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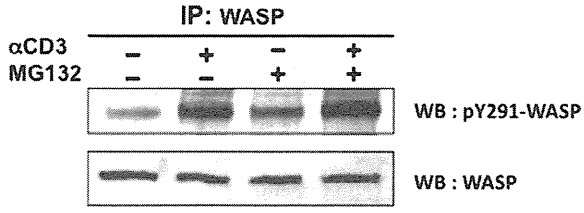


**FIG E1.** Tyrosine-phosphorylated WASP generated after TCR ligation is a target for proteasomal degradation. Effect of pretreatment of MG132 on the amount of tyrosine-phosphorylated WASP in anti-CD3–stimulated Jurkat T cells is shown. WASP immunoprecipitates were probed with anti-pY291-WASP antibody (Abcam). Similar results were obtained in 3 experiments. *IP*, Immunoprecipitate; *WB*, Western blot.

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**Abdominal and Lower Back Pain in Pediatric Idiopathic Stabbing Headache**

Yosuke Kakisaka, Tomoichiro Ohara, Naomi Hino-Fukuyo, Mitsugu Uematsu and Shigeo Kure

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# Abdominal and Lower Back Pain in Pediatric Idiopathic Stabbing Headache

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**KEY WORDS**
abdominal pain, back pain, idiopathic stabbing headache, valproic acid

**ABBREVIATIONS**
ISH—idiopathic stabbing headache
VPA—valproic acid

Dr Kakisaka drafted the initial manuscript; Drs Ohara, Hino-Fukuyo, Uematsu and Kure reviewed and revised the manuscript; and all authors approved the final manuscript as submitted.

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## abstract

Idiopathic stabbing headache (ISH) is a primary headache syndrome characterized by transient, sharp, stabbing pains located in the first division of the trigeminal nerve. Reports of pediatric ISH are rare, and extracephalic pain in pediatric ISH is extremely rare. Here we report the case of a 7-year-old male patient suffering from frequent, short, stabbing headache, which was occasionally associated with abdominal and lower back pain. Various investigations were normal. He was diagnosed with ISH, and valproic acid was administered to relieve his headache and accompanying symptoms. Our case demonstrates that abdominal and lower back pain may occur in pediatric ISH. This case may provide new evidence linking ISH and migraine by showing that extracephalic symptoms accompanying ISH are similar to those of migraine. We hypothesize that the mechanism underlying the headache and abdominal and lower back pain associated with ISH may be similar to that of a migraine headache. Accumulating additional cases by asking specific questions regarding the presence of the unusual symptoms presented in our case may help to establish a detailed clinical profile of these unfamiliar and peculiar symptoms in the pediatric ISH population. *Pediatrics* 2014;133:e245–e247

Idiopathic stabbing headache (ISH) is a primary headache syndrome classified under "other primary headaches" in the *International Classification of Headache Disorders, Second Edition*.[1] The pain is characterized as a transient, sharp, stabbing pain located in the first division of the trigeminal nerve.[2] Few studies have investigated pediatric ISH.[3–6] Several peculiar symptoms, such as extracephalic pain, associated with ISH have been reported in adult cases[7] but not in the pediatric population. We report a case of a pediatric patient with ISH associated with abdominal and lower back pain.

## CASE REPORT

A 7-year-old male patient presented at our hospital with severe stabbing headache in the left temporal region. Single episodes of stabbing pain several seconds in duration occurred about once a week and had begun 5 months before presentation. He was completely symptom-free between attacks. Before the initial visit, the attacks became more frequent, increasing to once or twice daily. Additionally, the headache was associated with antecedent abdominal pain in the epigastric to periumbilical region with or without bilateral lower back pain in approximately one-third of events. The extracephalic pain appeared ~20 seconds before headache onset and persisted in an intense manner, interfering with the patient's activities. He finally became incapacitated by simultaneous pain in multiple locations along with the stabbing headaches. Although he noted occasional hypersensitivity to sound during daily activity, his headache was not associated with nausea, vomiting, photophobia, osmophobia, or cranial autonomic symptoms (ie, lacrimation, conjunctival injection, eyelid edema, nasal congestion). The patient's mother had menstruation-related migraine headaches, but his history was not remarkable. The results of neurologic and physical examinations between attacks were normal. Laboratory examination and brain computed tomography results were normal. The patient was suspected to have idiopathic stabbing pain and administered valproic acid (VPA; 250 mg/day = 10 mg/kg/day) based on a previous report.[7] All symptoms resolved within a couple of days after VPA administration. He stopped the VPA after 2 weeks of medication and has been symptom free.

## DISCUSSION

Our case demonstrates that abdominal and lower back pain may occur in pediatric patients with ISH. The clinical course of our case, the appearance and disappearance of headache, and the associated extracephalic pain before and after VPA administration suggest that the mechanism mediating the abdominal and lower back pain may share a common pathway with that underlying ISH. Given that migraines have been associated with cutaneous allodynia or corpalgia[8,9] and abdominal pain and that a genetic predisposition may underlie ISH,[2] we hypothesize that the mechanism underlying the pain in our case is similar to the increased responsiveness (sensitization) of central pain neurons reported in migraine headaches.[10]

Soriani et al examined the clinical profiles of 83 pediatric ISH cases. They reported that some ISH patients had (1) a history of symptoms suggesting the presence of cyclic vomiting syndrome or abdominal migraine or migraine equivalent and (2) associated symptoms such as photophobia and nausea.[3] Their findings support previous reports suggesting that patients with ISH have a genetic predisposition for migraines.[2] Our case may provide new evidence connecting ISH and migraine by showing that extracephalic symptoms accompanying ISH are similar to those of migraine.

In contrast to pediatric ISH patients like ours, only a few reports of visceral pain in adult patients with ISH are known. This may be related not only to the rarity of ISH but also to the peculiar age-dependent nature of migraine and its symptoms. In other words, pediatric patients with migraine suffer recurrent abdominal pain years before the typical migraine headache appears, although the details of this trajectory remain unclear.

We believe that accumulating cases by asking specific questions[11] regarding the presence of the unusual symptoms presented in our case may help to establish a detailed clinical profile of these unfamiliar and peculiar symptoms in the pediatric ISH population.

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# Abdominal and Lower Back Pain in Pediatric Idiopathic Stabbing Headache

Yosuke Kakisaka, Tomoichiro Ohara, Naomi Hino-Fukuyo, Mitsugu Uematsu and Shigeo Kure

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# Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome

Horino S, Sasahara Y, Sato M, Niizuma H, Kumaki S, Abukawa D, Sato A, Imaizumi M, Kanegane H, Kamachi Y, Sasaki S, Terui K, Ito E, Kobayashi I, Ariga T, Tsuchiya S, Kure S. Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome.

Abstract: IPEX syndrome is a rare and fatal disorder caused by absence of regulatory T cells (Tregs) due to congenital mutations in the Forkhead box protein 3 gene. Here, we report a patient with IPEX syndrome treated with RIC followed by allogeneic BMT from an HLA-matched sibling donor. We could achieve engraftment and regimen-related toxicity was well tolerated. Although the patient was in mixed chimera and the ratio of donor cells in whole peripheral blood remained relatively low, selective and sustained expansion of Tregs determined as CD4+CD25+Foxp3+ cells was observed. Improvement in clinical symptoms was correlated with expansion of donor-derived Tregs and disappearance of anti-villin autoantibody, which was involved in the pathogenesis of gastrointestinal symptoms in IPEX syndrome. This clinical observation suggests that donor-derived Tregs have selective growth advantage in patients with IPEX syndrome even in mixed chimera after allogeneic BMT and contribute to the control of clinical symptoms caused by the defect of Tregs.

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Key words: allogeneic hematopoietic stem cell transplantation – enteropathy – Forkhead box protein 3 – immune dysregulation – polyendocrinopathy – reduced intensity conditioning – regulatory T cells – X-linked syndrome

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Abbreviations: ALL, acute lymphoblastic leukemia; APC, allophycocyanin; ATG, antithymocyte globulin; BMT, bone marrow transplantation; CyA, cyclosporine A; DAB, 3, 3′-diaminobenzidine; DLI, donor leukocyte infusion; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; IVIG, intravenous immunoglobulin; MLL, mixed lineage leukemia; PBMCs, peripheral blood mononuclear cells; PBSCT, peripheral blood stem cell transplantation; PE, phycoerythrin; PSL, prednisolone; RIC, reduced intensity conditioning; TBI, total body irradiation.

IPEX syndrome is primary immunodeficiency caused by the defects of regulatory T cells (Tregs). IPEX syndrome is often lethal in the first few months of life due to severe diarrhea associated with refractory enteropathy, infections, diabetes mellitus, dermatitis, and other autoimmune complications. This disorder is caused by mutations of Forkhead box protein 3 (*FOXP3*) gene located on chromosome Xp11.23. *FOXP3* encodes Forkhead box protein 3, which is essential for the development and maintenance of CD4+CD25+Foxp3+ Tregs (1, 2).

Established treatments for patients with IPEX syndrome include immunosuppressive therapy and allogeneic HSCT (3–6). Allogeneic HSCT serves as a curative therapy for patients with IPEX syndrome, and RIC regimens have been reported and resulted in better outcome than myeloablative conditioning regimen (7–11). In general, allogeneic HSCT with RIC regimen may increase the risk of rejection and mixed chimera. Some RIC regimens included the antibody against T lymphocytes such as alemtuzumab or ATG. However, these agents may increase the risk of viral reactivation after HSCT. To the best of our knowledge, only two cases of IPEX patients treated with allogeneic HSCT following RIC consisted of low-dose TBI instead of alemtuzumab or ATG have been reported (12).

Here, we report a patient with IPEX syndrome treated with RIC regimen consisted of fludarabine, cyclophosphamide, and low-dose TBI followed by allogeneic HSCT from an HLA-identical sibling donor. Although the patient was in mixed chimera, he was free from symptoms caused by the absence of Tregs. We could observe selective and sustained growth advantage of donor-derived Tregs and disappearance of anti-villin autoantibody in his serum, which was correlated with the improvement in refractory enteropathy.

## Patient and methods

### Patient

A Japanese male suffered from severe diarrhea at two months of age. He was diagnosed as IPEX syndrome by identifying a missense mutation of T1117G substitution in exon 10 of the *FOXP3* gene (13). We quantified CD4+CD25+Foxp3+ cells by flow cytometry, and positive cells were not identified at all in PBMCs. Autoantibodies examined were negative except anti-villin antibody in patient's serum. He was treated with immunosuppressive therapy of intravenous CyA and oral PSL. After complete remission was achieved, he was free from the symptom for six yr with oral low-dose CyA and PSL (14).

At the age of six, the patient suffered from severe diarrhea again and was referred to our hospital. Although he was treated with increased doses of CyA and PSL in addition to other immunosuppressive agents, these treatments were not effective enough to control his diarrhea completely. We next tried IVIG therapy, which resulted in the improvement in diarrhea, and we could taper immunosuppressive agents.

To control the disease without continuous immunosuppressive therapy, we considered to perform allogeneic BMT from an HLA-matched healthy sibling donor. The donor did not have the mutation in *FOPX3* gene. We used a RIC regimen consisted of 4 Gy (2 × 2 Gy) TBI (day 7), fludarabine at a dose of 30 mg/m$^2$ for five days (days 6 to day 2) and cyclophosphamide at a dose of 60 mg/kg for two days (days 3 and 2). Total nucleated bone marrow cells of $4.32 \times 10^8$/kg were transplanted. We selected CyA and short-term methotrexate as GVHD prophylaxis, and IVIG was continued weekly until autoimmune colitis was resolved.

### Chimerism assay

Chimerism assay was performed by polymerase-chain-reaction-based assays analyzing polymorphic short tandem repeat markers (15). The chimerism was examined in each fraction of T cells, total lymphocytes, and granulocytes in bone marrow or peripheral blood. We evaluated the chimerism in bone marrow before day 100 and in peripheral blood after day 100, because we had similar results in both samples before day 100 in the patient and avoided frequent bone marrow aspiration after day 100.

### Flow cytometry

PBMCs were stained with monoclonal antibodies of APC-conjugated human CD4, PE-conjugated human CD25, and FITC-conjugated human Foxp3 antibodies (BD Biosciences, San Jose, CA, USA) and analyzed by a FACSCanto II flow cytometer (BD Biosciences), as described previously (16).

### Immunoblot analysis of anti-villin antibody

Anti-villin autoantibody in patient's serum was analyzed as described previously (17). Briefly, 500 ng of GST-villin recombinant protein (121 kD) was transferred to the membrane and incubated with diluted serum at 1:160. Anti-villin antibody bound to GST-villin was detected by horseradish peroxidase-conjugated antibody and DAB system.

## Case report

### Clinical improvement after RIC and allogeneic HSCT

The patient achieved an engraftment on day 11, and the last transfusion of platelets was on day 7 and that of red blood cells was on day 1. He was complicated with transient acute GVHD of the skin (grade I) on day 35 but this resolved without additional immunosuppressive therapy. He had no episodes of significant infection and other severe regimen-related toxicity during the course of RIC and allogeneic HSCT.

Severe and bloody diarrhea settled down on day 14 after engraftment. The patient was

consistently free from symptoms of enteropathy and any other autoimmune diseases. Laboratory findings showed improvement in hypoalbuminemia and anemia caused by severe enteropathy on day 21. Colonoscopy examination on day 60 revealed disappearance of mucosal inflammation, multiple ulcerations and hemorrhage that were observed before the HSCT.

After the discharge on day 120, we had followed the patient every two wk. He had no episodes of autoimmune disorders and infection, and we could taper and stop immunosuppressive agents at six months. Unfortunately, he suffered from MLL gene-rearranged ALL at 24 months after transplantation. The origin of precursor B lymphoblasts was recipient cells. We treated him with chemotherapy and allogeneic PBSCT from the same donor. We used myeloablative conditioning regimen consisted of busulfan at a dose of 4 mg/kg for four days and melphalan at a dose of 90 mg/m$^2$ for two days for the second transplant from the same sibling donor to cure this secondary ALL. He has been in complete remission for more than two yr. Chimerism completely changed to donor-type and the number of Tregs increased to normal after the second transplant.

### Chimerism and immunological evaluation after first allogeneic HSCT

Because the ratio of donor T cells, total lymphocytes, and granulocytes in bone marrow was 74%, 48%, and 48%, respectively, on day 22 after HSCT, we reduced the dose of CyA immediately. The ratio of donor cells, however, was further declined to 5% on day 50, and the donor bone marrow was assumed to be rejected (Fig. 1). At that point, flow cytometric analysis of peripheral blood showed that 17.8% of PBMCs were CD4+CD25+Foxp3+ Tregs (Fig. 2a,b). This discordant result on day 50 was explained by selective expansion of donor-derived Tregs. After discontinuation of CyA on day 50, the ratio of donor T cells, total lymphocytes, and granulocytes was transiently increased up to 40% and then gradually decreased (Fig. 1). At 24 months after HSCT, donor cells were around 20% and CD4+CD25+Foxp3+ Tregs were at the range of 1.2–3.0% of PBMCs, which were comparable to healthy controls (Fig. 2a). We did not perform DLI because the ratio of donor cells was <50% and supposed that the patient was in high risk of bone marrow aplasia after repeated DLI.

The anti-villin autoantibody was detected by immunoblot analysis when the disease was active before HSCT. The antibody was under detectable levels both in clinical remission by immunosuppressive therapy before HSCT and after engraftment was achieved following HSCT even when immunosuppressive agents were not administrated (Table 1).

## Discussion

The defect of Tregs in patients with IPEX syndrome causes symptoms related to

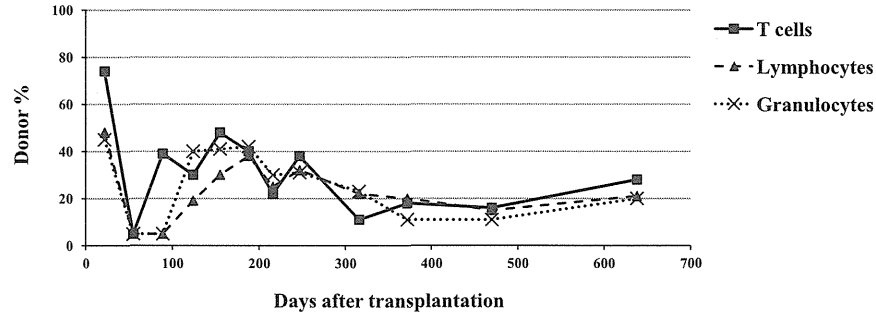


*Fig. 1.* Frequency of donor cells after allogeneic HSCT. Percentages of donor cells in each fraction of T cells, total lymphocytes, and granulocytes after HSCT are shown.

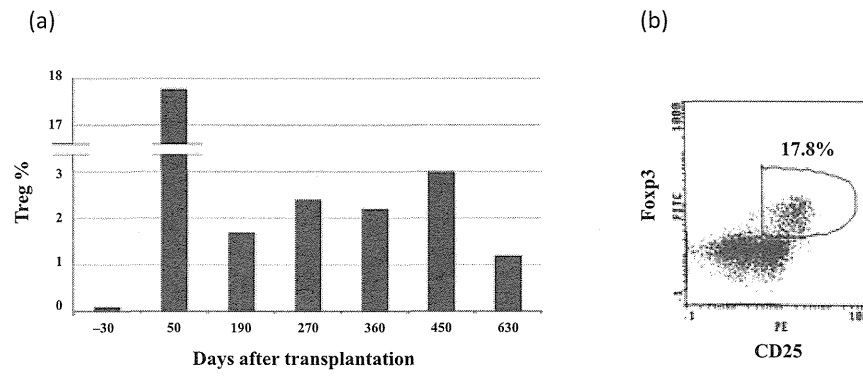


*Fig. 2.* Sustained expansion of donor Tregs after allogeneic HSCT. (a) Percentages of Tregs are evaluated as CD4+CD25+Foxp3+ cells in PBMCs. (b) Flow cytometric demonstration of CD25+Foxp3+ cells in CD4 gated PBMCs on day 50 after HSCT.

Table 1. Anti-villin autoantibody is correlated with clinical condition of enteropathy

| Clinical condition of enteropathy | Anti-villin autoantibody |
|---|---|
| Disease onset | + |
| Remission before HSCT | − |
| Relapse before HSCT | + |
| After HSCT | − |

GST-villin protein (121 kD) was transferred to the membrane, and immunoblot was performed with 1:160 diluted patient's serum at different disease condition of enteropathy as indicated. + indicates the presence of anti-villin antibody that recognizes GST-villin protein.

autoimmunity. However, clinical benefit of immunosuppressive therapy is often limited by its adverse effects and increased susceptibility to infection. At present, allogeneic HSCT is recognized as the curative therapy for patients with IPEX syndrome. We summarized all reported cases treated with HSCT in Table 2. Myeloablative regimen resulted in high fatality due to regimen-related toxicity or lethal infection (2, 5, 6). On the other hand, Rao et al. (7) first reported four patients who were successfully treated with non-myeloablative conditioning regimen consisted of fludarabine, melphalan, and alemtuzumab, and achieved high rate of donor chimerism above 84.6%. Non-myeloablative regimens with anti-T-lymphocyte antibody such as alemtuzumab or ATG have been used, and all patients are alive (7–11). However, it is known that alemtuzumab and ATG induce profound depletion of T cells and increase the risk of viral reactivation and fungal infection after HSCT. Therefore, we used low-dose TBI instead of anti-T-lymphocyte antibodies. The combination of low-dose TBI, fludarabine, and cyclophosphamide was well tolerated, and the patient was free from infections and severe regimen-related toxicities. Burroughs et al. (12) also reported that RIC regimen including low-dose TBI for IPEX syndrome resulted in stable engraftment of Tregs and better clinical outcome, proposing that this regimen was preferable for patients with IPEX syndrome.

The patient developed MLL-related secondary ALL in recipient cells. Although the dose of TBI was less than used in myeloablative conditioning, radiation and alkylating agents might cause DNA damage and increased the risk of secondary leukemia in recipient cells. Alternatively, the use of anti-T-lymphocyte antibody instead of low-dose TBI and/or dose reduction in alkylating agents should be carefully considered in IPEX syndrome.

Selected and sustained expansion of Tregs resulted in clinical improvement even though the patient was in mixed chimera after HSCT. Seidel et al. (11) reported a patient with IPEX

Table 2. Summary of IPEX patients treated with allogeneic HSCT reported in the literature

| Case | Age | Donor | Conditioning regimen | Complications after HSCT | Outcome | % Donor after HSCT | Reference |
|---|---|---|---|---|---|---|---|
| 1 | 13 yr | HLA-matched sibling | TBI 12 Gy + CY + ATG | Adenovirus infection, pneumonia | Dead | 50% | 2 |
| 2 | 9 yr | HLA-matched unrelated | TBI 12 Gy + CY + ATG | Cytomegalovirus infection, hemorrhagic cystitis, lymphoproliferative disorder | Dead | 70% | 2 |
| 3 | 4 months | HLA-matched sibling | BU + CY + ALG | Hemophagocytic syndrome | Dead | 30% in T cell | 5 |
| 4 | 1 yr | HLA-matched sibling | BU + CY + Flu + ATG | | Alive | 70% in T cell | 6 |
| 5 | 7 yr | HLA-matched unrelated | Flu + L-PAM + alemtuzumab | Cytomegalovirus infection | Alive | 100% | 7 |
| 6 | 1 yr | HLA-matched unrelated | Flu + L-PAM + alemtuzumab | Acute respiratory distress syndrome | Alive | 100% | 7 |
| 7 | 4 yr | HLA-matched sibling | Flu + L-PAM + alemtuzumab | Histoplasma infection | Alive | 89% | 7 |
| 8 | 5 months | HLA-matched unrelated | Flu + L-PAM + alemtuzumab | | Alive | 84.6% | 7 |
| 9 | 7 yr | HLA 5/6-matched cord blood | Flu + BU + ATG | Lymphoproliferative disorder | Alive | 81 ~ 98% | 8 |
| 10 | 7 months | HLA-matched unrelated | Flu + L-PAM + alemtuzumab | Sepsis of *Enterobacter cloacae* | Alive | 100% | 9 |
| 11 | 5 months | HLA-matched unrelated | Flu + L-PAM + alemtuzumab + anti-CD 45 monoclonal antibody | | Alive | 100% | 10 |
| 12 | 11 months | HLA-matched unrelated | Flu + L-PAM + alemtuzumab | | Alive | <10% | 11 |
| 13 | 9 months | HLA-matched unrelated | TBI 4 Gy + Flu | Bacteremia | Alive | 100% | 12 |
| 14 | 16 yr | HLA-matched related | TBI 4 Gy + Flu | Bacteremia | Alive | 20 ~ 60% in T cell | 12 |

Case series transplanted with RIC regimens were highlighted.
CY, cyclophosphamide; ALG, antilymphocyte globulin; BU, busulfan; Flu, fludarabine; L-PAM, melphalan.

syndrome who showed selective engraftment of Tregs for six yr after non-myeloablative transplantation. It has been reported that partial BMT or injection of T-enriched splenocytes resulted in the rescue of autoimmunity in Scurfy mice, a mouse model for IPEX syndrome in which *FOXP3* gene is naturally mutated. Sustained engraftment of relatively high frequency of CD4+CD25+Foxp3+ Tregs was observed even though the frequency of donor cells in whole peripheral blood ranged from 1.7% to 50% (18). These observations illustrate that the paradigm in the generation of Tregs is reinforced by the requirement and growth advantage regardless of chimerism of other hematopoietic cells in IPEX syndrome. However, we should still consider the possibility that mixed chimerism may result in subsequent development of autoimmune diseases observed in other primary immunodeficiency, as previously reported in some patients with Wiskott–Aldrich syndrome (19).

Intractable diarrhea is a major symptom in patients with IPEX syndrome. Villin, an actin-binding protein, is expressed as the 95 kD antigen in the small intestine, which is frequently targeted by autoantibodies in patients with IPEX syndrome (17). Anti-villin antibody was clearly correlated with the severity of clinical symptoms in our patient. Therefore, monitoring of anti-villin antibody might serve as a useful examination for evaluating gastrointestinal complications in patients with IPEX syndrome.

We reported here a unique phenomenon of selective growth advantage of Tregs in a patient with IPEX syndrome who was in mixed chimera after RIC and allogeneic HSCT. Sustained expansion of donor-derived Tregs resulted in the significant improvement in enteropathy. To determine optimal RIC regimen to achieve complete chimera and avoid secondary malignancy in residual recipient cells, further analysis in more patients and long-term follow-up study after HSCT are required to conclude this issue.

### Authors' contributions

Horino S and Sasahara Y designed the study, interpreted the data, wrote the paper, and treated the patient. Sato M, Kanegane H, Kamachi Y, and Kobayashi I performed experiments. All other authors treated the patient and collected clinical data.

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### Conflict of interest disclosure

The authors declare no conflict of interest.

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# Fatigue and quality of life in citrin deficiency during adaptation and compensation stage

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ABSTRACT

Citrin-deficient children and adolescents between adult-onset type II citrullinemia and neonatal intrahepatic cholestasis by citrin deficiency do not have clear clinical features except for unusual diet of high-fat, high-protein, and low-carbohydrate food. The aims of the present study are to characterize fatigue and quality of life (QOL) in citrin-deficient patients during adaptation and compensation stage, and to define the relationship between fatigue and QOL. The study subjects were 55 citrin-deficient patients aged 1–22 years (29 males) and 54 guardians. Fatigue was evaluated by self-reports and proxy-reports of the PedsQL Multidimensional Fatigue Scale. QOL was evaluated by the PedsQL Generic Core Scales. Both scale scores were significantly lower in child self-reports ($p<0.01$ and $p<0.05$, respectively) and parent proxy-reports ($p<0.01$ and $p<0.01$, respectively) than those of healthy children. Citrin-deficient patients with scores of 50 percentile or less of healthy children constituted 67.5% of the sample for the Fatigue Scale and 68.4% for the Generic Core Scales. The PedsQL Fatigue Scale correlated with the Generic Core Scales for both the patients ($r=0.56$) and parents reports ($r=0.71$). Assessments by the patients and their parents showed moderate agreement. Parents assessed the condition of children more favorably than their children. The study identified severe fatigue and impaired QOL in citrin-deficient patients during the silent period, and that such children perceive worse fatigue and poorer QOL than those estimated by their parents. The results stress the need for active involvement of parents and medical staff in the management of citrin-deficient patients during the silent period.



## 1. Introduction

Citrin deficiency is caused by mutations of the SLC25A13 gene on chromosome 7q21.3, encoding a calcium-binding mitochondrial solute carrier protein [1]. Citrin functions as a liver-type calcium ($Ca^{2+}$)-stimulated aspartate–glutamate carrier (AGC) by the electrogenic exchange of mitochondrial aspartate for cytosolic glutamate and proton [2]. AGC provides aspartate for the syntheses of urea, protein, and nucleotide, in addition to participating in gluconeogenesis from lactate and transporting cytosolic nicotinamide adenine dinucleotide (NADH) reducing equivalents into mitochondria as part of the malate–aspartate shuttle; thus, citrin (liver-type AGC) deficiency shows various symptoms [3–5]. The estimated prevalence of carriers of citrin deficiency in the Japanese population is 1 in 70, and that of patients is 1 in 17,000 [6,7].

Citrin deficiency is a recognized disorder that encompasses both adult-onset type II citrullinemia (CTLN2, OMIM 603471) and neonatal intrahepatic cholestasis by citrin deficiency (NICCD; OMIM 605814). The clinical characteristics of citrin deficiency vary with age. CTLN2 is characterized by frequent attacks of hyperammonemia, liver steatosis, and neuropsychiatric symptoms, such as disorientation,

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delirium, mental derangement, sudden unconsciousness, and ultimately death within a few years of onset [8,9]. The onset is sudden and affected individuals vary in age at diagnosis from 11 to 79 years (usually between 20 and 40 years). Liver transplantation is remarkably effective.

NICCD causes intrahepatic cholestasis (jaundice and acholic stool), failure to thrive, hypoproteinemia, hypoglycemia, and multiple amino acidemias including citrullinemia. [10–15]. About half of the patients with NICCD in Japan were found by newborn mass screening such as hypergalactosemia, hyperphenylalaninemia, and/or hypermethioninemia [14,15]. The condition in almost all patients is self-limiting between 6 and 12 months of age. However, a few patients with this disorder develop severe hepatic dysfunction requiring liver transplantation [13,16].

Most citrin-deficient patients between NICCD and CTLN2 do not have clear symptoms except for unusual diet of low-calorie, high-fat, high-protein, and low-carbohydrate food [17]. Therefore, this stage is considered to be the silent period for adaptation and compensation to deficient metabolism.

Recently, a follow-up study of the patients with NICCD reported the appearance of symptoms and metabolic abnormalities during the silent period. A subgroup of patients showed nonspecific clinical features such as inappetence (lack of appetite), general fatigue, growth disturbance, abdominal discomfort, hypoglycemia, and hyperlipidemia. Especially, citrin deficient patients during the silent period complain of fatigue during school life and therefore are considered to have impaired quality of life (QOL). These patients also exhibit mild citrullinemia, high lactate/pyruvate rate, hypercholesterolemia, and oxidative stress state [18]. It is estimated that the patients in the silent period lack sufficient compensation for metabolism.

In the last decade, the term pediatric health-related quality of life (HRQOL) was frequently used and closely examined. HRQOL is currently recognized as an essential point of reference in clinical trials, health care settings, and school health care. The Pediatric Quality of Life Inventory (PedsQL), developed in the United States, has demonstrated satisfactory psychometric properties in both diseased and healthy children [19,20]. The PedsQL includes well-established methods of child self-reporting and parent proxy-reporting, which have been utilized in clinical trials in Japan and other countries worldwide [21]. The Japanese-language version of the PedsQL Multidimensional Fatigue Scale and PedsQL Generic Core Scales has good reliability and validity and can be useful for evaluation of Japanese children in school and health care settings [21,22]. Fatigue is a health problem that significantly impacts HRQOL, and is a frequent and ubiquitous complaint among patients with chronic disease. Therefore, the aim of any treatment or intervention in chronic diseases includes improvement of fatigue [23]. Not only in children with chronic disease, fatigue is reported to affect non-school attendance in school-age children [24,25].

The aims of the present study are to characterize fatigue and QOL in patients with citrin deficiency during the silent period, and to define the relationship between fatigue and QOL.

## 2. Methods

### 2.1. Patients

The subjects of the present study were 55 patients with citrin deficiency (age: 1–22 years, 29 males and 26 females) and 54 guardians. For patients aged 1–4 years, only proxy-reports were examined, and for patients aged 5–22 years, self-reports and proxy-reports were examined. Self-reports were provided by 40 patients (21 males and 19 females), and proxy reports by 54 guardians (45 mothers, 5 fathers, and 4 nonresponders). Three patients (5.7%) had chronic conditions, excluding citrin deficiency, which required medications or hospitalization for treatment. The diagnosis of citrin deficiency was based on gene analysis for the SLC25A13 in all patients.

The study protocol was approved by the Institutional Review Boards of Osaka City University Graduate School of Medicine and Hyogo College of Medicine. Signed consent forms were obtained from the study subjects and/or their guardians.

### 2.2. The PedsQL Multidimensional Fatigue Scale (Japanese-language version)

The PedsQL Multidimensional Fatigue Scale (Japanese version) was used to evaluate fatigue in citrin-deficient patients [22]. The PedsQL Multidimensional Fatigue Scale consists of 18 items forming parts of three subscales: (i) general fatigue (six items), (ii) sleep/rest fatigue (six items), and (iii) cognitive fatigue (six items). The PedsQL Multidimensional Fatigue Scale comprises parallel child self-report and parent proxy-report formats. Child self-reports are available for children aged 5–7, 8–12, and 13–18. Parent proxy-reports exist for children aged 2–4, 5–7, 8–12, and 13–18. The instructions for children aged 8–18 years and their parents (guardians) included reporting a score for each item during the past month, using a 5-point Likert scale. For the self-report for children aged 5–7 years, the recall time was shortened to a few weeks; the response choice was simplified to a 3-point scale.

Scores were calculated using a linearly transformed reversed score range from 0 to 100 (0:100, 1:75, 2:50, 3:25, 4:0). Higher scores indicate less fatigue status. Scale scores were calculated as the sum of items divided by the number of items answered; however, the scale score was not calculated when more than 50% of the items on the scale were missing.

### 2.3. The PedsQL Generic Core Scales (Japanese-language version)

The Japanese-language version of the PedsQL Generic Core Scales was used for evaluation of QOL of citrin-deficient patients [21]. The original PedsQL 4.0 Generic Core Scales were developed according to the definition of health given by the World Health Organization [26] and consist of 23 items classified into four subscales: (i) physical functioning (eight items), (ii) emotional functioning (five items), (iii) social functioning (five items), and (iv) school functioning (five items). Higher scores represent a better HRQOL [19].

### 2.4. Statistical analysis

Total and subscale scores of the PedsQL Multidimensional Fatigue Scale and the PedsQL Generic Core Scale in citrin deficient patients were expressed as mean values and compared with those in healthy controlled child provided by preliminary research [22] using the Mann–Whitney $U$ test. The numbers of citrin-deficient patients were calculated for scores of less than 25 percentile, less than 50 percentile, less than 75 percentile, and more than 75 percentile of healthy controls, respectively, on PedsQL Multidimensional Fatigue Scale and the PedsQL Generic Core Scales.

Agreement between the PedsQL Fatigue Scale and Generic Core Scales was analyzed by Spearman rank correlation coefficient. According to Cohen [27], Spearman rank correlation coefficients effect sizes were considered small (0.10–0.29), moderate (0.30–0.49), and large ($\geq$0.50). Agreement between a child's self-report and a parent proxy-report was assessed with 36 to 38 pairs provided from both the citrin-deficient children and their guardians, by calculating Spearman rank correlation coefficients and intraclass correlation coefficients (ICCs). The ICC offers an index of absolute agreement given that it takes into account the ratio between subject variability and total variability [28,29]. Intraclass correlations are designated as 0.40 poor to fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement, and 0.81–1.00 excellent agreement [30].

In addition to correlation, the mean values of the absolute differences between self- and proxy-reports for total score and for all subscales of the Japanese-language version of the PedsQL Multidimensional Fatigue Scale were calculated as a further indicator of the level of agreement between child and parent responses. The directional differences can indicate bias of proxy-reported scores relative to those of self-reported scores [31]. The mean values of directional differences were standardized by relating a given score to its standard deviation to determine the statistical magnitude of any observed systematic bias. The size of bias of a standardized difference (d) was labeled as small when it was 0.2, medium when it was =0.5, and large when it was =0.8.

All statistical analyses were conducted using The Statistical Package for Social Sciences 14.0 for Windows (SPSS Inc., Chicago, IL).

## 3. Results

### 3.1. Effects of citrin deficiency on PedsQL Multidimensional Fatigue Scale and Generic Core Scales

Table 1 shows the mean values of the child self-report scores and proxy-report scores in citrin-deficient patients on the PedsQL Scales. On the PedsQL Multidimensional Fatigue Scale, the total fatigue scores and all subscales scores of both child self-report and parent proxy-report for citrin-deficient patients were significantly lower than the fatigue scores of healthy children examined in our previous study [22]. On the PedsQL Generic Core Scales, the total scores of self-report and parent proxy-report for citrin-deficient patients were significantly lower than the control. In the subscale evaluation, significant decreases were recognized in physical functioning and school functioning in child self-report, whereas significant decreases were noted on all subscales in parent proxy-report.

Fig. 1 shows the distribution of the child self-report score in citrin-deficient patients based on the percentile of the scores in healthy children on the PedsQL Fatigue Scale and Generic Core Scales. The numbers of citrin-deficient patients with scores of 50 percentile or less of those of healthy children were 27 (67.5%) on the Fatigue Scale and 26 (68.4%) on the Generic Core Scales. The above results demonstrated enhanced fatigue and low QOL in citrin-deficient patients in silent period around school life. Parents also recognized patient's fatigue and QOL impairment.

### 3.2. Correlation between PedsQL Fatigue Scale and Generic Core Scales

We also evaluated the correlation between the PedsQL Fatigue Scale and PedsQL Generic Core Scales in citrin-deficient patients using the Spearman rank correlation coefficient (Table 2). In the child self-report, there was a moderate correlation ($r=0.56$) between the total score on the Fatigue Scale and total score on the Generic Core Scales. The total score on the Generic Core Scales showed the strongest correlation with general fatigue among the subscales of the Fatigue Scale. Meanwhile, the total score on the Fatigue Scale showed the strongest correlation with physical functioning among the subscales of the Generic Core Scale (r=0.59). In the parent proxy-report, there was a good correlation between total fatigue score and total generic core score (r=0.71).

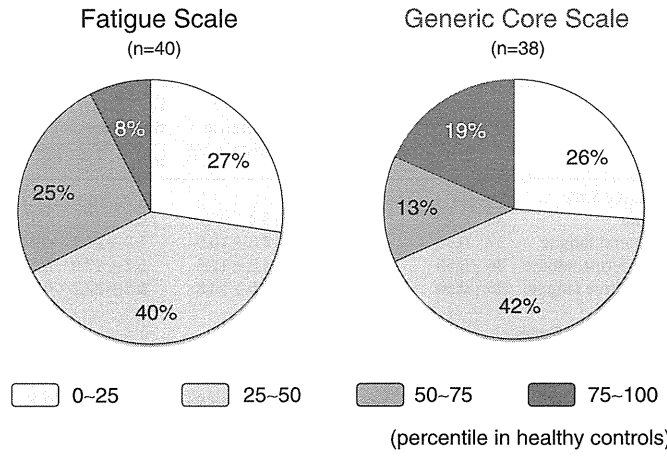


**Fig. 1.** PedsQL Multidimensional Fatigue Scale and Generic Core Scale of the child self-report in citrin-deficient patients, relative to those of the healthy control. A circle graph shows the number (%) of the citrin-deficient patients at the score of every 25 percentile of healthy controls on PedsQL Multidimensional Fatigue Scale and Generic Core Scales. For healthy controls, the score points of the Fatigue Scale for the 0–25, 26–50, 51–75, and 76–100 percentiles were 0–67.4, 67.5–80.9, 81–89.4, and 89.5–100, respectively, while those of the Generic Core Scale were 0–72.4, 72.5–83.2, 83.3–90, and 90.1–100, respectively.

### 3.3. Assessment of difference between citrin-deficient children and their parents

Table 3 summarizes the Spearman rank correlation coefficients (ρ) for child/parent assessment differences in fatigue and QOL recognition, ICC, absolute differences, and directional differences. The ICC of the child–parent agreement was moderate to good at 0.40–0.70 on the Fatigue Scale and fair to moderate at 0.36–0.49 on the Generic Core Scales. Large values in child–parent absolute differences and directional differences were noted in the cognitive fatigue on the Fatigue Scale and in the emotional functioning on the Generic Core Scales. These are items that are not easily shown in behaviors or daily living on the surface. Therefore, the above findings suggest

**Table 1**
Effects of citrin deficiency on PedsQL Multidimensional Fatigue Scale and Generic Core Scales.

| | Child self-report | | Parent proxy-report | |
|---|---|---|---|---|
| | Citrin deficiency mean±SD | Control mean±SD | Citrin deficiency mean±SD | Control mean±SD |
| Fatigue Scale | (n=40) | | (n=52) | |
| Total | 67.8±14.3¶ | 77.6±16.0 | 77.7±17.8¶ | 85.6±13.6 |
| General | 68.5±22.5* | 81.7±18.3 | 78.0±20.6¶ | 84.9±15.6 |
| Sleep/rest | 68.9±22.5¶ | 72.8±18.6 | 76.5±21.7¶ | 86.2±14.8 |
| Cognitive | 65.9±21.9¶ | 78.5±20.4 | 77.7±17.8* | 85.8±16.1 |
| Generic Core Scales | (n=38) | | (n=54) | |
| Total | 77.3±11.9* | 80.9±12.4 | 82.5±14.5¶ | 88.7±11.3 |
| Physical | 79.9±14.9¶ | 86.6±13.6 | 86.3±17.6¶ | 90.0±18.4 |
| Emotional | 69.9±16.4 | 67.8±21.5 | 78.6±18.3¶ | 87.7±13.1 |
| Social | 82.9±14.4 | 84.8±16.1 | 84.3±20.0* | 89.9±13.2 |
| School | 75.0±16.8* | 84.9±12.9 | 77.5±16.9¶ | 87.3±12.6 |

*$p<0.05$, ¶$p<0.01$, compared with the respective control (by Mann–Whitney $U$ test).

**Table 2**
Correlation between PedsQL Multidimensional Fatigue Scale and Generic Core Scales.

| | n | Fatigue Scale[a] | | | |
|---|---|---|---|---|---|
| | | Total | General | Sleep/rest | Cognitive |
| Generic Core Scales | | | | | |
| Child self-report | | | | | |
| Total | 38 | 0.56 | 0.45 | 0.22 | 0.27 |
| Physical | 38 | 0.59 | 0.60 | 0.15 | 0.26 |
| Emotional | 38 | 0.33 | 0.25 | 0.22 | 0.09 |
| Social | 38 | 0.39 | 0.27 | 0.06 | 0.40 |
| School | 38 | 0.37 | 0.17 | 0.40 | 0.05 |
| Parent proxy-report | | | | | |
| Total | 52 | 0.71 | 0.70 | 0.49 | 0.58 |
| Physical | 52 | 0.60 | 0.63 | 0.42 | 0.47 |
| Emotional | 52 | 0.60 | 0.60 | 0.44 | 0.46 |
| Social | 52 | 0.68 | 0.66 | 0.44 | 0.59 |
| School | 48 | 0.69 | 0.56 | 0.55 | 0.60 |

[a] Data are Spearman Rank Correlation Coefficient ρ.

**Table 3**
Agreement between child self-report and parent proxy-report on PedsQL Multidimensional Fatigue Scale and Generic Core Scales.

| | n | Spearman rank correlation coefficient (ρ) | ICC | Absolute difference (mean ± SD) | Directional difference | |
|---|---|---|---|---|---|---|
| | | | | | mean ± SD | *D* |
| *Fatigue Scale* | | | | | | |
| Total | 36 | 0.47 | 0.56 | 13.4 ± 9.7 | 6.6 ± 15.3 | 0.43 |
| General fatigue | 37 | 0.30 | 0.40 | 17.6 ± 16.9 | 5.0 ± 24.1 | 0.21 |
| Sleep/rest fatigue | 36 | 0.55 | 0.70 | 14.3 ± 12.5 | 6.7 ± 17.9 | 0.37 |
| Cognitive fatigue | 36 | 0.54 | 0.54 | 18.5 ± 14.8 | 8.8 ± 22.2 | 0.40 |
| *Generic Core Scales* | | | | | | |
| Total | 38 | 0.51 | 0.49 | 10.2 ± 9.3 | 3.6 ± 13.4 | 0.27 |
| Physical | 38 | 0.41 | 0.38 | 12.6 ± 13.9 | 6.3 ± 17.8 | 0.35 |
| Emotional | 38 | 0.42 | 0.36 | 15.6 ± 14.0 | 6.4 ± 20.1 | 0.32 |
| Social | 38 | 0.36 | 0.39 | 12.9 ± 11.7 | −0.5 ± 17.5 | 0.03 |
| School | 38 | 0.47 | 0.48 | 11.7 ± 12.3 | 0.7 ± 17.1 | 0.04 |

ICC: intraclass correlation coefficient.

that it is difficult even for parents to grasp the internal mental state of their children, resulting in a large recognition difference. On the PedsQL Fatigue Scale, the directional differences showed a positive score difference, indicating that parents assessed the conditions of their children more favorably than the children themselves.

## 4. Discussion

Fatigue is one of the three important body alarms, in addition to pain and fever, and is one of the important signals for the body to maintain health and life. In healthy individuals, physiological fatigue is a state where performance is temporarily decreased due to the load imposed on the mind or body. It often accompanies a desire for rest. Fatigue is caused not only by excessive exercise or chronic fatigue syndrome but also by various diseases and their therapies. Fatigue is a symptom associated with pain in various diseases. The following diseases were reported on the PedsQL fatigue scale so far: diabetes (73.5) [32]; rheumatism (73.8) [33]; cancer (71.0) [34]; obesity (67.7) [35]; and brain tumor (69.7) [36]. The fatigue scale score of citrin-deficient patients during silent period in this study was 67.8, which is one of the lowest in the above disease groups. The fatigue scale scores of 67.5% patients were 50 percentile or less of the scores of healthy children. Thus, citrin deficiency is a disease accompanied by a strong fatigue.

Fatigue is largely associated with biological oxidation caused by excessive activities of cells in the body, such as muscle cells and nerve cells. Oxidative stress reduces cell functions and energy generation in mitochondria, inducing fatigue due to the lack of energy source. Therefore, oxidative stress is regarded as a marker of fatigue [37,38]. The metabolic state of citrin-deficient patients in the silent period includes: 1) high level of oxidative stress, including increased activities of antioxidant enzymes (SOD and catalase) in red blood cells and high levels of urinary oxidative stress-related substances of acrolein-lysine and 8-hydroxy-2′-deoxyguanosine and; 2) high lactic acid/pyruvic acid ratio, in association with high NADH concentration in the cytoplasm; 3) hypercitrullinemia resulting from suppressed urea cycle; and 4) hypercholesterolemia [18]. These changes/abnormalities hamper full metabolic adaptation and compensation in citrin-deficient patients during the silent period. Although citrin-deficient patients during the silent period show no marked pathological symptoms, the metabolic dysfunction is likely to cause fatigue in citrin-deficient patients.

One of the characteristic results of PedsQL Multidimensional Fatigue Scale and Gemeric Core Scale was that fatigue impaired QOL in citrin-deficient patients during the silent period. In the child self-report, the QOL subscale score of physical and school functioning, which is easy to be affected by fatigue, was significantly lower than those in the control (Table 1). The results also showed a moderate correlation between the total score in Fatigue Scale and the total score in Generic Core Scales (Table 2). Furthermore, in the parent proxy-report, both the total fatigue score and the total generic core score in the citrin-deficient patients were significantly lower than those in the control, (Table 1), with a strong and significant correlation between the two variables (Table 2). These results indicate that fatigue correlates with QOL, and that parents of citrin-deficient patients believe that fatigue strongly correlates with QOL and affects QOL.

Other characteristic result of the PedsQL Fatigue Scale and Gemeric Core Scales in citrin deficiency was the presence of few assessment differences between child self-report and parent proxy-report. That is, the agreement level between child self-report and parent proxy-report was moderate to good agreement (0.51–0.73) on the Fatigue Scale, and moderate agreement (0.42–0.53) on the Generic Core Scales. To our knowledge, there is little or no information in the literature on the agreement level between child self-report and parent proxy-report on the Fatigue Scale. Concerning type 1 diabetes and obesity, the ICC of patient–parent agreement was poor to fair [32,35]. We reported previously in a fair to moderate agreement between Japanese children with chronic disease (asthma, atopic dermatitis) and their parents, compared with poor to fair agreement in the healthy control [22].

Although the assessments of fatigue and QOL by citrin-deficient patients and their parents correlated well, the parents underestimated fatigue compared with the patients themselves. Thus, the severity of fatigue experienced by the children during the silent period, and impairment of QOL and pain associated with the fatigue, exceeded that estimated by their parents. An important factor in the consultation of medical institution for children is how parents evaluate the health condition of their child [39]. In the case of citrin-deficient children, active involvement of the parents and medical profession is important. Diet therapy and sodium pyruvate are proposed as therapies for CTLN2 [40], and both therapies are reported to be effective in school-age children with marked growth disturbances [41]. It is expected that diet therapy and sodium pyruvate provided during the silent period improve the defective metabolic adaptation and compensatory processes in citrin-deficient children.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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# Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.



## 1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

*Abbreviations:* CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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**Table 1**
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

| Primer/probe set | Mutation | | Location | Nucleotide change | Effects of mutations | Allele frequency*[19] | References |
|---|---|---|---|---|---|---|---|
| A | Mutation [I] | :851del4 | exon 9 | c.851_854delGTAT | p.R284fs(286X) | 33.2% | [1] |
| B | Mutation [II] | :g.IVS11+1G>A | intron 11 | c.1019_1177del | p.340_392del | 37.6% | [1] |
| C | Mutation [III] | :1638ins23 | exon 16 | c.1638_1660dup | p.A554fs(570X) | 3.4% | [1] |
| D | Mutation [IV] | :S225X | exon 7 | c.675C>A | p.S225X | 5.3% | [1] |
| E | Mutation [V] | :g.IVS13+1G>A | intron 13 | c.1231_1311del | p.411_437del | 8.2% | [1] |
| F | Mutation [XIX] | :IVS16ins3kb | intron 16 | c. aberrant RNA | p.A584fs(585X) | 4.6% | [19] |
| G | Mutation [VI] | :1800ins1 | exon 17 | c.1799_1800insA | p.Y600X | 1.3% | [10] |
| | Mutation [VII] | :R605X | exon 17 | c.1813C>T | p.R605X | 0.90% | [10] |
| | Mutation [VIII] | :E601X | exon 17 | c.1801G>T | p.E601X | 1.2% | [11] |
| | Mutation [IX] | :E601K | exon 17 | c.1801G>A | p.E601K | 0.30% | [11] |
| | Mutation [XXI] | :L598R | exon 17 | c.1793T>G | p.L598R | 0% | [15] |
| | | | | | | Total 95.1% | |

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

## 2. Methods

### 2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

### 2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

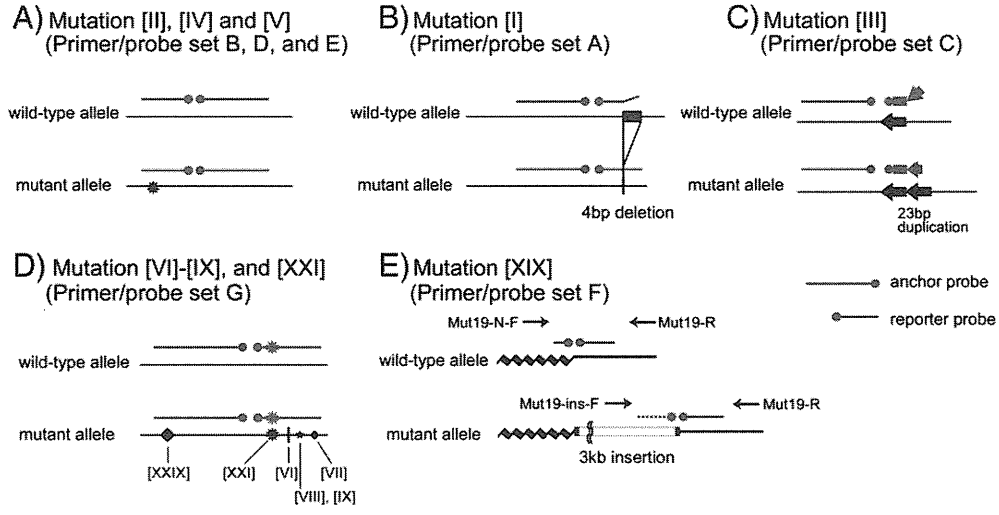


**Fig. 1.** Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.