays; H.O. and O.O. performed the genetic analyses; R.S. and H.H. prepared the anti-Munc13-4 and anti-Syntaxin11 antibodies; M.H., T.Y., K.I., T.K., R.N., S.M., and T.H. analyzed and discussed the results; T.Y. and S.M. performed the statistical analysis; and M.H. and T.Y. wrote the paper.

Compliance with Ethical Standards Informed consent was obtained from the patients and their parents in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Conflict of Interest The authors declare that they have no competing interests.

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primary immunodeficiency predisposing to dimorphic fungal infection.[5,6]

HLH is a life-threatening syndrome of hyperinflammation caused by genetic mutations affecting the cytolytic function of T cells and NK cells (primary HLH) or in response to various infections, rheumatologic disorders, or malignancy (secondary HLH). Infection with EBV and other herpesviruses is a common trigger of HLH and in some cases may indicate an underlying primary immunodeficiency.[7] This is exemplified by X-linked lymphoproliferative disease and the growing number of primary immunodeficiencies characterized by impaired control of EBV infection.[8] Similar to X-linked lymphoproliferative disease, patients with GATA2 deficiency have marked NK-cell dysfunction and susceptibility to herpesvirus infection, which may lead to aggressive HLH in some cases.[1,2] A previously reported patient with GATA2 deficiency developed HLH in the setting of marked EBV viremia and an EBV-driven T-cell lymphoproliferative disorder.[9] In contrast to our patient's fatal course, that patient was successfully treated with etoposide and dexamethasone followed by nonmyeloablative allogeneic hematopoietic stem cell transplantation.[9] These reports suggest that GATA2 deficiency should be considered in the differential diagnosis for herpesvirus-associated HLH and that early hematopoietic stem cell transplantation is critical.

This case further expands the spectrum of infectious and inflammatory complications of GATA2 deficiency. In particular, this case further confirms the importance of GATA2 in host defense against dimorphic fungi and identifies blastomycosis as an infection that can signify primary immunodeficiency. In addition, this case further supports the critical role of NK cells in the immune response to herpesvirus infections, dysfunction of which may lead to HLH.

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Clinical characteristics and genotype-phenotype correlations in C3 deficiency

To the Editor:

The complement system comprises 3 initiation pathways: the classical, lectin, and alternative pathways. Although each pathway is activated individually, they converge at the C3 step and subsequently follow a common pathway, indicating that C3 is a pivotal complement factor. A single-chain precursor (Pro-C3) of approximately 200 kDa is produced intracellularly, and subsequently proteolytically cleaved into 2 subunits, α (115 kDa) and β (70 kDa) subunits. Both α and β subunits assemble by a disulfide bond yielding the mature C3 protein. The C3 protein consists of 13 domains[1]: 8 macroglobulin domains (MG1-8); anaphylatoxin (ANA); linker (LNK); complement C1r/C1s, Uegf, Bmp1 (CUB); thioester-containing domain (TED); and C345 C domains.

C3 deficiency is a rare autosomal-recessive inherited disorder that is characterized by susceptibility to recurrent bacterial infections, although some cases are associated with autoimmune diseases.[2] However, no definite genotype-phenotype correlations have been established to date, possibly because of the extremely low prevalence of the disorder. In the present study, we investigated the clinical features of our 4 cases[3-6] of C3 deficiency and reviewed previously reported cases to clarify the genotype-phenotype correlations in this disorder. We searched for English and Japanese articles describing C3 deficiency in PubMed, Ovid, and Google Scholar from 1972 (first reported year of C3 deficiency) to December 2014. From 511 articles, we selected 43 articles reporting biochemically diagnosed C3 deficiency regardless of confirmation by molecular analysis, and collected their genetic and clinical information.

Thirty-seven cases (29 families) of various races have been identified from different regions in the world (Table I). Clinical features could be divided into 3 groups: severe infections, rheumatic diseases, and renal diseases. We arbitrarily defined the criteria for severe infections in this study as follows: infections

TABLE I. Clinical features, C3 values, C3 mutations, and sibling history in 37 patients with C3 deficiency from 29 families

Patient no.	Published year	Ethnicity	C	Age at diagnosis/ sex	C3 (mg/dL)	C3 molecular analysis	Clinical features	Sibling history	Reference
Severe infections									
1.	1972 1976 1989 1992	White South African	+	15 y/F	<0.25	800-bp deletion (homo)	Meningitis × 2, pneumonia × 14, recurrent otitis media, paronychia, impetigo, Sweet syndrome		E1-E4
2.	1976	White South African	−	4 y/M	0	NA	Meningitis × 6, recurrent tonsillitis, pneumonia × 2, deceased	Deceased, meningitis	E5
3.	1977	Unknown	+	2 y/M	<3	NA	Otitis media × 2, persistent fever (52 d), maculopapular rash, arthralgia	Deceased, encephalitis	E6
4.	1977	Unknown	−	5 y/F	<2.5∼5	NA	Pneumonia, septic arthritis, recurrent otitis media, and pharyngitis		E7
5.	1981 1994	Taiwanese Aborigines	−	10 y/F	0	IVS10 +1 g>t (homo)	Pneumonia × 12, otitis media × 5, septic arthritis, buttock abscess	Deceased, meningitis	E8,E9
6-1.	1983 1992	Dutch	?	26 y/F	ND	NA	Meningitis × 3, sepsis, recurrent otitis media, skin infections	Deceased, meningitis	E10,E11
6-3.				16 y/F	ND	NA	Septic osteomyelitis, recurrent otitis media, maculopapular rash during infectious episodes		
7.	1988 2002	Brazilian	+	6 y/M	ND	G1655A (K552X) (homo)	Meningitis × 3, pneumonia × 4, otitis media × 4, osteomyelitis × 2, skin infection, UTI, arthritis, giardiasis, fever of unknown origin × 5	Deceased, meningitis, pneumonia, prolonged diarrhea	E12,E13
8.	1990	Unknown	+	10 y/M	ND	IVS18 +1g>a (homo)	Recurrent otitis media, >20 episodes of erythematous plaques during upper respiratory tract infections		E14
9-1.	1992 1994 1995	New Zealander	−	19 y/M	ND	G1645A (D549N) (hetero s/o)*	Bacteremia at age 19 y, purpura		E15-E17
10.	1992	Unknown	+	4 y/F	0.8% of normal	NA	Meningitis × 4, recurrent otitis media	Deceased (2 brothers)	E18
11.	2001 2004	Brazilian	+	8 y/M	0.015	C2542T (R848X) (homo)	Lymphadenitis, sinusitis × 2, pneumonia, tonsillitis × 2, giardiasis	Deceased (4 siblings)	E19,E20
12.	2002	Turkish	+	4 y/F	4	NA	Meningitis × 2, recurrent otitis media	Immuno-compromised	E21
13.	2004	Unknown	−	4 y/M	<10	NA	Bacteremia, iliopsoas abscess, pneumonia × 2, recurrent otitis media, tonsillitis, pharyngitis, accidental death		E22
14-1.	2006	Unknown	?	7 y/F	6	NA	Meningitis × 2, otitis media × 2, UTI × 1	Deceased, meningitis	E23
14-2				10 mo/M	6	NA	Meningitis, pneumonia, recurrent otitis media		
15.	2008	Unknown	−	2 y/M	<4.3	T1648C (S550P) (hetero s/o)*	Meningitis, pneumonia, otitis media, sinusitis	Deceased, meningitis	E24
16.	2009	Unknown	?	5 y/F	11.2	NA	Meningitis × 4		E25
17.	2011	Arab	+	4 y/M	<10	3997delA (homo)	Pneumonia × 2, bacteremia × 3		E26
18.	2013	Turkish	+	16 y/M	8–19	C4554G (C1518W) (homo)	Recurrent pneumonia, otitis media, bronchiectasis, IgA deficiency	Deceased (2 siblings)	E27
Severe infections and IC-related diseases									
6-2.				19 y/F	ND	NA	Meningitis, recurrent otitis media, maculopapular rash during infectious episodes, subacute cutaneous lupus erythematosus		

(Continued)

TABLE I. *(Continued)*

Patient no.	Published year	Ethnicity	C	Age at diagnosis/ sex	C3 (mg/dL)	C3 molecular analysis	Clinical features	Sibling history	Reference
19.	1975 1983	White	?	4 y/F	ND	NA	Protracted diarrhea, recurrent otitis media, bacteremia × 2, recurrent UTI, sinusitis, type 1 MPGN		E28,E29
20-2.	1980	Lebanese	?	7 y/F	0	NA	Peritonitis, proteinuria, microhematuria	Nephrotic syndrome	E30
20-3.				5 y/M	0	NA	Peritonitis, proteinuria, left atrophic kidney due to renal artery stenosis		
21.	1985 1988 1996	Laotian	−	7 y/M	0.4	Reduced C3 mRNA	Meningitis × 2, pneumonia × 6, mesangiopathic glomerulonephritis		E31-E33
22-1.	1981	Japanese	+	16 y/M	<1	NA	Meningitis, IgA nephropathy	Deceased, butterfly rash, renal failure	E34,E35
22-2.	1991			14 y/F	ND	NA	Meningitis, SLE-like illness		
23.†	2008	Japanese	−	2 y/M	<2	3176dupT and C3243G (Y1081X) (hetero)	Meningitis, bacteremia × 4, pneumonia × 2, otitis media, focal segmental glomerulonephritis, SLE-like illness		E36
24.†	2011 2012	Japanese	−	4y/M	0.3	IVS9 -2a>t and C1432T (R478X) (hetero)	Bacteremia × 2, pneumonia × 2, otitis media, synovitis		E37,E38
25.†	Unpublished	Japanese	−	4 y/M	0	IVS11 +5 g>a and IVS12 −1 g>t (hetero)	Bacteremia, otitis media, sinusitis, membranous nephropathy, SLE-like illness		
IC-related diseases									
20-1.	1980	Lebanese	?	13 y/F	0	NA	Frequent earache, sore throat, abdominal pains, proteinuria, microhematuria/no severe infections	Nephrotic syndrome	E30
26-1.	1981 2001	Japanese	+	19 y/F	ND	C3243G (Y1081X) (homo)	SLE-like illness/no severe infections		E39,E40
26-2.				14 y/F	ND	C3243G (Y1081X) (homo)	SLE-like illness/no severe infections		
27.	1987	Unknown	+	7 y/F	ND	NA	Type 1 MPGN (renal transplantation)/ no severe infections		E41
28.	2005	Japanese	+	23 y/M	<2	IVS38 -2a>g (homo)	Tonsillitis and recurrent pneumonia in his late teens, SLE-like illness/ mild infections		E42
29.†	2005	Japanese	+	7 y/M	<0.5	3736_3737delTT (homo)	Bronchitis, otitis media, membranous nephropathy/no severe infections		E43
Neither severe infections nor IC-related diseases									
9-2.	1992 1994 1995	New Zealander	−	7 y/F	<5% of normal	G1645A (D549N) (hetero s/o)*	Asthma, rhinitis/no severe infections		E15-E17

Siblings are indicated by the same patient (pt) number with added hyphenated numbers in order of age. (Only No. 22-1 and 22-2 were cousins.)
C, Consanguinity; *F*, female; *M*, male; *MPGN*, membranoproliferative glomerulonephritis; *NA*, not available; *ND*, not detected; *UTI*, urinary tract infection.
*Incomplete analysis.
†Our case.

of normally sterile sites (blood, cerebrospinal fluid, peritoneal fluid, joint fluid, or bone marrow) or recurrent bacterial infections (≥3) before the age of 3 years regardless of the site of infection. Both rheumatic and renal diseases are possibly caused by immune complex (IC)-associated mechanisms and are grouped here as IC-related diseases.

Thirty cases (81%) developed severe infections. Fifteen cases had histories of septic meningitis, 9 (60%) of which suffered from recurrent meningitis. Among a total of 34 episodes of meningitis, *Streptococcus pneumoniae* and *Neisseria meningitides* were detected from cerebrospinal fluids in 14 and 6 episodes, respectively. Eight cases had histories of bacteremia or sepsis, 4 (50%) of which suffered from 2 or more episodes of bacteremia. Among a total of 15 episodes of bacteremia or sepsis, *S pneumoniae* was identified in 12 episodes (80%) and the remaining microorganisms were *N meningitides*, *Staphylococcus aureus*, and *Streptococcus milleri.* Twenty-four cases had recurrent respiratory tract infections and/or otitis media caused

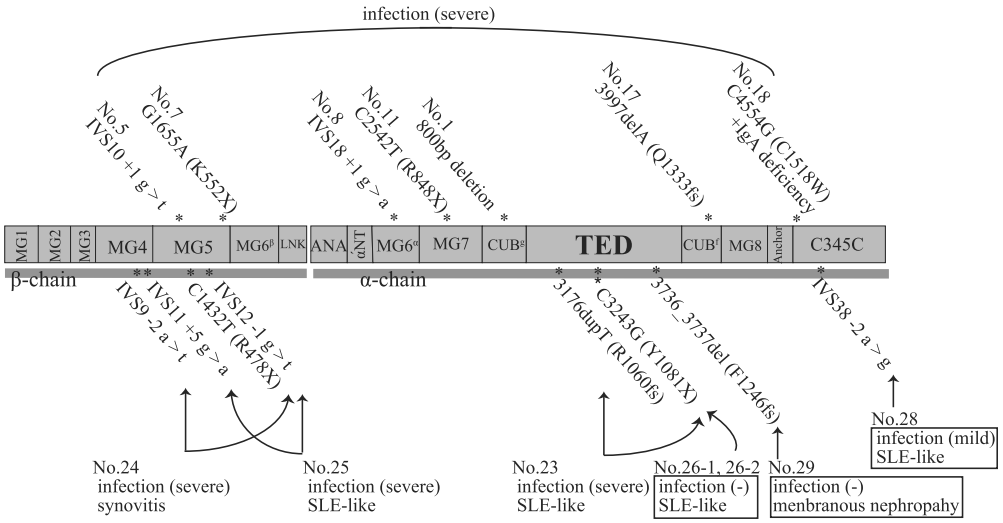

FIG 1. Severe infections preferentially develop in patients with *C3* mutations in the N-terminal end of the TED domain. Domain arrangement of the *C3* gene is shown. Both MG6 and CUB domains are formed by 2 separated parts, respectively. *C3* mutations are indicated by asterisk marks. The mutations in Japanese and other ethnicities are shown in the lower and upper rows, respectively. The patient numbering follows that used in Table I.

by *S pneumoniae, Haemophilis influenzae*, *Klebsiella aerogenes*, *S aureus*, and *Streptococcus pyogenes*.

Rheumatic diseases were observed in 8 cases (22%): 6 cases from 5 families displayed systemic lupus erythematosus (SLE)-like illness, 1 case had subacute cutaneous lupus erythematosus, and 1 case had synovitis.[6] Six cases were positive for antinuclear antibody at low titers (1:40∼1:320). Three of the 8 cases with rheumatic diseases (38%) had no history of severe infections.

Renal diseases (hematuria and/or proteinuria) were observed in 8 cases (22%). Renal biopsies were performed in 5 cases, showing membranoproliferative glomerulonephritis type 1 (2 cases), mesangiopathic glomerulonephritis (1 case), IgA nephropathy (1 case), and membranous nephropathy (1 case). Two patients with SLE-like illness who were grouped as suffering from rheumatic diseases had focal segmental glomerulonephritis and membranous glomerulonephritis, respectively, despite normal urinary analysis. Three of the 8 cases with renal diseases (38%) had no history of severe infections.

The median age of onset of any symptoms related to C3 deficiency was 2.0 years (range, 3 weeks-19 years): severe infections at 1.5 years ranging from 3 weeks to 10 years and IC-related diseases at 7.5 years ranging from 1 to 25 years. The overall mean age of diagnosis was 9.2 ± 6.5 years (range, 10 months-26 years). The patients with severe infections were diagnosed at a younger age than were those with only IC-related diseases (8.3 ± 6.3 years vs 13.8 ± 6.4 years; $P = .061$).

Genetic analyses had been carried out in only 18 cases (16 families) including the present 4 cases (4 families). Among these cases, complete analyses of both alleles were performed in 14 cases (13 families). Three cases (21%) had compound heterozygous mutations, whereas the remainder (79%) had homozygous mutations. We found 15 mutations: 6 splicing abnormalities, 4 nonsense mutations, 2 with 1 or 2 base deletion, 1 large deletion, 1 with 1 base insertion, and 1 missense mutation. We analyzed the genotype-phenotype correlation in 14 cases (13 families) with completely defined *C3* mutations. Fig 1 illustrates the domains of C3 and mutations of genetically confirmed patients. In patients with severe infections, *C3* mutations were located in the β-chain and upstream of the TED domain in the α-chain. However, patients without severe infections had *C3* mutations that were concentrated in and downstream of the TED domain (Nos. 26-1, 26-2, 28, and 29). Given that all mutations except 1 missense mutation (No. 18) were predicted to result in frame-shift or premature termination of protein translation, the mutations of the N-terminal half of the gene may cause large truncations and, possibly, lack of C3 protein. Although the small number of genetically confirmed cases is a limitation in our study, our results raise the possibility that mutations of the nucleotides encoding the TED domain or downstream of it causes production of mutant C3, which is able to control infections but not IC-related diseases, despite being undetectable by the conventional assay system. To address this possibility, it is necessary to develop a novel quantitative and functional assay system of mutant C3 molecules.

In conclusion, the clinical features of C3 deficiency were a combination of severe infections mainly caused by *S pneumoniae* and *N meningitides* and/or IC-related diseases such as SLE-like illness or renal diseases. Patients with *C3* mutations in the N-terminal half of the gene tend to be more susceptible to severe infections. The biological functions of the mutant C3 molecule remain to be elucidated.

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Efficacy of T-cell transcription factor–specific DNAzymes in murine skin inflammation models

To the Editor:

Most current therapies for inflammatory skin diseases are mainly symptomatic and do not interfere with the underlying pathomechanisms. In addition, they are often accompanied by moderate to severe adverse effects. Therefore, there is an unmet medical need for efficient and well-tolerated therapies that target disease-specific mechanisms. The involvement of different subtypes of T_H cells in skin inflammation has been intensively investigated[1] and different T_H subtype-specific transcription factors have been identified. These molecules orchestrate the differentiation and activation of the respective T_H subtype and influence the development of other subtypes, thereby modulating the nature of the inflammatory immune response.[2]

We generated deoxyribozymes (DNAzymes) directed against the central transcription factors of T_H2- and T_H1-cell differentiation and activation, namely, guanine adenine thymine adenine sequence-binding protein 3 (GATA-3) for T_H2 and T-box transcription factor TBX21 protein (Tbet) for T_H1 cells (see Table E1 in this article's Online Repository at www.jacionline.org). 10-23 DNAzymes represent a particular class of antisense molecules combining the specificity of antisense molecules with an inherent catalytic activity that makes them an attractive tool for highly specific interference with disease-causing target molecules. They are single-stranded DNA molecules with 2 sequence-specific RNA-binding domains flanking a central catalytic domain that exerts RNA cleavage activity after appropriate binding (see Fig E1 in this article's Online Repository at www.jacionline.org).[3] Targeting the transcription factors of disease-mediating T_H cells might be a versatile tool for therapeutic interventions in inflammatory skin diseases such as atopic dermatitis, allergic contact hypersensitivity, or psoriasis. Actually, efficacy of the GATA-3–specific DNAzyme approach has been shown in mouse models of allergic airway inflammation[4] and most recently in a phase IIa clinical trial in which inhalation of this compound resulted in significant abrogation of allergen-induced early- and late-phase allergic responses in patients with asthma.[5]

To investigate DNAzyme efficacy in inflammatory skin conditions, different mouse models were established. An oxazolone-induced contact hypersensitivity mouse model was modified from standard acute hapten-induced contact hypersensitivity mouse models to establish prolonged skin swelling reactions compared with acute models, thereby enabling the analysis of treatment effects on T-cell–mediated pathomechanisms (see Fig E2 in this article's Online Repository at www.jacionline.org). Three days after epicutaneous sensitization with the hapten oxazolone, mice were challenged by epicutaneous oxazolone application, and the prominent skin swelling reaction induced as a result is accompanied by an influx of inflammatory cells into the dermis consisting of mononuclear cells, eosinophils, and neutrophils, indicating a mixed T_H1-/T_H2-type inflammatory response and mild hyperkeratosis. Treatment with DNAzymes formulated in a water-in-oil-in-water emulsion, specifically developed for penetration enhancement and DNAzyme protection,[6,7] was performed by topical application.

Prophylactic treatment with the GATA-3–specific DNAzyme hgd40 significantly reduced oxazolone-induced skin swelling reactions compared with placebo and control DNAzyme ODNg3 (Fig 1, *A*). Immunohistologic analysis of $CD4^+$ T cells revealed significantly lowered cell numbers on hgd40 treatment in the dermis of oxazolone-induced mice (Fig 1, *B*). Target regulation was demonstrated by significantly reduced GATA-3 mRNA levels after topical application of hgd40 during the sensitization phase (Fig 1, *C*, and Table E2 in this article's Online Repository at www.jacionline.org). Inhibitory effects of hgd40 treatment on skin swelling reactions could also be detected after semi-therapeutic treatment starting a day before challenge in this model (Fig 1, *D*). These data are in line with the efficacy of inhaled hgd40 in mouse models of allergic airway inflammation,[4] indicating favorable therapeutic effects of topically applied hgd40 irrespective of the target organ. Furthermore, human T_H2-polarized $CD4^+$ T cells (see the Methods section and Tables E3 and E4 in this article's Online Repository at www.jacionline.org) were transfected with the GATA-3–specific DNAzyme hgd40 (the corresponding mRNA sequence of which is 100% homologous between mouse and human). This resulted in a significant reduction in intracellular GATA-3 protein levels at 22 hours after transfection paralleled by a decreased release of the T_H2 cytokine IL-13 compared with control DNAzyme-transfected cells (Fig 1, *E*).

Because the oxazolone model induced a mixed T_H1/T_H2 inflammatory phenotype based on T_H2- and T_H1-cell activities, potential effects of topical treatment with the mouse Tbet-specific

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