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ORIGINAL RESEARCH

Clinical and Immunological Characterization of ICF Syndrome in Japan

Chikako Kamae[1,2] · Kohsuke Imai[3] · Tamaki Kato[1,2] · Tsubasa Okano[4] · Kenichi Honma[1] · Noriko Nakagawa[1,2] · Tzu-Wen Yeh[4] · Emiko Noguchi[5] · Akira Ohara[6] · Tomonari Shigemura[7] · Hiroshi Takahashi[8] · Shunichi Takakura[9] · Masatoshi Hayashi[10] · Aoi Honma[11] · Seiichi Watanabe[11] · Tomoko Shigemori[12] · Osamu Ohara[13] · Hiroyuki Sasaki[14] · Takeo Kubota[15] · Tomohiro Morio[4] · Hirokazu Kanegane[16] · Shigeaki Nonoyama[1]

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Abstract

Objective Immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is a rare autosomal recessive primary immunodeficiency. Hypogammaglobulinemia is a major manifestation of ICF syndrome, but immunoglobulin replacement therapy does not seem to be effective for some ICF patients. Therefore, we aimed to reassess the immunological characteristics of this syndrome.

Methods Eleven Japanese patients with ICF syndrome were enrolled. We performed whole-exome sequencing in four cases and homozygosity mapping using SNP analysis in two. We evaluated their clinical manifestations and immunological status.

Results We newly diagnosed six ICF patients who had tentatively been diagnosed with common variable immunodeficiency. We identified two novel mutations in the *DNMT3B* gene and one novel mutation in the *ZBTB24* gene. All patients showed low serum IgG and/or IgG_2 levels and were treated by periodic immunoglobulin replacement therapy. Three of the six patients showed worse results of the mitogen-induced lymphocyte proliferation test. Analyses of lymphocyte subpopulations revealed that $CD19^+CD27^+$ memory B cells were low in seven of nine patients, $CD3^+$ T cells were low in three patients, CD4/8 ratio was inverted in five patients, $CD31^+$ recent thymic emigrant cells were low in two patients, and $CD19^+$ B cells were low in four patients compared with those in the normal controls. ICF2 patients showed lower proportions of $CD19^+$ B cells and $CD16^+56^+$ NK cells and significantly higher proportions of $CD3^+$ T cells than ICF1 patients. T cell receptor excision circles were

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✉ Chikako Kamae
shochikako310@gmail.com

[1] Department of Pediatrics, National Defense Medical College, 3-2, Namiki, Tokorozawa, Saitama 359-0042, Japan

[2] Department of Pediatrics, Self Defense Forces Central Hospital, Tokyo, Japan

[3] Department of Community Pediatrics, Perinatal and Maternal Medicine, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

[4] Department of Pediatrics and Developmental Biology, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

[5] Department of Medical Genetics, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan

[6] Department of Pediatrics, Toho University School of Medicine, Tokyo, Japan

[7] Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, Japan

[8] Department of Neurology, National Hospital Organization, Tottori Medical Center, Tottori, Japan

[9] Department of Infectious Diseases, Okinawa Chubu Hospital, Uruma, Japan

[10] Department of Pediatrics, Uwajima City Hospital, Uwajima, Japan

[11] Department of Pediatrics, Tsuchiura Kyodo General Hospital, Tsuchiura, Japan

[12] Department of Pediatrics, Nippon Medical School Tama Nagayama Hospital, Tama, Japan

[13] Department of Applied Genomics, Kazusa DNA Research Institute, Kisarazu, Japan

[14] Division of Epigenomics and Development, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

[15] Faculty of Child Studies, Seitoku University, Matsudo, Japan

[16] Department of Child Health and Development, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

undetectable in two patients. Despite being treated by immunoglobulin replacement therapy, three patients died of influenza virus, fatal viral infection with persistent Epstein–Barr virus infection, or JC virus infection. One of three dead patients showed normal intelligence with mild facial anomaly. Two patients presented with autoimmune or inflammatory manifestations. Infectious episodes decreased in three patients who were started on trimethoprim–sulfamethoxazole and/or antifungal drugs in addition to immunoglobulin replacement therapy. These patients might have suffered from T cell immunodeficiency.

Conclusion These results indicate that patients with ICF syndrome have a phenotype of combined immunodeficiency. Thus, to achieve a better prognosis, these patients should be treated as having combined immunodeficiency in addition to receiving immunoglobulin replacement therapy.

Keywords ICF syndrome · B cell immunodeficiency · T cell immunodeficiency · TREC · combined immunodeficiency

Abbreviations

CDCA7	Cell division cycle-associated protein 7
Con A	Concanavalin A
DNMT3B	DNA methyltransferase 3B gene
HELLS	Helicase, lymphoid-specific
HSCT	Hematopoietic stem cell transplantation
ICF	Immunodeficiency, centromeric instability, and facial anomalies
KREC	Kappa-deleting recombination excision circles
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PML	Progressive multifocal leukoencephalopathy
RTE	Recent thymic emigrant cells
TREC	T cell receptor excision circles
ZBTB24	Zinc finger and BTB domain-containing 24 gene

Introduction

Immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is a rare autosomal recessive inborn error of immunity characterized by hypogammaglobulinemia, centromere instability, and facial anomalies. Patients with this syndrome are described as presenting with facial anomalies such as ocular hypertelorism, epicanthic folds, broad flat nasal bridge, low-set ears, and macroglossia [1–3]. They are also associated with mental retardation, including motor delay, speech delay, and intellectual disability [1–3]. Approximately half of patients with ICF syndrome are classified into type 1 (ICF1) with mutation in the *DNMT3B* gene [4, 5], whereas about 30% of patients are classified into type 2 (ICF2) with mutation in the *ZBTB24* gene [6]. Recently, *CDCA7* and *HELLS* genes were identified as causative genes for ICF3 and ICF4, respectively [7]. *DNMT3B* is a de novo DNA methyltransferase that acts during early cell development [7]. The function of *ZBTB24* has not been definitively characterized, but it was reported to be involved in DNA methylation, particularly during B cell development [6]. *CDCA7* is reported to behave as a direct c-Myc target gene and its overexpression has been found to enhance the transformation of lymphoblastoid cells. *HELLS* is reported to be involved in regulation of the expansion or survival of lymphoid cells and is required for efficient DNA methylation.

Immunodeficiency is characterized by severe recurrent infections such as fatal respiratory and gastrointestinal infections as a result of hypo- or α-gammaglobulinemia in the presence of B cells [1–3, 7]. Diverse pathogens besides bacteria were reported in ICF patients, such as viruses, fungi, and parasites [8]. Defective humoral immunity characterizes ICF syndrome, and the patients are fundamentally treated with immunoglobulin replacement therapy. Additionally, $CD19^{+}CD27^{+}$ memory B cells were shown to be absent from patients with ICF1 and ICF2 [8–10]. However, T cell immunodeficiency in ICF syndrome is not fully understood. T cell apoptosis, decreased $CD4^{+}$ T cells, and decreased $CD4^{+}$ T cells associated with autoimmune/inflammatory diseases were reported in a small number of patients with ICF syndrome [11–13]. Hematopoietic stem cell transplantation (HSCT) might be useful for patients with ICF syndrome [8, 14, 15], whereas immunosuppressive therapy would be required for patients with autoimmune/inflammatory manifestations [8].

In this paper, we analyze the immunological profiles including T cells in Japanese patients with ICF syndrome, and disclose the existence of T cell immunodeficiency in these patients. In addition, we describe the infectious episodes and outcomes of the patients and reveal that some infections were consistent with T cell immunodeficiency.

Material and Methods

Study Approval and Samples

Eleven Japanese patients with ICF syndrome were enrolled in this study. Detailed clinical and biological data were collected from birth until June 2018. We defined “the age at final observation” as the age at June 2018 and “the age at immunological analysis” as the age when the immunological analysis was performed. We defined normal ranges of serum levels of globulins as from 10th (or − 1.28 SD) to 90th (or + 1.28 SD) [16]. Mental status was assessed by the physicians caring for the patients. When mental retardation was suspected, the

physicians evaluated the mental status using development quotient or intelligence quotient and defined mental retardation as when the score was below 70 or the patients had some difficulties attending an ordinary school or work. For 4 of the 11 patients, some clinical and biological data had previously been reported by Shirohzu et al. as Pt. 2 (Patient 3 in our study) and Pt. 3 (Patient 4 in our study) [17], and by Nitta et al. as P6 (Patient 8 in our study) and P7 (Patient 9 in our study) [10]. Written informed consent was obtained from the parents of the pediatric patients and from the adult patients, in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics board of the National Defense Medical College and Tokyo Medical and Dental University.

Genetic Analysis

We performed whole-exome sequencing using the samples of patients, parents, and/or siblings by HiSeq 1500 (Illumina, San Diego, CA, USA) and identified *DNMT3B* mutations in three cases (Patients 5, 6, and 11) and *ZBTB24* in one case (Patient 10). In two patients (Patients 1 and 7), we performed homozygosity mapping using SNP analysis by Human610-Quad (Illumina, San Diego, CA, USA) or Affymetrix CytoScan HD array (Thermo Fisher Scientific, Waltham, MA, USA). All mutations were ascertained to be homozygous (hemizygous) in the patients and heterozygous in the parents and/or siblings by Sanger sequencing with Applied Biosystems 3130xl Genetic Analyzer or Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Centromeric Instability

G-banding chromosome analysis was performed with the stimulation of phytohemagglutinin (PHA) in five patients (Patients 1, 5–7, and 11). Four cases (Patients 3, 4, 8, and 9) had previously been reported to show typical chromosomal anomalies at 1qh and/or 16qh. PHA (J-chemical, Tokyo, Japan)-stimulated heparinized whole blood was cultured at 37 °C with 5% CO_2 for 72 h in RPMI1640 with 10% FBS and 1% penicillin/streptomycin. During the last hour of culture, cells were exposed to colcemid (KaryoMAX Colcemid; Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 0.05 μg/ml. Hypotonic treatment was performed with 0.075 M KCL at 37 °C for 15 min, followed by fixation with Carnoy's fixative (acetic acid:methanol, 1:3). The metaphase chromosomes were treated with trypsin at 30 °C for 4 min and stained with 5% Giemsa stain for 7 min. At least 30 metaphases were analyzed. In three cases (Patients 1, 7, and 8), we reanalyzed the second G-banding chromosome analysis, with an increase in the examination time from 24 to 72 h after stimulation and an increase in analyses of metaphases to more than 30 cells.

Mitogen-Induced Lymphocyte Proliferation Tests

Tests of lymphocyte proliferation induced by mitogens (PHA and concanavalin A: Con A) were performed in five patients (Patients 1 and 6–9) and with PHA alone in one patient (Patient 10). Peripheral blood mononuclear cells were isolated from whole blood using Lymphosepar (Immuno-Biological Laboratories, Fujioka, Japan) density gradient centrifugation, in accordance with the manufacturer's instructions. The cells (1×10^5) were cultured with PHA (7 μg/ml; J-chemical, Tokyo, Japan) or Con A (7 μg/ml, L1104-50; EY Laboratories, San Mateo, CA, USA) in 200 μl of RPMI 1640 medium with 10% FBS at 37 °C with 10% CO_2 for 72 h. ^{3}H-thymidine was added. The cells were harvested using Filter Mate Cell Harvester (Perkin Elmer, Waltham, MA, USA) and counted by TopCount NXT (Perkin Elmer, Waltham, MA, USA).

Flow Cytometric Analysis

Peripheral blood samples of eight patients with ICF syndrome (ICF1, $n = 6$ and ICF2, $n = 2$) were analyzed in our study. Two patients were analyzed before this study [10]. Four patients (Patients 1, 3, 4, and 7) were analyzed by a FACS Calibur system (BD Biosciences, Franklin Lakes, NJ, USA) using anti-human monoclonal antibodies (mAbs): in a first tube, CD45RA (FITC, clone L48; BD Biosciences), CD31 (PE, clone WM59; BD Biosciences), CD4 (PerCP, clone SK3; BD Biosciences), and CD3 (APC, clone UCHT1; Beckman Coulter, Brea, CA, USA); in a second tube, CD3 (FITC, clone SK7; eBioscience, Waltham, MA, USA), CD16 (PE, clone B73.1; BD Biosciences), CD56 (PE-Cy5, clone N901; Beckman Coulter), and CD19 (APC, clone J3-119; Beckman Coulter); and in a third tube, IgD (FITC, clone IA6-2; BD Biosciences), CD27 (PE, clone 1A4CD27; Beckman Coulter), IgM (PE-Cy5, clone G20-127; BD Biosciences), and CD19 (APC, clone J3-119; Beckman Coulter). The data were analyzed by single platform analysis using Cell Quest (BD Biosciences). Four patients (Patients 5, 6, 10, and 11) were analyzed by the LSR-Fortessa system (BD Biosciences, Franklin Lakes, NJ, USA), as reported previously [18]. The data were analyzed using FlowJo flow cytometry analysis software (FlowJo LLC, Ashland, OR, USA).

The normal percentages of control data were taken from a previous report [18], as were the normal absolute counts of control data [19]. We defined normal ranges of control data as from 10th (or − 1.28 SD) to 90th (or + 1.28 SD) [18, 19].

Quantification of TREC and KREC by Real-time PCR

Quantification of T cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) was performed by TaqMan-based real-time quantitative

polymerase chain reaction (PCR), as previously described [20, 21], using samples of gDNA from nine patients with ICF syndrome (ICF1, $n = 6$ and ICF2, $n = 3$). Copy numbers of TREC and KREC were evaluated as the levels per microgram of DNA, and copy numbers of less than 100 copies/μg of DNA were defined as "undetectable."

Statistical Analysis

We conducted all statistical analyses by using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). When we needed to compare two groups, we used the unpaired *t* test with Welch's correction and considered a p value of < 0.05 as significant.

Results

Clinical Findings

Seven patients had ICF1 (Fig. 1, upper panel) and four patients had ICF2 (Fig. 1, lower panel). Four males and seven females were included. Six patients from four families (Patients 1–4, 9, and 11) were born to consanguineous parents (Fig. 1 and Table 1). The age at final observation and the age at immunological analysis did not differ between ICF1 and ICF2 [final observation age; ICF1, 23 ± 4 (range, 4–35 years) and ICF2, 22 ± 8 (range, 7–41 years), $p = 0.93$] [immunological analysis age; ICF1, 20 ± 4 (range, 4–33 years) and ICF2, 19 ± 8 (range, 5–39 years), $p = 0.92$].

Infectious Episodes and Inflammatory Manifestations

A 28-year-old female (Patient 1) and her twin sister (Patient 2) contracted recurrent pneumonia and sinusitis. Patient 2 had suffered from arthritis and mild hepatitis without detectable autoantibodies since the age of 17 years (Table 1). A 15-year-old girl (Patient 3) and her 13-year-old brother (Patient 4) contracted recurrent pneumonia and otitis media (Table 1). A 33-year-old female (Patient 5) and her 29-year-old sister (Patient 6) contracted recurrent pneumonia and otitis media. Additionally, Patient 5 suffered from myositis without detectable antibodies. Patient 6 suffered from bronchiectasis with middle lobe syndrome. A 5-year-old girl (Patient 7) suffered from recurrent pneumonia (Table 1). In addition, a 5-year-old boy (Patient 8) suffered from pneumococcemia, recurrent pneumonia, recurrent cytomegalovirus (CMV) viremia, and persistent Epstein–Barr virus (EBV) infection with refractory diarrhea. Moreover, a 39-year-old man (Patient 9) suffered from recurrent pneumonia, recurrent sinusitis, and progressive multifocal leukoencephalopathy (PML) due to JC virus (Table 1). A 27-year-old man (Patient 10) suffered from recurrent pneumonia and bronchitis. Finally, a 4-year-old girl (Patient 11) suffered from recurrent otitis media and contracted sepsis suspected of being caused by *Pseudomonas aeruginosa* with neutropenia (Table 1).

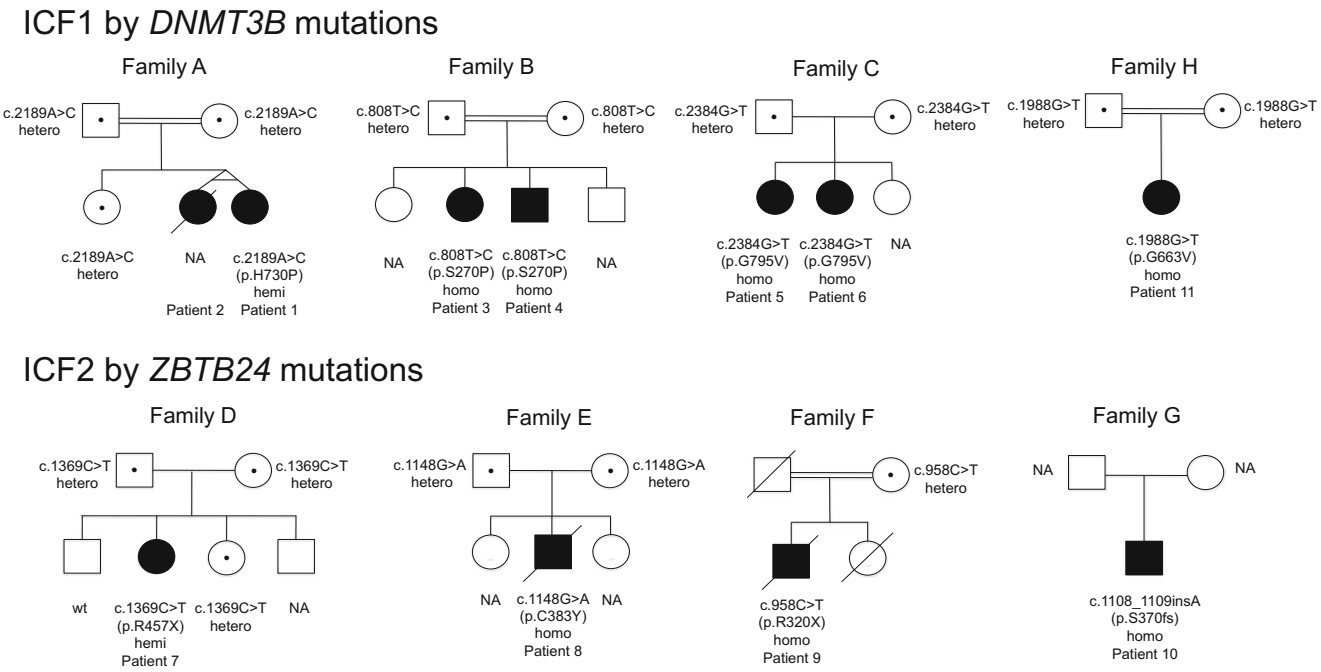

Fig. 1 Family pedigrees of the ICF1 or ICF2 patients. Upper panels (Families A–C and H) and lower panels (Families D–G) indicate family pedigrees of the ICF1 and ICF2 patients, respectively. Square symbols represent males and circular symbols represent females. Filled symbols show the patients and open symbols with a dot show the heterozygous mutated parents and/or siblings. Double line indicates consanguineous parents. wt, wild type; NA, not available

Treatment and Outcome

All 11 patients had been treated with intravenous or subcutaneous immunoglobulin replacement therapy. We analyzed the period of immunoglobulin replacement therapy, for which there was no significant difference between ICF1 and ICF2 [ICF1, 20.4 ± 4.0 (range, 0.9–33) and ICF2, 10.0 ± 5.9 (range, 2–27) years, $p = 0.20$] or surviving or deceased patients [surviving, 20.2 ± 3.9 (range, 0.9–33) and deceased, 7.0 ± 5.0 (range, 2–17) years, $p = 0.10$]. Four patients (Patients 1, 7, 8, and 11) received trimethoprim–sulfamethoxazole (TMP/SMX) and one patient (Patient 7) received antifungal drug, whereas Patient 9 was administered cytarabine against PML. Four of the eight surviving patients who were not using TMP/SMX had been suffering from recurrent infection, but hospitalization was unnecessary in most cases. That is, Patient 3 suffered from bronchitis and pneumonia, Patient 4 pneumonia, Patient 5 urosepsis, and Patient 10 pneumonia. Infectious episodes decreased in two patients (Patients 1 and 11) who started TMP/SMX and one patient (Patient 7) who started TMP/SMX and antifungal drugs (Table 1). Patient 1 had suffered from recurrent bronchitis and sinusitis even after the replacement of immunoglobulin, but the frequency of fever decreased after starting TMP/SMX.

Three patients (Patients 2, 8, and 9) died despite sufficient immunoglobulin replacement therapy. Patient 2 died during recovery from influenza virus type B infection at the age of 18 (Table 1). In Patient 8, at the age of 5, Epstein–Barr virus was detected from blood and skin, with a copy number in blood of 1.4×10^6 copies/μg DNA. The infected cells in blood were confirmed to be T cells, not B cells. He died of fatal infection with persistent EBV infection at the age of 7 [10] (Table 1). Patient 9 died of progressive PML due to JC virus at the age of 41 [10] (Table 1).

Genetic Analysis

A total of 6 of 11 patients (Patients 1, 5–7, 10, and 11) were tentatively diagnosed as having common variable immunodeficiency. In 2 patients (Patients 1 and 7), we identified loss-of-heterozygosity regions containing the *DNMT3B* and *ZBTB24* genes, respectively. c.1988G>T (p.G663V) mutation seen in Patient 11 was previously reported by others, whereas c.2189A>C (p.H730P) (Patient 1) and c.2384G>T (p.G795V) (Patients 5 and 6) were novel mutations in the *DNMT3B* gene (Fig. 2, upper panel). c.1369C>T (p.R457X) mutation seen in Patient 7 was previously reported by others, whereas c.1108_1109insA (p.S370fs) (Patient 10) was a novel mutation in the *ZBTB24* gene (Fig. 2, lower panel). The mutations of four patients (Patients 3, 4, 8, and 9) had previously been reported (Fig. 2 and Table 1) [10, 17]. Four patients (Patients 5–8) from three families (Families C–E) had homozygous and/or hemizygous mutations, even though they were from nonconsanguineous families (Fig. 1).

Centromeric Instability

All nine cases (Patients 1, 3–9, and 11) showed typical chromosomal anomalies at 1qh and/or 16qh (Table 1). Patients 1, 7, and 8 were ascertained to have centromeric instability in the second G-banding chromosome analysis and an increasing examination time of 72 h after stimulation.

Facial Anomalies

Facial anomalies were typical in six patients but were mild in five patients. Ocular hypertelorism was observed in six patients but was mild in five patients (Patients 1, 2, 5, 6, and 10). Epicanthic folds, broad flat nasal bridge, low-set ears, macroglossia, and micrognathia were observed in 7, 8, 5, 2, and 1 of 11 patients, respectively (Table 1).

Mental Status

Marked mental retardation was observed in three patients (one patient with ICF1 and two patients with ICF2) (Table 1). Eight patients (Patients 1–6, 8, and 10) showed normal intelligence levels, and five of them (Patients 1, 4–6, and 10) were able to attend an ordinary school or work without difficulty (Table 1).

Fertility

Two of six adult ICF patients (two ICF1 patients; Patient 5 and Patient 6) suffered from amenorrhea and one ICF2 patient (Patient 7) had no sign of secondary sexual characteristics at the age of 12. On the other hand, three of six adult ICF patients (two ICF1 and one ICF2; Patients 1, 2, and 10) showed normal gonadal function and one male ICF2 patient (Patient 10) had a child.

Immunological Analysis

A total of 9 of 11 patients with ICF syndrome showed low IgG levels [ICF1, 412 ± 145 (range, 138–1160) and ICF2, 513 ± 208 (range, 108–1088) mg/dL, $p = 0.71$] (Table 2, Fig. S1). Intriguingly, two patients with normal IgG levels (Patient 5, 1160 mg/dL and Patient 8, 1088 mg/dL) showed low IgG_2 levels (Patient 5, 33 mg/dL and Patient 8, 41 mg/dL). Serum IgG_2 was low in all five patients (Patients 5–9) (Table 2, Fig. S1B). Serum IgA levels were low in all patients [ICF1, 2 ± 1 (range, 0–7) and ICF2, 31 ± 14 (range, 0–69) mg/dL, $p = 0.13$], and IgA levels in patients with ICF1 were significantly lower than those in patients with ICF2 (Table 2, Fig. S1C).

Table 1 Clinical and genetical characteristics of ICF patients

ICF Type		ICF 1							ICF 2			
Family		Family A		Family B		Family C		Family H	Family D	Family E	Family F	FamilyG
Patient number		Patient 1	Patinet 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 11	Patient 7	Patient 8	Patient 9	Patinet 10
Sex		F	F	F	M	F	F	F	F	M	M	M
Age at Immunological Analysis (year)		28	18	15	13	33	29	4	5	5	39	27
Age at ICF Diagnosis (year)		28	After death	1	0.9	33	29	4	7	5	39	27
Age at Final Obeservation (year)		34	18	20	18	35	31	4	12	7	41	28
Consanguinity		Yes	Yes	Yes	Yes	No	No	Yes	No	No	Yes**	No
Gene Mutation		*DNMT3B* c.2189A>C (p.H730P) hemi	Identical twin of Pt.1	*DNMT3B* c.808T>C (p.S270P) homo*	*DNMT3B* c.808T>C (p.S270P) homo*	*DNMT3B* c.2384G>T (p.G795V) homo	*DNMT3B* c.2384G>T (p.G795V) homo	*DNMT3B* c.1988G>T (p.G663V) homo	*ZBTB24* c.1369C>T (p.R457X) hemi	*ZBTB24* c.1148G>A (p.C383Y) homo**	*ZBTB24* c.958C>T (p.R320X) homo**	*ZBTB24* c.1108-1109 insA (p.S370fs) homo
Centromeric instability		Yes	NA	Yes *	Yes *	Yes	Yes	Yes	Yes	Yes**	Yes**	NA
Infection episodes	Bacterial	Recurrent pneumonia, bronchitis, sinusitis	Recurrent pneumonia	Recurrent pneumonia, brochitis, otitis media	Recurrent pneumonia, otitis media, skin infection	Recurrent pneumonia, otitis media Urosepsis	Recurrent pneumonia	Recurrent otitis media Sepsis suspected of *Pseudomonas aeruginosa*	Recurrent pneumonia	Pneumococcemia Recurrent pneumonia, skin infection	Recurrent pneumonia, sinusitis	Recurrent pneumonia, bronchitis
	Viral	No	Dead after influenza viral infection	No	Neutropenia due to viral infection	No	No	No	No	CMV viremia Persistent EB viral infection Dead because of fatal viral infection	Dead because of PML due to JC viral infection	No
	Fungal	No	No	No	No	No	No	No	No	No	No	No
Autoimmune manifestations		No	Arthritis and mild hepatic disorder without detectable antibody	No	No	Myositis without detectable antibody	No	No	No	No	No	No
Immunoglobulin replacement therapy (treatment period)		Yes (33 years)	Yes (17 years)	Yes (19 years)	Yes (17 years)	Yes (28 years)	Yes (28 years)	Yes (0.9 years)	Yes (9 years)	Yes** (2 years)	Yes (2 years)	Yes (27 years)
Additional treatment (treatment periodt)		TMP/SMX (4 years)	No	No	No	No	No	TMP/SMX (0.3 years)	TMP/SMX (8.8 years) Antifungal drug (5.2 years)	TMP/SMX (2 years)	Cytarabine against PML (2 years)	Start TMP/SMX after diagnosis
Outcome		Alive	Dead at 18 years old	Alive	Alive	Alive	Alive	Alive	Alive	Dead at 7 years old	Dead at 41 years old	Alive
Facial Anomalies	ocular hypertelorism	Mild	Mild	Yes*	Yes*	Mild	Mild	Yes	Yes	Yes**	Yes**	Mild
	epicanthic folds	Mild	Mild	Yes*	Yes*	Mild	Mild	No	No	Yes**	No**	No
	broad flat nasal bridge	Mild	Mild	Yes*	Yes*	Mild	Mild	No	No	Yes**	No**	Mild
	low set ears	No	No	Yes*	Yes*	Mild	Mild	No	No	No**	Yes**	No
	macroglossia	No	No	Yes*	Yes*	No	No	No	No	No**	No**	No
	micrognathia	No	No	No *	No *	No	No	No	No	No**	Yes**	No
DQ/IQ Examination Score (Exam. Months)		Normal Work without difficulty	Normal	DQ88* (30) Apparently Normal	DQ84* (33) Normal Attend an ordinary school without difficulty	Normal Work without difficulty	Normal Work without difficulty	Speech Delay	IQ75 (57) Attend an ordinary school with difficulty to keep up with comlicated classes	DQ80** (60) Apparently Normal	IQ47**	Normal Work without difficulty
Reference		Present Study	Present Study	Shirohzu et.al.* Pt.2	Shirohzu et.al.* Pt.3	Present Study	Present Study	Present Study	Present Study	Nitta et.al.** P6	Nitta et.al.** P7	Present Study

Clinical and genetic characteristics of ICF1 patients (Patients 1–6 and 11) and ICF2 patients (Patients 7–9 and 10) are shown. "The age at final observation" indicates the age at June 2018 and "the age at immunological analysis" indicates the age when the immunological analysis was performed. Treatment period of Immunoglobulin replacement therapy and additional treatment indicated in parentheses. In examination score, the ages of the examination performed are indicated in parentheses with months expression. Data with * was referred to Shirohzu et al. [17]. Data with ** was referred to Nitta et al. [10]

DQ, development quotient; *IQ*, intelligence quotient; *CMV*, cytomegalovirus; *EB*, Epstein–Barr; *JC*, John Cunningham; *PML*, progressive multifocal leukoencephalopathy; *TMP/SMX*, trimethoprim–sulfamethoxazole; *NA*, not available

Serum IgM levels were variable in seven patients with ICF1; three patients showed low levels, one patient showed a normal level (Patient 6, 72 mg/dL), and two patients showed high levels (Patient 2, 300 mg/dL and Patient 11, 950 mg/dL). One patient (Patient 5) showed a high IgM level (265 mg/dL) at the age of 26, but this declined to a normal level (74 mg/dL) at the age of 33. In contrast, all patients with ICF2 showed significantly lower IgM levels [ICF1, 202 ± 131 (range, 4–950) and ICF2, 18 ± 8 (range, 0–40) mg/dL, $p = 0.21$] (Table 2, Fig. S1D).

Lymphocyte proliferation tests with PHA and Con A stimuli were performed in six patients with ICF syndrome (both in Patients 1, 5, and 7–9 and PHA only in Patient 10). Two of six patients showed low responses with PHA (Patient 9, 3150 cpm and Patient 10, 7910 cpm) [ICF1, 28,100 ± 7300 (range, 20,800–35,400) and ICF2, 21,640 ± 11,079 (range, 3150–52,300) cpm, $p = 0.66$] (Table 2) and two of five patients showed low responses with Con A (Patient 8, 10,400 cpm and Patient 9, 103 cpm) [ICF1, 31,101 ± 3910 (range, 27,200–35,002) and ICF2, 22,034 ± 17,044 (range, 103–55,600) cpm, $p = 0.66$] (Table 2).

Flow cytometric analyses were performed in 10 patients with ICF syndrome. Three of six patients, who were all adults, showed low percentages of $CD3^+$ T cells compared with the normal control (Patient 1, 54.3%; Patient 5, 43.8%; and Patient 6, 54.4%). All patients with ICF2 showed significantly higher percentages of $CD3^+$ T cells than the patients with ICF1 [ICF1, 61.8 ± 5.5% (range, 43.8–81.0%) and ICF2, 93.3 ± 1.9% (range, 90.0–97.0%), $p = 0.0016$] (Table 2, Fig. S2A). The CD4/8 ratio was inverted in two of six patients with ICF1 (Patient 1, 0.88 and Patient 3, 0.63) and three of four patients with ICF2 (Patient 7, 0.75; Patient 9, 0.32; and Patient 10, 0.54) [ICF1, 1.4 ± 0.4 (range, 0.6–3.0) and ICF2, 1.2 ± 0.6 (range, 0.3–3.0), $p = 0.80$] (Table 2, Fig. S2B). The level of $CD31^+$ recent thymic emigrant cells (RTE) among $CD3^+CD4^+CD45RA^+$ cells was low in one of six patients with ICF1 (Patient 3, 63.3%) and one of two patients with ICF2 compared with that in the normal control (Patient 10, 21.9%) (Table 2, Fig. S2C).

As for $CD19^+$ B cells, two of six patients with ICF1 (Patient 6, 3.3% and Patient 11, 6.3%) and three of four patients with ICF2 (Patient 8, 3.0%; Patient 9, 1.0%; and Patient 10, 0.7%) showed low percentages of $CD19^+$ B cells compared with the normal control. Three of six patients with ICF1 (Patient 1, 29.4%; Patient 3, 21.2%; and Patient 4, 25.0%) showed high percentages of $CD19^+$ B cells compared with the normal control. Mean percentages of $CD19^+$ B cells in patients with ICF2 were lower than those in patients with ICF1 [ICF1, 15.3 ± 4.6% (range, 3.3–29.4%) and ICF2, 3.0 ± 1.6% (range, 0.7–7.5%), $p = 0.069$] (Table 2, Fig. S2D). The percentages of $CD27^+$ memory B cells were low in seven of nine patients with ICF compared with that in the normal control. One patient with ICF1 (Patient 11, 7.7%) and one patient with ICF2 (Patient 10, 8.7%) showed normal percentages of $CD27^+$ memory B cells [ICF1, 3.7 ± 1.3% (range, 0.5–7.7%) and ICF2, 4.1 ± 2.4% (range, 0.3–8.7%), $p = 0.88$] (Table 2, Fig. S2E). The percentages of IgM^+ memory B cells ($CD27^+IgM^+IgD^+$) were low in 4 of 6 ICF1 patients and all 3 ICF2 patients compared with that in the normal control [ICF1, 3.08 ± 1.30% (range, 0.29–7.36%) and ICF2, 2.52 ± 1.52% (range, 0.34–5.45%), $p = 0.79$] (Table 2). The percentages of switched memory B cells ($CD27^+IgM^+IgD^-$) were low in all nine patients compared with that in the normal control [ICF1, 0.58 ± 0.16% (range, 0.07–1.10%) and ICF2, 1.61 ± 0.93% (range, 0.00–3.22%), $p = 0.39$] (Table 2).

As for $CD16^+56^+$ NK cells, two of six patients with ICF1 (Patient 5, 21.8%; Patient 6, 23.5%) showed high percentages, while one of three patients with ICF2 (Patient 10, 0.68%) showed low percentages of $CD16^+56^+$ NK cells compared with the normal control. Mean percentages of $CD16^+56^+$ NK cells in patients with ICF2 were lower than those in patients with ICF1 [ICF1, 11.3 ± 3.7% (range, 3.3–23.5%) and ICF2, 2.2 ± 1.4 (range, 0.7–5.0%), $p = 0.06$] (Table 2, Fig. S2E).

TREC and KREC were analyzed in nine patients with ICF syndrome. TREC were undetectable in two patients with ICF1 (Patients 5 and 6), who had been positive for them 7 years earlier. There was no significant difference in TREC between ICF1 and ICF2 [ICF1, 10.9 ± 10.6 (range, < 0.1–64.0) × 10^3; ICF2, 2.00 ± 0.63 (range, 0.73–2.80) × 10^3 copies/μg of DNA, $p = 0.44$]. KREC were positive in all patients [ICF1, 11.9 ± 5.73 (range, 1.63–39.00) × 10^3; ICF2, 2.3 ± 0.93 (range, 1.37–4.2) × 10^3 copies/μg of DNA, $p = 0.16$] (Fig. 3).

Discussion

In this study, we newly diagnosed six ICF patients who had tentatively been diagnosed as having common variable immunodeficiency. Furthermore, we identified three novel mutations of c.2189A>C (p.H730P) (Patient 1) and c.2384G>T (p.G795V) (Patients 5 and 6) in the *DNMT3B* gene and c.1108_1109insA (p.S370fs) (Patient 10) in the *ZBTB24* gene. In three families (Families C–E), we identified homozygous mutations even though they were nonconsanguineous families. The novel mutations found in *DNMT3B* (Patients 1, 5, and 6) were missense mutations in the catalytic domain, as previously reported [3], whereas the novel mutation found in *ZBTB 24* (Patient 10) was also a nonsense mutation presented in zinc fingers, as previously reported [3]. We diagnosed two ICF patients by the presence of memory B cells. Although memory B cells were present in Patient 10, IgA deficiency was observed and NK cells were also few in number; thus, we suspected ICF syndrome and performed diagnosis using whole-exome sequencing. Although memory B cells were present and extremely high levels of IgM were seen in Patient 11, we suspected ICF based on facial anomalies and mental retardation and performed diagnosis with G-banding chromosome analysis and genetic analysis.

Serum IgG, IgG_2, and IgA levels were all low in patients with ICF syndrome (ICF1 and ICF2), as reported previously [1–3]. Although serum IgM levels were reported to be low to normal in patients with both ICF1 and ICF2 [3], three patients with ICF1 were found to have increased levels of serum IgM. Notably, two of them (Patients 2 and 5) suffered from inflammatory manifestations. Although autoantibodies were not detected from both patients, these manifestations could be due to autoimmunity as a result of T cell immunodeficiency. $CD19^+$ B percentages were low in one of six patients with ICF1 and three of four patients with ICF2. Lack of $CD19^+CD27^+$ memory B cells was observed in 9 of 11 patients, as previously reported [8]. Two patients showed normal percentages of $CD19^+CD27^+$ memory B cells (Patient 10, 8.7% and Patient 11, 7.7%). However, they both had decreased numbers of B cells (Patient 10, 11/μL and Patient 11, 317/μL) and also decreased absolute numbers of $CD19^+CD27^+$ memory B cells.

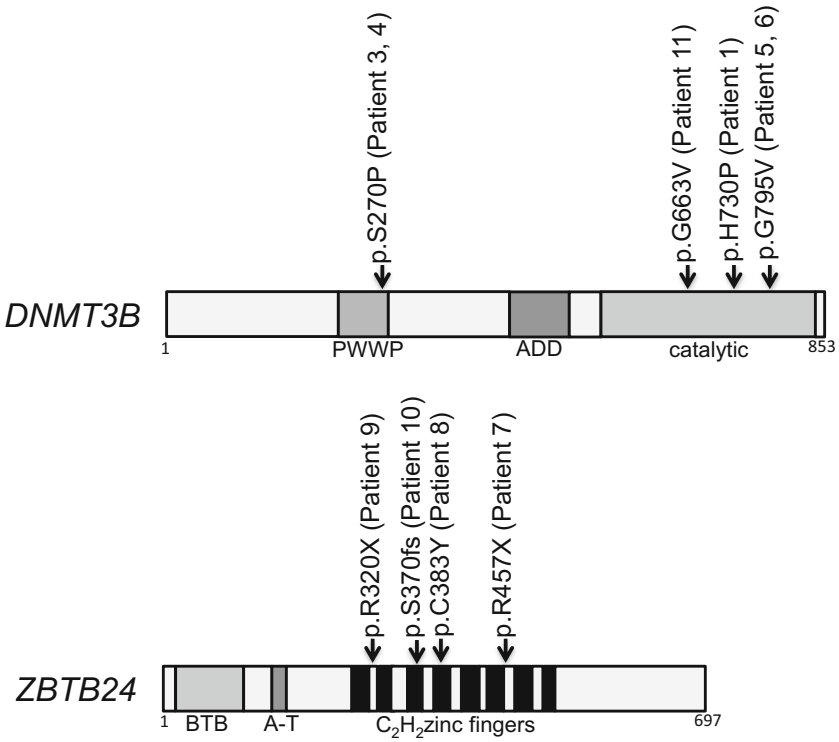

Fig. 2 Distributions of *DNMT3B* and *ZBTB24* mutations in patients with ICF syndrome. A schematic shows the protein structures associated with the *DNMT3B* (upper panel) and *ZBTB24* (lower panel) genes. Locations of the mutations identified in this study are shown by arrows. PWWP, PWWP domain; ADD, ADD domain; catalytic, catalytic domain; BTB, BTB domain; A-T, AT-hook; C_2H_2 zinc fingers, zinc finger domains

One patient (Patient 8) suffered from recurrent CMV viremia and persistent EB infection, and another (Patient 9) developed PML caused by JC virus infection. In addition, one patient (Patient 2) died during the recovery from influenza virus infection. These vulnerabilities to viral infection suggest the existence of T cell immunodeficiency in patients with ICF syndrome. Consistent with these observations, three patients (Patients 8–10) showed remarkably reduced responses in mitogen-induced lymphocyte proliferation tests. Three patients (Patients 1, 5, and 6) showed low percentages of $CD3^+$ T cells, five patients (Patients 1, 3, 7, 9, and 10) showed an inverted CD4/CD8 ratio, and two patients showed decreased RTE (Patients 3 and 10). TREC progressively decreased over years in two patients (Patients 5 and 6), indicating poor T cell neogenesis [22]. Moreover, infectious episodes decreased in three patients who started taking TMP/SMX and/or antifungal drugs in addition to immunoglobulin replacement therapy. T cell abnormalities such as T cell apoptosis and a reduction of $CD4^+$ T cells with aging had been reported in ICF syndrome [11–13]. It has been reported that three patients developed pneumocystis pneumonia and six patients did persistent candida stomatitis [2]. Three patients with ICF syndrome required HSCT because of the complications of persistent small round structured virus and candida infection, *Pneumocystis jirovecii* infection, and persistent enteritis with unknown cause [14]. In addition, one patient with ICF1 underwent HSCT because of *P. jirovecii* infection [15]. Previous reports were of individual cases showing T cell immunodeficiency in ICF syndrome. In this study, we used multiple methods such as clinical course analysis, lymphocyte proliferation test, lymphocyte subset analysis, and TREC and demonstrated T cell immunodeficiency in ICF syndrome. Therefore, patients with ICF syndrome should be treated as having T cell immunodeficiency in addition to receiving immunoglobulin replacement therapy.

Two patients with ICF1 (Patients 2 and 5) presented autoimmune or inflammatory manifestations that are uncommon in ICF syndrome. Patient 5 exhibited a decreased number of $CD3^+$ T cells and undetectable TREC. One patient with ICF2 who suffered from autoimmune or inflammatory features also had decreased $CD4^+$ T cells as a combined immunodeficiency [13]. It has been reported that two patients with ICF1 who were associated with autoimmune/inflammatory manifestations had a poor outcome, for whom the need for additional immunosuppressive treatment was proposed [8]. Patient 2 died during the recovery from influenza virus infection at the age of 18. These findings suggest that T cell disorders occur in ICF syndrome, especially in cases with autoimmune/inflammatory manifestations.

ICF syndrome is well known to be associated with facial anomaly. Regarding mental status, Weemaes et al. [3] reported mental retardation in all 13 ICF2 patients, but reported that mental retardation was not observed in almost half of the ICF1 patients. Sterlin et al. [8] reported marked mental retardation in one ICF2 patient, although intelligence was almost normal in ICF1 and ICF3 patients. In our study, 5 of 11 patients (four ICF: Patients 1, 2, 5, and 6; one ICF2: Patient 10) showed mild facial anomaly and normal intelligence (Table 1). Notably,

Table 2 Immunological characteristics of ICF patients

ICF type	ICF 1							ICF 2				*p* value (ICF1 between ICF2)
Family	Family A		Family B		Family C		Family H	Family D	Family E	Family F	Family G	
Patient number	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 11	Patient 7	Patient 8	Patient 9	Patient 10	
Age at analysis (year)	28	18	15	13	33	29	4	5	5	39	27	
IgG (mg/dl)	**140** (381–941)	**614** (768–1548)	**155*** (381–941)	**175*** (381–941)	1160 (768–1548)	**505** (768–1548)	**138** (637–1221)	**357** (637–1221)	1088** (637–1221)	**108**** (768–1548)	**500** (768–1548)	0.71
IgG2 (mg/dl)	NA	NA	NA	NA	**33** (208–754)		NA			**34**** (208–754)	NA	0.45
IgA (mg/dl)	**< 10** (14–60)	**< 10** (122–278)	**7*** (14–60)	**< 4*** (14–60)	**3** (122–278)	**1** (122–278)	**< 5** (58–128)	**27** (58–128)	**29**** (58–128)	**69**** (122–278)	**< 3** (122–278)	0.13
IgM (mg/dl)	**10** (25–83)	*300* (64–134)	**6*** (25–83)	**4*** (25–83)	74 (64–134)	72 (64–134)	*950* (33–79)	**< 5** (33–79)	**16**** (33–79)	**16**** (64–134)	**40** (64–134)	0.21
$CD3^+$ lymph ($CD3^+$ % of lymph cells)	**54.3** (61–75)	NA	69.0 (59–91)	68.0 (59–91)	**43.8** (61–75)	**54.4** (61–75)	81.0 (58–81)	*90.2* (58–81)	*90.0*** (58–81)	*97.0*** (61–75)	*96.0* (61–75)	***0.0016***
CD4/8 ratio (normal range, 0.9–3.2)	**0.88**	NA	**0.63**	1.69	2.95	0.95	1.00	**0.75**	3**	**0.32****	**0.54**	0.8
RTEs ($CD31^+$ % of $CD3^+$ $CD4^+CD45RA^+$ lymph cells)	67.4 (59–88)	NA	**63.3** (78–99)	90.8 (78–99)	71.5 (59–88)	76.3 (59–88)	81.7 (77–99)	86.1 (77–99)	NA	NA	**21.9** (59–88)	0.63
$CD19^+$ lymph ($CD19^+$ % of lymph cells)	*29.4* (6.6–17.8)	NA	*21.2* (4.3–20.5)	*25.2* (4.3–20.5)	6.61 (6.6–17.8)	**3.33** (6.6–17.8)	**6.3** (6.6–25.6)	7.5 (6.6–25.6)	**3.00**** (6.6–25.6)	**1.00**** (6.6–17.8)	**0.65** (6.6–17.8)	0.069
$CD27^+$ memory B cells (% of $CD19^+$ lymph cells)	**0.48** (8.0–29.0)	NA	**0.64** (6.6–24.2)	**1.43** (6.6–24.2)	**4.45** (8.0–29.0)	**7.29** (8.0–29.0)	7.69 (7.2–26.6)	**3.39** (7.2–26.6)	**0.34**** (7.2–26.6)	NA	8.67 (8.0–29.0)	0.88
IgM^+ memory B cells ($CD27^+$ IgD^+ % of $CD19^+$ lymph cells)	**0.41** (6.0–16.3)	NA	**0.29** (1.4–13.3)	**0.65** (1.4–13.3)	**3.35** (6.0–16.3)	6.44 (6.0–16.3)	7.36 (5.3–12.1)	**1.77** (5.3–12.1)	**0.34** (5.3–12.1)	NA	**5.45** (6.0–16.3)	0.79
Switched memory B cells ($CD27^+$ gD^- % of $CD19^+$ lymph cells)	**0.07** (4.0–22.5)	NA	**0.35** (2.1–12.7)	**0.78** (2.1–12.7)	**1.1** (4.0–22.5)	**0.85** (4.0–22.5)	**0.33** (5.2–15.3)	**1.62** (5.2–15.3)	**0** (5.2–15.3)	NA	**3.22** (4.0–22.5)	0.39
$CD16^+56^+$ lymph ($CD16^+$ $CD56^+$ % of lymph cells)	9.50 (8.2–18.6)	NA	5.94 (0–14.5)	3.84 (0–14.5)	*21.8* (8.2–18.6)	*23.5* (8.2–18.6)	3.3 (0.5–17.1)	0.99 (0.5–17.1)	5 (0.5–17.1)	NA	**0.68** (8.2–18.6)	0.06
PHA (cpm) (normal range, 20,500–56,800)	35,400	NA	NA	NA	NA	20,800	NA	52,300	23,200**	**3150****	7910	0.66
Con A (cpm) (normal range, 20,300–65,700)	35,002	NA	NA	NA	NA	27,200	NA	55,600	**10,400****	**103****	NA	0.66
Outcome	Alive	Dead at 18 years old	Alive	Alive	Alive	Alive	Alive	Alive	Dead at 7 years old	Dead at 41 years old	Alive	

Immunological characteristics of seven ICF1 patients (Patients 1–6 and 11) and four ICF2 patients (Patients 7–9 and 10) are shown. Mitogen-induced lymphocyte proliferation tests of two ICF1 patients (Patient 1 and 6) and four ICF2 patients (Patients 7–9 and10) are shown by PHA or Con A. *p* values indicate the unpaired *t* test with Welch's correction between ICF1 and ICF2. *p* value < 0.05 in bold letters indicate significant. Data with * was referred to Shirohzu et al. [17]. Data with ** was referred to Nitta et al. [10]

Normal control ranges are indicated in parentheses. Values shown in bold are higher than normal control range, values shown in underline and italic are lower than normal control range

The normal values of serum levels globulins were referred from [16]. We defined normal ranges of serum levels of globulins as from 10% (or − 1.28 SD) to 90% (or + 1.28 SD) [16]. The normal control percentages of lymphocyte subsets were referred from Takashima et al. reported previously [18]. We defined normal ranges of control data as from 10% (or − 1.28 SD) to 90% (or + 1.28 SD) [18]

RTEs, recent thymic emigrant cells; *PHA*, phytohemagglutinin; *Con A*, concanavalin A; *cpm*, counts per minute; *NA*, not available

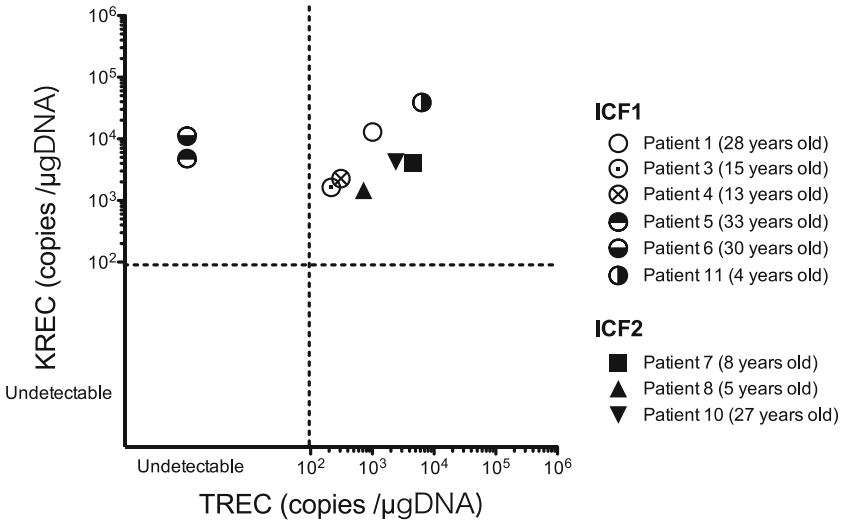

Fig. 3 TREC and KREC levels of patients with ICF syndrome. Copy numbers of TREC and KREC in six ICF1 patients (Patients 1, 3–6, and 11) and three ICF2 patients (Patients 7, 8, and 10) are shown. The age in parentheses indicates the age at the time of analysis of TREC and KREC. Copy numbers less than 100 copies/μg of DNA were defined as "undetectable." TREC, T cell receptor excision circles; KREC, kappa-deleting recombination excision circles

four patients (Patients 1, 5, 6, and 10) are able to work without difficulty. All five patients were associated with typical immunodeficiency and/or immune dysregulation. That is, all five patients showed hypogammaglobulinemia. In total, one patient with ICF2 (Patient 10) showed a decreased mitogen-induced lymphocyte proliferation test result, three patients with ICF1 (Patients 1, 5, and 6) showed low percentages of $CD3^+$ T cells, two patients (Patients 1 and 10) showed an inverted CD4/8 ratio, one patient with ICF2 (Patient 10) showed a low percentage of RTE, and two patients with ICF1 (Patients 5 and 6) showed undetectable TREC. Patient 2 with mild facial anomaly and normal intelligence died of viral infection. Therefore, immunodeficiency might not be closely related to facial anomaly and mental retardation in ICF syndrome.

Regarding the differences in clinical findings between ICF1 and ICF2, it was reported that intellectual impairment was mild in ICF1 and severe in all ICF2 patients [3] [8]. In our study, two ICF2 patients with normal mental intelligence were reported and one patient (Patient 10) was working without difficulty. We reported two ICF1 patients with autoimmune/inflammatory manifestations, which was the same as previously reported [8]. As a feature that had not been reported previously, we reported hypogonadism for the first time in two ICF1 patients and suspicion of this in one ICF2 patient. Regarding the differences of immunological findings between the two ICF subtypes, it was reported that the humoral immunodeficiency of ICF1 was more prominent than ICF2 [3]. As in a previous report, agammaglobulinemia and hypogammaglobulinemia were more prominent in ICF1, but three ICF1 patients showed extremely high levels of IgM and were also found to have autoimmune/inflammatory manifestations. Regarding the results of the mitogen-induced lymphocyte proliferation test, they were reported to worsen in some ICF cases (subtype unknown) [2], while normal results were also reported in both ICF1 and ICF2 types [8]. Our study showed that this test result worsened in half (3 out of 6 patients) of the cases examined, all of which were ICF2 patients. In this study, we newly discovered that ICF2 patients show lower percentages of $CD19^+$ B cells and $CD16^+56^+$ NK cells and significantly higher percentages of $CD3^+$ T cells than ICF1 patients. In addition, although normal TREC results were reported in a single ICF1 patient [12], we newly discovered that TREC declined and became undetectable in two ICF1 patients (Patients 5 and 6).

Our results suggest that a poor prognosis of ICF syndrome might be caused by the associated T cell immunodeficiency. We propose that ICF syndrome should be managed and treated as a combined immunodeficiency, in addition to the administration of immunoglobulin replacement therapy (i.e., antibacterial drugs including trimethoprim–sulfamethoxazole, antiviral drugs, and/or HSCT). The number of patients examined was limited in this study, and further studies are necessary to clarify the detail of the T cell immunodeficiency in ICF syndrome and what types of treatments are needed for particular kinds of ICF patients.

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Authorship Contributions C.K., K.I., H.K., and S.N. designed the study and wrote the manuscript. T.K., K.H., N.N., and T-W.Y. performed the flow cytometric analysis and collected the data. T.O., E.N., and O.O. performed the gene analysis. A.O., T.S., H.T., S.T., M.H., A.H., S.W., and T.S. cared for the patients. H.S., T.K., and T.M. contributed to the critical discussion.

Compliance with Ethical Standards

Written informed consent was obtained from the parents of the pediatric patients and from the adult patients, in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics board of the National Defense Medical College and Tokyo Medical and Dental University.

Conflict of Interest The authors declare that they have no conflicts of interest.

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