

**Fig. 4.** Sequential analysis of neutrophil differentiation from hiPSCs. (A–C) Sequential morphological analysis of day 10 + 10 (A), day 10 + 20 (B) and day 10 + 30 (C). Scale bars: 10  $\mu$ m. (D) Surface antigen expression at each level of differentiation of hiPSC-derived cells was analyzed by flow cytometry. All adherent cells including OP9 cells were harvested and stained with antibodies. Human CD45<sup>+</sup>DAPI<sup>-</sup> cells were gated as hiPSC-derived viable leukocytes ( $n = 3$ ; bars represent SDs). (E–F) Sequential RT-PCR analysis of a pluripotency marker (E), genes associated with neutrophil development and neutrophils-specific granules (F) during differentiation. Human GAPDH was used as a loading control. Abbreviations: BM, human bone marrow cells; 253G4, undifferentiated 253G4 cells; 10 + 0, sorted VEGFR2<sup>high</sup>CD34<sup>+</sup> cells after 10 days differentiation; 10 + 10, 20, 30, all cells after 10, 20, 30 days differentiation after cell sorting; hGAPDH, human GAPDH; mGAPDH, mouse GAPDH. The figures are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

## Discussion

The analysis of the differentiation process of neutrophils can provide helpful information for the elucidation of the pathogenesis of hematopoietic diseases that affect neutrophils and/or myeloid differentiation, including inherited bone marrow failure syndromes and neutrophil function disorders. Traditionally, HL-60, an acute promyelocytic cell line, has been used as a neutrophil differentiation model (Collins et al., 1978; Newburger et al., 1979). Although this cell line grows well and differentiates easily into neutrophils, the neutrophil differentiation model is not suitable for the analysis of neutrophil-affected disorders because of its leukemic cell-origin. Development of a neutrophil differentiation system based on iPS cells would provide a better model for the analysis of such diseases, because iPS cells can be generated from the somatic cells of patients suffering from these diseases.

The current study aimed to investigate two issues in hiPSC-derived neutrophil differentiation: tracking the step-wise maturation in vitro and evaluating the wide spectrum of neutrophil functions. Through the use of a modified OP9 co-culture system, the directed and step-wise differentiation from hiPSCs to mature neutrophils containing neutrophil specific granules was first accomplished. The expression of surface antigens, transcription factors and granule proteins during differentiation exhibited the characteristic pattern of normal granulopoiesis. The biological functions of hiPSC-derived neutrophils were demonstrated through the quantitative assessment of granule enzyme activities and biological bactericidal activities such as chemotaxis and phagocytosis.

Defects in the maturation and function of neutrophils are associated with certain blood diseases including inherited bone marrow failure syndromes and neutrophil function disorders.

Among bone marrow failure syndromes, certain conditions affect a specific maturation stage, such as the maturation arrest at the promyelocyte/myelocyte stage seen in severe congenital neutropenia. Neutrophil function disorders can affect specific bactericidal activities, such as the absence of MPO activity characteristic of MPO deficiency disorders. The use of hiPSCs for the investigation of these diseases requires sequential analyses that can identify each neutrophil maturation stage and include a functional analysis to evaluate each bactericidal activity separately on disease-specific, iPSC-derived neutrophils. Although previous studies have reported neutrophil differentiation models from hESCs (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009) and hiPSC-derived neutrophils have been shown before (Choi et al., 2009), evidence showing that hiPSCs, which are artificially reprogrammed somatic cells, can follow the normal developmental pathway into fully functional mature neutrophils is of great significance, and the description of methods for identifying each neutrophil maturation step and analyzing each bactericidal pathway separately is important for clinical applications.

Although flow-cytometric analysis combined with RT-PCR identified the neutrophil maturation step relatively successfully, discrepancies between the neutrophil differentiation system in this study and normal granulopoiesis were noted such as the lower expression of CD16 than that shown by previous reports on hESC-derived neutrophils (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009). As CD16 is a mature neutrophil marker in peripheral blood, two reasons could explain this phenomenon. First, residual precursors could have been more significant contaminants in the present system than in previously reported methods due to the function of cytokines and stroma supporting immature hematopoietic cells. Another possible reason is the shift of protein types between membrane-bound and soluble forms. Calluri previously reported that G-CSF is not only a myeloid cell growth factor, but also a modulator of neutrophil behavior (Carulli, 1997), and its stimulation decreases the membrane bound CD16 and increases its soluble form. Low CD16 expression has been documented in neutrophils derived *in vitro* from bone marrow CD34<sup>+</sup> cells by stimulation with G-CSF (Kerst et al., 1993b), and it has been observed *in vivo* when G-CSF is administered to healthy volunteers (Kerst et al., 1993a). This phenomenon, which is also documented in a report of hESC-derived neutrophils (Yokoyama et al., 2009), is unavoidable in differentiation culture systems using recombinant cytokines. The combination of flow cytometric and PCR analyses enables a more accurate staging of progenitors that could be of importance in the investigation of maturation arrest in future studies.

The culture system presented in this study is considered ineligible for clinical applications due to the use of xenogenic factors such as OP9 cells and FCS. To overcome this problem, a xeno-free hematopoietic differentiation system from pluripotent cells is currently being established.

In conclusion, the present study shows the establishment of a fully functional mature neutrophil differentiation system from hiPSCs and the detailed analysis of their function and differentiation process. This system could become a useful tool for the investigation of various hematological diseases with defects in maturation and function of neutrophils.

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# 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,  $\geq 2$  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.

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  $0.5 \times 10^9/l$  for three consecutive days. Platelet recovery was defined by a count of at least  $20 \times 10^9/l$ , 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)  $\pm$  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 ( $\times 10^7/\text{kg}$ ) (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- $\kappa$ -B essential modulator) deficiency, IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome, Idiopathic CD4 lymphopenia and Blau syndrome.

(TLI), BU/CY + ATG  $\pm$  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)  $\pm$  low-dose TBI or TLI, Flu/BU  $\pm$  TLI or Flu/CY (50–60 mg/kg)  $\pm$  low-dose TBI/TLI, Flu + low-dose TBI or Flu + ATG were categorized as receiving reduced-intensity conditioning (RIC). L-PAM dose was  $\leq 140 \text{ mg/m}^2$  in patients receiving RIC.

GVHD was graded according to the standard criteria (Przepiora *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 ( $P < 0.05$ ) or marginally significant ( $P < 0.1$ ) 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  $< 8.2 \times 10^7/\text{kg}$  vs.  $\geq 8.2 \times 10^7/\text{kg}$  and CD34 cell dose of  $< 2.1 \times 10^5/\text{kg}$  and  $\geq 2.1 \times 10^5/\text{kg}$ , 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  $31.1 \times 10^7/\text{kg}$ , with a median of  $8.60 \times 10^7/\text{kg}$ . No correlation was observed between the cell dose infused and engraftment.

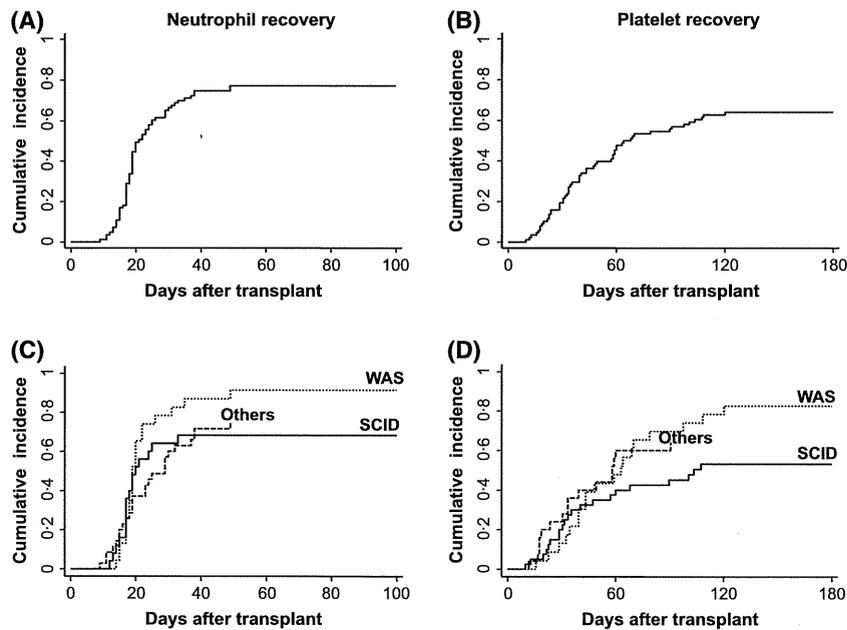


Fig 1. Cumulative incidence of neutrophil and platelet recovery after UCBT. (A) The cumulative incidence of neutrophil recovery 77% (95% CI, 66–85%). (B) The cumulative incidence of platelet recovery 56% (95% CI, 45–65%). The cumulative incidence of neutrophil (C) and platelet (D) recovery according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

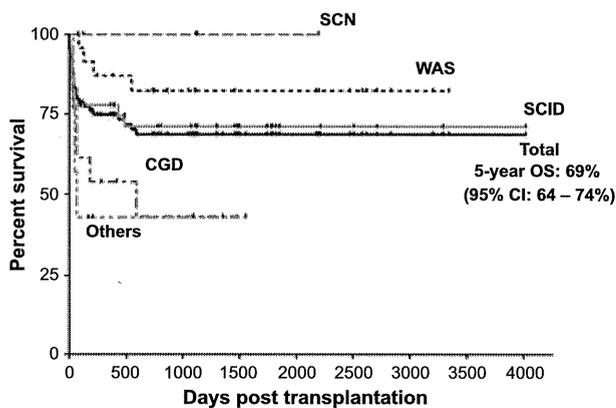


Fig 2. Kaplan–Meier estimates of overall survival after umbilical cord transplantation.

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 ( $\leq 28$  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)			Infection at CBT (N)	Cause of death (<day 100)			Cause of death (≥day 100)	
			<28 (N)	<100 (N)	≥100 (N)		Bac/Fung infection (N)	Viral infection (N)	Others (N)	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; PTLT, 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 ( $P = 0.071$ ) (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.

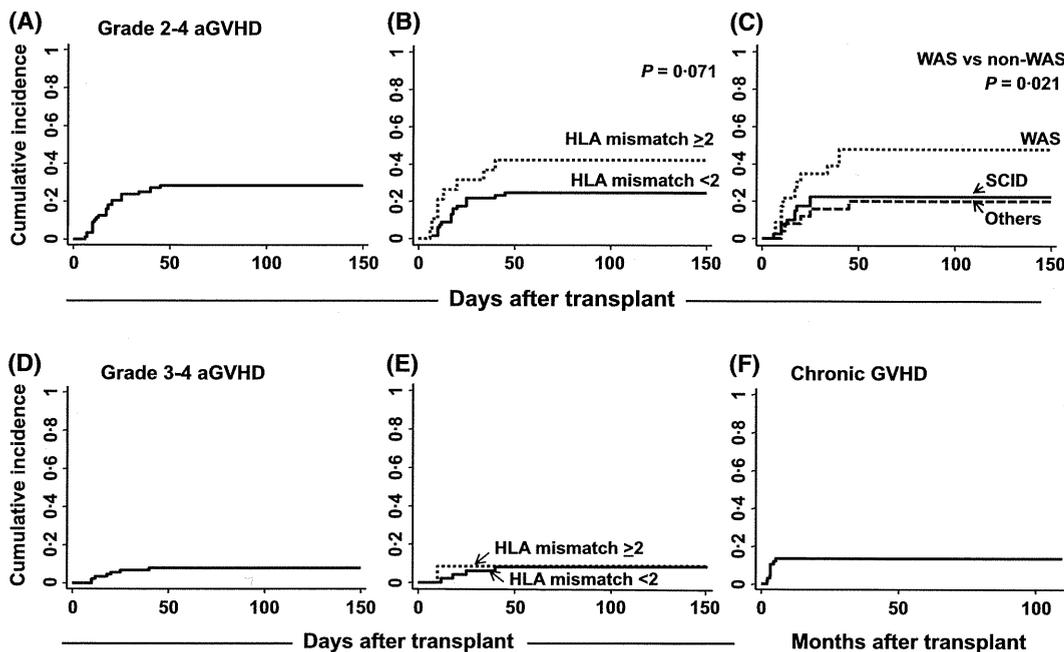


Fig 3. Cumulative probability of acute and chronic GVHD after UCBT. The cumulative incidence of grade 2–4 acute GVHD (aGVHD) at day 100 was 28% (95% CI, 19–38%) (A). The incidence was higher in transplantation mismatched for ≤2 antigens (B) and in that for WAS patients (C). The cumulative incidence of 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,  $P = 0.021$ . 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  $\geq 2$  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

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

Factors	Hazard ratio	95% CI	P-value
Age: $\geq 12$ 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: $\geq 8.2 \times 10^7$ /kg	1.51	(0.69–3.29)	0.299
CD34 cell dose: $\geq 2.1 \times 10^5$ /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: $\geq 180$ 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.

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 ( $P = 0.01$ ) (Fig 4 and Table IV).

### 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
<b>Diagnosis</b>			
WAS and SCN	1.00		
SCID	1.71	(0.39–7.38)	0.475
Other diseases	7.50	(2.06–27.19)	0.002*
<b>HLA disparity</b>			
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*
<b>Conditioning</b>			
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.

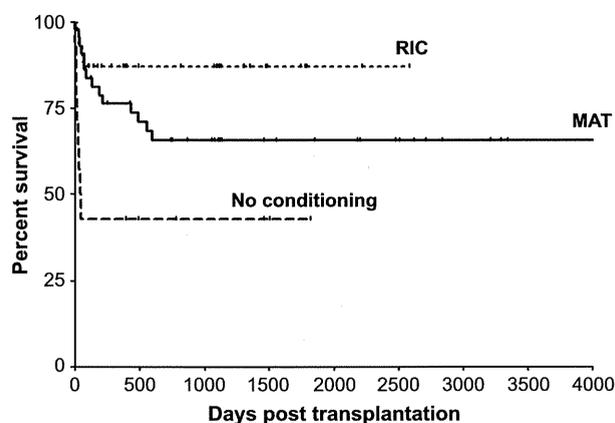


Fig 4. Kaplan–Meier estimates of overall survival after umbilical cord transplantation. Comparison of overall survival between reduced intensity conditioning (RIC), myeloablative therapy (MAT), and no conditioning is shown. For 5-year OS, MAT versus RIC,  $P = 0.111$ , MAT versus no conditioning,  $P = 0.017$  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 ( $P < 0.0001$ ), 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  $\geq 2$  antigen-mismatched graft compared with those transplanted with a  $< 2$  antigen-mismatched graft [Hazard Ratio (HR) = 3.87,  $P = 0.002$ ]. 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,  $P < 0.0001$  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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Y. A., D. T., T. N.-I., K. Kato, and S. K. analysed the data; K. Kato, T. N.-I., T. A., T. N.-I., K. Kawa, K. Koike, T. H., and M. K. contributed to the acquisition and interpretation of data; Y. A. and S.K. edited the manuscript; T. M. designed research and wrote the manuscript; all authors reviewed and approved the manuscript.

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## ADA-SCID with 'WAZA-ARI' mutations that synergistically abolished ADA protein stability

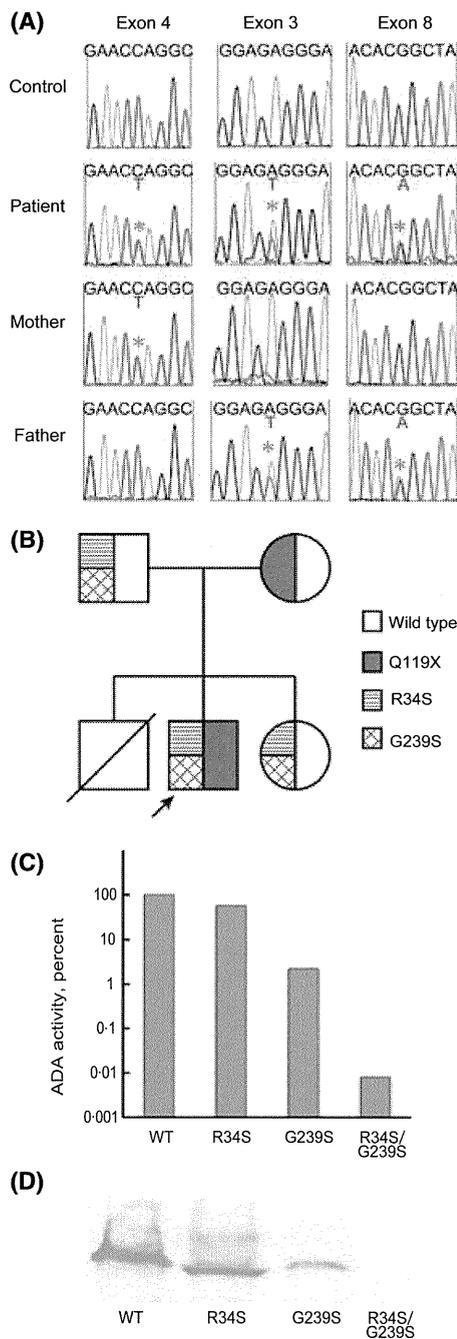
Adenosine deaminase (ADA) deficiency is a systemic purine metabolic disorder in which toxic levels of ADA substrates, particularly deoxyadenosine (dAdo), primarily affects lymphocyte development and functions (Hershfield & Mitchell, 2001). The affected patients present with varying degrees of immunodeficiency, such as severe combined immunodeficiency (ADA-SCID), delayed-onset ADA deficiency, and late-onset ADA deficiency. 'Partial ADA deficiency' has also been identified in healthy individuals with abolished ADA activity in erythrocytes but at greater levels in other cells (0.9–70% of normal) (Daddona *et al*, 1983; Hirschhorn & Ellenbogen, 1986). To date, more than 70 ADA mutations have been identified, including deletions, missense, nonsense, and splicing mutations (Hershfield, 2003). Correlation between the effect of mutations on ADA activity and clinical phenotype has been demonstrated by systematic expression studies of mutant ADA cDNAs in *Escherichia coli* (Arredondo-Vega *et al*, 1998; Hershfield, 2003). Here, we report an ADA-SCID patient with two mutations on the same allele, each of which retained detectable levels of ADA activity.

A Japanese 1-month-old boy was referred to our hospital because of poor sucking and failure to thrive. His elder brother had died of recurrent pneumonia at 4 months of age. His non-consanguineous parents and his younger sister were all in good health. Based on the history of his affected sibling, initial investigations were performed soon after birth. Although the initial haematological examination showed no lymphopenia (absolute lymphocyte count:  $2.79 \times 10^9/l$ ), profound lymphopenia (absolute lymphocyte count:  $0.13 \times 10^9/l$ ) was noticed when he was 40 d old. T-cell receptor excision circles were undetectable (Morinishi *et al*, 2009). After obtaining informed consent for genetic analysis under a protocol approved by the Institutional Review Board of Hokkaido University Graduate School of Medicine, we performed genetic analysis of

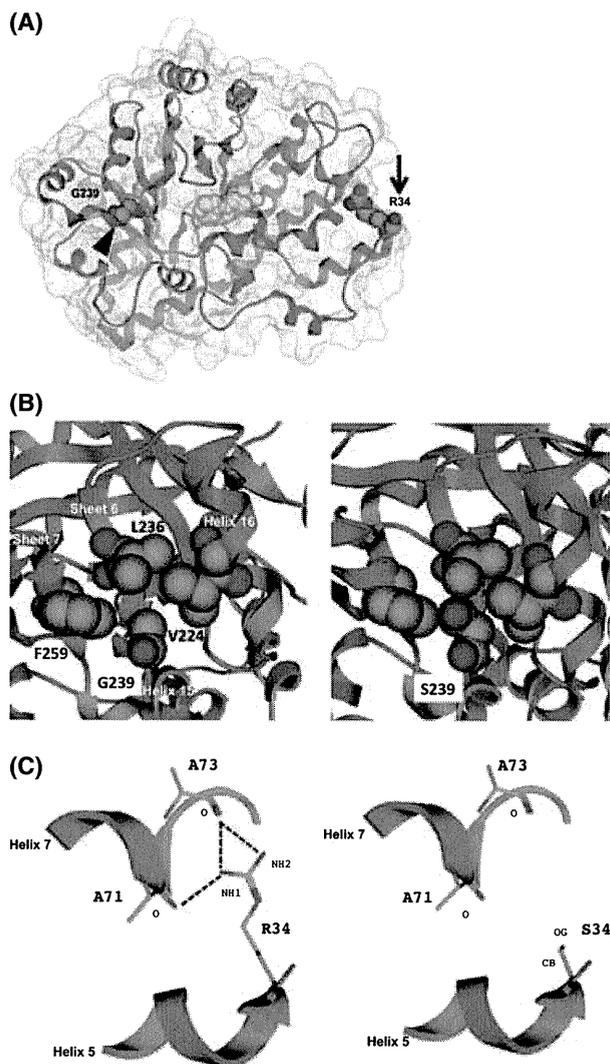
T-B-SCID (SCID with a virtual lack of circulating mature T and B lymphocytes)-related genes, such as ADA, RAG1, RAG2, and DCLRE1C, and found mutations in ADA gene. He was diagnosed with ADA-SCID based on his clinical severity, immune dysfunction, and the presence of ADA mutations. He died of respiratory distress 22 d after unrelated umbilical cord blood stem cell transplantation.

Direct sequence analysis of the patient's genomic DNA demonstrated three base changes in the ADA gene: 355C>T (Q119X) in exon 4, 102A>T (R34S) in exon 3, and 715G>A (G239S) in exon 8 (Fig. 1A,B). Studies of his family members demonstrated that his mother was heterozygous for 355C>T encoding Q119X, whereas his father and younger sister were heterozygous for 102A>T and 715G>T encoding R34S and G239S, respectively (Fig. 1A,B, data not shown). Q119X has been identified as one of the mutations for ADA-SCID (Ariga *et al*, 2001a), while G239S is a 'partial mutation' observed in a patient with partial ADA deficiency (Ariga *et al*, 2001b). There have been no reports of R34S mutation in ADA deficiency. To further determine whether the father's R34S and G239S mutations were both on the same allele, the reverse transcription polymerase chain reaction (RT-PCR) products consisting of full-length ADA cDNA were cloned and analysed for their nucleotide sequences. Only two clones were found: one with wild-type sequence and the other containing both R34S and G239S mutations. These results indicated that ADA-SCID in the patient was caused by compound heterozygous mutations: Q119X inherited from his mother and R34S/G239S from his father (Fig. 1B).

To study the effect of R34S/G239S mutations on ADA activity, the cDNAs containing the base changes individually and in combination were recloned into the pZ plasmid and introduced into the bacterial ADA-defective *E. coli* strain, SØ3834. ADA activity of each mutant cDNA expressed in



**Fig 1.** Studies of ADA sequence, activity, and expression. (A) Direct sequence analysis of the ADA gene in a control, the patient, mother, and father. The sites of mutations are indicated by asterisks. Left panels: Forward sequence of exon 4. 355C>T (Q119X) was present in the patient and his mother. Middle panels: Forward sequence of exon 3. 102A>T (R34S) was present in the patient and his father. Right panels: Forward sequence of exon 8. 715G>A (G239S) was present in the patient and his father. (B) Pedigree of the patient's family. Genotypes of the ADA are shown. An arrow indicates the patient. (C) ADA activity of mutants (R34S, G239S and R34S/G239S), expressed in *Escherichia coli*. Each mutant resulted in 56.4%, 2.2%, and 0.008% of the normal ADA activity, respectively. (D) Western blot analysis of ADA expression in lysates of SØ3834 in which wild-type, R34S, G239S, and R34S/G239S were expressed. R34S and G239S exhibited mildly and moderately reduced expression, respectively. R34S/G239S mutations in combination resulted in undetectable expression. WT, wild-type.



**Fig 2.** The effect of mutations on the tertiary structure of ADA. (A) Location of mutated residues, R34 and G239, are shown by an arrow and an arrowhead, respectively. The complexed ligand within the active site is represented by yellow spheres. (B) An enlarged view of regions neighbouring G239 (left) and S239 (right). Substitution of Gly to Ser, which has a hydrophilic side chain, weakens the hydrophobic force of the core. (C) An enlarged view of regions neighbouring R34 (left) and S34 (right). R34 is located in Helix 5. Substitution of Arg with Ser, which has a shorter side chain and lacks a positive charge, eliminates the hydrogen bonds, thereby affecting the ligand gating function of Helix 7. Hydrogen bonds are shown by dotted lines.

SØ3834 was quantitated as previously described (Arredondo-Vega *et al*, 1998). Each R34S and G239S mutation resulted in 56.4% and 2.2% of normal ADA activity, respectively. However, the combination of R34S/G239S mutations resulted in 0.008% of normal ADA activity, indicating that the two mutations have synergistic effects on the loss of this activity (Fig. 1C).

Western blot analysis of the *E. coli* transformed with each mutant cDNA as previously described (Arredondo-Vega *et al*, 1998) revealed reduced expression of R34S or G239S mutant

protein, whereas the combination of R34S/G239S resulted in no detectable protein expression (Fig. 1D). These results suggest that abolished ADA activity in R34S/G239S, is attributed to impaired protein expression. Direct sequence analysis of cDNA derived from his father demonstrated comparable signals of both wild-type and R34S/G239S mutant. In addition, sequence analysis of cloned RT-PCR products of ADA cDNA derived from his father showed wild-type and R34S/G239S mutant at a ratio of 7–5 (data not shown). These suggest that the lack of the mutant ADA protein expression is a result of protein instability, rather than mRNA instability.

Next, we analysed the tertiary structure of the mutant ADA based on the crystal structure of human ADA (PDB code: 3IAR) as previously described (Montano *et al*, 2007) (Fig. 2A). G239 is a part of the hydrophobic core formed by Helices 15 and 16 and Sheets 6 and 7. Substitution of this amino acid with Ser, which has a hydrophilic side chain, retains the overall structure but weakens the hydrophobic force of the core (Fig. 2B). R34 is a positively charged peripheral residue located in Helix 5. Helix 5 forms hydrogen bonds with Helix 7, which is enables for the substrate to enter the active site. Substitution of Arg with Ser, which has a shorter side chain and is uncharged, loses the hydrogen bonds. This causes a presumably unstable local structure, despite retained overall structure (Fig. 2C).

Jiang *et al* (1997) described the presence of two missense mutations on the same allele (L106V/Y97C), which synergistically abolished ADA activity independent of the protein instability. Therefore, the present report is the first to ADA-SCID in which two partial mutations on the same allele, R34S/G239S, synergistically abolished ADA protein stability. We named the combination of partial mutations that resulted in complete deficiency of the gene product 'WAZA-ARI' mutations, following a scoring system of Judo's competition. A 'waza-ari' is a half point; two 'waza-ari' scoring constitute the full point needed for win. In cases that lack correlation between genotype and phenotype, another mutation on the same allele should be assessed for 'WAZA-ARI' mutations.

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**Keywords:** adenosine deaminase deficiency, severe combined immunodeficiency, partial mutation, allelic mutations, 'WAZA-ARI' mutations.

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## **Autoimmune lymphoproliferative syndrome–like disease with somatic *KRAS* mutation**

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## Brief report

# Autoimmune lymphoproliferative syndrome–like disease with somatic *KRAS* mutation

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**Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (type I-III). Germline *NRAS* mutation was recently identified in type IV ALPS. We report 2 cases with ALPS-like disease with somatic *KRAS* mutation. Both cases were characterized by prominent autoimmune cytopenia and lymphadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia and are probably defined as a new disease entity of RAS-associated ALPS-like disease (RALD). (*Blood*. 2011;117(10):2887-2890)**

## Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway,<sup>1,2</sup> currently categorized as: type Ia, germline *TNFRSF6/FAS* mutation; type Ib, germline *FAS ligand* mutation; type Is, somatic *TNFRSF6/FAS* mutation; and type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases, such as immune cytopenia and hyper- $\gamma$ -globulinemia. An additional subclassification has been proposed that includes types III and IV, whereby type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis and type IV as one showing germline *NRAS* mutation.<sup>3</sup> Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to interleukin-2 (IL-2) depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS-associated leukoproliferative disease.<sup>4</sup>

Juvenile myelomonocytic leukemia (JMML) is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. Approximately 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells, including mutations of *NF1*, *RAS* family,<sup>5</sup> *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood mononuclear cells (MNCs) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of CD34<sup>+</sup> BM-MNCs.<sup>6</sup>

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively<sup>7</sup>; among these tumors, the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present 2 cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic *KRAS* G13D mutation without any clinical features of germline RAS mutation, such as cardio-facio-cutaneous or Noonan syndrome.

## Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

### Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (supplemental Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Blood test revealed the presence of hemolytic anemia and autoimmune thrombocytopenia. Hyper- $\gamma$ -globulinemia with various autoantibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in "Results and discussion."

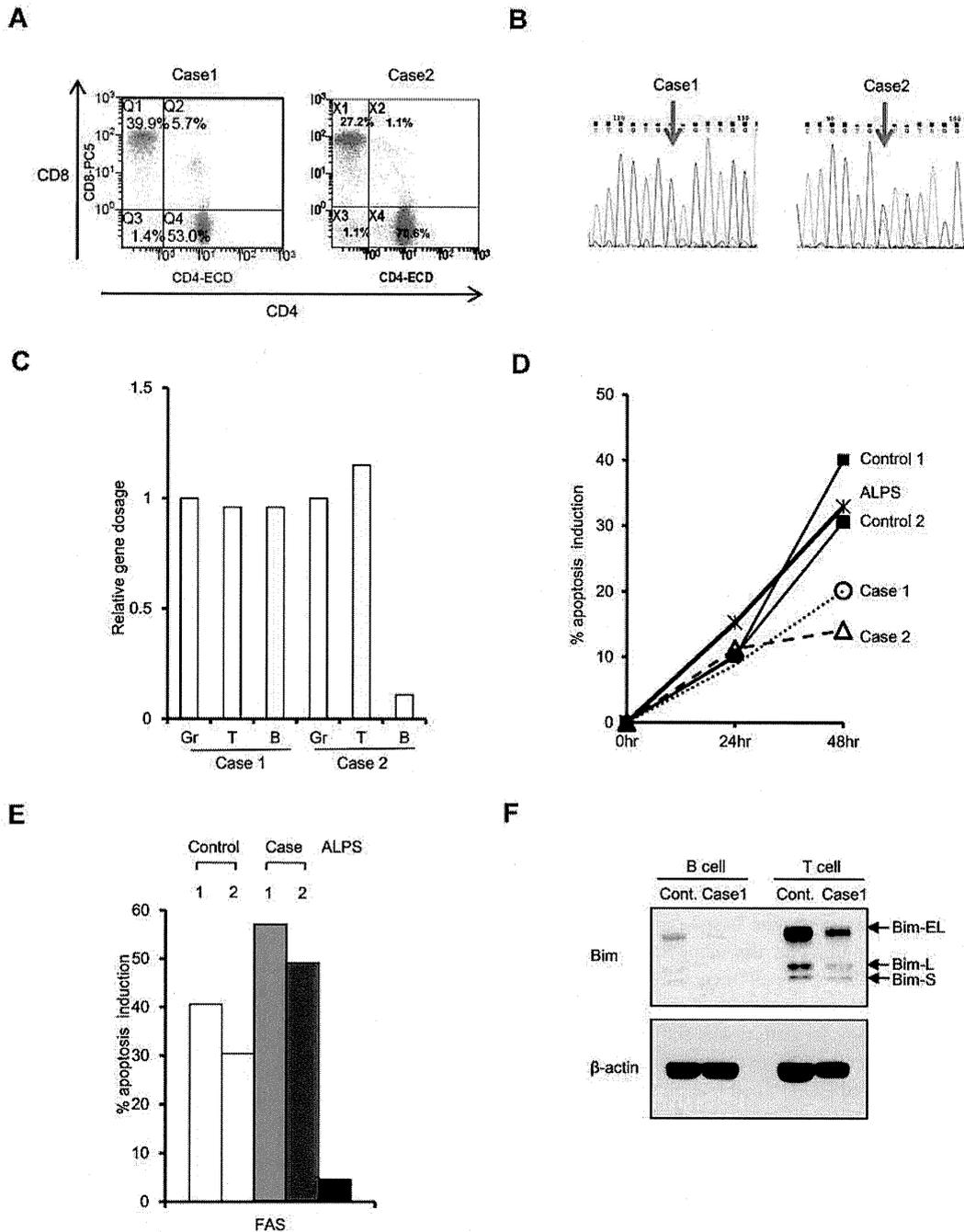
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**Figure 1. Molecular cell biologic assay of RALD.** (A) Flow cytometric analysis of double-negative T cells. CD8 and CD4 double staining was performed in T-cell receptor- $\alpha\beta$ -expressing cells. (B) Electropherogram showing KRAS G13D mutation in BM-MNCs in case 1 (left panel) and case 2 (right panel). (C) Gene dosage of mutated allele in granulocytes (Gr), T cells (T), and B cells (B). Relative gene dosage was estimated by a mutant allele-specific polymerase chain reaction method in cases 1 and 2 using albumin gene as internal control. (D) Apoptosis assay using activated T cells. Apoptosis percentage was measured by flow cytometry with annexin V staining 24 and 48 hours after IL-2 depletion. (E) Apoptosis percentage was measured 24 hours after addition of anti-FAS CH11 antibody (final 100 ng/mL). (F) Western blotting analysis of Bim expression.

**Case 2**

A 5-month-old girl had a fever and massive hepatosplenomegaly (supplemental Figure 1D). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper- $\gamma$ -globulinemia and autoantibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNCs showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive monocytosis or increased fetal hemoglobin. Detailed clinical history and laboratory data are provided in supplemental data.

Detailed methods for experiments are described in supplemental data.

**Results and discussion**

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (supplemental Table 1), except for number of double-negative T cells, which was only 1.4% of T-cell receptor- $\alpha\beta$  cells (Figure 1A). JMML was also nominated as a disease to be differentiated because remarkable hepatosplenomegaly with thrombocytopenia and moderate monocytosis was