In the United States the regulation of nonstandardized AEs presented some similarities with our approach. AEs were classified into 4 categories according to scientific data supporting their use in diagnosis and treatment, and the extracts were regularly evaluated by the regulatory agencies. The last update was conducted between 2003 and 2011, and the process was recently reviewed by Slater et al.[1] It was shown that for nearly half of nonstandardized AEs there were, in fact, little or no data to support their effectiveness. We had similar results: 66 of 84 AEs were validated for diagnosis, but only for 29 of 66 was there at least 1 published piece of data to support their effectiveness for immunotherapy (Table I). Among those 66 authorized AEs, approximately one third are standardized. There is no consensus about the standardization methods, and the European approaches present some differences compared with the US approach (see Table E1 in this article's Online Repository at www.jacionline.org). Briefly, in-house reference preparation (IHRP) AEs are standardized *in vivo* and *in vitro*. Each manufacturer has its own IHRP, and there is no national standard. Batch-to-batch standardization is performed *in vitro* through a comparison of the AEs with the IHRP.[9]

In the future, the NPP list will be updated every 5 years, and requests for MA will be made and processed for standardized AEs produced industrially and frequently used for immunotherapy.

In conclusion, for the first time in Europe, this work guarantees that available AEs are clinically relevant and safe. Moreover, it guarantees that all AEs comply with recent European guidelines on APs, including rare allergens for which it is not possible to obtain large clinical studies requested for MA. The process involved all the representatives of allergists and manufacturers and is still ongoing.

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Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles

To the Editor:

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency associated with hypogammaglobulinemia and other various clinical manifestations. CVID was originally reported to be a disease primarily caused by defective B-cell function, with defective terminal B-cell differentiation rendering B cells unable to produce immunoglobulin. However, combined immunodeficiency (CID) involving both defective B and T cells is often misdiagnosed as CVID.[1] Indeed, one study reported that $CD4^+$ T-cell numbers were decreased in 29% of 473 patients with CVID[2]; similarly, another study found that naive T-cell numbers were markedly reduced in 44% (11/25) of patients with CVID.[3] These observations indicated that a subgroup of patients with clinically diagnosed CVID is T-cell deficient. Consistently, some patients with CVID have complications that might be related to T-cell deficiency, including opportunistic infections, autoimmune diseases, and malignancies, which is similar to that observed in patients with CID.[1,4] Therefore identifying novel markers to better classify CVID and distinguish CID from CVID will be required to best manage medical treatment for CVID.

We recently performed real-time PCR-based quantification of T-cell receptor excision circles (TREC) and signal joint immunoglobulin κ-deleting recombination excision circles (KREC) for mass screening of severe combined immunodeficiency (SCID)[5] and B-lymphocyte deficiency[6] in neonates. TREC and KREC are associated with T-cell and B-cell neogenesis, respectively.[7] Here we retrospectively report that TREC and KREC are useful for classifying patients with clinically diagnosed CVID.

Hypogammaglobulinemic patients (n = 113) were referred to our hospital for immunodeficiency from 2005-2011, and the following patients were excluded from the CVID pool by estimating their SCID genes based on clinical manifestations and lymphocyte subset analysis: 18 patients with SCID diagnoses; 14 patients less than 2 years of age (transient infantile hypogammaglobulinemia); 10 patients with IgM levels of greater than 100 mg/dL (hyper-IgM syndrome); 26 patients with diseases other than CVID caused by known gene alterations (10 with X-linked agammaglobulinemia and 11 with hyper-IgM syndrome

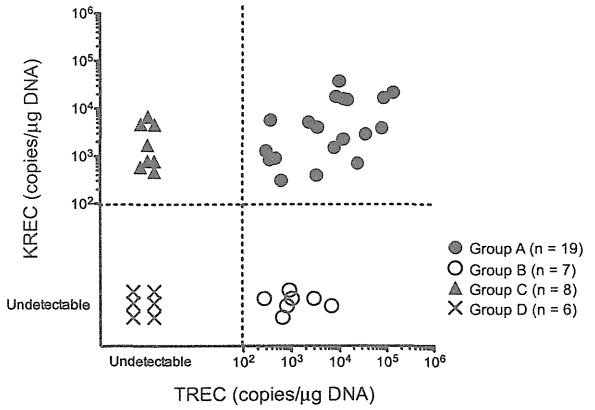

FIG 1. Quantifying TREC and KREC classifies patients with CVID into 4 groups. Patients with CVID were classified as follows: TREC(+)/KREC(+), group A (19 patients); TREC(+)/KREC(−), group B (7 patients); TREC(−)/KREC(+), group C (8 patients); and TREC(−)/KREC(−), group D (6 patients). *Undetectable*, Less than 100 copies/μg DNA.

[*CD40L* or *AICDA* mutated]), (2 with DiGeorge syndrome, and 3 with *FOXP3*, *IKBKG*, or *6p* deletions); and 5 patients with drug-induced hypogammaglobulinemia. The remaining 40 patients with decreased IgG (≥2 SDs below the mean for age), IgM, and/or IgA levels, as well as absent isohemagglutinins, poor response to vaccines, or both were included in this study as patients with CVID and analyzed for TREC/KREC levels, retrospectively.

Ages of patients with CVID ranged from 2 to 52 years (median age, 15.5 years). The sex ratio of the patients was 21 male/19 female patients. Serum IgG, IgA, and IgM levels were 370 ± 33 mg/dL (0-716 mg/dL), 30 ± 7 mg/dL (1-196 mg/dL), and 40 ± 6 mg/dL (2-213 mg/dL), respectively. TREC and KREC quantification was performed by using DNA samples extracted from peripheral blood, as reported previously.[5,6] Clinical symptoms were then assessed retrospectively. The study protocol was approved by the National Defense Medical College Institutional Review Board, and written informed consent was obtained from adult patients or parents of minor patients in accordance with the Declaration of Helsinki.

Based on TREC and KREC copy numbers, the 40 patients with CVID were classified into 4 groups (groups A, B, C, and D; Fig 1). Comparing lymphocyte subsets, $CD3^+$ T-cell numbers were similar among groups A, B, and D but were significantly lower in group C ($P < .05$; group A, 1806 ± 204 cells/μL; group B, 1665 ± 430 cells/μL; group C, 517 ± 124 cells/μL; and group D, 1425 ± 724 cells/μL; $P = .0019$, Tukey multiple comparison test based on 1-way ANOVA). $CD3^+CD4^+CD45RO^+$ memory T-lymphocyte percentages in groups B, C, and D were significantly higher than those in group A ($P < .0001$; group A, 37% ± 16%; group B, 67% ± 13% [$P = .0006$]; group C, 92% ± 8.2% [$P < .0001$]; and group D: 83% ± 14% [$P < .0001$]; see Fig E1 in this article's Online Repository at www.jacionline.org); additionally, the percentages of these cells in groups C and D were higher than in group B ($P = .0115$). These results indicate that group C and D patients have markedly decreased $CD4^+CD45RA^+$ naive T-cell counts than group A patients and that counts in group B are also significantly decreased, although less so than in groups C or D, which is consistent with a report showing lower TREC copy numbers in $CD4^+CD45RO^+$ cells. Some patients in groups B, C, and D exhibited normal $CD4^+CD45RO^+$ percentages, although TREC levels, KREC levels, or both decreased. This discrepancy indicates that TREC/KREC levels could be independent markers to determine the patient's immunologic status in addition to $CD4^+CD45RA^+$; the reasons underlying the discrepancy between $CD4^+CD45RA^+$ and TREC/KREC levels remain unsolved.

$CD19^+$ B-cell numbers in group A were significantly higher ($P < .05$) than those in groups B and D (group A, 269 ± 65 cells/μL; group B, 35 ± 16 cells/μL; group C, 60 ± 11 cells/μL; and group D, 29 ± 16 cells/μL; $P = .0001$). However, B-cell subpopulations, including $CD27^-$, IgD^+CD27^+, and IgD^-CD27^+ cells, were not significantly different among the groups. Standardizing KREC copy numbers for each patient by dividing their $CD19^+$ by their $CD27^+$ percentages revealed the same patient classification as that shown in Fig 1 (data not shown), indicating that the original classification was independent of $CD19^+$ B-cell or $CD27^+$ memory B-cell percentages.

Because TREC and KREC levels decrease with age (see Fig E2 in this article's Online Repository at www.jacionline.org)[5,6] and age distribution was wide in this study, we compared patients' ages among groups at the time of analysis to determine whether classification was associated with age. TREC/KREC-based classification was independent of both age and sex because age distribution was not significantly different among groups ($P > .05$; group A, 12.7 ± 2.3 years [2-30 years]; group B, 23.4 ± 4.2 years [6-39 years]; group C, 21.5 ± 6.1 years [4-52 years]; and group D, 25.5 ± 4.4 years [15-46 years]; data not shown) nor was male/female sex ratio (overall, 21/19; group A, 10/9; group B, 2/5; group C, 5/3; and group D, 4/2; $P = .4916$, χ^2 test; data not shown).

We next evaluated whether any correlation existed between TREC/KREC-based classification and clinical symptoms in each patient group. All patients in the study had been treated with intravenous immunoglobulin (IVIG) substitution at the time of analysis. We found that the cumulative events of complications (opportunistic infections, autoimmune diseases, and malignancies) per 10 patient-years were highest in group D (0.98 events/10 patient-years), followed by group C (0.63 events/10 patient-years), group B (0.30 events/10 patient-years), and group A (0.04 events/10 patient-years), where events in groups D and C were significantly higher than group A (group A vs group D, $P = .0022$; group A vs group C, $P = .0092$; group A vs group B, $P = .0692$; Fig 2). Furthermore, we found similar results when evaluating only patients 19 years old or older for group D (1.01 events/10 patient-years), group C (0.56 events/10 patient-years), group B (0.32 events/10 patient-years), and group A (0.06 events/10 patient-years; group A vs group D, $P = .0074$; group A vs group C, $P = .0407$; group A vs group B, $P = .1492$; data not shown). Categorizing patients by using several different previously reported CVID classifications (focused primarily on separating patients based on levels of circulating B-cell subsets), we found that no classification scheme showed any significant event increases in any particular group (see Fig E3 in this article's Online Repository at www.jacionline.org). Assessing longitudinal cumulative opportunistic infection incidence among the groups, group D and C values were significantly higher than in group A (see Fig E4, A, in this article's Online Repository at www.jacionline.org; $P = .0059$). Autoimmune and malignant diseases ($P = .5168$ and $P = .6900$, respectively) were observed in groups B and D but not in group A (see Fig E4, *B* and *C*). Cumulative events were significantly different between groups ($P = .0313$, log-rank test; group A, 5.3% and 5.3%; group B, 14.3% and

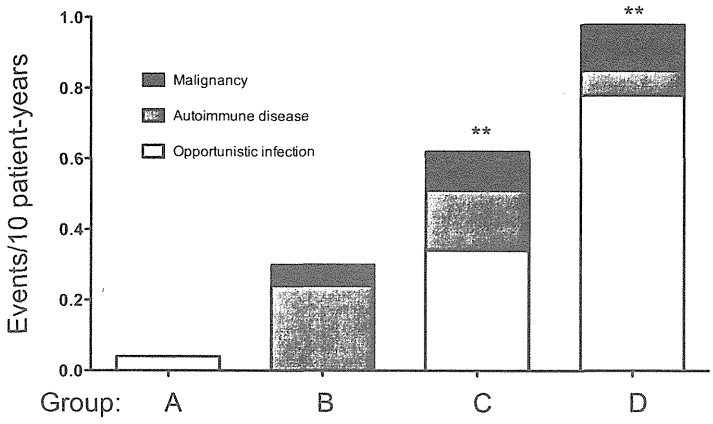

FIG 2. Cumulative incidence of complication events per 10 patient-years differs among groups. Opportunistic infections, autoimmune diseases, and malignancies were evaluated for each patient group. Complication incidences in group D (0.98 events/10 patient-years), group C (0.63 events/10 patient-years), and group B (0.30 events/10 patient-years) were higher than in group A (0.04 events/10 patient-years). Group A versus group D: $^{**}P = .0022$; group A versus C: $^{**}P = .0092$; group A vs group B: $P = .0692$.

57.1%; group C, 27.1% and 63.5%; and group D, 33.3% and 83.3% at 10 and 30 years of age, respectively; see Fig E4, *D*). One patient in group D died of *Pneumocystis jirovecii* pneumonia, and 2 other patients in the same group received hematopoietic stem cell transplantation after complications caused by EBV-related lymphoproliferative disorder.

Assessing these data, TREC/KREC-based classification matches clinical outcomes. Because group D patients exhibited the most frequent complications (opportunistic infections, autoimmune diseases, and malignancies), they could receive a diagnosis of CID based on these symptoms. If they are indeed determined to have CID, then TREC/KREC analysis is helpful to distinguish between CID and CVID. Their TREC(−)/KREC(−) phenotype might relate to defective V(D)J recombination in T- and B-cell development[8] because patients with B-negative SCID (*RAG1*, *RAG2*, *Artemis*, and *LIG4*), as well as patients with ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS; see Fig E5 in this article's Online Repository at www.jacionline.org),[5,6] were also negative for both TREC and KREC; it is intriguing to speculate that an unknown V(D)J recombination gene or genes is responsible. As for treatment, hematopoietic stem cell transplantation should be considered the preferred treatment to "cure" group D patients, as reported in patients with severe CVID/CID, because event-free survival is poor.[9]

In contrast to group D patients, TREC(+)/KREC(+) group A patients treated with IVIG substitution therapy remained healthy. One possible explanation is that these patients harbor defects only in terminal B-cell differentiation, but not in T cells, and represent typical patients with CVID, as originally reported.

Group C patients had a high frequency of both opportunistic infections and malignancies, suggesting that these TREC(−) patients have T-cell defects. Although group C patients had a similar TREC/KREC pattern to patients with SCID with B cells (*IL2RG* and *JAK3*; see Fig E5, *A*), they do not fulfill the European Society for Immunodeficiencies criteria for SCID, and no mutation was identified in the SCID genes estimated from clinical manifestation and lymphocyte subset analysis. However, from our data, they would likely benefit from undergoing similar treatment to patients with SCID or CID to prevent these complications.

Although opportunistic infections were rare in group B patients, autoimmune diseases were often observed. This is consistent with this group being TREC(+)/KREC(−) and the idea that balance between T and B cells is important to prevent autoimmune diseases in patients with CVID.[1] Intriguingly, a group of patients with AT and NBS were also TREC(+)/KREC(−) (see Fig E4, *B*), which is similar to group B patients. Additionally, $CD45RA^{+}CD4^{+}$ naive T-cell numbers were reduced in most group B patients, which is similar to the phenotype exhibited by patients with AT and NBS. This finding raises the possibility that although some group B patients are also T-cell deficient, as well as B-cell deficient, and should be treated similarly to patients with CID, other patients have only B-cell deficiency and are effectively treated with IVIG substitution therapy.

By analyzing a large CVID patient cohort, the overall survival rate of patients with more than 1 complication was worse than that for patients without other complications.[4] Our findings indicate that low TREC levels, KREC levels, or both are useful markers that correlate well with the overall survival rate in patients with CVID. Therefore we conclude that TREC and KREC are useful markers to assess the clinical severity and pathogenesis of each patient with CVID and to distinguish CID from CVID. Thus patient classification based on TREC/KREC levels would provide a helpful tool for deciding on an effective treatment plan for each patient with CVID.

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Homing frequency of human T cells inferred from peripheral blood depletion kinetics after sphingosine-1-phosphate receptor blockade

To the Editor:

Naive and central memory (CM) T cells home through lymph nodes (LNs), whereas T cells with an effector memory (EM) phenotype preferentially screen peripheral tissues in search of cognate antigen.[1] LN entry and egress are distinct and highly regulated processes mediated by an orchestrated interplay of chemokines/chemokine receptors and adhesion molecules.[2] Interaction of peripheral node addressins with L-selectin on T cells allows tethering/rolling along high endothelial venules (HEVs).[2] Interaction of the chemokine receptor CCR7 with its ligands CCL19/CCL21 and CXCR4 with CXCL12 then mediates firm adhesion to HEVs through high-affinity interactions of lymphocyte function–associated antigen 1 and intercellular adhesion molecule 1, permitting transmigration of T cells across the HEV cell layer.[2] Within the LNs, T-cell migration is directed through T-cell zones toward the cortical sinuses.[3] A sphingosine-1-phosphate (S1P) gradient established across the endothelial cells of the cortical sinuses is directing LN egress of T cells through efferent lymph back to the peripheral blood circulation.[4] Acting as a functional antagonist on the S1P receptor, the pharmacologic compound fingolimod, which has shown efficacy in the treatment of multiple sclerosis (MS), blocks this egress.[4,5] As a consequence, in fingolimod-treated subjects naive and CM T cells are trapped in LNs and reduced in the blood circulation.[6]

Here, by studying depletion kinetics of T cells in the blood of *de novo* fingolimod-exposed subjects in combination with *in vitro* migration experiments, homing frequencies and LN access hierarchy between T-cell subsets were derived indirectly. First, we defined the effect of *de novo* fingolimod exposure on the number of circulating $CD4^+$ and $CD8^+$ phenotypic T-cell subsets in patients with MS during a 6-hour observation period (hourly measurements, 1 time before and 6 times after drug exposure) by using flow cytometry (detailed information on patients and methods is provided in the Methods section and Table E1 in this article's Online Repository at www.jacionline.org). In fingolimod-treated subjects, 6 hours after the first drug dose, numbers of $CD4^+$ T-cell subsets with an LN homing phenotype (ie, naive and CM T cells) were significantly reduced (Fig 1, *A* [representative example; absolute cell counts], and Fig 1, *B* [pooled data; proportional change]). Intriguingly, the kinetics of reduction differed between phenotypic naive (CD62 ligand [CD62L]–positive $CD45RA^+$) and CM ($CD62L^+CD45RA^-$) $CD4^+$ T cells. Specifically, compared with baseline measurements, naive $CD4^+$ T-cell counts started to decrease earlier than CM $CD4^+$ T-cell counts (2 vs 5 hours after fingolimod exposure; Fig 1, *B*). In $CD8^+$ T cells, contrasting $CD4^+$ T cells, only naive ($CD62L^+CD45RA^+$) $CD8^+$ T-cell counts decreased significantly (after 3 vs 2 hours in naive $CD4^+$ T cells) after the first dose of fingolimod (Fig 1, *C* [representative example; absolute cell counts], and Fig 1, *D* [pooled data; proportional change]).

On the basis of these *ex vivo* depletion kinetics, *in vitro* chemotaxis experiments were performed, as described in the Methods section in this article's Online Repository. In a transwell system spontaneous migration of bulk $CD4^+$ and $CD8^+$ T cells was comparably low in healthy control subjects and untreated patients with MS (and was further decreased in the presence of fingolimod; see Fig E1 in this article's Online Repository at www.jacionline.org). Gradients of CXCL12, CCL19, and CCL21 mediated a clear increase in migration of bulk $CD4^+$ and $CD8^+$ T cells from healthy control subjects and untreated patients with MS, which was not significantly influenced by fingolimod (see Fig E1). Dot plot distribution (as a percentage) of migrated versus nonmigrated, phenotypic naive, CM, EM, and (for $CD8^+$ T cells) CD45RA re-expressing EM cells (EMRA) was then compared between control cells (spontaneous migration) and cells that migrated toward CXCL12, CCL19, or CCL21. An example of CXCL12-mediated changes in the

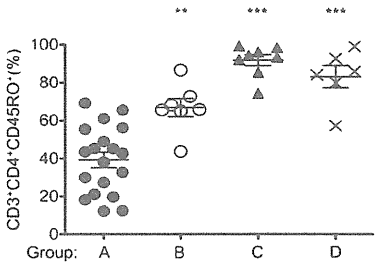
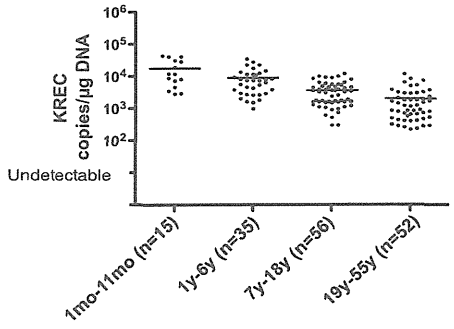


FIG E1. $CD45RO^+CD3^+CD4^+$ T-cell frequency within $CD4^+CD3^+$ lymphocytes was analyzed among groups. $CD45RO^+CD3^+CD4^+$ lymphocyte counts were significantly higher in groups B, C, and D compared with those in group A ($P < .0001$). Group A: 37% ± 16%; group B: 67% ± 13% (**$P < .01$); group C: 92% ± 8.2% (***$P < .001$); and group D: 83% ± 14% (***$P < .001$).



mean:	17.9	8.9	3.6	2.0	
max:	442	349	12.1	12.0	x10³ copies/μg DNA
min:	2.8	0.99	0.29	0.22	

FIG E2. KREC levels were analyzed in genomic DNA samples extracted from peripheral blood of control subjects at different age groups (n = 158; age range, 1 month to 55 years). KREC levels were significantly higher in infants (17.9 ± 3.9 × 10^3 copies/μg DNA) compared with other children's age groups (8.9 ± 1.3 × 10^3 copies/μg DNA in the 1- to 6-year-old group and 3.6 ± 3.8 × 10^3 copies/μg DNA in the 7- to 18-year-old group) and adults (2.0 ± 3.3 × 10^3 copies/μg DNA; $P < .0001$).

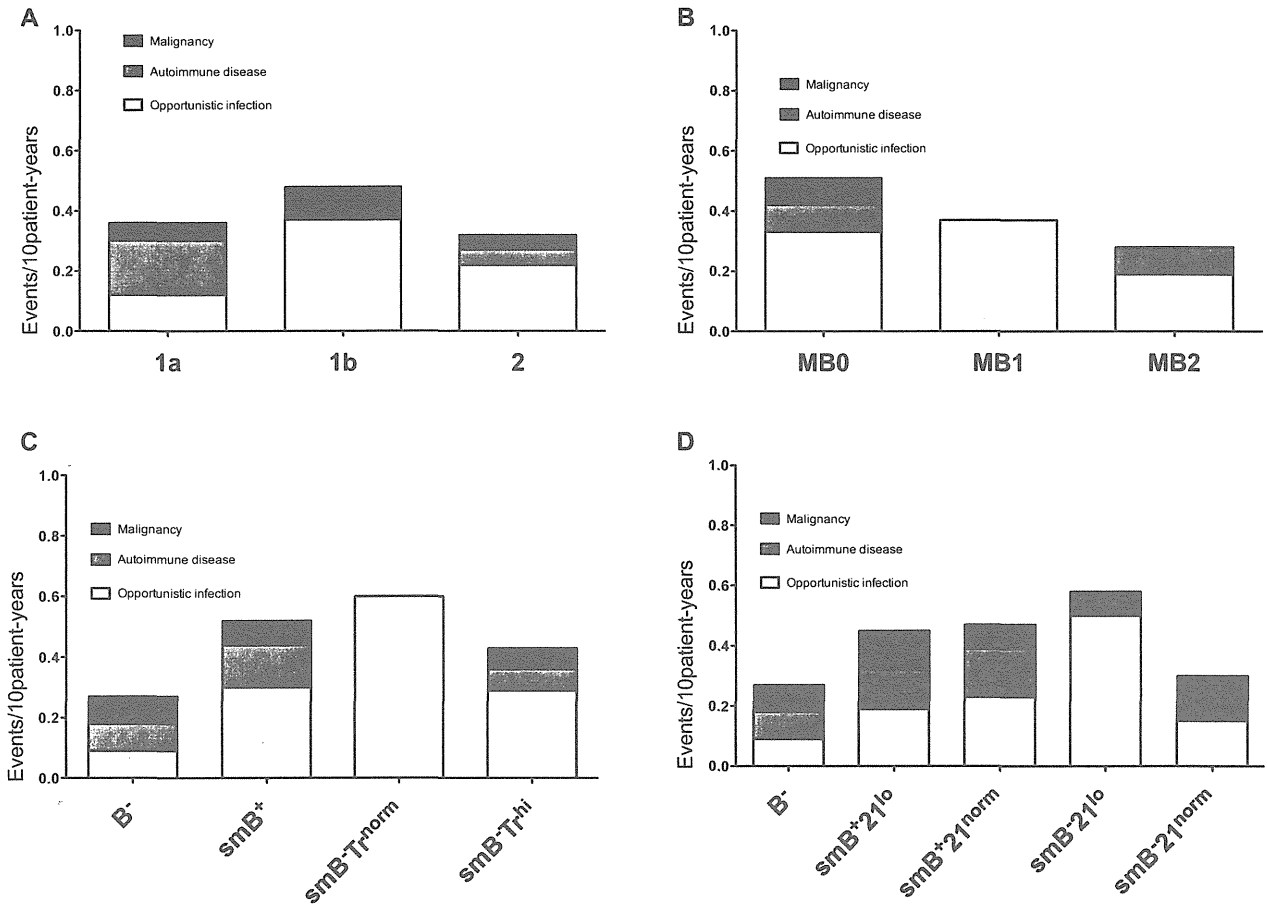


FIG E3. Patients were classified in the following way and analyzed for cumulative incidence of complications: **A**, Freiburg; **B**, Paris; and **C**, EUROclass classifications, according to $CD38^{hi}IgM^{hi}$ transitional B cells (Fig E3, *A-C*) or $CD21^{low}$ B cells (**D**). Five patients were excluded from the Freiburg and Paris classifications because of decreased B-cell numbers (<1%). Additionally, we excluded 4 patients in the Freiburg classification, 1 patient in the Paris classification, and 4 patients in the EUROclass classification for transitional B cells and 8 in the EUROclass classification for $CD21^{low}$ B cells because of lack of data. The following cumulative events/10 patient-years were found. Freiburg classification: 1a, 0.36; 1b, 0.48; 2, 0.32. Paris classification: MB0, 0.50; MB1, 0.37; MB2, 0.28. EUROclass classification according to transitional B cells: B^-, 0.27; smB^+, 0.52; smB^-Tr^{norm}, 0.60; smB^-Tr^{high}, 0.43. EUROclass classification according to $CD21^{lo}$ B cells: B^-, 0.27; smB^+21^{lo}, 0.45; smB^+21^{norm}, 0.47; smB^-21^{lo}, 0.58; smB^-21^{norm}, 0.30. No classification showed any significantly increased events in any particular group according to calculated *P* values, as follows—Freiburg classification: 1a vs 2 = .898, 1b vs 2 = .479, 1a vs 1b = .838; Paris classification: MB0 vs MB2 = .179, MB1 vs MB2 = .654, MB0 vs MB1 = .764; EUROclass classification according to transitional B cells: B^- vs smB^+ = .298, smB^-Tr^{norm} vs smB^+ = .809, smB^-Tr^{hi} vs smB^+ = .702, smB^-Tr^{hi} vs smB^-Tr^{norm} = .641, smB^-Tr^{norm} vs B^- = .329, smB^-Tr^{hi} vs B^- = .508; EUROclass classification according to $CD21^{lo}$ B cells: B^- vs smB^+21^{norm} = .443, smB^+21^{lo} vs smB^+21^{norm} = .930, smB^-21^{lo} vs smB^+21^{norm} = .695, smB^-21^{norm} vs smB^+21^{norm} = .575, B^- vs smB^-21^{norm} = .926, smB^+21^{lo} vs smB^-21^{norm} = .609, smB^-21^{lo} vs smB^-21^{norm} = .399, B^- vs smB^+21^{lo} = 0.474, B^- vs smB^-21^{lo} = 0.270, smB^+21^{lo} vs smB^-21^{lo} = 0.618.

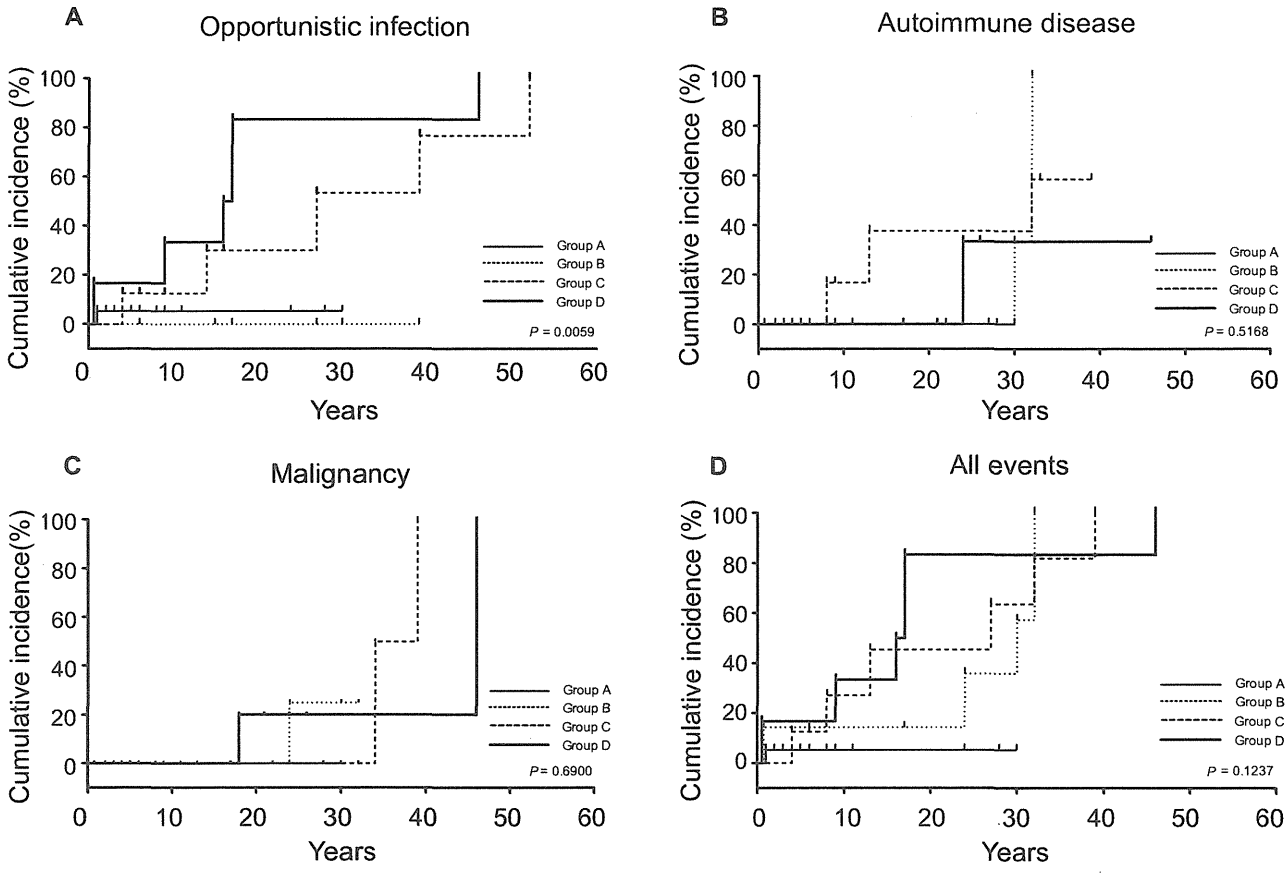


FIG E4. Comparing longitudinal cumulative incidence of complication events among groups. Cumulative incidence was estimated separately and longitudinally by using the Kaplan-Meier method and statistically compared between groups by using the log-rank test. The cumulative incidence of opportunistic infections **(A)**, autoimmune diseases **(B)**, malignancies **(C)**, and all events **(D)** is shown.

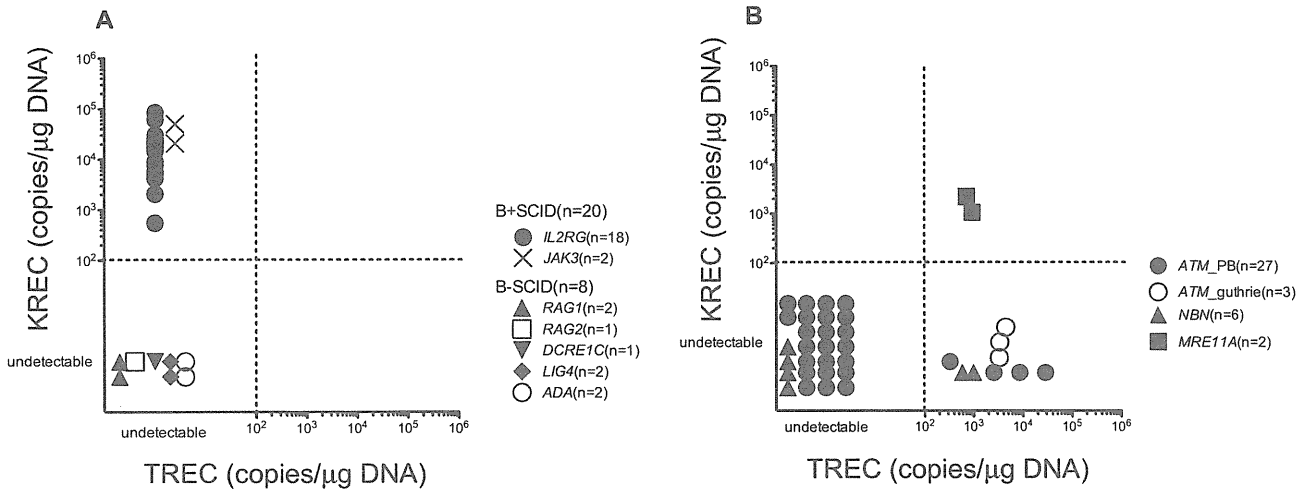


FIG E5. TREC and KREC quantification classifies patients with SCID, AT, NBS, or ataxia-telangiectasia–like disease *(ATLD)* into 4 groups. **A**, Patients with B^+SCID (n = 20) were classified as group C, and patients with B^-SCID (n = 8) were classified as group D; these patients were included in the previous studies.[5,6] **B**, Although most patients with AT (n = 23) and patients with NBS (n = 4) were classified as group D, TRECs were detected in peripheral blood samples (n = 4 in patients with AT and n = 2 in patients with NBS) and neonatal Guthrie cards (n = 3) of some patients with AT, who were classified as group B. Patients with ATLD with *MRE11A* mutations were classified as group A.

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CASE REPORT

A novel Wiskott–Aldrich syndrome protein mutation in an infant with thrombotic thrombocytopenic purpura

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Abstract

Thrombotic thrombocytopenic purpura (TTP) has not yet been reported to be associated with mutations in the Wiskott–Aldrich syndrome (WAS) gene. WAS is an X-linked recessive disorder characterized by thrombocytopenia, small platelet size, eczema, recurrent infections, and increased risk of autoimmune disorders and malignancies. A broad spectrum of mutations in the WAS protein (WASP) gene have been identified as causing the disease. In this study, we report on a 2-month-old Japanese boy who presented with cytomegalovirus (CMV) infection and TTP. The activity of von Willebrand factor cleaving metalloproteinase, ADAMTS13 was low and the antibody against ADAMTS13 was positive (3.6 Bethesda U/mL). Although TTP was improved by plasma exchange and steroid pulse therapy, thrombocytopenia persisted and regular transfusions of irradiated platelets were needed. Tiny platelets were found on a peripheral blood smear. CMV genome was positive in peripheral blood by polymerase chain reaction and the CMV viremia continued to persist despite intravenous gancyclovir therapy. Through direct sequencing of genomic DNA of the WASP gene in the patient, we identified a novel mutation of WASP gene: the seventh nucleotide in exon 11 (G) had been deleted (1345delG). This mutation causes a frameshift and a stop codon at amino acid 470. Western blotting demonstrated a truncated WAS protein. To our knowledge, this is the first report describing TTP in WAS patients with novel mutation in the WASP gene.

Key words Wiskott-Aldrich syndrome; thrombotic thrombocytopenic purpura; autoimmunity

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Wiskott–Aldrich syndrome (WAS) is a rare X-linked disorder with variable clinical phenotypes that correlate with the type of mutations in the WAS protein (WASP) gene (1). The WASP gene is composed of 12 exons containing 1823 base pairs and encodes a 502-amino acid protein that appears to be of central importance for the function of hematopoietic stem cells (2). Mutations of WASP gene are located throughout the gene, although some hot spots have been identified (3). The type of mutation strongly influences the clinical severity of WAS (3). Mutations that abolish WASP expression are mainly associated with a severe clinical phenotype (full blown WAS) and a life expectancy below 20 yr of age (4). Mutations, on the other hand, result in residual expression of a full-length point-mutated WASP, are often associated with X-linked thrombocytopenia (XLT) (5), corresponding to a longer life expectancy (6). A scoring system based on clinical symptoms has been developed to differentiate these distinct clinical phenotypes caused by WASP gene mutations (2, 3, 7). Autoimmune complications are frequently observed in WAS and patients who develop autoimmune diseases are assigned to a high-risk group with poor prognosis (1). The incidence of autoimmunity in WAS is high in the US and European populations (40–72%), whereas a lower incidence was reported in Japan (22%)

(1, 6). The most common autoimmune manifestation in WAS is hemolytic anemia (36%), followed by vasculitis (including cerebral vasculitis; 29%), arthritis (29%), neutropenia (25%), inflammatory bowel disease (9%), and IgA nephropathy (3%) (8). Henoch–Schönlein purpura, dermatomyositis, recurrent angioedema, and uveitis have also been reported in some patients (6, 9). Moreover, in some cases, multiple autoimmune manifestations are observed.

Autoimmune hematological diseases are characterized by the production of antibodies against blood proteins and cells, and comprise immune thrombocytopenia, autoimmune hemolytic anemia, acquired hemophilia, and thrombotic thrombocytopenic purpura (TTP). TTP is a rare but severe disease characterized by mechanical hemolytic anemia and consumptive thrombocytopenia leading to disseminated microvascular thrombosis that causes signs and symptoms of organ ischemia and functional damage. von Willebrand factor (vWF) is synthesised in endothelial cells and assembled in larger multimers that are present in normal plasma. The larger multimers, called unusually large vWF (ULvWF), are rapidly degraded in the circulation into the normal size range vWF multimers by a specific vWF-cleaving protease, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13) (10). ADAMTS13 deficiency leads sequentially to the accumulation of ULvWF multimers, platelet aggregation and platelet clumping, which is characteristic of the disease. ULvWF multimer accumulation in TTP is associated with absent or markedly diminished ADAMTS13 activity due to an inherited or acquired deficiency (11). An inhibitory autoantibody to the ADAMTS13 metalloproteinase has been found in patients with acquired TTP (11).

Here, we report a male infant who presented with cytomegalovirus (CMV) infection and acquired TTP which led to the diagnosis of WAS. A novel mutation, one nucleotide deletion at position 1345 (1345delG) in exon 11 was identified. To our knowledge, this is the first report regarding WAS with TTP as an autoimmune disease.

Materials and methods

Flow cytometric analysis of WASP expression

Intracellular staining with anti-WASP mAb was performed as described by Kawai *et al.* (12) In brief, peripheral blood mononuclear cells (PBMCs) from both a healthy control and the patient were first fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and stained with phycoerythrin (PE)-labeled CD3 (PharMingen, San Diego, CA, USA), CD19 (Beckman Coulter, Fullerton, CA, USA), or CD56 (PharMingen) mAb. Then cells were permeabilized in 0.1% Triton X-100 in Tris-buffered saline (pH 7.4) with 1% fetal calf serum (FCS) and 0.1% NaN_3 for 5 min. Subsequently, these cells were reacted with 10 mg/mL of anti-WASP (5A5) (12) or isotype-matched control mouse IgG2a mAb (PharMingen) for 20 min on ice, washed, and then incubated with 10 mg/mL of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG2a antibody (Southern Biotechnology Associates, Birmingham, AL, USA). The stained cells were immediately analyzed on an EPICS XL (Beckman Coulter).

Anti-WASP antisera and Western blot analysis

B-Lymphoblatoid cell lines (B-LCLs) were established by inoculating PBMCs from healthy controls and the patient with Epstein–Barr virus (EBV) – containing supernatant (6). B-LCLs from healthy control and the patient were suspended at 1.0×10^7/mL in lysis buffer containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% aprotinin, and 10 μg/mL leupeptin at pH 7.5 and were kept on ice for 30 min. From each sample, 40 μg total protein was loaded onto a sodium dodecyl sulfate polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were incubated with rabbit anti-WASP antibody (Ab 503) against a synthetic peptide (aa's 209–226 of WASP) (6) at 1 : 5000 dilutions. The membranes were incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Promega, Madison, WI, USA). Results were visualized by incubation with AP buffer (100 mM Tris–HCl, pH 9.5; 100 mM NaCl; and 5 mM $MgCl_2$).

DNA purification and sequencing of genomic DNA

Genomic DNA was extracted from the patient's PBMCs using Sepa-Gene (Seikagaku kogyo, Tokyo, Japan). Purified genomic DNA samples were amplified with primer pairs designed to span each exon and exon/intron junction, and the specific causative mutation was identified by direct sequencing as described previously (6). For gene sequencing, informed consent by the patient's family and approval by institutional review boards was obtained.

Patient and results

The patient was the first son of healthy and non-consanguineous Japanese parents, born at term following an uncomplicated pregnancy, and his body weight at birth was 2888 g. His past medical history was unremarkable. At the age of 2 months, he presented with fever, intermittent tachypnea, and general petechiae. On examination, he looked pale and icteric. He had hepatosplenomegaly, but did not have lymphoadenopathy or eczema. Peripheral blood analysis disclosed severe anemia and thrombocytopenia with hemoglobin (Hb) of 3.9 g/dL (normocytic), reticulocytes of 37.8% and platelet count of 11×10^9/L. The mean platelet volume was 5.8–8.1 fL (normal range, 9.0–10.7 fL) and morphology

showed small platelets. White blood cell count (WBC) was 12.3 × 10^9/L. Laboratory investigations revealed the following: serum total bilirubin (T-bil) 3.5 mg/dL (indirect 2.4 mg/dL), lactate dehydrogenase (LDH) 3264 IU/L, aspartate aminotransferase (AST) 210 IU/L, alanine aminotransferase (ALT) 73 IU/L, gamma-glutamyltranspeptidase (γGTP) 257 IU/L, blood urea nitrogen (BUN) 12 mg/dL and creatinine (Cre) 0.22 mg/dL. His prothrombin time, activated partial thromboplastin time and fibrinogen were normal. D-dimer was 7.8 μg/mL (normal range, 0–0.5 μg/mL) and haptoglobin was 8.9 mg/dL with a negative Coombs' test. Furthermore, peripheral blood smears showed fragmented red blood cells. Urinalysis revealed microscopic hematuria.

The patient was diagnosed as having TTP and treated with steroid pulse and plasma exchange (PE) therapy (40 mL/kg/d) for six consecutive days. The patient responded with elevations in the Hb to 8.0 g/dL. LDH decreased to 600 IU/L. Further serum analysis on admission showed a noticeable decrease in ADAMTS13 activity to <0.5% (normal, 70–120%), with the existence of anti-ADAMTS13 IgG autoantibody. Anti-ADAMTS13 IgG autoantibody was evaluated with the chromogenic ACT enzyme-linked immunosorbent assay (ELISA) with the Bethesda method in the Department of Blood Transfusion, Nara Medical University. One Bethesda unit is defined as the amount of inhibitor that reduces the enzymatic activity by 50% of the control value, and values >0.5 U/mL are considered significant (13, 14). Our patient showed markedly decreased ADAMTS13 activity (<0.5%) and tested positive for anti-ADAMTS13 IgG autoantibody (3.6 Bethesda U/mL) at the onset of TTP.

Viral serology study showed a positive result for CMV IgM. CMV was subsequently identified by a urine shell vial culture method and a plasma polymerase chain reaction test for CMV (PCR-CMV) demonstrated significant viremia with 7.0 × 10^5 copies/mL. Administration of intravenous ganciclovir (10 mg/kg/d) was initiated. Gancyclovir therapy was continued until viral loads were stable at around 1000 copies/mL and did not seem to further decline. His platelet counts, however, did not rise and the child required repeated platelet transfusions. A trial of intravenous immunoglobulin (IVIG) as well as a trial of systemic prednisone failed to induce a rise in platelet counts. Antiplatelet antibodies were negative. He also developed several episodes of gastroenteritis due to norovirus and methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia secondary to soft tissue infection or pneumonia, despite the monthly administration of prophylactic treatment with intravenous immunoglobulin. The presence of thrombocytopenia, small sized platelets, frequent potentially life-threatening infections and autoimmune disease led to the consideration of WAS. WASP expression was examined by flow cytometric analysis of intracellular WASP expression and a reduced expression level was detected (Fig. 1A). Western blot analysis of lysates from the normal control showed that WASP was normally expressed (66 kDa), but a truncated WASP was expressed in the patient (Fig. 1B). Sequencing of WASP genomic DNA identified a one-nucleotide (G) deletion at the position of exon 11, that cause a frameshift, resulting in the generation of a premature stop signal at codon 470 (Fig. 1C and 1D). This mutation has not been previously described. Immunological analysis of peripheral blood revealed normal percentages and numbers of $CD3^+$ T cells (1.35 × 10^9 cells/L), $CD19^+$ B cells (0.85 × 10^9 cells/L) and $CD16^+CD56^+$ NK cells (0.78 × 10^9 cells/L). Analysis of cytolytic activity against K562 target cells demonstrated a normal functional activity of the patient's NK cells compared with that from control.

Discussion

The 502-amino acid protein, WASP, consists of five functional domains: an N-terminal *Drosophila*-enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain, a basic region (BR), a GTPase-binding domain (GBD), a polyproline-rich region (PRR) and a C-terminal verpolin cofilin homology domains/acidic region (VCA) domain (3) (Fig. 1D). Since the causative gene was first isolated and cloned in 1994(15), various unique mutations have been reported in the WASP gene, spanning all 12 exons. Here, we report a novel WASP gene mutation identified in a Japanese boy, that is, deletion of one nucleotide (G) in exon 11 (1345delG), which leads to a frameshift, resulting in a stop codon at amino acid 470. Most missense mutations are localized to the EVH1 domain, and a mutated WASP often cannot bind to WASP-interacting protein (WIP), leading to defective WASP expression (16). However, since 1345delG mutation causes the partial deletion of WASP in VCA domain, but still maintains an intact EVH1 domain for WIP binding, we can assume that the mutant WASP can bind to WIP and is relatively stable, which protects the truncated WASP from being degraded. But, due to the lack of the VCA area, the truncated WASP cannot combine with the actin-related protein (ARP) 2/3 complex, which plays a key role in cytoskeletal remodeling. WASP, in the active form, binds the ARP 2/3 complex, which gives rise to nucleation of actin filaments at the side of pre-existing filaments, thus creating a branching network of actin at the plasma membrane (8). The activity of the ARP2/3 complex was shown to contribute to a variety of cellular functions, including change of cell shape, motility, endocytosis, and phagocytosis (17).

While many thought that autoimmunity was more common in patients with complete WASP deficiency, recent reports show that autoimmunity can occur in both severe and attenuated cases of the disease (6). Antibody-mediated cytopenias are the most frequent manifestation of autoimmune reactions but various vascular and organ-based autoimmune processes have also been reported (18). Although 22–72% of reported WAS cases suffered from autoimmune disorders,

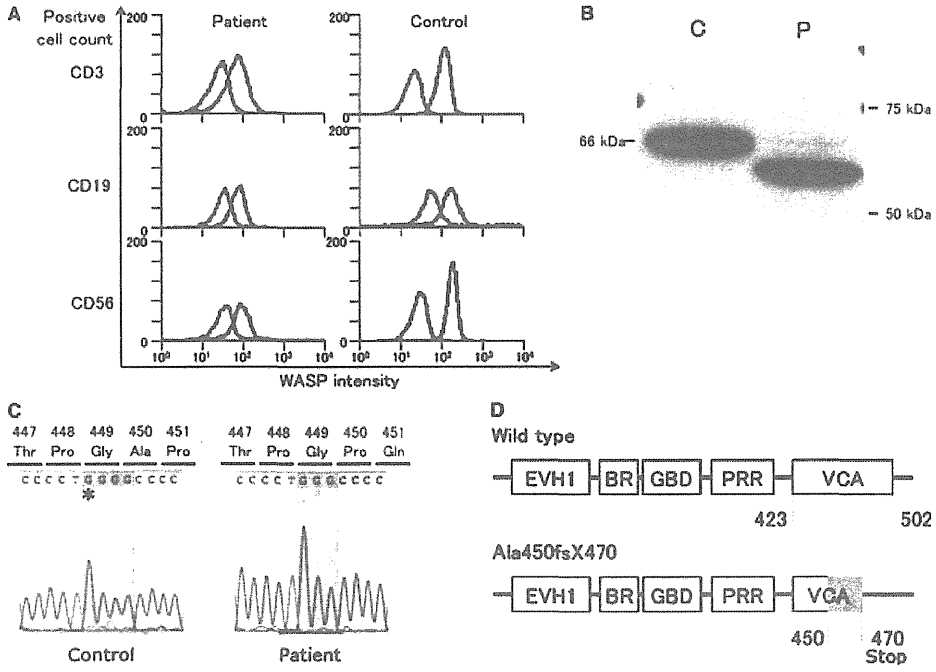

Figure 1 (A) Intracytoplasmic Wiskott–Aldrich syndrome (WAS) protein (WASP) expression analysis by flow cytometry. Histograms represent anti-WASP staining compared with isotype control in different lymphocyte subsets as indicated. (B) Anti-WASP Western blot analysis from peripheral blood mononuclear cells (PBMCs). The lysate from normal individual expressed WASP at a normal size (66 kDa), and a truncated WASP was expressed in the patient's PBMCs. C: normal control, P: patient. (C) Mutation analysis of the WASP gene. Electropherogram shows the deletion in exon 11 of the WASP gene. The position of the deletion is indicated by the asterisk on the wild-type sequence, and the changes of amino acids in the patient are shown. (D) Wild type and 1345delG-mutated WASP. EVH1, Ena/VASP homology 1 domain; BR, basic region; GBD, GTPase-binding domain; PRR, proline-rich region; VCA, verpolin cofilin homology domains/acidic region.

none of them developed TTP (8, 19). Why the present case developed TTP as an autoimmune disorder is not clear. Thrombotic microangiopathy (TMA) including TTP has been shown to occur in the setting of bacterial infections, viral infections, autoimmune diseases, malignancies, pregnancy related complications, and certain medications such as ticlopidine, cyclosporine, and tacrolimus (20). To date, there are several case reports of active CMV infection associated with TMA in both immunocompetent and immunosuppressed individuals. Although the exact pathogenesis by which CMV infection results in TMA is unknown, CMV has been shown to injure endothelial cells either by direct infection or indirectly by initiating an abnormal immune response (20, 21).

Thrombocytopenic purpura concurrently occurs in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, scleroderma, Still's disease, polymyositis, and myasthenia gravis (22). While the present case has no autoimmune disorders other than TTP, Monteferrante *et al* (23). presented a patients with WAS who developed SLE at the age of 12 yr. The definitive phenotype in patients with mutations in the WAS gene may manifest only late in life and never reach the medical literature (6). Nikolov *et al.* and Humblet-Baron *et al.* (24, 25) have found that older WASP deficient mice develop anti-nuclear and anti-dsDNA antibodies at much higher rates than isogenic controls with titers approaching those of other autoimmune-prone mouse strains. In WASP deficient mice over 6 months of age, Nikolov *et al.* (24) found circulating immune complexes, immune complex deposition in the kidney, and mild nephritis resembling the IgA nephropathy seen in some patients with WAS. As infants with WAS may not yet have developed the final clinical phenotype, careful observation for unexpected clinical phenotypes is warranted.

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Effects of idursulfase enzyme replacement therapy for Mucopolysaccharidosis type II when started in early infancy: Comparison in two siblings

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ABSTRACT

Mucopolysaccharidosis type II (MPS II) is a lysosomal storage disorder that is progressive and involves multiple organs and tissues. While enzyme replacement therapy (ERT) with idursulfase has been shown to improve many somatic features of the disease, some such as dysostosis multiplex and cardiac valve disease appear irreversible once established, and little is known about the preventative effects of ERT in pre-symptomatic patients. We report on two siblings with severe MPS II caused by an inversion mutation with recombination breakpoints located within the *IDS* gene and its adjacent pseudogene, *IDS-2*. The siblings initiated treatment with idursulfase at 3.0 years (older brother) and 4 months (younger brother) of age, and we compared their outcomes following 2 years of treatment. At the start of treatment, the older brother showed typical features of MPS II, including intellectual disability. After 34 months of ERT, his somatic disease was stable or improved, but he continued to decline cognitively. By comparison, after 32 months of ERT his younger brother remained free from most of the somatic features that had already appeared in his brother at the same age, manifesting only exudative otitis media. Skeletal X-rays revealed characteristic signs of dysostosis multiplex in the older brother at the initiation of treatment that were unchanged two years later, whereas the younger brother showed only slight findings of dysostosis multiplex throughout the treatment period. The younger brother's developmental quotient trended downward over time to just below the normal range. These findings suggest that pre-symptomatic initiation of ERT may prevent or attenuate progression of the somatic features of MPS II. Follow-up in a larger number of patients is required to confirm the additive long-term benefits of ERT in pre-symptomatic patients.

1. Introduction

Mucopolysaccharidosis (MPS) type II (Hunter syndrome, OMIM #309900), is an inborn error of glycosaminoglycan (GAG) metabolism caused by deficient activity of lysosomal iduronate 2-sulfatase (IDS, EC 3.1.6.13). The responsible gene, IDS, is located on chromosome Xq28, and the disease shows classic X-linked recessive inheritance. Rarely, females may be affected as a result of biallelic mutations, skewed X-inactivation, uniparental isodisomy, or X-autosome translocations [1,2]. Dermatan sulfate and heparan sulfate, the substrates for IDS, accumulate in the lysosomes of various tissues and organs of affected patients, leading to the development of characteristic signs and symptoms of MPS II after the first year of life. (HOS reference). Somatic features include coarse facies, straw-like hair, rough and thickened skin, macrocephaly, disproportionate short stature due to dysostosis multiplex, decreased joint mobility, cardiac valve disease and left ventricular hypertrophy, hepatosplenomegaly, obstructive sleep apnea, and restrictive lung disease. Frequent otitis media and hernias (inguinal and umbilical) may be the earliest presenting signs, but are non-specific. Patients with little to no IDS activity (severe form) exhibit progressive somatic disease, cognitive decline, and death during adolescence (HOS). Patients with some residual IDS activity (mild form) have largely somatic disease with normal intellectual development [3].

In recent years, enzyme replacement therapy (ERT) with recombinant human iduronate-2sulfatase (idursulfase, Elaprase®, Genzyme, a Sanofi Company and Shire Human Genetic Therapies, Cambridge, MA) has been available for the treatment of MPS II. Weekly infusions of idursulfase have been shown to improve walking capacity, hepatosplenomegaly, and urinary GAG levels [4]. However, ERT appears to be less effective in correcting disease manifestations once developed in the skeletal system and heart valves [5,6]. Intravenously administered ERT has not been shown to slow or prevent the deterioration of the central nervous system in patients with the severe phenotype, most likely because it does not cross blood–brain barrier at the labeled dose [7]. Although idursulfase is approved for use only in patients who are at least 5 years of age, a recent report from the Hunter Outcome Survey (HOS) suggests that it can safely reduce urinary GAG levels and hepatomegaly in young children, some

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of whom were below 1 year of age [8]. Recognizing that MPS II is a progressive disease that has some irreversible features, a panel of MPS II experts has recommended starting ERT as early as possible to achieve the best outcomes [9].

In this report, we describe our treatment experience in two Japanese brothers with the severe form of MPS II who started ERT at 4 months of age (pre-symptomatic) and 3 years of age (symptomatic). Our findings suggest that early, pre-symptomatic treatment is associated with a better clinical outcome as evidenced by the amelioration or prevention of certain somatic manifestations, e.g. dysostosis multiplex and cardiac valve disease, which once established, appear to be irreversible.

1.1. Case report

1.1.1. Patient 1 (older brother)

A 2 year 7 month old boy presented to our metabolism clinic with dysmorphic features, cardiac and skeletal disease, and severe developmental delay. He was the first child born to non-consanguineous Japanese parents. Following an uneventful pregnancy and neonatal period, he was noted to have a small ventricular septal defect during a febrile illness at 3 months of age. At 9 months of age, the ventricular septal defect had closed but mild mitral valve regurgitation was present. His parents noticed a gibbus deformity at approximately 1 year of age, and by age 2 he had developed stiffness in his elbow and fingers. His psychomotor development was moderately delayed: he walked at 1.5 years and was still non-verbal. Other past medical history was notable for a febrile seizure, umbilical hernia, enlarged adenoids, and bilateral otitis media. On physical examination, the boy had a coarse facies and disproportionately short limbs. His was above average in height (92.2 cm, +0.6 SD), overweight (17.0 kg, +3.2 SD), and had macrocephaly (50 cm, +0.5 SD). He had marked hepatomegaly and a nonpalpable spleen. Urinary GAG analysis revealed an elevated uronic acid level of 254 mg/g creatinine (normal mean ± SD, 30.0 ± 12.8) with increased amounts of dermatan sulfate (63%) and heparan sulfate (12%) relative to chondroitin sulfate (25%), consistent with MPS I or II. The diagnosis of MPS II was confirmed by the absence of detectable I2S activity in leukocytes.

No potential disease-causing mutation was found by sequencing all 9 exons of the IDS gene and their intron-exon junctions by conventional PCR-based methods [10]. To detect a recombination mutation between IDS and its adjacent putative pseudogene, IDS-2, that leads to an inversion and non-functional *IDS* gene, we performed a simple and rapid assay involving two PCR reactions. The first reaction selectively amplifies a 2.8 kb DNA fragment from the recombinant gene but not the wild type IDS gene, while the second reaction selectively amplifies a 3.5 kb DNA fragment from the wild type IDS gene but not the recombinant gene (Fig 1a) [11]. Genetic testing of the patient revealed an abnormal banding pattern indicative of recombination between the IDS gene and the IDS-2 pseudogene (Fig 1b).

1.1.2. Patient 2 (younger brother)

The younger brother was born just after his older brother was diagnosed with MPS II. Birth weight (2.966 kg) and length (47 cm) were normal for his gestational age of 39 weeks. There were no abnormal findings on initial physical examination, but the urinary uronic acid level was elevated at 423 mg/g creatinine (normal mean ± SD, 43.4 ± 12.9), and urinary GAG analysis showed increased amounts of dermatan sulfate (55%) and heparan sulfate (11%) relative to chondroitin sulfate (34%). IDS activity in leukocytes was below the detectable limit. As expected, Patient 2 had the same recombination mutation as his older brother.

1.1.2.1. Enzyme replacement therapy. Treatment with intravenous recombinant idursulfase was started at 3.0 years of age for Patient 1 and 4 months of age for Patient 2. Although the recommended dose of idursulfase is 0.5 mg/kg/week, Patient 1 received only 0.3–0.4 mg/kg/week for the first 1.5 years until his weight reached 20 kg (4.5 years of age) because of a restriction by the health insurance system; subsequently, he received 0.5 mg/kg/week of idursulfase. The dose for Patient 2 was 0.5 mg/kg/week from the start of treatment. As of December 2012, Patients 1 and 2 had received ERT for 34 and 32 months, respectively. Both patients have tolerated ERT well with only mild and intermittant urticaria.

2. Results

2.1. Urinary GAG

The uronic acid in urine was measured at several time points after initiation of ERT using the carbazole reaction method (SRL Medisearch, Tokyo, Japan). Fig. 2 shows the changes observed in both patients over time. In Patient 1, the uronic acid level decreased to approximately half of the baseline level after 3 months and then plateaued at 130–180 mg/g creatinine (29–49% reduction from baseline) (Fig. 2a). The uronic acid level in Patient 2 showed a continuous decrease to below 100 mg/g creatinine (76% reduction from baseline), but remains above the normal range (Fig. 2b).

2.2. Liver and spleen size

The liver edge of Patient 1 extended 4 cm below the right costal margin at baseline, and it rapidly became non-palpable after the initiation of ERT. The spleen was not palpable at any time, and by ultrasound, it was at the upper limit of normal size for age and remained stable during the first 28 months of ERT. Patient 2's liver and spleen were normal in size before and during ERT.

2.3. Cardiac function

At baseline, Patient 1's echocardiogram revealed moderate mitral valve regurgitation and a mildly distorted left ventricular wall, although the ejection fraction was normal at 69 %. These findings showed little change after 22 months of ERT. In Patient 2, no abnormalities were detected by echocardiography before and after 11 months of ERT.

2.4. Respiratory and Hearing

Patient 1 had bilateral exudative otitis media and adenoid hypertrophy at baseline that did not respond well to ERT. Although an adenoidectomy was performed at 3.5 years of age, exudative otitis media and hearing impairment persisted. Patient 2 also had exudative otitis media during the ERT period. Neither patient developed sleep apnea.

2.5. Skeletal X-rays

At baseline, dysostosis multiplex was already apparent in Patient 1. The most prominent findings were hypoplastic changes of the vertebral bodies giving rise to a characteristic protrusion of the antero-inferior surface, the so-called inferior tongue. Other mild signs of dysostosis multiplex included oar-like ribs, bullet-shaped phalanges, and iliac flaring. After 27 months of ERT, these findings showed little change. Similar, but milder findings of oar-like ribs and bullet-shaped phalanges were present in Patient 2 at 3 months of age. After 25 months of ERT, "inferior tongue" had become notable and oar-like ribs had progressed (data not shown).

2.6. Joints

Patient 1 had stiffness in multiple joints of his extremities at baseline. There was no obvious change with ERT, although accurate

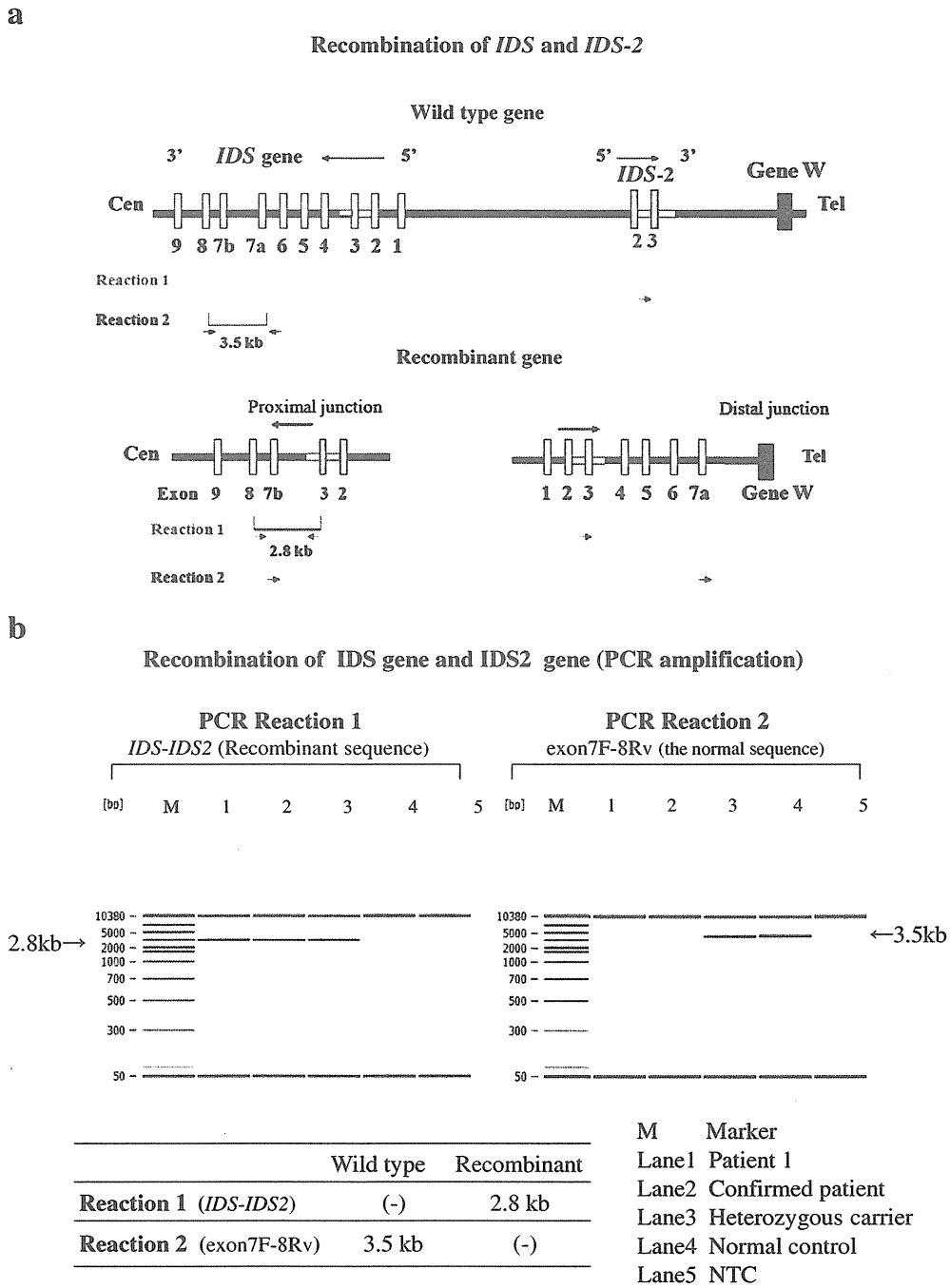

Fig. 1. Genetic diagnosis of MPS II by detecting recombination of the IDS and IDS-2 genes. The pseudogene IDS-2, which consists of sequences that are homologous to exons 2 and 3 and intron 7 of the IDS gene, is located ~20 kb telomeric to *IDS* in Xq27.3–q28. In the recombinant gene, exons 1, 4, 5, 6, 7a are translocated to the IDS-2 locus, thereby grossly altering the structure of the IDS gene. PCR reaction 1 amplified a 2.8 kb fragment of the recombinant gene in Patients 1 and 2 and the heterozygous carrier, but not in the normal control. PCR reaction 2 amplified a 3.5 kb fragment of the wild-type *IDS* gene in the heterozygous carrier and normal control, but not in the two patients.

measurement was difficult. Patient 2 had normal joint mobility that was maintained during ERT.

3. Magnetic resonance imaging of the central nervous system

By MRI, Patient 1 had dilated perivascular spaces in the cerebral white matter both at baseline and after 22 months of ERT, and at the latter timepoint, mild dilatation of the lateral ventricles also was apparent. At baseline, Patient 2's MRI showed only subtle changes in the corpus callosum that were suggestive of dilated perivascular spaces. After 14 months ERT, the dilated perivascular spaces became more typical and resembled those of his brother. Patient 2 did not show any evidence of hydrocephalus or cerebral atrophy (Data not shown).

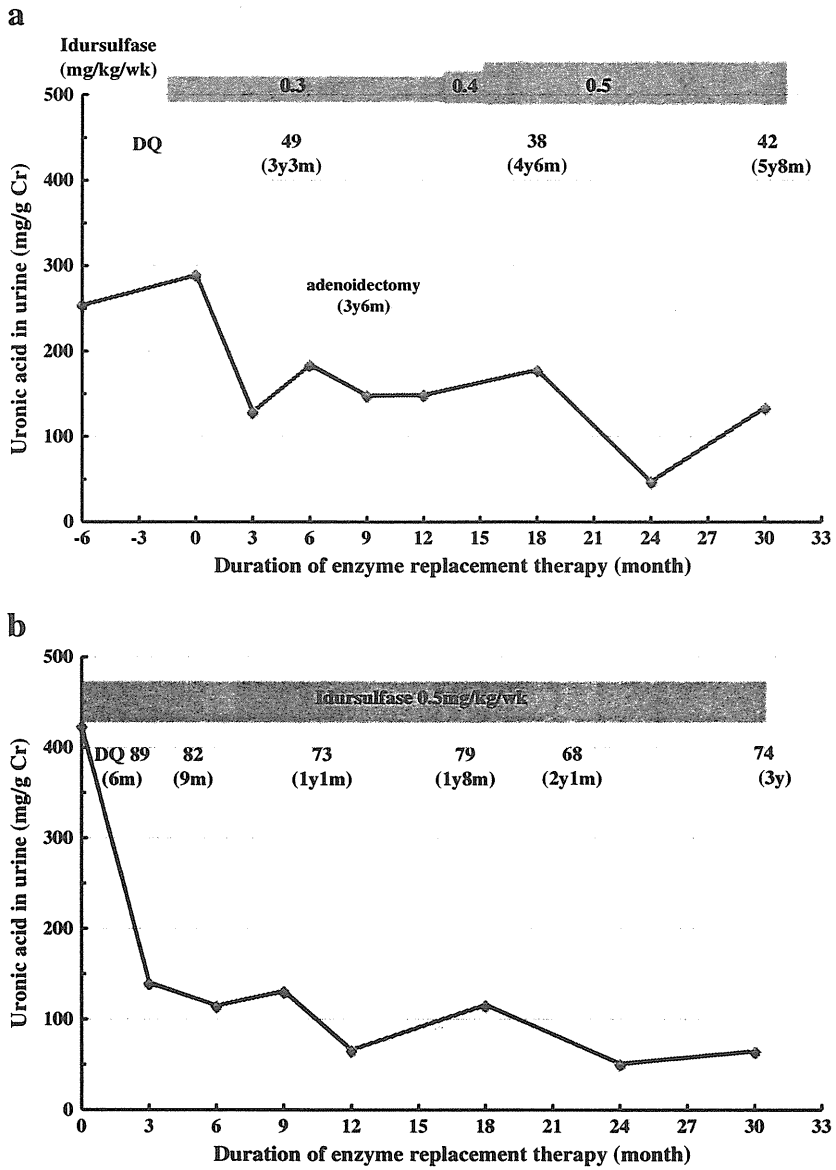

Fig. 2. Uronic acid level in urine and developmental quotient (DQ) during ERT for (a) Patient 1 and (b) Patient 2. Idursulfase was administered to Patient 1 at a reduced dose for more than 1 year because of a restriction from the health insurance system.

3.1. Psychomotor development

The developmental quotient (DQ) score of Patient 1 was already low at baseline (DQ=49), and it declined further after 34 months of ERT (DQ=42). He barely acquired any words, and behavioral problems, such as hyperactivity, were becoming significant. The DQ score of Patient 2 was initially normal, but then fell to just below the normal range during ERT. His DQ scores at 6, 9, 13, 18, 21 and 34 months of age were 89, 82, 73, 79, 68 and 74 respectively. He recently learned to walk, but he does not yet speak any words.

These observations are summarized in Table 1.

3.2. Facial appearance

The preventative effects of ERT on facial appearance were evident in Patient 2 (Fig. 3b; 2 years and 10 months old) compared with

Table 1
Clinical features of the patients.

	Patient 1 (elder brother)		Patient 2 (younger brother)	
Start of ERT	3y0m		0y4m	
Duration of ERT	34 m		32 m	
Clinical symptoms	before ERT (3y0m)	after ERT (5y10m)	before ERT (0y4m)	after ERT (3y0m)
Coarse features of face	yes	stable	no	no
Thick and coarse skin	yes	improved	no	no
Hepatosplenomegaly	yes	improved	no	no
Cardiac dysfunction	yes	stable	no	no
Problems in joints	yes	stable	no	no
Dysostosis multiplex	yes	slowly progressive	mild	slowly progressive
Exudative otitis media	yes	persistent	no	yes
Developmental quotation	49	42	89	74

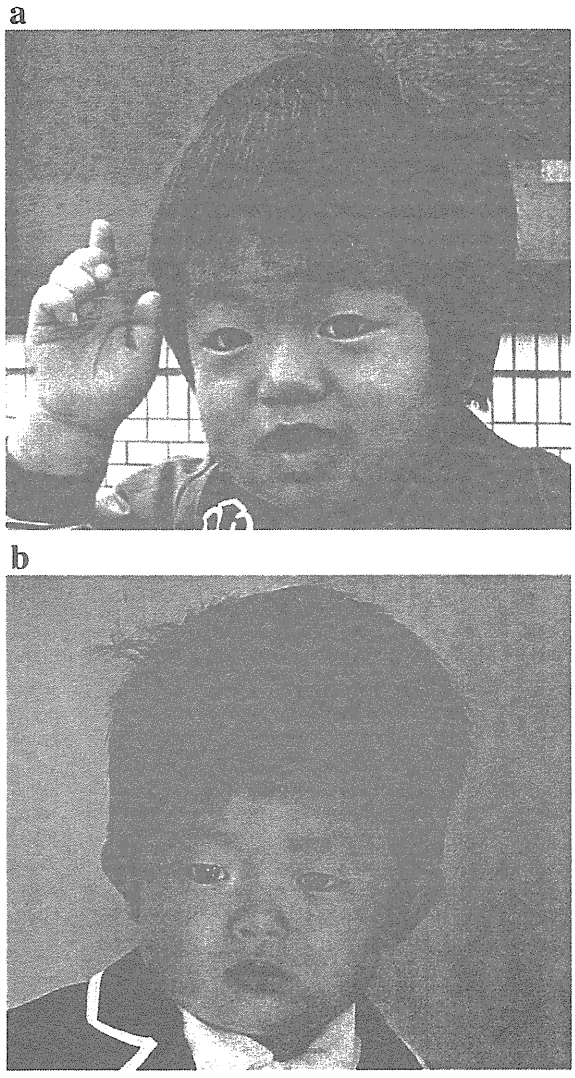

Fig. 3. Facial appearances of the brothers. (a) Patient 1 at the age of 2 years and 9 months, before initiation of ERT. (b) Patient 2 at the age of 2 years and 10 months, after 31 months of ERT.

Patient 1 (Fig. 3a; 2 years and 9 months old). Patient 1 had coarse features affecting his nose, lips, and tongue, whereas Patient 2 did not show any MPS II-related facial features.

4. Discussion

Since idursulfase ERT for MPS II became commercially available (2006 in the US; 2007 in the European Union and Japan), there has been an increasing number of reports on its clinical effects. Idursulfase has been shown to improve walking capacity while reducing hepatosplenomegaly and urinary GAG levels. The most common adverse events have been infusion-related reactions, including some reports of anaphylactic reactions [5]. However, most of the treatment effects described to date have been in patients above age 5 who manifested typical symptoms of MPS II before initiation of ERT [12,13]. The results suggest that once established, pathological changes in certain organs and tissues, e.g. the bones, joints, heart valves, and central nervous system are difficult to correct [9]. A recent analysis of the effects of ERT in patients younger than 6 years old enrolled in the Hunter Outcome Survey (HOS) has shown a similar safety profile and reduction in hepatomegaly as in older patients [8]. Of these 124 children treated ERT, 11 initiated treatment during the first year of life, and the youngest treated was 1 month of age. However, no individual outcome data have been reported.

There is limited information on the ability of idursulfase to prevent the occurrence of disease manifestations in pre-symptomatic MPS II patients. MPS II is difficult to diagnose in early infancy before the development of typical signs and symptoms due to the insidious progression of disease [14,15]. The few patients that have been diagnosed early usually had a previously affected relative that prompted pre-symptomatic testing, as was the case for our siblings. Only one recent case report has described the effects of idursulfase ERT initiated in an asymptomatic infant with MPS II [16]. This boy was diagnosed at 14 days of life on the basis of an older affected sister, who interestingly, was found to have low IDS activity and be heterozygous for a missense mutation, p.Tyr523Cys/c.1568A>G in exon 9, with almost totally skewed X-inactivation of the normal *IDS* gene. Idursulfase (0.5 mg/kg/wk) was initiated at 3 months of life and 3-year follow-up was provided. The affected boy did not develop coarse facial features, joint disease, or organomegaly, and his cardiac function remained normal; the only abnormal finding was a mild deformity of one vertebrae. In contrast, the older sister showed typical clinical features of MPS II when she was diagnosed at age 3, including severe intellectual disability (IQ=50) that worsened over time (IQ=24 at age 10) despite 5 years of ERT. Considering her severe phenotype, it is surprising that her affected brother has maintained a normal IQ of 98 at 3 years of age. An earlier report had described this mutation as mild [17]. It is possible that the sister had other unknown central nervous system complications or effects of skewed X-inactivation that affected her cognitive status, or that the original assignment as a mild mutation was incorrect. Another possibility is that ERT started in early infancy had a protective effect on the central nervous system, but animal data suggest that intravenously administered idursulfase is unable to cross the blood–brain barrier at this dose.

Our experience has been similar to this recent case report, with a better outcome observed when treatment was initiated at 4 months of age instead of at 3 years of age, a difference of 2.7 years. The reduced dose that the older brother received for the initial 15 months of treatment may have contributed to some of the differences in outcomes. Nevertheless, somatic symptoms were present in Patient 1 before 2 years of age, but none were seen in Patient 2 at the same age except for possibly exudative otitis media. The only other somatic finding has been slight signs of dysotosis multiplex by X-ray. The prognosis for his mental development seems less promising, given the gradual decline in DQ from normal to slightly below normal. Although hearing problems due to chronic exudative otitis media may have contributed to the apparent decline in DQ, it has been reported that speech development is less affected in patients with mild compared to severe MPS II despite similar otological findings [18]. The inversion mutation is predicted to be a severe mutation that leads to a non-functional *IDS* gene, and in one series it was present in 13% of boys with MPS II [19]. Treatment options to prevent further deterioration of his intellectual abilities appear limited at this time. Previous reports on the therapeutic effects of hematopoietic stem cell transplantation (HSCT) in MPS II patients have generally been negative, but most patients had pre-existing CNS disease and little clinical data exists on the use of this procedure as a preventative measure in patients with normal cognitive function [20]. According to a recent report, donor-derived cells were detected in the brain of a transplanted MPS II patient [21]. To determine whether HSCT may be beneficial to MPS II patients at risk for CNS involvement, additional data must be collected on cases in which HSCT is performed as early as possible. Intrathecal delivery of ERT to treat MPS II-related CNS disease is currently being investigated in an ongoing Phase 1 clinical trial, and the results have not yet been published.

In summary, this is the second detailed case report of idursulfase ERT started in early infancy in a patient with MPS II. In contrast to

the older brother who had typical features of MPS II at the initiation of ERT that did not completely resolve after 2 years of treatment, we believe that the near absence of somatic findings in the younger brother after 2 years of treatment is attributable to early ERT administered in the pre-symptomatic state. The effect of early ERT on the younger brother's intellectual development is less clear. Long-term observation of these and other similar cases should help to clarify the extent of the preventative effects of ERT on the somatic and CNS aspects of MPS II as well as to define the optimal timing of treatment to achieve the best possible outcomes.

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