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ORIGINAL ARTICLE

Endocrine complications in primary immunodeficiency diseases in Japan

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Summary

Background In spite of the accumulating evidence on the interaction between the immune and endocrine systems based on the recent progress in molecular genetics, there have been few epidemiological studies focused on the endocrine complications associated with primary immunodeficiency diseases (PID).

Objective To investigate the prevalence and clinical features of endocrine complications in patients with PID in a large-scale study.

Design and participants This survey was conducted on patients with PID who were alive on 1 December 2008 and those who were newly diagnosed and died between 1 December 2007 and 30 November 2008 in Japan. We investigated the prevalence and the clinical data of the endocrine complications in 923 patients with PID registered in the secondary survey.

Results Among 923 PID patients, 49 (5·3%) had endocrine disorders. The prevalence of the endocrine diseases was much higher in patients with PID than in the general population in the young age group, even after excluding patients with immune dysregulation.

Conclusions Endocrine disorders are important complications of PID. Analysis of the endocrine manifestations in patients with PID in a large-scale study may provide further insights into the relationship between the immune and endocrine systems.

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Introduction

A wide variety of clinical complications have been described in primary immunodeficiency diseases (PID).[1,2] PID have been reported to be associated with an increased risk of cancer, in particular non-Hodgkin lymphoma,[2] and the contribution of immune dysfunction in PID to cancer risk is receiving much attention. It is also well known that patients with PID often have complications such as autoimmune and allergic disorders.[1,3] Recently, the interaction between the immune and endocrine systems has been getting increasing attention.[4,5] However, there have so far been no reports focusing on the endocrine complications associated with PID in a large-scale survey.

Many endocrine disorders in patients with PID are thought to be due to the development of the autoimmunity, which is closely related to the pathophysiology of PID.[6] However, it is not known how the immunological and molecular defects in individual PID contribute to the development of various autoimmune endocrine disorders. In addition, the genetic defects in some PID can lead to these complications directly or indirectly via nonimmunological mechanisms.[6]

We analysed the endocrine complications in PID from the information obtained from the nationwide PID survey in Japan conducted in 2008. This is the first large-scale survey focusing on the endocrine complications in PID.

Materials and methods

This survey was performed according to the nationwide epidemiological survey manual of patients with intractable diseases (2nd edition 2006, Ministry of Health, Labour and Welfare of Japan) as described previously.[7] PID classification was based on the criteria of the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee in 2007.[8] The survey was conducted on patients with PID who were alive on 1 December 2008 and those who were newly diagnosed and died between 1 December 2007 and 30 November 2008 in Japan. The initial survey covered 1224 paediatric departments and 1670 internal medicine departments, which were randomly selected according to the number of beds among the 2291 paediatric departments and 8026 internal medicine departments in Japan. Primary questionnaires regarding the number of patients and the disease names based on the PID classification

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were sent to the selected hospitals. The initial survey was conducted to investigate the prevalence of the respective PID. The secondary survey was performed to study the detailed clinical features of individual patients with PID. Secondary questionnaires regarding age, gender, clinical manifestations and complications other than those related to haematopoietic stem cell transplantation of individual patients with PID were sent to the respondents who answered that they observed at least one PID patient with characteristics listed in the primary questionnaires. The details of the methods of the questionnaire investigation, the response rates and the breakdown of the number of patients in both paediatric and internal medicine departments were described elsewhere.[9] The questionnaires were designed to elucidate the clinical characteristics including the manifestations and laboratory data of the patients. In this study, all endocrine manifestations in patients with PID were included as complications of PID, even if they were well known major symptoms of PID.

Results

Detailed clinical information was available from 923 (secondary survey) out of 1240 patients with PID (initial survey).[9] Among the 923 patients with PID, 49 (5·3%) had endocrine disorders. As shown in Table 1, more than two thirds of the patients with PID were <20 years old and the prevalence of endocrine diseases was much higher in the young population of patients with PID than that in the general young population,[7,10–14] even after excluding patients with immune dysregulation (PID category IV). As expected, hypoparathyroidism was the most common endocrine disorder, because it is very frequently observed in patients with DiGeorge syndrome. Endocrine manifestations were also common in patients with diseases of immune dysregulation, such as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Although the number of patients with defects in innate immunity was small, endocrine complications seemed to be more common than expected. Interestingly, endocrine disorders were not observed in patients with complement deficiencies. In addition, Graves' disease and Addison's disease were not observed in any of the patients with PID in this study.

Type 1 diabetes mellitus (T1D) was observed in six patients with PID (Tables 1 and 2) including four with type 1A (autoimmune) and two with type 1B (autoantibody-negative, idiopathic). Type 1A diabetes mellitus occurred frequently in patients with IPEX or IPEX-like syndrome (two of six patients, 33·3%) (Table 1). One patient of unknown aetiology in PID category IV showed type 1A diabetes and Hashimoto's thyroiditis along with recurrent viral infections (Tables 1, 2 and S1). In the cases of type 1A diabetes mellitus, anti-glutamic acid decarboxylase (GAD) autoantibodies and anti-insulin autoantibodies (IAA) were positive in all patients and in two of four patients, respectively (Table 2). The patients with IPEX and IPEX-like syndrome had a history of diabetic ketoacidosis with poor glycaemic control, and they developed T1D at a younger age than the other patients with PID. The first case of warts, hypogammaglobulinaemia, infections, and myelokathexis (WHIM) syndrome with T1D and hypothyroidism was included (Tables 2 and S2).[15] With regard to type 1B diabetes mellitus, the patient with hypogammaglobulinaemia of unknown aetiology had diabetic ketoacidosis (Table 2). On the other hand, type 2 diabetes mellitus (T2D) was observed in two patients with PID (Table 1).

Hashimoto's thyroiditis was observed in five patients with PID (Tables 1 and S1). The onset was very early in the patient with IPEX syndrome (at birth). All patients had at least 1 autoantibody among the anti-thyroid peroxidase (TPO), anti-thyroglobulin (Tg) and thyroid stimulating hormone receptor autoantibodies (TRAb).

Nonautoimmune hypothyroidism was reported in seven patients with PID (Tables 1 and S2). Anti-thyroid autoantibodies were all negative when measured. Among these, three patients with X-linked agammaglobulinaemia (XLA), IgG subclass deficiency or WHIM syndrome had primary (congenital) hypothyroidism detected by newborn mass screening. Hypothyroidism in the other four patients with normal TSH levels was considered to be due to central hypothyroidism, a disorder of the pituitary, hypothalamus or hypothalamic-pituitary portal circulation. Two patients with severe combined immunodeficiency (SCID) developed hypothyroidism before they received haematopoietic stem cell transplantation.

Growth hormone deficiency (GHD) was observed in six patients with PID (Tables 1 and S3), whose heights at the diagnosis of GHD ranged from −11·3 SD to −2·5 SD. Five patients were treated with growth hormone. One patient with SCID received cord blood transplantation when she was 20 months old, without conditioning chemotherapy or radiation.

Hypogonadism was observed in three patients with PID (Tables 1 and S4). Among them, two had hypergonadotrophic (primary) hypogonadism, whereas the other had hypogonadotrophic (central) hypogonadism. None of the patients received haematopoietic stem cell transplantation.

One common variable immunodeficiency disease (CVID) patient had isolated ACTH deficiency (Table 1). The other endocrine complications included hypophosphataemia, pseudohypoaldosteronism, adrenal crisis, hypoglycaemia and hypophosphataemic rickets as shown in Table 1.

Discussion

This is the first nationwide survey focusing on the endocrine complications of PID. Among these, hypoparathyroidism was the most common, observed in patients with DiGeorge syndrome and APECED.[16,17] In APECED, the calcium-sensing receptor has been reported to be the autoantigen responsible for hypoparathyroidism.[18] Although it has been reported that 79% of patients with APECED have hypocalcaemia due to hypoparathyroidism,[17] only 1 (25%) among four patients with APECED developed hypoparathyroidism in this study, which might be one of the clinical characteristics of patients with APECED in Japan.

The prevalence (33·3%) of T1D in patients with IPEX syndrome in this study seemed to be lower than that (>70%) of the previous reports.[19,20] The low prevalence of T1D might be due to

Table 1. Endocrine complications in PID patients

PID category	Hypopara-thyroidism	Diabetes mellitus			Thyroid disease		GHD	Hypogonadism	Isolated ACTH deficiency	Others	The number of PID patients			
		T1D		T2D	Autoimmune hypothyroidism (Hashimoto's thyroiditis)	Non-autoimmune hypothyroidism					*n*	0–19 years	Total	Percent in total
		1A	1B											
I. Combined T and B cell immunodeficiencies											**4**	**67**	**75**	**5·3**
RAG1 deficiency						1					1	6	6	16·7
CD4 deficiency					1						1	2	2	50·0
Undetermined											2	10	10	20·0
T-B-SCID						1	1				2	4	4	50·0
II. Predominantly antibody deficiencies											**13**	**231**	**378**	**3·4**
X-linked agammaglobulinaemia						1				2*	3	93	138	2·2
Common variable immunodeficiency disorders			1		1††		1		1	2†	6	29	93	6·5
IgG subclass deficiency						2					2	45	50	4·0
Undetermined			1				1**	1**			2	9	9	22·2
III. Other well-defined immunodeficiency syndromes											**20**	**126**	**165**	**12·1**
Hyper-IgE syndrome							1	1		1‡	3	31	46	6·5
DiGeorge syndrome	14										14	29	32	43·8
Ataxia telangiectasia				1							1	8	13	7·7
Chronic mucocutaneous candidiasis					1††						1	9	13	7·7
ICF syndrome								1			1	0	1	100·0
IV. Diseases of immune dysregulation											**6**	**31**	**38**	**15·8**
IPEX syndrome		2			1					1§	4	5	6	66·7
APECED	1										1	3	4	25·0
Undetermined		1**			1**						1	2	2	50·0
V. Congenital defects of phagocyte number, function or both											**3**	**106**	**153**	**2·0**
Chronic granulomatous disease						1	1				2	54	87	2·3

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Table 1. *(continued)*

PID category	Hypopara-thyroidism	Diabetes mellitus: T1D 1A	T1D 1B	Diabetes mellitus: T2D	Thyroid disease: Autoimmune hypothyroidism (Hashimoto's thyroiditis)	Thyroid disease: Non-autoimmune hypothyroidism	GHD	Hypogonadism	Isolated ACTH deficiency	Others	The number of PID patients: *n*	0–19 years	Total	Percent in total
Shwachman–Diamond syndrome							1				1	2	2	50·0
VI. Defects in innate immunity											**2**	**9**	**12**	**16·7**
NEMO deficiency										1¶	1	7	7	14·3
WHIM syndrome		1**				1**					1	2	3	33·3
VII. Autoinflammatory disorders											**1**	**54**	**74**	**1·4**
Familial Mediterranean fever				1††							1	23	36	2·8
VIII. Complement deficiencies											**0**	**18**	**23**	**0**
IX. Undetermined											**0**	**3**	**5**	**0**
Total	15	6		2	5	7	6	3	1	7	49	645	923	5·3
Estimated prevalence per 10 000 in the young population (0–19 years) of PID patients (95% CI)	232·6 (141·4–380·1)	93·0 (42·7–201·5)		15·5 (2·7–87·3)	46·5 (15·8–135·9)	108·5 (52·7–222·3)	93·0 (42·7–201·5)	46·5 (15·8–135·9)	15·5 (2·7–87·3)					
Prevalence per 10 000 in the general young Japanese population	0·072‡‡	1·19		0·461§§	30·0§§	13·5¶¶	1·47	ND	0·035					
References	[7]	[10]		[10]	[11]	[12]	[13]	ND	[14]					

SCID, severe combined immunodeficiency; ICF, immunodeficiency with centromeric instability and facial anomalies; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; APECED, autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy; NEMO, NF-κB essential modulator; WHIM, warts, hypogammaglobulinaemia, infections, and myelokathexis; T1D, type 1 diabetes; T2D, type 2 diabetes; GHD, growth hormone deficiency.

*Hypophosphatemia 1, Obesity 1.

†Obesity 2.

‡Pseudohypoaldosteronism 1.

§Adrenal crisis, Hypoglycaemia 1.

¶Hypophosphatemic rickets 1.

**Two endocrine disorders were observed in the same patient.

††the case whose onset age of a endocrine complication is 20 years or older, *n*: number of PID patients who had endocrine disorders, CI: confidence interval.

‡‡prevalence in all age groups.

§§incidence data.

¶¶prevalence in the United States, ND: no data available.

Table 2. Clinical data of T1D patients

Case		1	2	3	4	5	6
Disease		IPEX syndrome	IPEX-like syndrome	Immune dysregulation (undetermined)	WHIM syndrome	CVID	Hypogammaglobulinaemia (unknown aetiology)
Genetic mutations (gene name)		+ (*FOXP3*)	Unknown	Unknown	+ (*CXCR4*)	Unknown	NT
HSCT		–	–	–	–	–	–
Sex		M	M	F	F	F	M
Present age		8 years 5 months	14 years 5 months	21 years 8 months	18 years 9 months	19 years 1 month	25 years 3 months
Onset age of T1D		3 months	10 months	7 years 9 months	5 years 7 months	7 years 9 months	6 years 5 months
Type of T1D		1A	1A	1A	1A	1B	1B
Clinical symptoms		Polydipsia, polyuria	Polydipsia, weight loss	ND	Polydipsia, polyuria	None	None
Diabetic ketoacidosis		+ (pH 7·112)	+ (pH 7·012)	–	–	–	+ (urine ketone body (4+))
Laboratory data	Normal range						
Fasting blood glucose (mmol/l)	3·9–6·1	31·7	29·1	6·1*	7·6	8·3	7·7
HbA1c (%)	4·3–5·8	7·9	8·3	8·7*	8·9	5·6	9·1
Plasma CPR (nmol/l)	0·33–0·93	ND	0·27	0·10*	ND	0·27	ND
Urinary CPR (μg/day)	20–100	ND	ND	2·5*	15	NT	ND
Anti-GAD Ab							
Result		+	+	+*	+	None	None
Value (U/ml)	<1·5	69·1	4860	9·3*	92	ND	ND
Anti-IAA Ab							
Result		–	ND	+*	+	ND	ND
Value (nIU/ml)	<125	2·8		ND	ND		
Treatment							
Age at the start		3 months	10 months	7 years 9 months	5 years 7 months	8 years 1 month	6 years 5 months
Content		Insulin	Insulin	Insulin	Insulin	Insulin	Insulin

NT, not tested; ND, no data available; FOXP3, forkhead box P3; CXCR4, CXC chemokine receptor 4; HSCT, haematopoietic stem cell transplantation; CPR, C-peptide immunoreactivity; GAD, glutamic acid decarboxylase; IAA, insulin autoantibody.
*Post-treatment data.

some genetic factor, because the Japanese have been have reported to be one of the races with the lowest incidence of T1D.[21] With regard to the patient with WHIM, Takaya *et al.*[15] have reported that mutations of *CXCR4*, the gene responsible for WHIM syndrome, might be closely related to the development of T1D, because recent findings have suggested that impaired CXCR4 signalling is involved in the pathogenesis of T1D. The prevalence of T1D in patients with CVID was 1·1% (one in 93 patients) in our study, which was almost equal to that in the previous report.[3]

The development of T2D was observed in only one of 13 patients with ataxia telangiectasia (AT) (7·7%) in contrast to the high prevalence of T2D in the previous report (five of eight patients),[22] suggesting the unique clinical characteristics of patients with AT in Japan.

Hashimoto's thyroiditis is a relatively common endocrine manifestation in patients with IPEX syndrome.[19,20] The prevalence of Hashimoto's thyroiditis in patients with CVID in our study was 1·1% (one in 93 patients), which was similar to that of the previous report.[23] There have been only a few reports of Hashimoto's thyroiditis in patients with (S) CID.[24,25] Interestingly, this was the first report of Hashimoto's thyroiditis in a patient with CD4 deficiency, while autoimmune cytopenia is frequently associated with this disease (19%).[26] The patient with a patient with CD4 deficiency and Hashimoto's thyroiditis did not receive stem cell transplantation, suggesting that this complication was caused by autoimmunity based on the combined immunodeficiency. Nagpala *et al.*[25] reported an infant with autoimmune thyroiditis and hypothyroidism with SCID due to adenosine deaminase deficiency despite an extremely low number of T cells and a low level of IgG, which suggested that the leaky SCID phenotype permitted the survival of a few T cells with autoimmune potential.[27]

Central hypothyroidism (no TSH elevation) was observed in two patients with SCID before they received haematopoietic stem cell transplantation (Table S2), also suggesting the possibility that this complication was related to the combined immunodeficiency itself. In addition, this was the first report of primary hypothyroidism (elevated TSH levels at birth) in patients with

XLA or IgG subclass deficiency, although the aetiologies remain to be determined.

Of note, the prevalence of GHD in patients with PID seemed much higher than that in the general population (Table 1). Until now, GHD has been reported in patients with several diseases in PID including SCID, CVID and Shwachman–Diamond syndrome, as shown in our study.[28–30] However, to the best of our knowledge, this was the first report of GHD in patients with hyper-IgE syndrome (HIES) and chronic granulomatous disease (CGD). Some SCID patients with GHD have been reported to have *STAT5b* gene mutations.[31] However, the gene was not investigated in our patient with SCID. With respect to the mechanism underlying the development of GHD in patients with CVID, common impairment in the IGF-1 and IgG pathways has been suggested as a cause of the growth retardation in some patients with CVID.[32] In addition, anti-pituitary antibodies have been detected in some of these patients.[33] The patient with congenital agammaglobulinaemia had various other complications in addition to GHD (Table S3), suggesting that this patient might have had a novel primary immunodeficiency.

Hypogonadism in patients with immunodeficiency with centromeric instability and facial anomalies (ICF) syndrome has been reported previously[34], although the mechanism is unclear. On the other hand, this was the first report of hypogonadism in patients with congenital agammaglobulinaemia and HIES. It is possible that hypogonadism has not been a major concern in PID for clinicians.

Isolated ACTH deficiency usually occurs during adult life, and only a few cases have been reported in childhood.[35] However, the development of isolated ACTH deficiency in a 14-year-old girl with CVID has been reported[35], in addition to the present case (Table 1). Therefore, a common pathological background is suspected in some of the patients with CVID.

Several limitations of this study should be considered. First, there were only a small number of adult patients with PID reported in this study, from which we could not estimate the accurate prevalence of endocrine manifestations in adults. Second, not all of the patients with PID were given sufficient examinations by endocrinologists and different examination methods were used at the respective hospitals.

There has been growing evidence of the interaction between the immune and endocrine systems.[4,5] In this study, we have found an increased prevalence of endocrine complications in patients with PID, which appear to be caused by immune dysregulation or by the underlying genetic disorders of the respective PID. Although various endocrine abnormalities have been reported to occur after stem cell transplantation,[36] therapy-related endocrine abnormalities were not included in the present study. A large-scale study such as a nationwide survey, focusing on the endocrine diseases, may have the potential to provide further insights into the mechanisms or pathophysiology of endocrine disorders in non-PID as well as patients with PID.

Conflicts of interest/financial disclosure

We declare that we have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical data of patients with Hashimoto's thyroiditis.

Table S2. Clinical data of patients with nonautoimmune hypothyroidism.

Table S3. Clinical data of patients with GHD.

Table S4. Clinical data of patients with hypogonadism.

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Letter to the Editor

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

To the Editor:

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

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

Hypogammaglobulinemic patients (n = 113) were referred to our hospital for immunodeficiency from 2005-2011, and the following patients were excluded from the CVID pool by estimating their SCID genes based on clinical manifestations and lymphocyte subset analysis: 18 patients with SCID diagnoses; 14 patients less than 2 years of age (transient infantile hypogammaglobulinemia); 10 patients with IgM levels of greater than 100 mg/dL (hyper-IgM syndrome); 26 patients with diseases other than CVID caused by known gene alterations (10 with X-linked agammaglobulinemia and 11 with hyper-IgM syndrome [*CD40L* or *AICDA* mutated]), (2 with DiGeorge syndrome, and 3 with *FOXP3*, *IKBKG*, or *6p* deletions); and 5 patients with drug-induced hypogammaglobulinemia. The remaining 40 patients with decreased IgG (≥2 SDs below the mean for age), IgM, and/or IgA levels, as well as absent isohemagglutinins, poor response to vaccines, or both were included in this study as patients with CVID and analyzed for TREC/KREC levels, retrospectively.

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

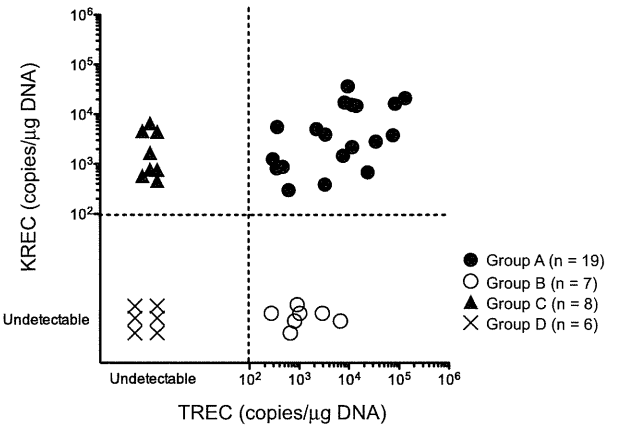

FIG 1. Quantifying TREC and KREC classifies patients with CVID into 4 groups. Patients with CVID were classified as follows: TREC(+)/KREC(+), group A (19 patients); TREC(+)/KREC(−), group B (7 patients); TREC(−)/KREC(+), group C (8 patients); and TREC(−)/KREC(−), group D (6 patients). *Undetectable*,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

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

$CD19^+$ B-cell numbers in group A were significantly higher ($P < .05$) than those in groups B and D (group A, 269 ± 65 cells/μL; group B, 35 ± 16 cells/μL; group C, 60 ± 11 cells/μL; and group D, 29 ± 16 cells/μL; $P = .0001$). However, B-cell subpopulations, including $CD27^-$, IgD^+CD27^+, and

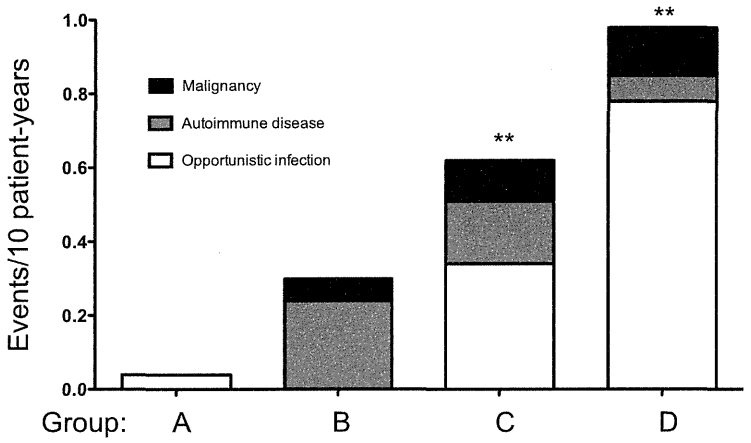

FIG 2. Cumulative incidence of complication events per 10 patient-years differs among groups. Opportunistic infections, autoimmune diseases, and malignancies were evaluated for each patient group. Complication incidences in group D (0.98 events/10 patient-years), group C (0.63 events/10 patient-years),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

IgD^-CD27^+ cells, were not significantly different among the groups. Standardizing KREC copy numbers for each patient by dividing their $CD19^+$ by their $CD27^+$ percentages revealed the same patient classification as that shown in Fig 1 (data not shown), indicating that the original classification was independent of $CD19^+$ B-cell or $CD27^+$ memory B-cell percentages.

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

We next evaluated whether any correlation existed between TREC/KREC-based classification and clinical symptoms in each patient group. All patients in the study had been treated with intravenous immunoglobulin (IVIG) substitution at the time of analysis. We found that the cumulative events of complications (opportunistic infections, autoimmune diseases, and malignancies) per 10 patient-years were highest in group D (0.98 events/10 patient-years), followed by group C (0.63 events/10 patient-years), group B (0.30 events/10 patient-years), and group A (0.04 events/10 patient-years), where events in groups D and C were significantly higher than group A (group A vs group D, $P = .0022$; group A vs group C, $P = .0092$; group A vs group B, $P = .0692$; Fig 2). Furthermore, we found similar results when evaluating only patients 19 years old or older for group D (1.01 events/10 patient-years), group C (0.56 events/10 patient-years), group B (0.32 events/10 patient-years), and group A (0.06 events/10 patient-years; group A vs group D, $P = .0074$; group A vs group C, $P = .0407$; group A vs group B, $P = .1492$; data not shown). Categorizing patients by using several different previously reported CVID classifications (focused primarily on separating patients based on levels of circulating B-cell subsets), we found that no classification scheme showed any significant event increases in any particular group (see Fig E3 in this article's Online Repository at www.jacionline.org). Assessing longitudinal cumulative opportunistic infection incidence among the groups, group D and C values were significantly higher than in group A (see Fig E4, A, in this article's Online Repository at www.jacionline.org; $P = .0059$). Autoimmune and malignant diseases ($P = .5168$ and $P = .6900$, respectively) were observed in groups B and D but not in group A (see Fig E4, *B* and *C*). Cumulative events were significantly different between groups ($P = .0313$, log-rank test; group A, 5.3% and 5.3%; group B, 14.3% and 57.1%; group C, 27.1% and 63.5%; and group D, 33.3% and 83.3% at 10 and 30 years of age, respectively; see Fig E4, *D*). One patient in group D died of *Pneumocystis jirovecii* pneumonia, and 2 other patients in the same group received hematopoietic stem cell transplantation after complications caused by EBV-related lymphoproliferative disorder.

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

In contrast to group D patients, TREC(+)/KREC(+) group A patients treated with IVIG substitution therapy remained healthy. One possible explanation is that these patients harbor

defects only in terminal B-cell differentiation, but not in T cells, and represent typical patients with CVID, as originally reported.

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

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

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

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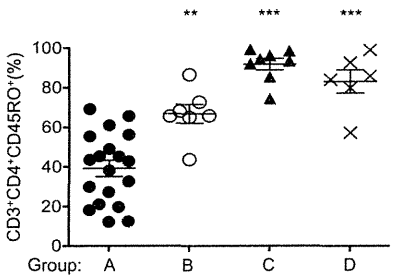

FIG E1. $CD45RO^+CD3^+CD4^+$ T-cell frequency within $CD4^+CD3^+$ lymphocytes was analyzed among groups. $CD45RO^+CD3^+CD4^+$ lymphocyte counts were significantly higher in groups B, C, and D compared with those in group A ($P < .0001$). Group A: 37% ± 16%; group B: 67% ± 13% (**$P < .01$); group C: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

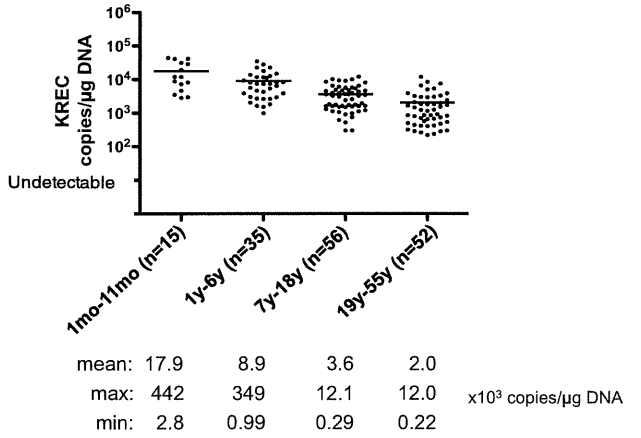

FIG E2. KREC levels were analyzed in genomic DNA samples extracted from peripheral blood of control subjects at different age groups (n = 158; age range, 1 month to 55 years). KREC levels were significantly higher in infants (17.9 ± 3.9 × 10^3 copies/μg DNA) compared with other children's age groups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

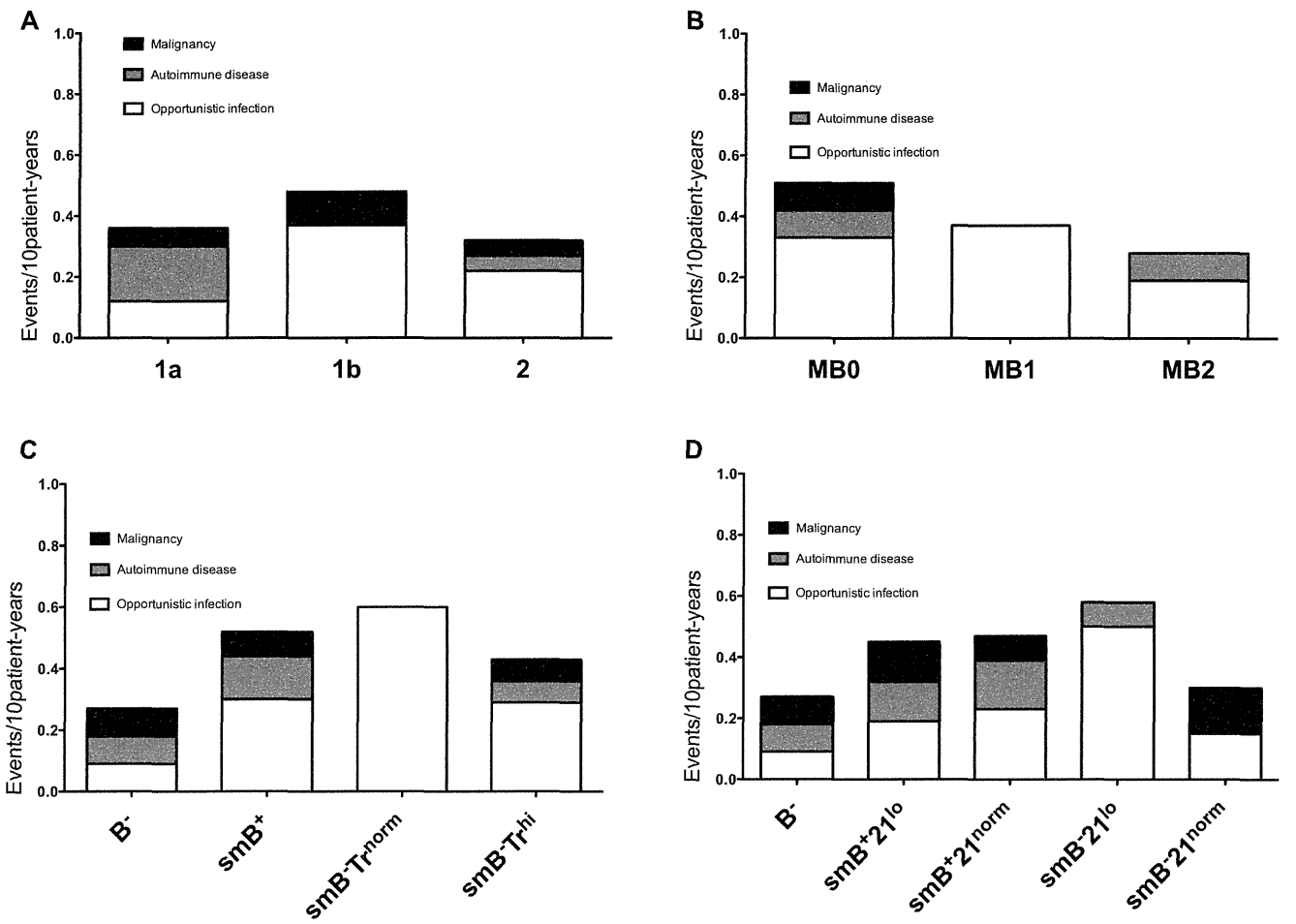

FIG E3. Patients were classified in the following way and analyzed for cumulative incidence of complications: **A,** Freiburg; **B,** Paris; and **C,** EUROclass classifications, according to $CD38^{hi}IgM^{hi}$ transitional B cells (Fig E3, *A-C*) or $CD21^{low}$ B cells **(D)**. Five patients were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

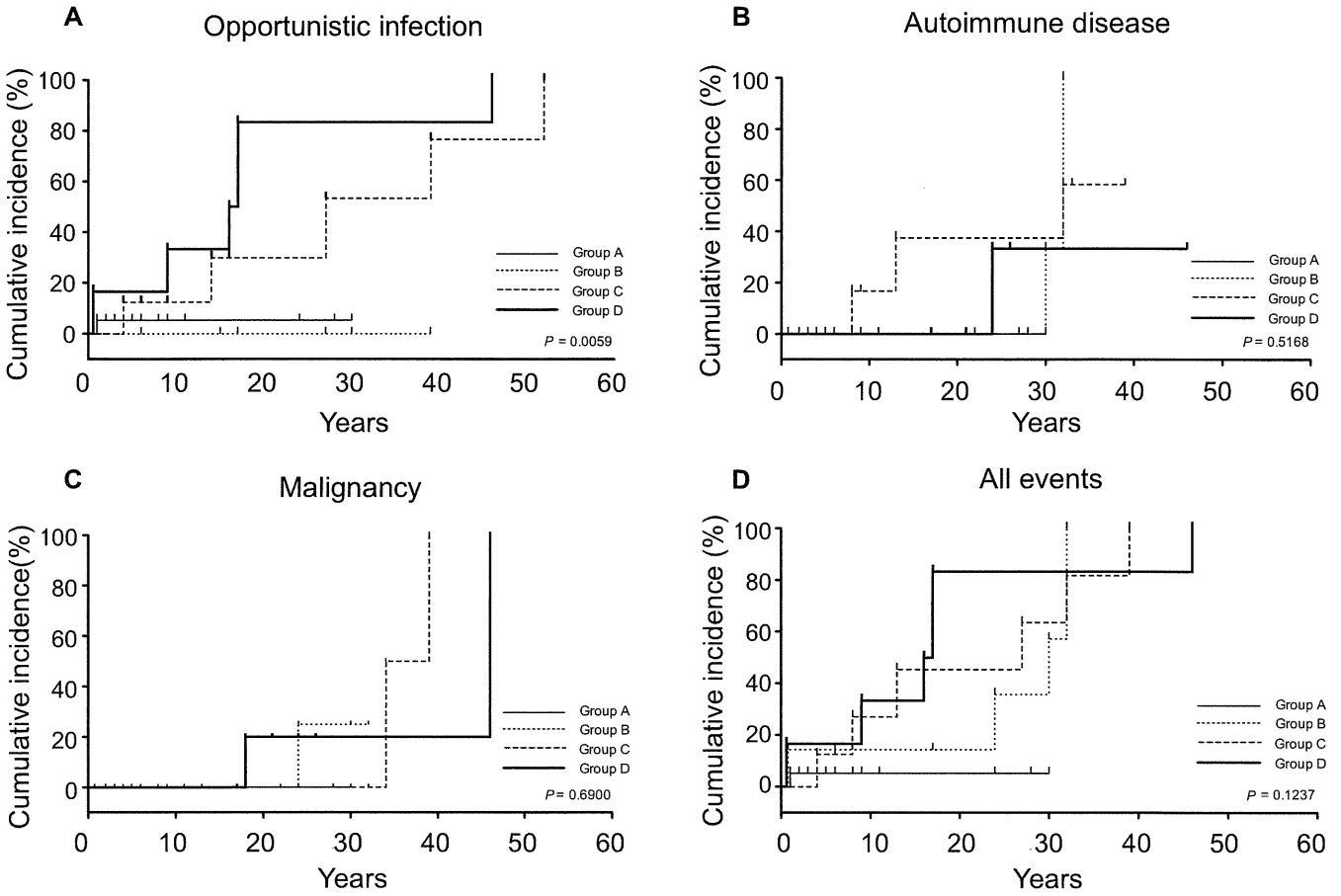

FIG E4. Comparing longitudinal cumulative incidence of complication events among groups. Cumulative incidence was estimated separately and longitudinally by using the Kaplan-Meier method and statistically compared between groups by using the log-rank test. The cumulative incidence of opportunistic infections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

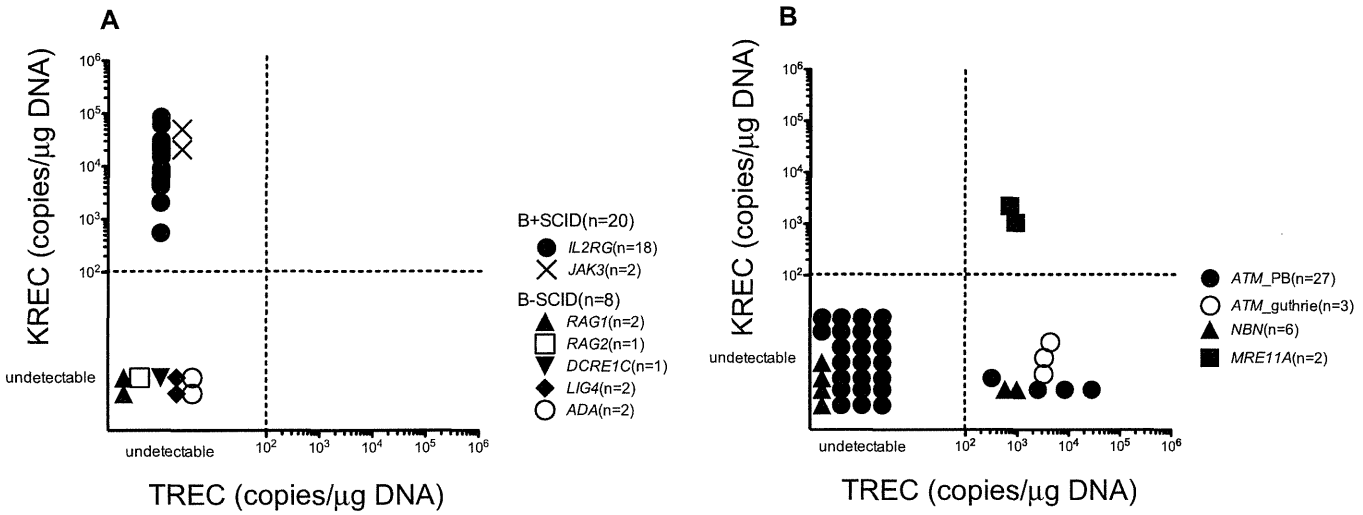

FIG E5. TREC and KREC quantification classifies patients with SCID, AT, NBS, or ataxia-telangiectasia–like disease *(ATLD)* into 4 groups. **A,** Patients with B^+SCID (n = 20) were classified as group C, and patients with B^-SCID (n = 8) were classified as group D; these patients were included in the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

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Detection of Base Substitution-Type Somatic Mosaicism of the *NLRP3* Gene with >99.9% Statistical Confidence by Massively Parallel Sequencing

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Abstract

Chronic infantile neurological cutaneous and articular syndrome (CINCA), also known as neonatal-onset multisystem inflammatory disease (NOMID), is a dominantly inherited systemic autoinflammatory disease and is caused by a heterozygous germline gain-of-function mutation in the *NLRP3* gene. We recently found a high incidence of *NLRP3* somatic mosaicism in apparently mutation-negative CINCA/NOMID patients using subcloning and subsequent capillary DNA sequencing. It is important to rapidly diagnose somatic *NLRP3* mosaicism to ensure proper treatment. However, this approach requires large investments of time, cost, and labour that prevent routine genetic diagnosis of low-level somatic *NLRP3* mosaicism. We developed a routine pipeline to detect even a low-level allele of *NLRP3* with statistical significance using massively parallel DNA sequencing. To address the critical concern of discriminating a low-level allele from sequencing errors, we first constructed error rate maps of 14 polymerase chain reaction products covering the entire coding *NLRP3* exons on a Roche 454 GS-FLX sequencer from 50 control samples without mosaicism. Based on these results, we formulated a statistical confidence value for each sequence variation in each strand to discriminate sequencing errors from real genetic variation even in a low-level allele, and thereby detected base substitutions at an allele frequency as low as 1% with 99.9% or higher confidence.

Key words: next generation sequencing; mosaicism; DNA diagnosis; chronic infantile neurological cutaneous and articular syndrome

1. Introduction

Chronic infantile neurological cutaneous and articular syndrome (CINCA; MIM #607115), also known as neonatal-onset multisystem inflammatory disease (NOMID), is a dominantly inherited autoinflammatory disease that is characterized by neonatal onset and a triad of symptoms, including an urticarial-like skin rash, neurological manifestations, and arthritis/arthropathy.[1–3] Patients often experience

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recurrent fever and systemic inflammation. CINCA/NOMID is the most severe clinical phenotype in the spectrum of cryopyrin-associated periodic syndromes (CAPS), which also include two less severe but phenotypically similar syndromes, familial cold autoinflammatory syndrome (FCAS; MIM #120100), and Muckle–Wells syndrome (MWS; MIM #191900). CAPS are caused by mutations in the *NLRP3* gene, which is a member of the Nod-like receptor (NLR) family of the innate immune system.[4–6]

Approximately 60% of CINCA/NOMID patients carry heterozygous germline missense mutations in the *NLRP3* coding region (mutation-positive patients).[7] More than 80 different disease-causing mutations have been reported to date.[8] However, the remaining clinically diagnosed CINCA/NOMID patients (~40%) show no heterozygous germline *NLRP3* mutation based on conventional DNA sequencing-based genetic analyses (mutation-negative patients). In a previous international collaborative study, we found that there was a high incidence of somatic *NLRP3* mosaicism in mutation-negative CINCA/NOMID patients worldwide.[9] The level of mosaicism ranges from 4.2 to 35.8% (median = 10.2%). Rapidly diagnosing somatic *NLRP3* mosaicism is important to ensure proper treatment. However, the conventional approach used to identify somatic mosaicism of the *NLRP3* gene is time and labour intensive due to the subcloning of the *NLRP3* exon polymerase chain reaction (PCR) products, hereafter designated as amplicons, followed by capillary DNA sequencing of more than 100 subclones for each patient. Thus, this approach is not suitable to routinely diagnose somatic mosaicism of the *NLRP3* gene and additional labour and time will be required to reliably identify somatic mosaicism that occurs at a lower rate. The aim of the present study was to establish a new method that can be used to reliably diagnose somatic mosaicism using the *NLRP3* gene as a model. Massively parallel DNA sequencing (MPS) technology is an obvious method of choice to identify somatic mosaicism, and this approach has been already reported by other groups.[10–12] However, a well-known caveat of MPS is the high rate of sequencing errors, which cannot be disregarded when identifying low-level somatic mosaicism. To our knowledge, there have been no reports of a reliable method to discriminate MPS sequencing errors from somatic mosaicism with statistical confidence.

In this study, we first analysed the patterns of sequencing errors in *NLRP3* coding exons at a single-residue resolution by MPS using a Roche 454 GS-FLX sequencer and then constructed an error rate map for each base position in the *NLRP3* exons. Based on the error rate map, we could formulate a discrimination pipeline of somatic mosaicism from sequencing errors and thereby detect new somatic mosaicism in mutation-negative CINCA/NOMID patients, whose somatic mutations were subsequently confirmed by subcloning and Sanger sequencing. This approach can also be generally used to identify low-level somatic mosaicism in other genes.

2. Patients and methods

2.1. *Patients and DNA materials*

Patients were clinically diagnosed with CAPS by their referring physicians and the *NLRP3* gene was examined using the conventional Sanger sequencing method. DNA samples were obtained from Japanese *NLRP3* somatic mosaic patients ($n = 5$) who have been previously described,[9,13] CAPS patients ($n = 5$) with heterozygous *NLRP3* mutations, and healthy donors ($n = 50$). Genomic DNA samples from mutation-negative CINCA/NOMID patients ($n = 10$) were obtained from the National Institute of Health, Bethesda, USA. To generate DNA samples with no mosaicism, we constructed a set of subcloned plasmids containing each exon and its flanking intronic regions in the *NLRP3* gene from healthy donor genomic DNA using a Topo TA cloning kit (Invitrogen, San Diego, CA, USA). The cloned plasmids containing each exon and the flanking regions were validated by Sanger sequencing. Written informed consent was obtained from all the patients and their families. The study was approved by the ethical committees of Kyoto University and Kazusa DNA Research Institute and was conducted in accordance with the Helsinki Declaration.

2.2. *MPS of* NLRP3 *gene amplicons*

Genomic DNA samples were extracted from whole blood or peripheral blood mononuclear cells as previously described. We used a two-step PCR assay and pooled sample libraries for MPS. To cover the entire *NLRP3* coding exonic regions and flanking intronic regions, 14 amplicons were designed to be as long as an average read length for a 454 GS-FLX sequencer (up to 450 bases) and then amplified from each genomic DNA sample (Fig. 1A). The sequences of the PCR primers that were used to generate these 14 amplicons are provided in Supplementary Table S1. The upper and lower amplicon-specific primer sequences were flanked by common 15-base adapter sequences (TGTAAAACGACGGCC and GGAAA CAGCTATGAC for the upper and lower primers, respectively) at the 5′ end in order to fuse the primer-binding sequence for MPS in the second-step PCR. The first PCR amplifications were performed in 50-μl reactions using 30 ng of genomic DNA, 1× PrimeSTAR GXL buffer, 0.2 mM of each dNTP,

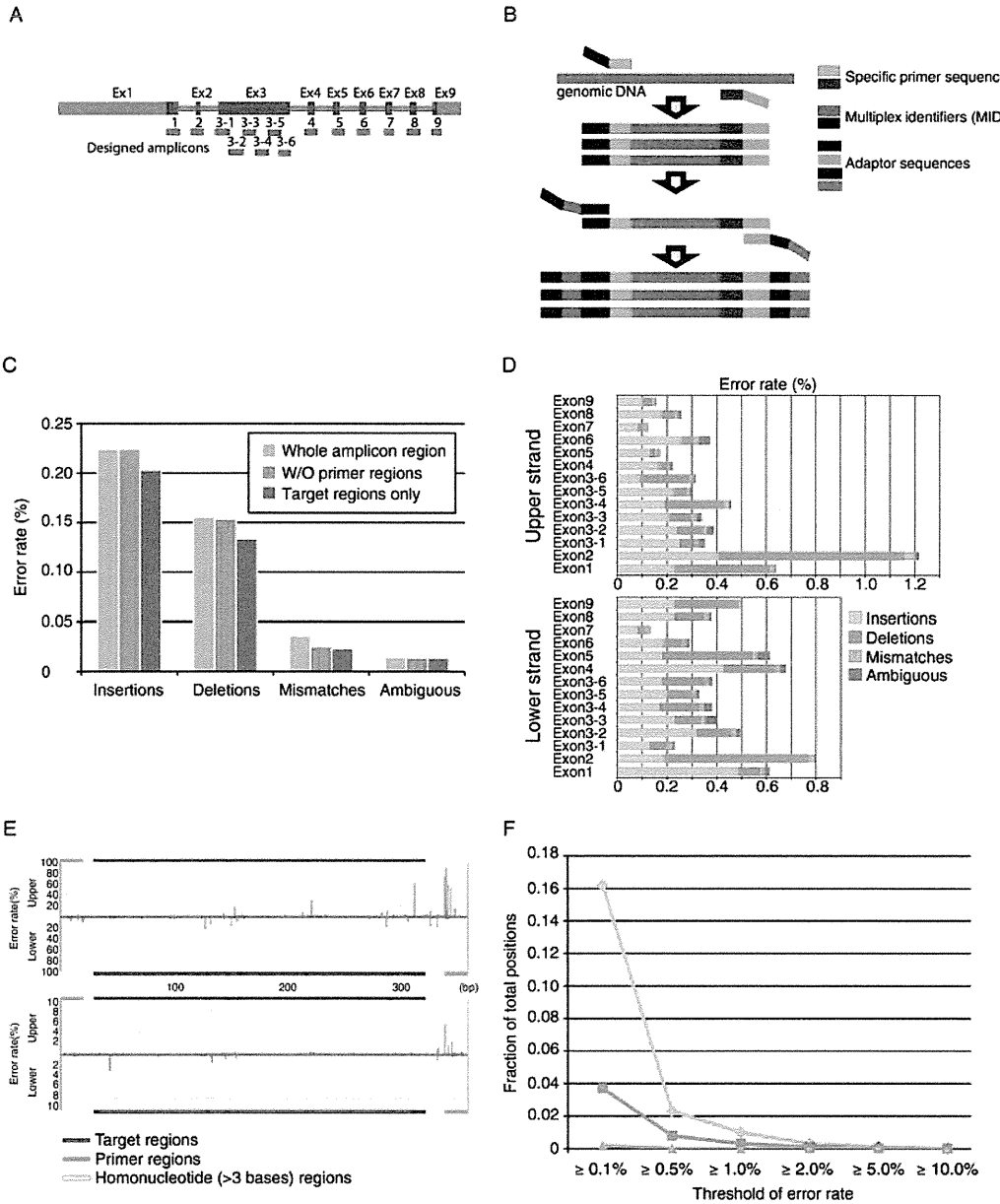

Figure 1. The amplicon analysis for *NLRP3* exons and its error rate. (A) Exon–intron structure of the *NLPR3* gene. Thick and thin rectangles depict exons and introns, respectively. Blue thick rectangles indicate the CDS region. The 14 designed amplicons (red) for nine exons are shown under the exon–intron structure. (B) Amplicon design schema. (C) Error rate for each error category in the region of entire amplicon (pale blue), that without designed primer regions (light blue), and the target regions (CDS + flanking intron; dark blue), respectively. (D) Strand-wise error rate for each amplicon. (E) Error rates along the amplicon sequence of exon 1 in each strand for insertions and deletions in the upper panel and mismatches and ambiguous base calls in the lower panel. The orange and blue lines depict the primer and target regions, respectively. The yellow shaded area depicts the homonucleotide ($n > 3$) region. The colour representation for the bars is the same as (D). (F) Co-occurrence error rate in both strands. The fraction of positions where a certain error occurred with the error rate for insertions, deletions, and mismatches. The colour representation is the same as in (D) and (E).

12.5 pmol of each forward and reverse primer, and 1.25 U of PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan). The thermal cycling profile consisted of an initial denaturation step at 98°C for 1 min, followed by 28–32 cycles of 10 s denaturation at 98°C, 15 s of annealing at 60°C, and a 30 s extension at 68°C. The lengths of the PCR products ranged from 291 to 421 bp. The second PCR amplifications were performed using primers with adapter sequences at the 3′ end and Multiplex Identifier (MID) sequences at the 5′ end (Fig. 1B), which was used as a tag for each sample. The PCR reactions were performed in 50-μl volumes using 0.5 μl of the first PCR products, 1× PrimeSTAR GXL buffer, 0.2 mM of each dNTP, 12.5 pmol of each forward and reverse primer, and 1.25 U of PrimeSTAR GXL

DNA polymerase to attach the anchor sequences for MPS. The thermal cycling profile consisted of an initial denaturation step at 98°C for 20 s, followed by 20 cycles of 10 s denaturation at 98°C, 15 s of annealing at 60°C, and a 40 s extension at 68°C.

After confirming the amount and integrity of the PCR products by agarose gel electrophoresis, we mixed virtually equal amounts of the respective PCR amplicons that were generated using the same genomic DNA and applied the samples to a 454 Genome Sequencer (GS)-FLX system (Roche Diagnostics Corp., USA). All amplicons were amplified by emPCR and sequenced together in a multiplex fashion. MPS on this platform was performed as instructed by Roche. The sequencing reads from each of the pooled libraries were identified by their MID tags.

2.3. *Sequence data analysis*

The sequence read data were generated using GS RunProcessor ver.2.5.3 with default settings. Reads were sorted according to the MID tag sequences and were mapped to the reference amplicon sequences using the BLAT program[14] with the '-fine' option. In order to identify positions where the bases in a read differed from those in the reference sequence, each read was aligned to its reference sequence with the dpAlign module in the BioPerl package (http://www.bioperl.org/). The 454 pyrosequencing-related errors were categorized as insertions, deletions, mismatches, or ambiguous base calls. When aligning sequences, insertions/deletions are allocated based on the sequence context and strand orientation. To eliminate alignment artefacts due to insertion/deletion positions, the lower strand reads were converted to the reverse complement sequence, i.e. keeping the same strandness as the upper strand reads, when aligned with the reference sequence. A sequence error was defined as discordance in an equivalent position between the reference and control (from the 49 healthy individuals and a cloned plasmid vector). The error rate for a specified category was defined as the number of errors divided by the total number of bases in a read. The error rates of a base position on each strand were calculated from 50 control samples.

2.4. *Confirmation of somatic mosaicism of the* NLRP3 *gene by subcloning and subsequent capillary DNA sequencing*

To confirm the somatic mutational frequency that was identified based on the 454 sequencing data, we subcloned the PCR products and performed capillary DNA sequencing as previously described.[9] A Topo TA cloning kit (Invitrogen, San Diego, CA, USA) was used to subclone each of the 14 amplicons.

2.5. *Functional analysis*

To determine whether the identified *NLRP3* mutants are disease-causing, we assessed both ASC [apoptosis-associated speck-like protein containing a caspase recruitment domain; PYCARD, an approved symbol from the HUGO Gene Nomenclature Committee (HGNC) database]-dependent NF-κB activation in HEK293FT cells and transfection-induced cell death in THP-1 cells, a human monocytic cell line, as previously described.[9,13,15] cDNAs encoding carboxy-terminal green fluorescent protein (GFP)-tagged NLRP3 and its mutants were subcloned into pcDNA5/TO (Invitrogen). Before being introduced into THP-1 cells (10^6) using a Cell Line Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany), phorbol myristate acetate (10 ng/ml) was added to enhance transient expression of *NLRP3* gene with minimizing spontaneous cell death.[15] Four hours after the introduction of plasmids (0.5 μg), cell death of GFP-positive THP-1 cells was measured by flow cytometry.

Expression plasmids for NLRP3 and ASC in the pEF-BOS vector background have been previously described.[13] HEK293FT cells (10^5) were transfected using TransIT-293 Transfection Reagent (Milus Bio, Madison, WI, USA) with an NF-κB reporter construct (pNF-κB-luc; 20 ng; BD Biosciences Clontech, Palo Alto, CA, USA), an internal control construct (pRL-TK; 5 ng; Toyo Ink, Tokyo, Japan), and wild-type or mutant NLRP3 expression plasmid (20 ng) in the presence or absence of ASC expression plasmid (20 ng). The amounts of total plasmid DNA used for transfection experiments were kept constant by adding pEF-BOS vector DNA. Twenty-four hours later, the transfected cells were harvested and subjected to dual luciferase assay by which the ability of each construct to induce NF-κB activation was assessed as previously described.[9]

3. Results

3.1. *Construction of base- and strand-specific error rate maps of* NLRP3 *exons from the MPS data of 50 control samples*

Errors in sequence reads generated by a Roche 454 GS-FLX sequencer are not randomly distributed along the sequence and depend on various factors.[16] Although this is a well-known characteristic of 454 sequencing, the occurrence pattern of these errors has not been explored in detail simply because these sequencing errors are considered noise that can be filtered out in most cases. However, it is highly critical to understand the occurrence pattern of sequencing errors on the MPS platform because low-level somatic mosaicism might appear at a rate close to that of sequencing errors. To address this, we collected

~1 million sequence reads using the 454 GS-FLX sequencer for 14 amplicons of *NLRP3* exons from 50 control samples that were thought to be free from somatic mosaicism, and ~94% of those reads were mapped to one of the reference *NLRP3* exon sequences. The number of sequencing depths for each amplicon of each sample on each strand was between 65 and 2139 (mean = 565.3, Supplementary Table S2). We found that the average error rate for each mutation category (insertion, deletion, mismatch, and ambiguous base calls) at each base position on each strand of the amplicons in the control samples was 0.22, 0.16, 0.036, and 0.014%, respectively (Fig. 1C). These values were consistent with those reported in a recent study on the error rates with 454 sequencing data.[16] The sequencing error in the 454 GS-FLX system tends to occur at the beginning and end of the reads,[11,16] and we confirmed this trend in our amplicon sequencing data (Supplementary Fig. S1). Moreover, after removing the end regions of the read sequences, we found that the error rates of the target regions for each category were 0.20, 0.134, 0.023, and 0.014%, respectively (Fig. 1C and Supplementary Table S3). When generating the amplicon sequences for the *NLRP3* exons, the target sequence (CDS region and flanking intron in 10-bp length) was designed to be 300−400 bp and not adjacent to primer sequences in order to obtain relatively low sequencing error rates (Fig. 1C). However, when the base- and strand-specific error rates of the respective amplicons were compared, we noticed that there were large variations in the error rate among amplicons in a strand-specific manner (Fig. 1D). We further examined the occurrence pattern of sequencing errors, as shown in Fig. 1E; the average sequencing error rates at each base in the 50 control amplicons are shown in a bar graph, where the bars in the upper or lower direction show the sequence error rates at the base position on the upper or lower strand of the amplicons, respectively. As evident in Fig. 1E, the error rates at most residues were low (<1%) with some hotspots for each type of error. Most of the insertion/deletion errors preferentially occurred at a homonucleotide region (yellow regions in Fig. 1E) as previously described,[17] but it was not always the case for all of homonucleotide regions. We could not find any tight relationship between other sequence patterns and the error rate. In addition, there was almost no position where sequencing errors occurred at a similar rate on both strands. This is more clearly shown in Fig. 1F, which indicates the numbers of positions with sequence variations in both strands that were higher than the threshold along the horizontal axis. These results indicate that the sequence errors can be discriminated from real genetic alterations when the sequence is read in both directions. However, it is important to keep in mind that PCR errors are not distinct from real genetic alterations. We did not observe any base substitution at a rate higher than 1% in our experiments (Fig. 1F), and the overall PCR error rate under MPS conditions was lower than 1% as long as a high-fidelity DNA polymerase was used to generate the amplicons.

Because Gilles *et al.*[16] recently reported that the occurrence of sequencing errors using the Roche 454 GS-FLX DNA sequencer depends on various factors, we first examined variations in the sequencing error rates of *NLRP3* exons among samples in the same run. For each mutation category, we found a similar trend in the error distribution rate in the amplicon sequences among the control samples (Supplementary Figs S2−S4). We confirmed that, for almost all residues, the error rate distributions among the 50 control samples fitted a Poisson distribution (data not shown). We next examined the run-to-run variation of the sequencing error rate for *NLRP3* exons. For this purpose, we performed an additional MPS run with seven amplicons (exons 3, 4, and 6) that were newly prepared and compared the number and positions of the sequencing errors between two independent sequencing runs. Out of 1993 base positions in the target regions, there was a low occurrence rate of mismatch errors in both runs and this seemed to fit a Poisson distribution. However, insertion/deletion errors (>1% error rate) were observed at ~100 base positions (<5% in the target regions) in each run, and only a half of these errors were shared between both runs (Table 1).

Table 1. Run-to-run variations in the error occurrence (>1% frequency)

Error category	Upper strand			Lower strand			All[a]
	First run	Second run	Overlap	First run	Second run	Overlap	
Insertions	63	73	42	76	96	52	10
Deletions	36	44	24	29	65	20	2
Mismatches	0	0	0	3	0	0	0
Ambiguous base calls	6	8	6	12	10	10	0

[a]The number of positions where the error rates in each category were commonly >1% for both strands in two independent runs.