Table II (continued)

Category	Total number	Pediatric department	Internal medicine department
Others	3	3	0
Untested or undetermined	22	20	2
VIII. Complement deficiencies	32 (3%)	29 (3%)	3 (3%)
IX. Undetermined	18 (1%)	14 (1%)	4 (4%)
Total	1,240	1,146	94

APECED Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy, *IPEX* immune dysregulation, polyendocrinopathy, enteropathy, X-linked

increasing age (Fig. 2b). The median age of CID, BTK deficiency, CVID, and CGD patients was 5.2, 12.8, 25.1, and 14.7 years, respectively.

It is well known that PID patients are susceptible to many pathogens and experience community-acquired or opportunistic infections. In this study, we focused on noninfectious complications of PID because they have been less well studied on a large scale and may provide important information for improving the quality of life of PID patients. Twenty-five PID patients developed malignant disorders (2.7%; Table III). Lymphoma, in particular, Epstein–Barr virus-related, and leukemia were dominant, while there were no patients with gastric carcinoma. CVID, Wiskott–Aldrich syndrome (WAS), and ataxia telangiectasia were more frequently associated with malignant diseases among PID patients. A case of Mendelian susceptibility

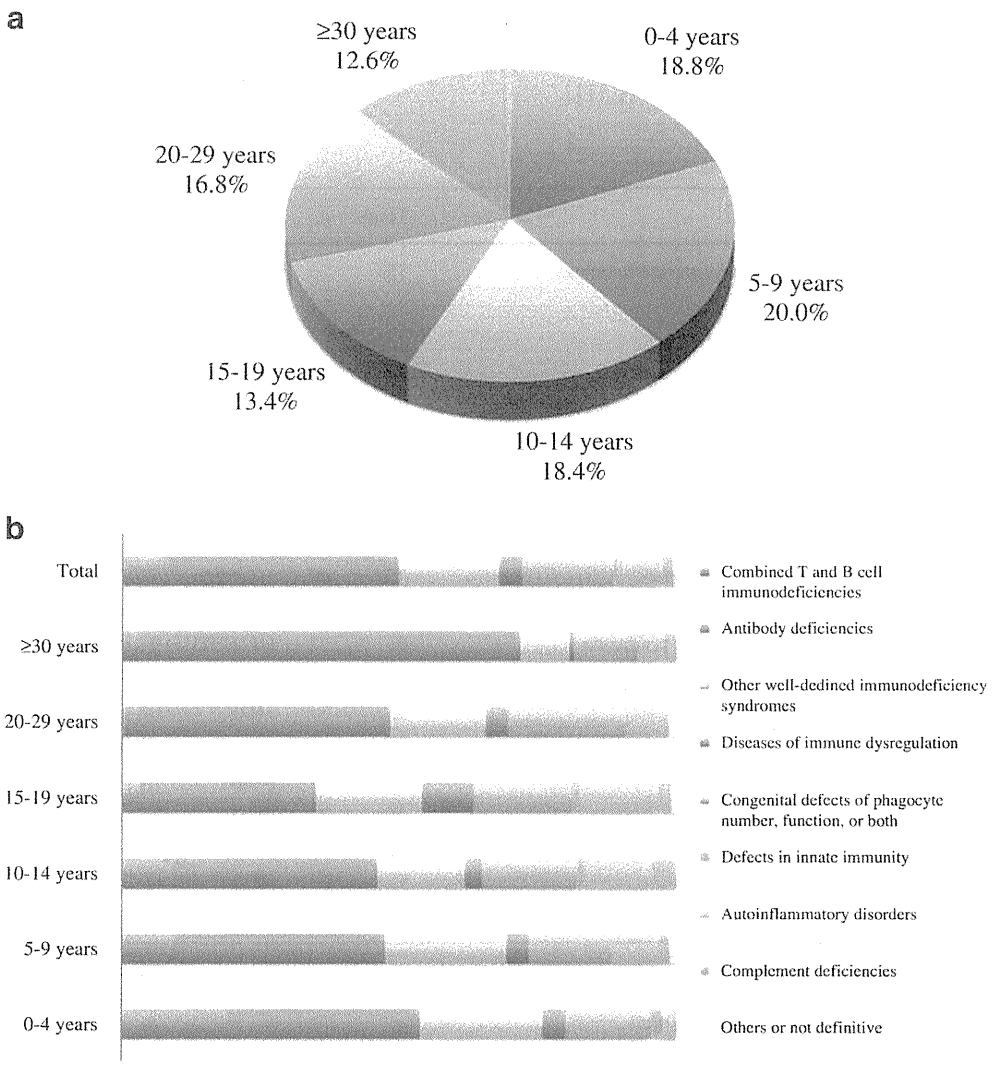

Fig. 2 **a** Age distribution of PID patients. **b** Distribution of PID in each age group

to mycobacterial disease with squamous cell carcinoma was also observed [9] (Table III).

Seventy-eight PID patients had immune-related (autoimmune) diseases (8.5%; Table IVa). Autoimmune lymphoproliferative syndrome, immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, and nuclear factor kappa B essential modulator (NEMO) deficiency were associated with immune-related diseases at a very high incidence. In addition, immune-related diseases were relatively common in CGD and CVID patients (Table IVa). The most commonly observed immune-related disease was inflammatory bowel disease (33 cases), which was most frequently observed in CGD patients, followed by immune thrombocytopenic purpura (13 cases), autoimmune hemolytic anemia (8 cases), and systemic lupus erythematosus (SLE; 8 cases; Table IVa and b). Kawasaki disease occurred in WAS and CGD patients. In addition, this is the first report of Kawasaki disease in patients with complement deficiency (C9) and familial Mediterranean fever (FMF). A patient with warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome and a patient with tumor necrosis factor receptor-associated periodic syndrome (TRAPS) were first reported as cases of type 1 diabetes mellitus and SLE, respectively [10, 11].

Discussion

We conducted a nationwide survey of PID for the first time in 30 years and report the prevalence of PID in Japan. We registered 1,240 PID patients and found that the estimated prevalence of PID (2.3/100,000) is higher than that previously reported (1.0/100,000) in Japan. Our results are equivalent to those reported in Singapore (2.7/100,000) and Taiwan (0.77–2.17/100,000) [12–14]. However, our values are lower than those reported in Middle Eastern countries such as Kuwait (11.98/100,000) or in European countries such as France (4.4/100,000) [5–7, 15]. The high rate of consanguinity may be a cause of the high prevalence rate of PID reported in Middle Eastern countries [6, 15]. There may has been sample selection bias in this study because some asymptomatic cases (SIgAD, etc.), clinically recovered cases (transient hypogammaglobulinemia of infancy, etc.), and cases in which patients were deceased were not registered. In addition, lack of recognition of PID in internal medicine departments, not just the low response rate, might also have influenced the estimated prevalence of PID as well as the age and disease distribution. The regional prevalence of PIDs in Japan was homogenous, unlike in other countries in which a higher prevalence was

Table III Malignancies in PID patients

Primary immunodeficiency	Total	*n*	Malignancy
I. Combined T and B cell immunodeficiencies	75	2	(2.7%)
Ommen syndrome	3	1	NHL (EBV+) 1[a]
Adenosine deaminase deficiency	4	1	Breast carcinoma 1
II. Predominantly antibody deficiencies	378	8	(2.1%)
Common variable immunodeficiency disorders	93	7	HL 2, ML 2, ALL 1, Basal cell carcinoma 1, Cervical carcinoma 1
Good syndrome	4	1	Double primary carcinoma of breast and colon 1
III. Other well-defined immunodeficiency syndromes	165	7	(4.2%)
Wiskott–Aldrich syndrome	57	5	NHL 3, NHL/HL 1, LPD (EBV-) 1
Ataxia telangiectasia	13	2	T-ALL 1, MDS 1
IV. Diseases of immune dysregulation	38	4	(10.5%)
X-linked lymphoproliferative syndrome	5	2	Burkitt lymphoma 2
Autoimmune lymphoproliferative syndrome	6	2	HL (EBV+) 1, Brain tumor 1
V. Congenital defects of phagocyte number, function, or both	153	4	(2.6%)
Severe congenital neutropenia	35	3	MDS 3 (including 2 cases with monosomy 7)
MSMD	7	1	Squamous cell carcinoma of finger 1
VI. Defects in innate immunity	12	0	(0%)
VII. Autoinflammatory disorders	74	0	(0%)
VIII. Complement deficiencies	23	0	(0%)
IX. Undetermined	5	0	(0%)
Total	923	25	(2.7%)

n Number of PID patients who had malignant disorders, *ALL* acute lymphoblastic leukemia, *EBV* Epstein-Barr virus, *HL* Hodgkin lymphoma, *LPD* lymphoproliferative disease, *MDS* myelodysplastic syndrome, *ML* malignant lymphoma, *MSMD* Mendelian susceptibility to mycobacterial disease, *NHL* non-Hodgkin lymphoma

[a] The number of patients

Table IV Immune-related diseases in PID patients

(a) Immune-related diseases with each PID

Primary immunodeficiency	Total	*n*	Immune-related disease
I. Combined T and B cell immunodeficiencies	75	2	(2.6%)
MHC class II deficiency (suspected)	1	1	ITP with AIHA 1[a]
CD4 deficiency	1	1	Hashimoto disease 1
II. Predominantly antibody deficiencies	378	24	(6.3%)
Common variable immunodeficiency disorders	93	16	ITP 3, RA 2, AIHA 2, Hashimoto's disease 2, IBD 2, SLE 1, MG 1, ADEM 1, Autoimmune hepatitis 1, Uveitis 1
Hyper-IgM syndrome	32	3	JIA 1, SLE (complicated with C1q deficiency) 1, IBD 1
Selective IgA deficiency	28	3	SLE 1, SLE with Kikuchi disease 1, RA 1
IgG subclass deficiency	50	2	ITP with AIHA 1, ITP with MS 1
III. Other well-defined immunodeficiency syndromes	165	5	(3.0%)
Wiskott–Aldrich syndrome	57	3	AIHA 2, Kawasaki disease 1
DiGeorge syndrome	33	2	AIHA 1, ITP 1
IV. Diseases of immune dysregulation	38	10	(26.3%)
X-linked lymphoproliferative syndrome	5	1	IBD 1
Autoimmune lymphoproliferative syndrome	6	4	ITP 3, Graves' disease with IBD 1
APECED	5	1	T1DM with Hashimoto's disease and Vogt–Koyanagi–Harada disease 1
IPEX syndrome	6	4	T1DM 1, T1DM with ITP, AIN and IBD 1, Autoimmune enteritis 1, AIHA with Autoimmune enteritis and Hashimoto's disease 1
V. Congenital defects of phagocyte number, function, or both	153	25	(16.3%)
Chronic granulomatous disease	87	25	IBD 20, ITP 2, JIA 1, MCTD 1, Kawasaki disease 1
VI. Defects in innate immunity	12	5	(41.7%)
NEMO deficiency	7	4	IBD 3, IBD with JIA 1
WHIM syndrome	3	1	T1DM 1
VII. Autoinflammatory disorders	74	3	(4.0%)
Familial Mediterranean fever	36	2	SLE 1, Kawasaki disease 1
TNF receptor associated periodic syndrome	9	1	SLE 1
VIII. Complement deficiencies	23	3	(13.0%)
C4 deficiency	1	1	SLE with RA 1
C6 deficiency	1	1	IBD 1
C9 deficiency	11	1	Kawasaki disease 1
IX. Undetermined	5	1	(20%)
Nakajo syndrome	1	1	SLE 1
Total	923	78	(8.5 %)

(b) Immune-related manifestations associated with PID

Immune-related diseases	*n*
IBD (including autoimmune enteritis)	33
ITP	13
AIHA	8
SLE	8
RA/JIA	6
Hashimoto's disease/Graves' disease	5
Kawasaki disease	4
T1DM	4
Uveitis (including Vogt–Koyanagi–Harada disease)	2
ADEM/MS	2
Others	5

n Number of PID patients who had immune-related disorders, *ADEM* acute disseminated encephalomyelitis, *AIHA* autoimmune hemolytic anemia, *AIN* autoimmune neutropenia, *APECED* autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, *IBD* inflammatory bowel disease, *IPEX* immunodysregulation, polyendocrinopathy, enteropathy X-linked, *ITP* immune thrombocytopenic purpura, *JIA* juvenile idiopathic arthritis, *MCTD* mixed connective tissue disease, *MG* myasthenia gravis, *MS* multiple sclerosis, *RA* rheumatoid arthritis, *SLE* systemic lupus erythematosus, *T1DM* type 1 diabetes mellitus, *WHIM* warts, hypogammaglobulinemia, infections, and myelokathexis

[a] The number of patients

observed in urban areas [5, 7, 16]. This may be because many PID patients were treated or followed by PID specialists distributed nationwide in Japan; this is assumed by the location of hospitals with which they were affiliated.

The distribution ratios of BTK deficiency (14.7%) and CGD (11.9%) in Japan were higher than those in a previous report from Europe (5.87% and 4.33%, respectively), while those of CIDs and other well-defined immunodeficiency syndromes were comparable [17]. The prevalence of BTK deficiency was previously reported to be 1/900,000–1,400,000 in a European cohort study [18]. In contrast, this value was estimated to be 1/300,000 in Japan in our study. BTK deficiency appears to be common in Japan, although this may be partially because more patients, including those showing atypical clinical manifestations, were diagnosed more accurately by the recently established genetic diagnostic network in Japan [19]. This is supported by the highest proportion of Japanese patients in the international mutation database for X-linked agammaglobulinemia (BTKbase) [20]. The reason for the low number of registered CGD patients in Europe in a recent report (1/620,000) [17] is unknown; the prevalence of CGD was 1 in 250,000 in a previous European survey [21], which was similar to our results (1 in 380,000 in this study and 1 in 280,000 in our previous study [22]). The percentage of BTK deficiency and CGD would be lower if more adult cases were registered because the prevalence of these disorders is low in adults. CVID was the most commonly reported PID (20.7%) in Europe, and the onset of symptoms was observed most commonly in the third decade of life in these patients [17, 23]. In this study, CVID constituted 11.0% (136 cases) of PID cases, and only 29 cases were reported from internal medicine departments (Table II). A lower number of registered CVID patients may have led to a lower number of reported patients with antibody deficiency and a lower prevalence of PID, although it is still possible that CVID is not as common in Japan as in European countries. There was no significant difference in the distribution rate of SIgAD between Japanese and Europeans, although SIgAD is rare in Japanese (1/18,500) compared with Caucasians (1/330–2,200) according to seroepidemiologic studies [24]. This may be because most SIgAD patients lack clinical manifestations. The distribution ratio of autoinflammatory disorders in Japan (9%) was much higher than that in Europe (1.02%) [17] (Table II). Considering the disease type of the autoinflammatory disorders was not specified in 22 cases (20%), it is possible that many other patients with autoinflammatory disorders remain undiagnosed in Japan as well as in other countries.

The percentage of men (69.7%) with PID is higher in Japan than in Europe (60.8%) or Kuwait (61.8%), but is equivalent to that in Taiwan (70.2%) [6, 13, 17]. The higher ratio of men, particularly in younger generation (<15 years), appears to be due to the larger number of X-linked PID patients (BTK deficiency, X-CGD, γc deficiency, etc.) in this study compared to that in Europe or Kuwait. Adolescents or adults (≥15 years) constituted 42.8% of the patients in this study, which is equivalent to the number in the European study (≥16 years: 46.6%), while those >16 years constituted only 10.9% in the previous survey [3, 17]. In this study, it was found that CVID and SIgAD are common in adults (Table II) and that antibody deficiencies are more common with increasing age (Fig. 2b). A reason for the increased number of adult PID patients may be long-term survival of PID patients due to improved treatments such as immunoglobulin replacement therapy. In addition, an increased likelihood of patients being diagnosed by internists as having late-onset PID, e.g., CVID and SIgAD, may have contributed to these values [17, 25, 26]. Therefore, it is important for internists to be well-informed regarding PID. In contrast, CIDs are fatal during infancy without hematopoietic stem cell transplantation or gene therapy. Because hematopoietic stem cell transplantation has been widely performed in Japan since the 1990s, surviving patients with CID are limited to the younger generation, similar to French patients (Fig. 2b) [5, 27, 28].

It has been reported that PID patients are at increased risk of developing malignant diseases, in particular, non-Hodgkin lymphoma, leukemia, and stomach cancer [29]. Although lymphoma and leukemia were relatively common, stomach cancer was not observed in our study. In the previous survey in Japan, eight of nine PID patients with malignant disorders (including one gastric cancer patient) died [3]. It is possible that some PID patients with malignant disorders were not registered because they were deceased. PID is also associated with immune-related diseases because of a defect in the mechanisms to control self-reactive B and T cells. The frequency of immune-related manifestations varied among individual PID patients, as reported previously [30, 31]. Four PID patients who had developed Kawasaki disease, one patient with WHIM syndrome and type 1 diabetes mellitus, and one patient with TRAPS and SLE in our study may provide new pathophysiological insights of these diseases and the association between PID and autoimmune diseases.

Conclusions

We report the prevalence and clinical characteristics of PIDs in Japan. Although the advances in diagnostic technologies and treatments have improved the prognoses of PID, many patients continue to experience severe complications such as malignancy and immune-related diseases as well as infections. To improve the quality of life of PID patients, it is necessary to pay attention to

complications and treat them appropriately. Web-based PID databases and consultation systems have been created in Japan (Primary Immunodeficiency Database in Japan [4] and Resource of Asian Primary Immunodeficiency Diseases in Asian countries [32]) to reveal precise information regarding PID and to promote cooperation between doctors and researchers [19].

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Conflict of Interest There is no actual or potential conflict of interest in relation to the study.

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bjh research paper

Outcome of unrelated umbilical cord blood transplantation in 88 patients with primary immunodeficiency in Japan

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Summary

We report the results of umbilical cord blood transplantation (UCBT) performed in 88 patients with primary immunodeficiency (PID) between 1998 and 2008 in Japan; severe combined immunodeficiency (SCID, $n = 40$), Wiskott–Aldrich syndrome (WAS, $n = 23$), chronic granulomatous disease ($n = 7$), severe congenital neutropaenia (SCN, $n = 5$) and other immunodeficiencies ($n = 13$). Five-year overall survival (5-year OS) for all patients was 69% [95% confidence interval (CI), 57–78%], and was 71% and 82% for SCID and WAS, respectively. The main cause of death before day 100 was infection (17/19), while that after day 100 was graft-versus-host disease (GVHD) (5/7). Using multivariate analyses, pre-transplant infection, no conditioning, ≥2 human leucocyte antigen (HLA) mismatches or diagnosis other than SCID, SCN or WAS were all associated with poor prognosis. Reduced-intensity conditioning was associated with decreased overall mortality compared with myeloablative therapy. The cumulative incidence of grade 2–4 acute GVHD at day 100 was 28% (95% CI, 19–38%), and that of chronic GVHD at day 180 was 13% (95% CI, 7–23%). We conclude that UCBT should be considered for PID patients without an HLA-matched sibling. The control of pre-transplant infection and selection of HLA-matched donors will lead to a better outcome.

Keywords: primary immunodeficiency, severe combined immunodeficiency, Wiskott-Aldrich syndrome, cord blood transplantation, reduced-intensity conditioning.

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Allogeneic haematopoietic stem cell transplantation (HSCT) has been successfully used as a curative therapy for most severe forms of primary immunodeficiency (PID) (Zeidler *et al*, 2000; Antoine *et al*, 2003; Sakata *et al*, 2004; Rao *et al*, 2005; Kobayashi *et al*, 2006; Mazzolari *et al*, 2007; Dvorak & Cowan, 2008; Griffith *et al*, 2008; Cuvelier *et al*, 2009). Stem cell transplantation from a human leucocyte antigen (HLA)-identical family donor provides better prognosis than bone marrow transplantation from an unrelated donor (Antoine *et al*, 2003). Survival with this type of transplantation from a matched unrelated donor has improved significantly over the years in patients with severe combined immunodeficiency (SCID), whereas no improvement in survival has been observed with this transplantation in non-SCID patients (Antoine *et al*, 2003). The optimal stem cell source for PID patients with no HLA-identical sibling remains to be determined (Dvorak & Cowan, 2008; Griffith *et al*, 2008; Cuvelier *et al*, 2009).

Umbilical cord blood grafts from unrelated donors have been successfully used, primarily in children and subsequently in adults (Kurtzberg *et al*, 1996; Wagner *et al*, 1996; Gluckman *et al*, 1997; Rubinstein *et al*, 1998; Rocha *et al*, 2000, 2004; Laughlin *et al*, 2004). Theoretically, unrelated cord blood transplantation (UCBT) has the following distinct advantages in PID patients: (i) the cord blood product is rapidly accessible in most cases; (ii) the incidence and severity of graft-versus-host disease (GVHD) is not excessive, even in mismatched transplantation and (iii) the risk of latent viral transmission is low. The disadvantages of UCBT include slower haematopoietic/immunological reconstitution and graft failure, which have been observed with UCBT for malignant disorders, and naivety of lymphocytes to pathogens (Brown *et al*, 2008; Griffith *et al*, 2008; Szabolcs *et al*, 2008). Rapid immune reconstitution is particularly important in PID patients with ongoing infection who undergo UCBT.

The limited data available show that UCBT can be a curative measure in patients with SCID, Wiskott–Aldrich syndrome (WAS), chronic granulomatous disease (CGD) and severe congenital neutropaenia (SCN) (Knutsen & Wall, 2000; Bhattacharya *et al*, 2003, 2005; Fagioli *et al*, 2003; Knutsen *et al*, 2003; Kobayashi *et al*, 2006). Most of the available data have come from a single centre, and thus, detailed information on the outcome and problems associated with UCBT in PID patients is still lacking. In this study, we report the results of UCBT performed in 88 PID patients between 1998 and 2008 in Japan.

Methods

Collection of data

All UCBTs carried out for PIDs through the Japan Cord Blood Bank Network (JCBBN) between August 1998 and January 2008 was enrolled in this study. Eighty-eight patients with PID underwent UCBT during this period. All data were provided by JCBBN, which collects recipients' clinical information at day 100 after transplantation. Recipients' data on survival, disease status and long-term complications are renewed annually by administering follow-up questionnaires. Latest data acquisition was performed in November 2009. The present study was approved by the institutional ethical and data management committees of JCBBN.

Patients

A summary of patients enrolled in this study is shown in Table I. Forty patients had SCID (45%) and 48 (55%) had non-SCID. Patients with familial haemophagocytic syndrome were not included in this study. The median age at the time of transplantation was 10 months (range, 0–248 months).

Procedures

Cryopreserved, unrelated cord blood cells were used as the source of haematopoietic stem cells. The type of conditioning used and median cell dose infused are shown in Table I.

In most cases, HLA matching was performed by both serological and DNA typing for HLA-A, HLA-B and HLA-DRB1. In this study, HLA mismatch was defined according to serological or low-resolution molecular typing for HLA-A and HLA-B and high-resolution molecular typing for HLA-DRB1. Of the UCB donors, 29 (33%) were HLA fully compatible. Of the mismatched donors, 40 (45%) were 1-antigen mismatched, 15 (17%) were 2-antigen mismatched and four (5%) were 3-antigen mismatched (Table I). In 48 patients in whom high-resolution genotypical typing was performed for HLA-A, HLA-B and HLA-DRB1, 11 were fully matched, 13 were 1-antigen mismatched, 16 were 2-antigen mismatched, five were 3-antigen mismatched and three were 4-antigen mismatched.

Immunosuppressive prophylaxis against GVHD after UCBT consisted of ciclosporin A (CyA)- and tacrolimus-based regimens in 48 and 35 patients, respectively. Five patients were not administered any immunosuppressive drug after UCBT.

Various techniques including karyotyping, HLA typing and fluorescence *in situ* hybridization for the XY chromosome and variable number of tandem repeats were used to confirm the engraftment of donor cells.

Definitions

Neutrophil recovery was defined by an absolute neutrophil count of at least $0{\cdot}5 \times 10^9$/l for three consecutive days. Platelet recovery was defined by a count of at least 20×10^9/l, unsupported by transfusion for 7 d. Reticulocyte recovery was defined by a count of at least 20‰.

Patients without conditioning or with only anti-thymocyte globulin (ATG) were categorized as receiving no conditioning. Patients administered busulfan (BU)/cyclophosphamide (CY) ± total body irradiation (TBI) or total lymphoid irradiation

Table I. Age at the time of transplantation, type of conditioning and HLA disparity.

	Patients (*N*)	Median age at transplantation (months) (range)	Median cell dose ($\times 10^7$/kg) (range)	Second or third transplantation (*N*)	Conditioning			HLA disparity			
					No (*N*)	RIC (*N*)	MAT (*N*)	0 (*N*)	1 (*N*)	2 (*N*)	3 (*N*)
Total	88	9 (0–248)	8·60 (1·89–31·1)	8	14	31	43	29	40	15	4
SCID	40	6·5 (0–27)	11·4 (4·55–31·1)	1	12	18	10	17	15	5	3
WAS	23	14 (4–84)	6·49 (2·89–13·6)	1	0	2	21	7	10	6	0
CGD	7	63 (31–248)	6·00 (1·89–12·3)	5	1	4	2	2	4	1	0
SCN	5	10 (4–124)	5·99 (4·16–9·19)	0	0	1	4	1	4	0	0
Others	13	37 (6–194)	8·11 (3·01–19·8)	1	1	6	6	2	7	3	1

RIC, reduced-intensity conditioning; MAT, myeloablative therapy. Definition of conditioning regimens are described in *Methods* section. 'Others' include four CD40L deficiency, two common variable immunodeficiency and one of each of the following disorders: Major histocompatibility complex (MHC) class II deficiency, DiGeorge syndrome, X-linked lymphoproliferative disorder, NEMO (NF-κ-B essential modulator) deficiency, IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome, Idiopathic CD4 lymphopenia and Blau syndrome.

(TLI), BU/CY + ATG ± TLI, BU/CY + fludarabine (Flu) or CY/etoposide/high-dose cytarabine were categorized as receiving myeloablative therapies (MATs). CY dose ranged from 120 to 240 mg/kg (median, 200 mg/kg) in patients receiving MAT.

TBI < 4 Gy was classified as 'low-dose TBI'. Patients administered Flu/melphalan (L-PAM) ± low-dose TBI or TLI, Flu/BU ± TLI or Flu/CY (50–60 mg/kg) ± low-dose TBI/TLI, Flu + low-dose TBI or Flu + ATG were categorized as receiving reduced-intensity conditioning (RIC). L-PAM dose was ≤140 mg/m^2 in patients receiving RIC.

GVHD was graded according to the standard criteria (Przepiorka *et al*, 1995).

Statistical analyses

The probability of survival was estimated by the product-limit method, and the log-rank test was used for group comparisons. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of neutrophil, platelet and reticulocyte recovery and that of acute and chronic GVHD. Death before recovery was the competing event for haematological recovery, and death without GVHD was the competing event for GVHD. Gray's test was used for group comparisons of cumulative incidence (Gray, 1988; Gooley *et al*, 1999). The Cox regression model was used to analyse data for the identification of prognostic factors. Factors found to be significant ($P < 0{\cdot}05$) or marginally significant ($P < 0{\cdot}1$) in univariate analysis were included in multivariate analysis. The variables considered were patient age at the time of transplantation, diagnosis, duration from diagnosis to transplantation, second or third transplantation, HLA disparity, presence of infection at the time of transplantation, conditioning regimen and cell dose infused. Variables with >2 categories were included in the final model using dichotomized dummy variables when at least one of the categories showed significant effect on survival. Continuous variables were dichotomized for the prognostic factor analyses. Variables were dichotomized as follows; patient age greater or <12 months at transplantation, dichotomized at a median nucleic cell dose of $<8{\cdot}2 \times 10^7$/kg vs. $\geq 8{\cdot}2 \times 10^7$/kg and CD34 cell dose of $<2{\cdot}1 \times 10^5$/kg and $\geq 2{\cdot}1 \times 10^5$/kg, shorter than or equal to or longer than 180 d for time to transplant. All *P*-values were two-sided.

Results

Engraftment

Sixty-seven patients (76%) achieved stable engraftment. The cumulative incidence of neutrophil, platelet and reticulocyte recovery at day 100 after transplantation was 77% [95% confidence interval (CI), 66–85%], 56% (95% CI, 45–65%) and 64% (95% CI, 53–73%) respectively (Fig 1A, B; data not shown). The median time for neutrophil, platelet and reticulocyte recovery was 19 d (range, 9–104 d), 40 d (range, 10–122 d) and 27 d (range, 12–98 d), respectively. The cumulative incidences of neutrophil recovery were not statistically different among the disease groups (SCID, 74%; WAS, 91% and others, 68% at day 100 after transplantation) (Fig 1C), although incidence was low in CGD patients ($N = 7$, 43%).

The time required for neutrophil recovery was similar in all disease groups, while that required for platelet recovery varied to some extent among the different disease groups. Platelet engraftment was slightly delayed in WAS patients, but the time required for engraftment in these patients was not significantly different from that required in other patients (Fig 1D).

Forty-three, 31 and 14 patients received MAT, RIC and no conditioning, respectively. No difference was observed in the incidence of neutrophil recovery between the MAT and RIC groups (84% vs. 87% at day 100). Similarly, no difference was observed in platelet recovery between these two groups (data not shown).

The cell dose infused ranged from 1·89 to $31{\cdot}1 \times 10^7$/kg, with a median of $8{\cdot}60 \times 10^7$/kg. No correlation was observed between the cell dose infused and engraftment.

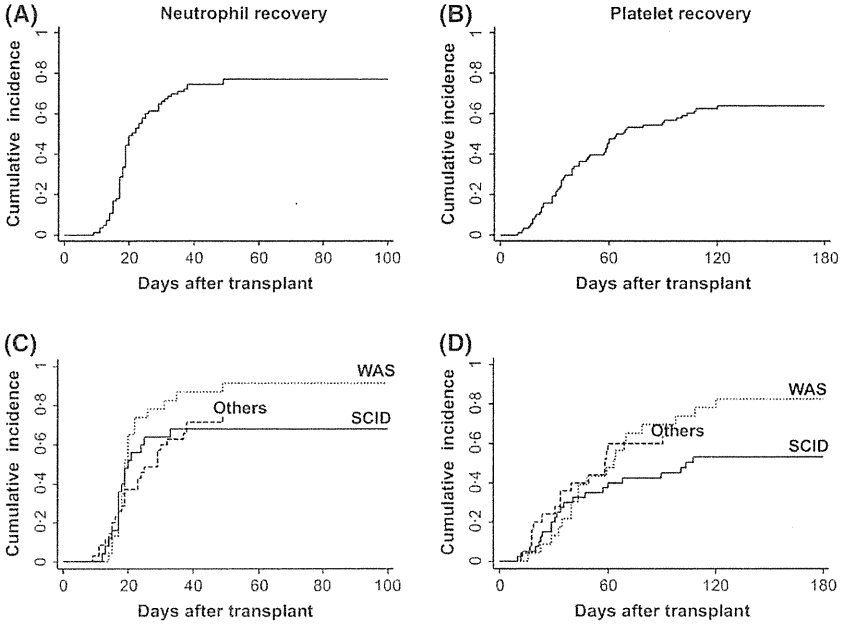

Fig 1. Cumulative incidence of neutrophil and platelet recovery after UCBT. (A) The cumulative incidence of neutrophil recovery 77% (95% CI, 66–85%). (B) The cumulative incidence of platelet recovery 56% (95% CI, 45–65%). The cumulative incidence of neutrophil (C) and platelet (D) recovery according to disease category is shown.

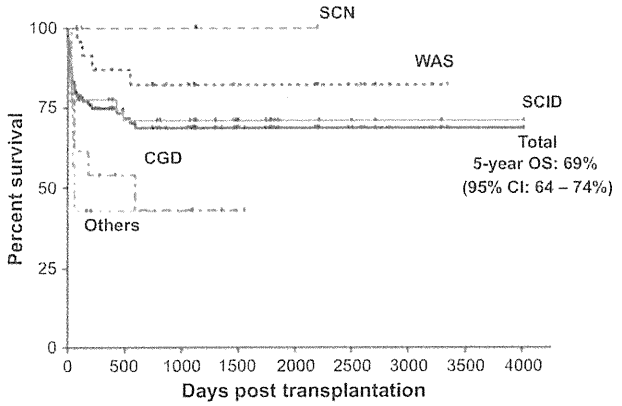

Fig 2. Kaplan–Meier estimates of overall survival after umbilical cord transplantation.

Five of 21 patients with engraftment failure received a second transplantation. Two WAS patients achieved successful engraftment in the second transplantation, while one SCID and two CGD patients did not survive the second transplantation. Only two of the remaining 16 patients who rejected the UCB graft remained alive at the latest data analysis.

Survival and causes of death

Of the 88 PID patients who underwent UCBT, 62 remained alive at the latest follow-up. Five-year OS for all patients was 69% (95% CI, 57–78%) (Fig 2), while that for SCID and WAS patients was 71% and 82%, respectively. All five SCN patients remained alive, although one patient had rejected the graft on day 79 after UCBT. Three of seven CGD patients survived UCBT; this low survival rate may be due to the fact that UCBT was selected in five patients after the first or second failed bone marrow transplantation (BMT). Seven of 14 patients categorized as 'other diseases' remained alive at the latest follow-up.

Table II summarizes the survival and causes of death after UCBT. Of the 26 patients who died, 19 had died within day 100 (17 from infection) and seven (SCID, six and congenital CD4 lymphopenia, one) had died within day 28 after UCBT.

Causes of early death (≤28 d) were cytomegalovirus (CMV) disease (three patients), *Pneumocystis* pneumonia (one patient), interstitial pneumonia (one patient), bacterial infection (one patient) and veno-occlusive disease (VOD) (one patient). All those who died of CMV disease had CMV pneumonia before transplantation.

The cause of death between days 28 and 100 in the remaining 12 patients was bacterial infection (seven had concomitant fungal infection, one also had VOD and one had CMV disease), CMV disease (two patients), fungal infection (one patient), multiple organ failure (one patient) and VOD (one patient). Four of seven CGD patients died of bacterial or fungal infection without engraftment. Although detailed data on bacterial/fungal infections at the time of transplantation were not collected, all the CGD patients were administered both antimicrobial and antifungal agents at the time of transplantation.

The causes of death after day 100 were GVHD (five patients), Epstein–Barr virus (EBV)-associated post-transplant lymphoproliferative disorder (EBV-PTLD, one patient) and

Table II. Survival and causes of death.

	Cases (*N*)	Alive (*N*)	Death (day) <28 (*N*)	<100 (*N*)	≥100 (*N*)	Infection at CBT (*N*)	Cause of death (<day 100) Bac/Fung infection (*N*)	Viral infection (*N*)	Others (*N*)	Cause of death (≥day 100) GVHD (*N*)	Others (*N*)
Total	88	62	7	19	7	18	10	7	VOD 3 MOF1	5	PTLD 1 AI 1
SCID	40	29	6	9	2	11	2	6	1 (VOD)	1	1 (AI)
WAS	23	19	0	1	3	1	1	0	0	3	0
CGD	7	3	0	4	0	5	4	0	1 (VOD)	0	0
SCN	5	5	0	0	0	0	0	0	0	0	0
Others	13	6	1	5	2	1	3	1	1 (VOD) 1 (MOF)	1	1 (PTLD)

Bac/Fung infection, bacterial and/or fungal infection. VOD, veno-occlusive disease; MOF, multiple organ failure; AI, adrenal insufficiency; PTLD, post-transplant lymphoproliferative disorder. Cause of death total does not equal the number of deceased patients because one patient died of VOD and bacterial infection.

adrenal insufficiency (one patient). None of the other patients died of infection after day 100.

GVHD

All but five patients in the present study received either CyA- or tacrolimus-based immunosuppressant prophylaxis for GVHD. The cumulative incidence of grade 2–4 acute GVHD at day 100 was 28% (95% CI, 19–38%), and that of grade 3–4 GVHD was 8% (95% CI, 4–15%) (Fig 3A, D).

The incidence of grade 2–4 GVHD was higher in patients who underwent 2- or 3-antigen-mismatched UCBT compared with those who underwent HLA-matched or HLA-1-antigen–mismatched UCBT, but it was not statistically significant ($P = 0{\cdot}071$) (Fig 3B). On the other hand, no difference was observed in the incidence of grade 3–4 GVHD between <2-antigen-mismatched and >2-antigen-mismatched transplants (Fig 3E), although grade 3–4 GVHD was not observed by high-resolution DNA typing in patients who underwent genotypically HLA-matched transplantation.

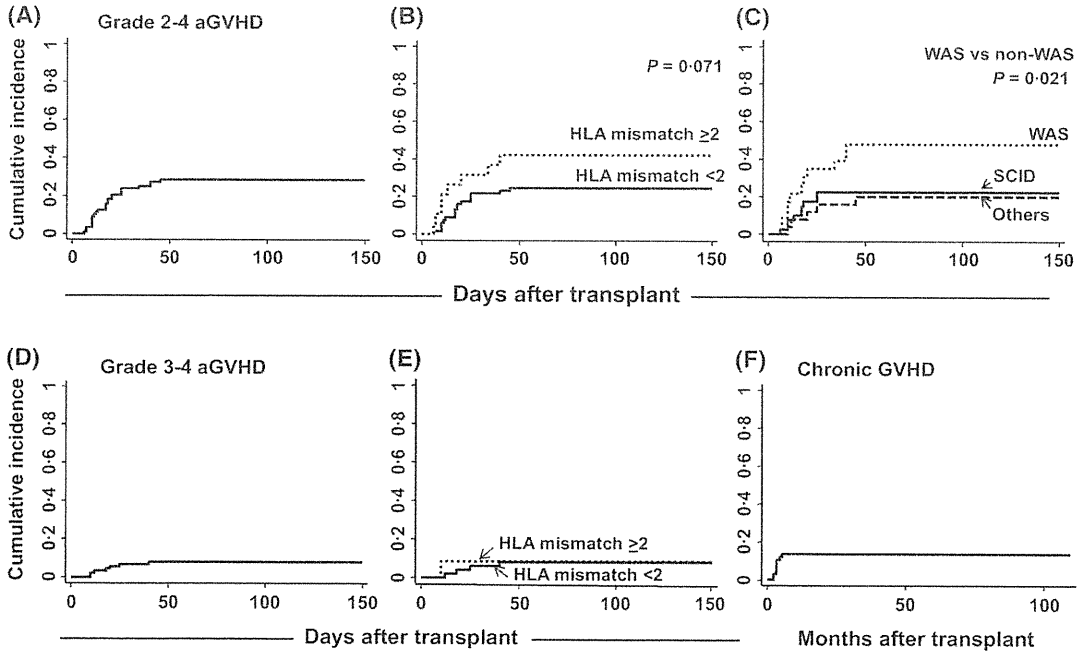

Fig 3. Cumulative probability of acute and chronic GVHD after UCBT. The cumulative incidence of grade 2–4 acute GVHD (aGVHD) at day 100 was 28% (95% CI, 19–38%) (A). The incidence was higher in transplantation mismatched for ≤2 antigens (B) and in that for WAS patients (C). The cumulative incidence of grade 3–4 acute GVHD at day 100 was 8% (95% CI, 4–15%) (D) and the incidence was not different between patients undergoing transplantation for ≥2-antigen mismatched transplant and those undergoing <2-antigen mismatched transplant (E). The cumulative incidence of chronic GVHD at day 180 was 13% (95% CI, 7–23%) (F).

When focusing on differences among the disease groups (Fig 3C), a significantly higher incidence of grade 2–4 GVHD was observed in WAS patients than in non-WAS patients, $P = 0{\cdot}021$. In addition, three of five WAS patients who developed grade 3–4 GVHD died of either GVHD (two patients) or VOD (one patient).

Chronic GVHD was observed in nine patients, and its cumulative incidence at day 180 was 13% (95% CI, 7–23%) (Fig 3F).

Infections

Twenty-eight patients (SCID, 11; WAS, eight; CGD, three and other diseases, six) developed bacterial infection after UCBT. Sixteen of the 28 patients remained alive at the time of data collection.

Fungal infection mainly caused by *Aspergillus* species was observed in eight patients (CGD, three; SCID, two; WAS, two and X-linked hyperIgM syndrome, one). Three of the eight patients died of bacterial infection, bacterial/fungal infection or GVHD.

Twenty patients (SCID, eight; WAS, four; CGD, two; SCN, two and others, four) developed CMV disease after UCBT. CMV was detected before conditioning in all eight SCID patients of which four patients died of CMV disease after transplantation. Twelve of the 20 patients remained alive at the time of analysis.

Other notable virus-related complications were respiratory syncytial virus bronchiolitis accompanied by chronic GVHD in one SCID patient and EBV-PTLD in one patient with Blau syndrome; both infections led to a fatal outcome. One WAS patient had severe haemorrhagic colitis caused by Coxsackie virus B infection, which was treated successfully by infusion of expanded CD4 T cells prepared from the infusion residual of donor cord blood (Tomizawa *et al*, 2005). Another WAS patient had persistent norovirus infection. Interstitial pneumonia not due to CMV or *Pneumocystis* was noted in three patients of which one patient had parainfluenza/rhinovirus infection, while the causative agent for infection in the remaining two patients was not identifiable.

Risk factors for overall mortality

Lastly, we analysed the factors contributing to overall survival. Using univariate analyses, the following were found to be significant contributory factors to a poor prognosis: HLA mismatch of ≥2 antigens, time to transplant >180 d, second or third transplantation, ongoing infection at the time of transplantation, no conditioning for UCBT and diagnosis other than SCID, SCN or WAS (Table III). The dose of transfused nucleated cells or CD34-positive cells did not affect the 5-year OS.

Using multivariate regression analyses, the following were found to be significant contributory factors to patient death: infection at the time of transplantation, no conditioning, HLA mismatch of >2 antigens and diagnosis other than SCID, SCN or WAS (Table IV). RIC was determined to be the favourable factor for patient survival ($P = 0{\cdot}01$) (Fig 4 and Table IV).

Table III. Univariate analyses of factors that contributed to 5-year OS.

Factors	Hazard ratio	95% CI	*P*-value
Age: ≥12 months	1·73	(0·78–3·83)	0·175
Diagnosis			
WAS and SCN	1·00		
SCID	2·34	(0·75–7·36)	0·145
Other diseases	5·39	(1·70–17·0)	0·004*
Nucleic cell dose: ≥$8{\cdot}2 \times 10^7$/kg	1·51	(0·69–3·29)	0·299
CD34 cell dose: ≥$2{\cdot}1 \times 10^5$/kg	0·86	(0·36–2·08)	0·744
HLA disparity			
6/6 matched	1·00		
5/6 matched	1·68	(0·58–4·83)	0·337
4/6 matched	3·78	(1·23–11·60)	0·020*
3/6 matched	3·24	(0·63–16·74)	0·160
4/6 or 3/6 matched	2·64	(1·20–5·83)	0·016*
Time to transplant: ≥180 d	1·89	(0·85–4·17)	0·117
Infection at transplant	6·24	(2·61–14·9)	<0·0001*
Second or third transplantation	3·37	(1·26–9·02)	0·016*
Conditioning			
MAT	1·00		
RIC	0·41	(0·13–1·23)	0·111
No conditioning	2·89	(1·21–6·93)	0·017*

*Significant contributory factors to the poor prognosis.

Discussion

This paper reports the outcome of UCBT for 88 PID patients, the largest cohort of PIDs to receive UCBT to date. The overall survival rate for PID patients undergoing UCBT was comparable to that previously reported for 46 Japanese PID patients undergoing BMT from either HLA-identical siblings or unrelated donors (Sakata *et al*, 2004), and also to that reported by the European Society of Immunodeficiency and other stem cell transplantation centres for PID patients receiving BMT from HLA-matched related donors, HLA-mismatched related donors or unrelated donors (Antoine *et al*, 2003; Rao *et al*, 2005; Dvorak & Cowan, 2008). The time for haematopoietic recovery was comparable to or better than the median recovery time observed in a large cohort of UCBT in children with haematopoietic disorders (Thomson *et al*, 2000; Michel *et al*, 2003) and in adults with leukaemia (Laughlin *et al*, 2004; Atsuta *et al*, 2009). The incidence of grade 2–4 GVHD (28%) in UCBT was lower compared with that reported in unrelated donor BMT in PID patients in Japan (47%) (Sakata *et al*, 2004), with that reported in BMT in 90 SCID patients (34%) (Neven *et al*, 2009) and with that observed in the studies of UCBT for childhood haematological malignancies (Thomson *et al*, 2000; Michel *et al*, 2003; Sawczyn *et al*, 2005). The incidence of chronic GVHD (13%) after UCBT was slightly

Table IV. Multivariate analyses of factors that contributed to 5-year OS.

Factors	HR	95% CI	*P*-value
Diagnosis			
WAS and SCN	1·00		
SCID	1·71	(0·39–7·38)	0·475
Other diseases	7·50	(2·06–27·19)	0·002*
HLA disparity			
6/6 matched	1·00		
5/6 matched	1·53	(0·50–4·66)	0·454
4/6 matched	5·64	(1·66–19·14)	0·006*
3/6 matched	1·04	(0·68–23·96)	0·124
4/6 or 3/6 matched	3·87	(1·63–9·19)	0·002*
Infection at transplant	4·61	(1·74–12·16)	0·002*
Conditioning			
MAT	1·00		
RIC	0·20	(0·06–0·69)	0·011†
No conditioning	4·87	(1·79–13·3)	0·002*

*Significant contributory factors to an unfavourable prognosis.
†Significant contributory factors to a favourable prognosis.

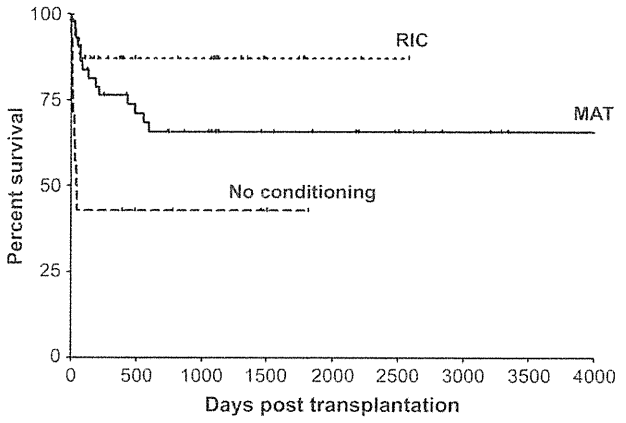

Fig 4. Kaplan–Meier estimates of overall survival after umbilical cord transplantation. Comparison of overall survival between reduced intensity conditioning (RIC), myeloablative therapy (MAT), and no conditioning is shown. For 5-year OS, MAT *versus* RIC, $P = 0{\cdot}111$, MAT *versus* no conditioning, $P = 0{\cdot}017$ in univariate analysis.

lower than that after URBMT in PID patients in Japan (20%) (Sakata *et al*, 2004), and was lower compared to that in UCBT studies for childhood leukaemia (Michel *et al*, 2003; Sawczyn *et al*, 2005). Thus, UCBT in PID patients in the present study was associated with a good survival rate, good engraftment rate, rapid haematological recovery and a lower incidence of acute and chronic GVHD.

Given that the 5-year OS for SCID patients (71%) was better than that for SCID patients receiving bone marrow from HLA-mismatched related donors in both Japan (5-year OS, 36%, Imai, Morio, Kamachi, Kumaki, Ariga, Nonoyama, Miyawaki, and Hara, unpublished observations) and Europe (5-year OS, 52%, Antoine *et al*, 2003), UCBT would be particularly beneficial for patients requiring rapid access to donor units yet lacking a matched related donor.

The present study found that several key risk factors were associated with overall mortality. First, infection was the major cause of mortality during the first 100 d after UCBT in PID patients, and the frequency was much higher than that observed in other disorders following UCBT (Rocha & Gluckman, 2006; Kurtzberg *et al*, 2008, Szabolcs *et al*, 2008). As predicted and reported in previous studies (Antoine *et al*, 2003; Cuvelier *et al*, 2009), infection at the time of transplantation was associated with poor survival ($P < 0{\cdot}0001$), suggesting that the control of pre-existing infection at the time of UCBT is critically important.

Eight of 11 SCID patients who had active infection, mainly CMV pneumonia, died before day 50, while 26 of 28 patients without infection at the time of UCBT remained alive at the time of data collection. UCBT without conditioning was selected for 12 patients, of which seven had CMV infection and one had *Pneumocystis* pneumonia at the time of transplantation. Six out of the seven patients died of CMV infection; and one patient with *Pneumocystis* pneumonia did not survive UCBT.

UCBT in WAS patients achieved a good 5-year OS, as reported in a previous study of 15 cases (Kobayashi *et al*, 2006). One of the key factors would have been the time from diagnosis to transplantation. In our WAS patients, UCBT was performed at a median age of 14 months (range, 4–84 months), when most patients were thrombocytopenic, but did not yet have uncontrolled infection or autoimmunity.

Four CGD patients died of bacterial or fungal infection without engraftment. Although these patients were not categorized as those with active infection at the time of transplantation, they required intravenous administration of antimicrobial and antifungal agents before and after transplantation.

Second, HLA disparity was a risk factor associated with overall mortality. Lower survival was observed in UCB recipients transplanted with a ≥2 antigen-mismatched graft compared with those transplanted with a <2 antigen-mismatched graft [Hazard Ratio (HR) = 3·87, $P = 0{\cdot}002$]. Although no difference was observed in 5-year OS between recipients of HLA-matched and those of HLA 1-antigen mismatched UCBT in the present study, we would need data from a larger number of patients with information on more extensive and sensitive HLA typing to discuss the impact of fully matched HLA on transplant outcome.

Finally, non-SCID/SCN/WAS patients showed a significantly lower survival rate (HR = 5·40, $P < 0{\cdot}0001$ by multivariate analyses). Although a previous large-scale study showed that results of HSCT according to disease did not show obvious disease-specific findings (Antoine *et al*, 2003), it is not yet known if UCBT is suitable for all types of PIDs. This may indicate donor source other than UCB is preferable for certain types of PID. Although the success of UCBT noted for X-linked hyperIgM syndrome, bare lymphocyte syndrome and

X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency (Tono *et al*, 2007) is encouraging, optimization of transplantation procedures and determination of suitable timing for UCBT may be necessary for this group of patients. Alternatively, this may simply indicate an expansion of transplantation to less favourable clinical conditions or to less favourable transplantation conditions. Studies on a larger cohort are necessary for drawing any conclusion on whether diagnosis is significant overall.

Recent studies suggest improved survival after BMT for PID with the RIC regimen; however, to date, comparison of CBT using RIC *versus* MAT has not been made. In our study, 87% of patients on the RIC regimen and 66% on the MAT regimen remained alive at the latest follow-up. Multivariate analyses revealed that the RIC regimen is associated with a higher 5-year OS than the MAT regimen (HR = 0·20, $P = 0·011$). Although it is premature to conclude that RIC provides an equal or superior outcome to MAT for all PID patients, non-myeloablative treatment may be beneficial at least for certain types of PID. RIC was selected preferentially in SCID and CGD patients, with good survival rates: 17 of 18 SCID patients and three of four CGD patients remain alive. As a result of this, we are in the process of initiating a clinical trial of UCBT with RIC in SCID patients. On the other hand, only two of 23 WAS patients received RIC. Our previous data showed that a conditioning regimen other than BU/CY or BU/CY/ATG was the only independent factor associated with failure in HSCT for WAS patients (Kobayashi *et al*, 2006). However, whether this holds true for UCBT in younger WAS patients should be determined.

Notably, although the outcome of UCBT for WAS in this cohort was excellent compared with that from previously reported HSCT results using different donor sources (Kobayashi *et al*, 2006; Friedrich *et al*, 2009), UCBT in WAS patients was associated with a high rate of grade 2–4 acute GVHD (11 of 23 patients) and a post-transplant infectious episode (13 of 23 patients). Eight patients experienced bacteraemia/sepsis and six suffered a viral infection (CMV pneumonia, four; Coxsackie virus enterocolitis, one and persistent norovirus infection, one). The high rate of serious infections and GVHD in WAS patients after transplantation warrants further study in search of preventive measures that might include RIC for severe, transplantation-related toxicities.

Long-term follow-up of the clinical and immunological status is necessary when considering the lifespan of PID patients. Recent studies on the long-term outcome after HSCT for SCID revealed the presence of relatively late complications, such as chronic GVHD, autoimmune events, severe or recurrent infections, chronic human papilloma virus infection, nutritional problems and late rejection in 50% of patients (Mazzolari *et al*, 2007; Neven *et al*, 2009). Similarly, long-term follow-up of HSCT in WAS patients revealed that 20% of patients developed chronic GVHD-independent autoimmunity (Ozsahin *et al*, 2008). One possible measure that might be taken to avoid the chronic problems associated with CBT would be to select a HLA-matched UCB unit, as HLA disparity was a risk factor for both overall survival and the development of GVHD in our study. The advantage of RIC over MAT in preventing late complications needs careful assessment, together with data on mortality, engraftment and early post-transplant complications.

Finally, the issue of SCID patients who died before or without receiving SCT, most likely due to uncontrolled infection, still remains unresolved. This suggests that the early diagnosis of SCID and prevention of opportunistic infection within a protected environment and the administration of appropriate prophylactic drugs is critically important for the improvement of survival in SCID patients in general. To that end, neonatal screening with the employment of T cell receptor excision circles should be beneficial for an improved outcome in SCID patients (McGhee *et al*, 2005; Morinishi *et al*, 2009).

We report the results of UCBT for 88 PID patients in Japan. Despite the limitations of a retrospective, non-randomized study, our study suggests that unrelated umbilical cord blood can be considered as a promising stem cell source for children with congenital immunodeficiency when a HLA-matched related donor is not available.

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Analysis of mutations and recombination activity in RAG-deficient patients

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Abstract Mutations in the recombination activating genes (*RAG1* or *RAG2*) can lead to a variety of immunodeficiencies. Herein, we report 5 cases of RAG deficiency from 5 families: 3 of Omenn syndrome, 1 of severe combined immunodeficiency, and 1 of combined immunodeficiency with oligoclonal TCRγδ⁺ T cells, autoimmunity and cytomegalovirus infection. The genetic defects were heterogeneous and included 6 novel *RAG* mutations. All missense mutations except for Met443Ile in *RAG2* were located in active core regions of *RAG1* or *RAG2*. V(D)J recombination activity of each mutant was variable, ranging from half of the wild type activity to none, however, a significant decrease in average recombination activity was demonstrated in each patient. The reduced recombination activity of Met443Ile in *RAG2* may suggest a crucial role of the non-core region of RAG2 in V(D)J recombination. These findings suggest that functional evaluation together with molecular analysis contributes to our broader understanding of RAG deficiency.

1. Introduction

V(D)J recombination mediated by the recombination activating genes (RAG) 1 and RAG2 leads to the generation of diverse antigen receptors [1]. A complete lack of RAG activity causes severe combined immunodeficiency (SCID) with the

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absence of mature T and B cells, but the presence of natural killer (NK) cells (T^-B^- SCID) [2], whereas partial loss results in variant syndromes, such as Omenn syndrome (OS) [3] or combined immunodeficiency (CID) presenting with oligoclonal TCR$\gamma\delta^+$ T cells, autoimmunity and cytomegalovirus (CMV) infection (CID with $\gamma\delta$/CMV) [4,5]. OS is characterized by early-onset generalized erythroderma, lymphadenopathy, hepatosplenomegaly, protracted diarrhea, failure to thrive, eosinophilia, hypogammaglobulinemia, elevated serum IgE levels, the absence of B cells, and the presence of activated and oligoclonal T cells [6]. In contrast to T^-B^- SCID and OS, patients affected with CID with $\gamma\delta$/CMV exhibit autoimmune cytopenias, B cells, normal immunogulobulin levels, oligoclonal TCR$\gamma\delta^+$ T cells, and disseminated CMV infections [4,5]. Very recently, another distinct clinical syndrome caused by hypomorphic *RAG* mutations has been described. Schuetz et al. [7] reported 3 patients with late age of onset of illness characterized by hypogammaglobulinemia, diminished numbers of T and B cells, and the formation of granulomas in the skin, mucous membranes and internal organs. De Ravin et al. [8] described an adolescent patient presenting with destructive midline granulomatous disease who also exhibited autoimmunity, relatively normal numbers of T and B cells, and a diverse T-cell receptor (TCR) repertoire.

Herein, we report the identification of 8 *RAG* mutations including 6 novel mutations in a group of patients presenting with a variety of clinical phenotypes, and discuss the functional significance of these mutations by using the V(D)J recombination assay.

2. Materials and methods

2.1. Patients

We studied five patients with RAG deficiency from five families. Table 1 presents the immunological features of the patients. All patients except for patient 5 were born to non-consanguineous Japanese parents. The clinical and immunological data of patient 1 and patient 3 have been reported elsewhere [9]. Patient 2 was a 1-month-old boy who presented with generalized erythroderma, hepatosplenomegaly and *Pseudomonas aeruginosa* sepsis. Laboratory studies revealed hypereosinophilia, hypogammaglobulinemia, lack of B cells, and oligoclonal expansion of activated TCR$\alpha\beta^+$ T-cells. These findings were consistent with typical features of OS. Patient 4 was a 2-year-old girl who presented with prolonged diarrhea, bronchopneumonia, liver dysfunction and CMV infections. CMV was detected in her stool and sputum. Laboratory analysis revealed lymphopenia with normal immunoglobulin levels, an increased percentage of TCR$\gamma\delta^+$ T cells (61.7% of $CD3^+$), and multiple autoantibodies including anti-nuclear, anti-DNA, and antiparietal cell antibodies and Coombs test. In addition, IgG antibody against CMV was detected (20.7; normal, <2.0). Her elder sister suffered from autoimmune hemolytic anemia and immune mediated thrombocytopenia, and died of fatal interstitial pneumonia of adenovirus at age of 1 year. Patient 5 was the fourth child born to non-consanguineous parents of Indian origin. All of her 3 siblings were affected with immunodeficiency and died within the first year of life. Patient 5 showed lymphopenia, very low numbers of autologous T and B cells, preserved numbers of NK cells, and the presence of maternal $CD4^+$ T cell engraftment. At the age of 2 months, she remained asymptomatic except for oral thrush and microcephaly.

Table 1 Immunological features of the patients at diagnosis.

Patient	1[a]	2	3[a]	4	5
Diagnosis	OS	OS	Atypical OS	CID with $\gamma\delta$/CMV	Atypical SCID with MFT
Age at onset (month)	0	0	7	8	0
WBC	26,900	19,000	2800	3900	3280
Lymphocytes (/mm^3)	8339	5700	1300	546	459
$CD3^+$ (%)	84.8	41.3	20.0	53.9	7.8
$CD4^+$ (%)	56.7	16.6	17.3	9.9	7.4
$CD8^+$ (%)	27.0	37.8	1.3	35.4	0.1
$CD19^+$ or 20^+ (%)	0.0	0.2	0.1	11.6	0.1
IgG (mg/dl)	461	220	328	678	1475
IgA (mg/dl)	<4	<1	62	63	114
IgM (mg/dl)	<4	<2	31	65	147
IgE (IU/ml)	7	<2	16	NA	NA

OS, Omenn syndrome; CID, combined immunodeficiency; $\gamma\delta$, TCR$\gamma\delta^+$ T cells; CMV, cytomegalovirus; SCID, severe combined immunodeficiency; MFT, maternal T-cell engraftment; WBC, white blood cells; NA, not available.

[a] Data of patient 1 and patient 3 have been reported previously [9].

Approval for this study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

2.2. Mutation analysis of RAG1 and RAG2

DNA was extracted from blood samples using standard methods. The *RAG1* and *RAG2* genes were amplified in several segments from genomic DNA using specific primers, as previously described [10,11]. Sequencing was performed on purified polymerase chain reaction (PCR) products using the ABI Prism BigDye Terminator Cycle sequencing kit on an ABI 3100 automated sequencer (Applied Biosystems, Foster, CA).

2.3. V(D)J recombination assay

In vivo V(D)J recombination assay was performed by using the recombination substrate pJH200 as described previously with modifications [3,12]. The complete open reading frames of human *RAG1* and *RAG2*, and the active core regions of mouse *RAG1* (aa 330–1042) and *RAG2* (aa 1–388) were subcloned into the mammalian expression vector pEF-BOS [13]. PCR products carrying the patients' mutations were also subcloned into the vector. Cotransfections of full-length human *RAG1*, the mouse *RAG2* active core, and pJH200, or of full-length human *RAG2*, the mouse *RAG1* active core, and pJH200 into 293T cells were performed using 1 μg of each plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Cells were harvested after 48-hours of culture, and the recombined products of signal joints were analyzed for recombination frequency by PCR using primers RA-CR2 and RA-14 [14]. After 30 cycles, the amplified products were visualized by ethidium bromide staining, and the intensity of each band was quantified using Image J software (NIH, Bethesda, MD).

2.4. Analysis of IgE production and somatic hypermutation (SHM) in variable regions of IgM

Peripheral blood mononuclear cells were isolated and incubated with 500 ng/ml of anti-CD40 (Diaclone, Besançon, France) and 100 U/ml of recombinant interleukin-4 (IL-4; R&D Systems, Minneapolis, MN) for 12 days. IgE production in culture supernatants was determined by enzyme-linked immunosorbent assay as previously described [15,16]. The frequency and characteristics of SHM in the V_H3-23 region of IgM were studied in purified $CD19^+$ $CD27^+$ B cells as previously described [15,16].

3. Results

3.1. RAG mutations

As shown in Table 2, we found 2 missense and 1 nonsense mutations in *RAG2* and 4 missense and 1 nonsense mutations in *RAG1*. Two distinct novel *RAG2* mutations, R73H and Q278X, were demonstrated in patient 1. Patient 2 was found to be homozygous for a novel M443I mutation in *RAG2*. Patient 3 was a compound heterozygote bearing R142X and R396H mutations in *RAG1*. The latter mutation has been repeatedly reported in OS patients [17]. Patient 4 was a compound heterozygote bearing R474C and L732P mutations in *RAG1*. These missense mutations are novel, although similar missense mutations, R474S, R474H and L732F, have been reported in patients with RAG deficiency [17–19]. Patient 5 carried a homozygotic novel E770K mutation in *RAG1*. All missense mutations but one (M443I in *RAG2*) were located in the active core regions of *RAG1* or *RAG2*, and all patients had at least one missense mutation. None of these mutations were found in 100 alleles of healthy controls.

Table 2 *RAG* mutations and recombination activity.

Patient	Gene	Nucleotide mutation	Effect	Relative recombination activity (%)[a]
1	RAG2	1419 G>A	R73H	59.3±4.7
		2033 C>T	Q278X	0.4±0.3
2	RAG2	2530 G>T[b]	M443I	8.7±1.2
3	RAG1	536 C>T	R142X	51.2±9.2
		1299 G>A	R396H	1.0±0.5
4	RAG1	1532 C>T	R474C	47.2±7.9
		2307 T>C	L732P	0.5±0.4
5	RAG1	2420 G>A[b]	E770K	15.6±9.1
Control	RAG2	wild type	–	100
	RAG1	wild type	–	100

[a] Data are expressed as the percentage of activity as compared with that of the wild type protein, and represent the mean±standard deviation of three independent experiments.
[b] Homozygous mutation.

3.2. Recombination activity of RAG mutants

To elucidate the pathogenic significance of these novel mutations, we performed V(D)J recombination assay using the artificial extrachromosomal rearrangement substrate (Table 2). As expected, the recombined products were amplified from 293T cells transfected with both wild type RAG1 and RAG2, and no products were obtained from 293T cells transfected with either RAG1 or RAG2 (Fig. 1). Although the relative recombination activity of each mutant was variable, ranging from about half of the wild type activity to none, a significant decrease in average recombination activity was demonstrated in each patient (Fig. 1 and Table 2). The effects of the patients' missense mutations were also evaluated by the web-based analysis tools including Mutation@A Glance (http://rapid.rcai.riken.jp/mutation/) [20] and MutationTaster (http://www.mutationtaster.org/) [21]. Mutation@A Glance predicted all the mutation except for the E770K in *RAG1* to be deleterious on the basis of the SIFT program [22], whereas MutationTaster predicted all the missense mutations to be disease-causing.

3.3. B cell analysis of patient 4

The percentages of IgD^- $CD27^+$ and IgD^+ $CD27^+$ cells within $CD19^+$ B cells from patient 4 were found comparable to controls (Fig. 2A) [23]. After stimulation with anti-CD40 and IL-4, B cells from patient 4 produced levels of IgE equivalent to normal, indicating their capability of undergoing class

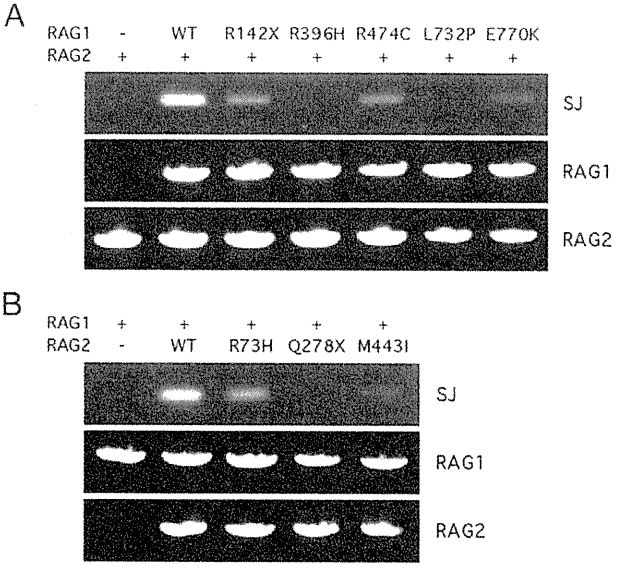
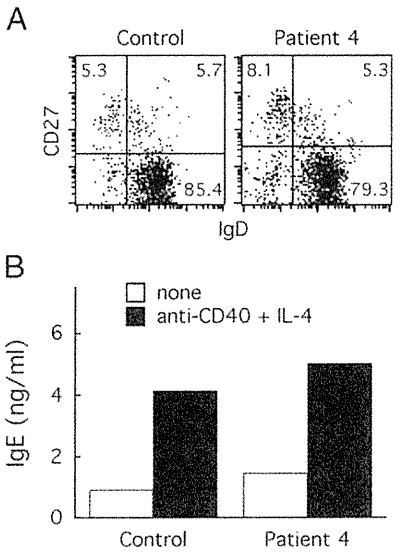


Figure 1 V(D)J recombination assay. V(D)J recombination activity was assessed by using the recombination substrate pJH200 in 293T cells that were cotransfected with mutant RAG1 and wild type RAG2 (A), or with wild type RAG1 and mutant RAG2 (B). Recombined products (signal joints, SJ) were analyzed by PCR (top). The presence of RAG1 and RAG2 was verified by vector specific PCR (middle and bottom).



C

%	Patient 4	Controls
SHM	4.8	2.6-6.3
GC target	56.0	62-66
GC transition	71.4	57-63
AT transition	50.0	33-67

To / From

%	A	C	G	T	Total
A		5	13	10	28
C	2		6	11	19
G	29	6		2	37
T	2	9	5		16

Patient 4

%	A	C	G	T	Total
A		6.2	12.9	4.1	23.2
C	2.6		6.0	11.4	20.0
G	26.1	13.2		4.3	43.6
T	4.8	5.5	3.0		13.2

Control

Figure 2 B cell analysis of patient 4. (A) B cell subpopulations. Peripheral bloods were stained with FITC-labeled anti-IgD, PE-labeled anti-CD27, and APC-labeled anti-CD19 monoclonal antibodies. The dot plot of immunofluorescence profiles of IgD and CD27 expression within $CD19^+$ B cells is shown. The number indicates the percentage of cells in each quadrant. (B) IgE production. After stimulation of peripheral blood mononuclear cells with anti-CD40 and IL-4 for 12 days, concentrations of IgE in the culture medium were quantified. (C) The frequency and pattern of somatic hypermutation in the V_H3-23 region of the IgM in memory B cells. RT-PCR products amplified from purified $CD19^+$ $CD27^+$ B cells by using V_H3-23 and $C\mu$ primers were subcloned and sequenced. Nucleotide changes were evaluated and shown as percentages.

switch recombination and IgE synthesis *in vitro* (Fig. 2B). In addition, the frequency and nucleotide substitution patterns of SHM were similar to those of healthy individuals (Fig. 2C).

4. Discussion

RAG deficiency has been considered to display a range of phenotype from classical T^-B^- SCID (complete RAG deficiency) to OS (partial RAG deficiency), depending on residual V(D)J recombination activity [24]. Atypical SCID/OS or leaky SCID may be also diagnosed in patients who show incomplete clinical and immunological characteristics and do not fulfill the criteria for SCID or OS [17]. However, it has recently been recognized that the clinical spectrum of RAG deficiency is much broader and includes CID with γδ/CMV [4,5], and CID with granulomatous inflammation [7], or destructive midline granulomatous disease [8]. In the present study, we studied 5 cases of RAG deficiency including 3 of OS, 1 of CID with γδ/CMV, and 1 of SCID with maternal T-cell engraftment, and identified 6 novel and 2 recurrent *RAG* mutations in these patients.

Hypomorphic RAG mutations leading to immunodeficiency have been shown to have up to 30% of wild type RAG activity by V(D)J recombination assay [7]. Although the R73H mutation in *RAG2* from patient 1, the R142X mutation in *RAG1* from patient 3, and the R474C mutation in *RAG1* from patient 4 exhibited around half of the wild type activity, all of these patients also had mutations with extremely low levels of recombination activity on the other allele, resulting in a substantial decrease in the average recombination activity due to a tetrameric complex formation of RAG1 and RAG2 during V(D)J recombination [1]. Similar results were obtained from an investigation of a RAG-deficient patient with destructive granulomatous disease who carried a W522C mutation with half of the recombination activity and a L541CfsX30 mutation with no recombination activity in *RAG1* [8]. It therefore seems reasonable that the clinical phenotype of partial RAG deficiency in patients 1, 3 and 4 is a consequence of these combinations of the mutations.

Biochemical studies have identified the core regions of RAG1 and RAG2 that are the minimal regions necessary for recombination of exogenous plasmid substrates *in vivo* and for DNA cleavage *in vitro* [1]. The M443I missense mutation demonstrated in patient 2 was located in the noncanonical plant homeodomain (PHD) of the non-core region of RAG2. Recent evidence indicates the importance of the non-core regions of RAG1 and RAG2 in V(D)J recombination and lymphocyte development [25]. The PHD of RAG2 has been shown to play crucial roles for chromatin and phosphoinositide binding, regulation of protein turnover, and cellular localization of RAG2 [26]. Additionally, the PHD of RAG2 is known to recognize histone H3 that has been trimethylated at the lysine at position 4 by interacting with 4 essential amino acids, Y415, M443, Y445, and W453 [27]. To date, 8 mutations of the non-core region in *RAG2* (W416L, K440N, W453R, A456T, C446W, N474S, C478Y, and H481P) have been reported in patients with T^-B^- SCID or OS [28]. A significant decrease in recombination activity of the M443I mutation from our patient further supports the important role of PHD of RAG2 in regulating V(D)J recombination.

Although the R142X nonsense mutation found in the N-terminal domain of RAG1 in patient 3 should have resulted in a complete loss of function, it remained partially functional for recombination unlike the Q278X mutation in RAG2 in our assay. On the other hand, the same R142X mutation has been described in a typical OS patient who also had a nonfunctional frameshift mutation in the core region of RAG1 on the other allele, thus suggesting that the residual V(D)J recombination activity exists

with the R142X mutation [29]. One explanation for these findings is alternative usage of methionine as a translation start site, which has been reported in OS patients with N-terminal RAG1 frameshift mutations [30,31]. A translation start prediction program NetStart 1.0 also indicated that methionines at codon 183 and 202, which were the first and second methionines found after the R142X mutations, could be alternative translation start sites with scores comparable to the conventional initiator codon 1 (http://www.cbs.dtu.dk/services/NetStart/) [32]. Therefore, it is possible that an N-terminal truncated and partially functional RAG1 protein generated by alternative usage of methionine led to the OS phenotype in our patient.

The clinical features of patient 4 were consistent with CID with γδ/CMV. Despite decreased recombination activity, patient 4 exhibited normal immunogulobulin levels and a normal percentage of peripheral B cells. These findings were in contrast to SCID and OS, but were in agreement with previously described cases of this disease [4,5]. Moreover, our B cell analysis of patient 4 revealed normal maturation, normal production of IgE after stimulation with anti-CD40 and interleukin-4, and normal somatic hypermutation in $CD27^+$ B cells. Taken together, our case provided additional data of the genetic and immunological features of this unique disease.

RAG mutations found in patients with typical T^-B^- SCID have been usually shown to abrogate recombination activity almost completely [2,33]. The residual V(D)J recombination activity resulting from the E770K mutation in RAG1 was associated with the SCID phenotype in patient 5. Despite trends towards more severe mutations, such as nonsense and frameshift mutations in SCID patients, missense mutations can lead to the SCID phenotype [33]. It is also known that the same mutations may cause different clinical phenotypes, presenting as either T^-B^- SCID or OS [18], and as either T^-B^- SCID or CID with γδ/CMV even within one family [34,35]. These findings suggest that that residual V(D)J recombination activity may not be solely responsible for the disease development. Further studies will be necessary to assess additional factors that influence the clinical phenotype of RAG deficiency.

In summary, our studies demonstrated the pathogenic significance of the 8 RAG mutations including 6 novel mutations from 5 patients with RAG deficiency. The characterization of the genetic defects and functional abnormalities in RAG-deficient patients will help define the role of RAG in V(D)J recombination and may lead to a better understanding of the variable phenotypic expression in RAG deficiency.

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