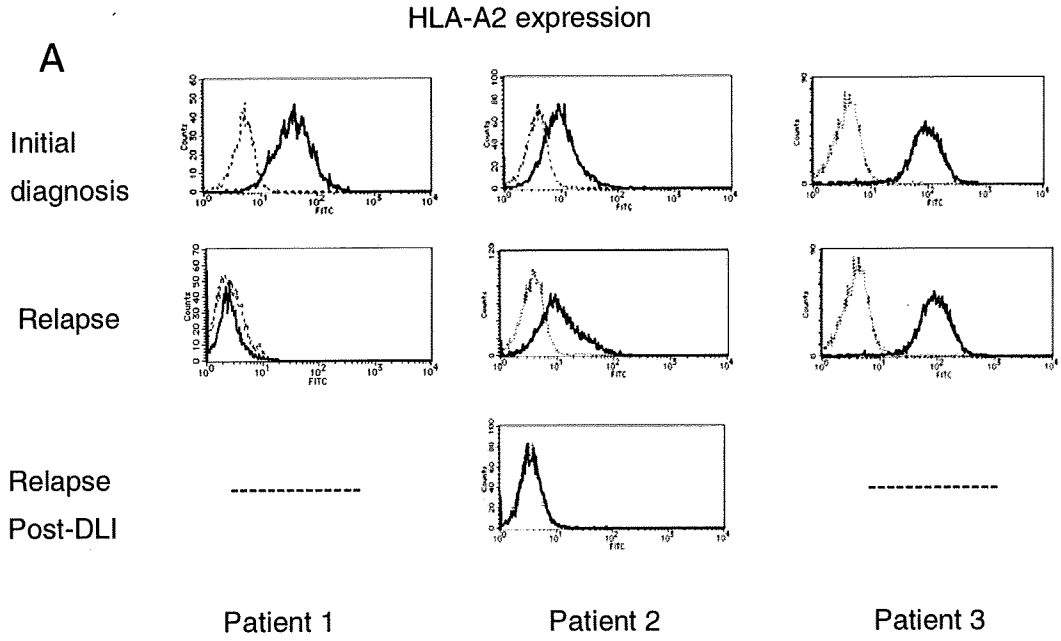
production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

Table 1. The cytotoxic T-lymphocyte precursor frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Purified $CD8^+$ T cells from the peripheral blood mononuclear cells obtained post-transplant from Patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated 3×10^4 leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI-1640 medium supplemented with 4% pooled human serum, IL-6, and IL-7 (10 ng/ml, both from R&D Systems, Minneapolis, MN). The IL-2 (50 U/ml) was added on Day 7 with a half medium change. For each dilution, there were at least 12 replicates. On Day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against ^{51}Cr-radiolabeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after donor lymphocyte infusion if indicated. The supernatants were measured in a gamma counter after 4-h incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was $>3 \times$ SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The cytotoxic T-lymphocyte precursor (CTLp) frequency was calculated using L-Calc software (StemCell Technologies, Vancouver, Canada). The CTLp frequencies reactive with recipient T-cell blasts in $CD8^+$ T cells obtained around Days 100, 180, and 300 (4 months before relapse) were undetectable, while the CTLp frequency obtained at Day 520 (1 month after the third donor lymphocyte infusion or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor $CD8^+$ cells. Complete remission and more than 99% donor chimerism were confirmed on those days.

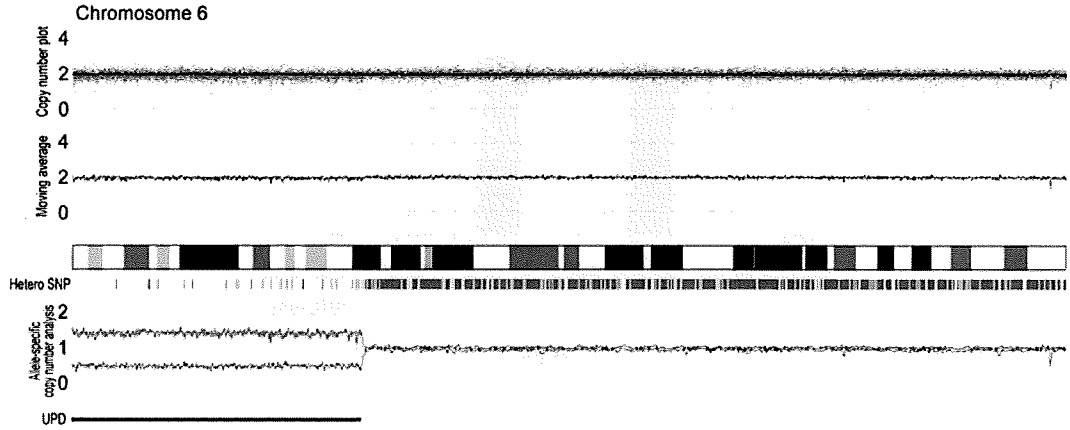
Figure 1


HLA-A2 expression
A
Initial diagnosis
Relapse
Relapse Post-DLI

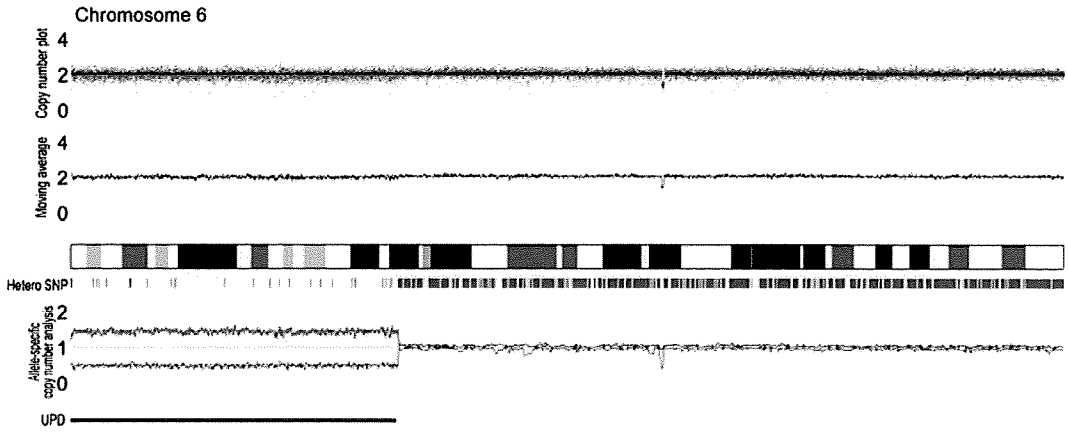
Patient 1
Patient 2
Patient 3

B

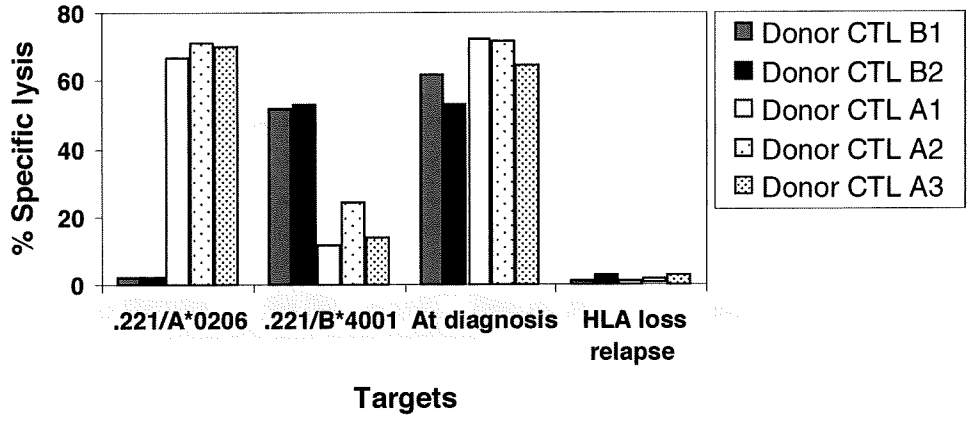
Patient 1



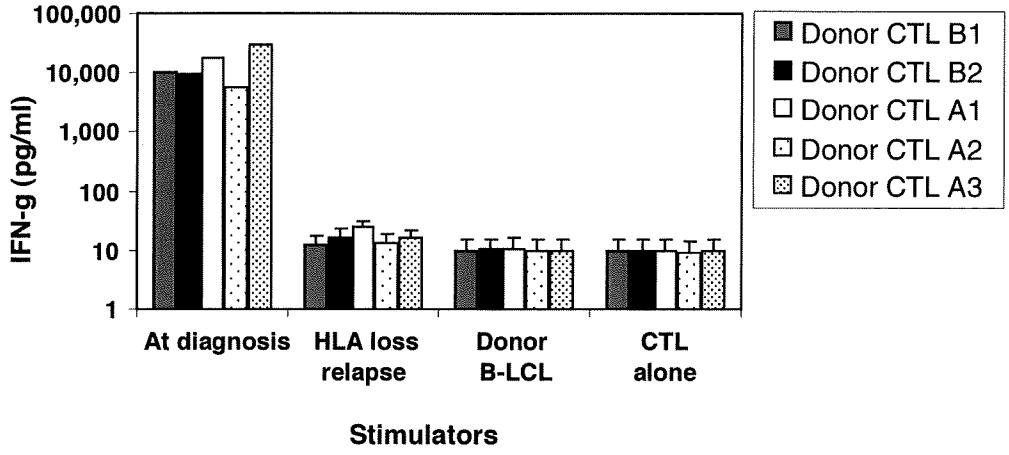
Patient 2



C


80
60
40
20
0
% Specific lysis
Donor CTL B1
Donor CTL B2
Donor CTL A1
Donor CTL A2
Donor CTL A3
.221/A*0206
.221/B*4001
At diagnosis
HLA loss relapse
Targets

D


100,000
10,000
1,000
100
10
1
IFN-g (pg/ml)
At diagnosis
HLA loss relapse
Donor B-LCL
CTL alone
Stimulators
Donor CTL B1
Donor CTL B2
Donor CTL A1
Donor CTL A2
Donor CTL A3

Blood (ASH Annual Meeting Abstracts) 2008 112: Abstract 2331

Poster Session

Experimental Transplantation - Basic Biology, Immune Function, and Engraftment Poster I

Cyclosporine, but Not mTOR Inhibitors, Hampers the Reconstitution of Bone Marrow-Derived Tregs in Long-Term Complete Donor chimeras .

Haruko Sugiyama, MD[1,*], Yoshinobu Maeda, MD[1,*], Hisakazu Nishimori, MD[1,*], Koichiro Kobayashi, MD[1], Miyuki Nishie-Kataoka, MD[1,*], Takanori Teshima, MD[2] and Mitsune Tanimoto, MD[1]

[1] Department of Hematology, Oncology and Respiratory Medicine, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan, [2] Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan

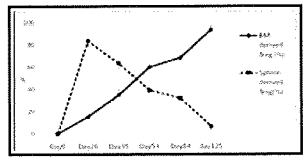
Abstract

Chronic graft-versus-host disease (GVHD) is the most common complication in the late stage after allogenic hematopoietic-stem-cell-transplantation (SCT), but the pathophysiology and treatment strategy of chronic GVHD remain poorly defined. Prolonged administration of cyclosporine (CSA) did not decrease the risk of chronic GVHD. Recent studies using a mouse model have shown that regulatory T cells (Tregs) can influence immune responses, and Tregs in the grafts can prevent acute GVHD when injected together with donor T cells. However, it is not known whether Tregs remain in the grafts in the late stage of SCT and play a role in preventing chronic GVHD.

First, we examined the origin of Tregs using a major histocompatibility complex (MHC) mismatched mouse SCT model. Lethally irradiated C3H/HeN(H-2k) recipient mice received $10x10^6$ T-cell-depleted bone marrow (BM) cells from B6.Ly-5a(H-2b, CD45.1) mice and $1x10^6$ spleen cells from C57BL/6(B6, H-2b, CD45.2) mice. Spleen cells were collected from SCT recipient mice at serial time points and subjected to fluorescence-activated cell sorting (FACS) analysis. Transplanted mice displayed complete donor hematopoietic chimerism and mild acute GVHD at day14. On day 21 (early stage) after SCT, host type Tregs (CD4+FoxP3+ H-2k) were no longer detectable, and most of the Tregs (83±3%) were derived from donor spleen Tregs (H-2b, CD45.2). However, the homeostatic expansion of spleen Tregs gradually contracted and newly arising donor BM-derived Tregs (H-2b CD45.1) became dominant (93.8±0.5%) in the late stage of SCT (day 120). As in the spleen, BM-derived Tregs reconstitution in the late stage was seen in the thymus and mesenteric lymph nodes. Moreover, in a minor MHC-mismatched SCT model (B6 into C3H.SW), Tregs in the late stage were derived from donor BM cells (97.0±0.2%). These BM-derived Tregs suppress alloreactivity in the same manner as naturally occurring Tregs isolated from naïve mice in the MLR.

Next, we compared the effects of CSA and the mTOR inhibitor rapamycin (RAPA) on Tregs reconstitution. Mice receiving CSA or RAPA showed the same Tregs reconstitution pattern: in the early and late stages, Tregs were derived from donor spleen and BM cells, respectively. However, the number of Tregs in the spleen was reduced significantly in mice receiving CSA, as compared to control mice receiving phosphate-buffered saline (PBS; $1.3±0.2x10^6$ vs. $2.4±0.6x10^6$) at day 110. In particular, the number of Tregs in the thymus was reduced dramatically in mice receiving CSA ($0.7±0.2\ x10^5$ vs. $2.6±0.5x10^5$, $P<0.02$). By contrast, the numbers of Tregs in both the thymus and spleen from RAPA-treated mice were the same as those from PBS-treated mice. Mice treated with everolimus, another mTOR inhibitor, also showed no reduction in the numbers of Tregs. Histologic examination revealed that CSA-treated mice showed pathogenic features of chronic GVHD, including sclerodermatous skin changes, bile duct loss, fibrosis in the portal area of the liver and fibrosis and atrophy of acinar tissue in the salivary glands, while RAPA-treated mice showed no sign of chronic GVHD.

Our findings indicate that a) Tregs cannot remain in grafts in the late stage, and newly arising donor BM-derived Tregs became dominant; b) CSA hampers BM-derived Tregs reconstitution and may be associated with the development of chronic GVHD; and c) mTOR inhibitors do not hamper Tregs reconstitution and might prove beneficial for the treatment of both acute and chronic GVHD.



Figure

View larger version (15K):
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Footnotes

Corresponding author

Disclosures: No relevant conflicts of interest to declare.

X-linked thrombocytopenia (XLT) due to *WAS* mutations: clinical characteristics, long-term outcome, and treatment options

Michael H. Albert,[1] Tanja C. Bittner,[1] Shigeaki Nonoyama,[2] Lucia Dora Notarangelo,[3] Siobhan Burns,[4] Kohsuke Imai,[2] Teresa Espanol,[5] Anders Fasth,[6] Isabelle Pellier,[7] Gabriele Strauss,[8] Tomohiro Morio,[9] Benjamin Gathmann,[10] Jeroen G. Noordzij,[11] Cristina Fillat,[12] Manfred Hoenig,[13] Michaela Nathrath,[14] Alfons Meindl,[15] Philipp Pagel,[16] Uwe Wintergerst,[17] Alain Fischer,[18] Adrian J. Thrasher,[4] *Bernd H. Belohradsky,[1] and *Hans D. Ochs[19]

[1]Dr von Haunersches Kinderspital, Ludwig-Maximilians-Universität, Munich, Germany; [2]National Defense Medical College, Tokorozawa, Japan; [3]University of Brescia, Brescia, Italy; [4]University College London Institute of Child Health, London, United Kingdom; [5]Vall d'Hebron Hospital, Barcelona, Spain; [6]The Queen Silvia Children's Hospital, Göteborg, Sweden; [7]Centre Hospitalier Universitaire Angers, Angers, France; [8]Charité Campus Virchow-Klinikum, Otto-Heubner-Zentrum für Kinder- und Jugendmedizin, Berlin, Germany; [9]Tokyo Medical and Dental University, Tokyo, Japan; [10]Universitätsklinikum Freiburg, Freiburg, Germany; [11]St Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [12]Centre de Regulació Genòmica, Centro de Investigación Biomédica en Red de Enfermedades Raras, Barcelona, Spain; [13]Universitätsklinik für Kinder- und Jugendmedizin Ulm, Ulm, Germany; [14]University Children's Hospital, Technische Universität, Munich, Germany; [15]Frauenklinik am Klinikum rechts der Isar, Technische Universität, Munich, Germany; [16]Lehrstuhl für Genomorientierte Bioinformatik, Wissenschaftszentrum Weihenstephan, Technische Universität, Freising, Germany; [17]Krankenhaus St. Josef, Braunau, Austria; [18]Hôpital Necker Enfants Malades, Paris, France; and [19]University of Washington, Seattle Children's Hospital

A large proportion of patients with mutations in the Wiskott-Aldrich syndrome (WAS) protein gene exhibit the milder phenotype termed X-linked thrombocytopenia (XLT). Whereas stem cell transplantation at an early age is the treatment of choice for patients with WAS, therapeutic options for patients with XLT are controversial. In a retrospective multicenter study we defined the clinical phenotype of XLT and determined the probability of severe disease-related complications in patients older than 2 years with documented *WAS* gene mutations and mild-to-moderate eczema or mild, infrequent infections. Enrolled were 173 patients (median age, 11.5 years) from 12 countries spanning 2830 patient-years. Serious bleeding episodes occurred in 13.9%, life-threatening infections in 6.9%, autoimmunity in 12.1%, and malignancy in 5.2% of patients. Overall and event-free survival probabilities were not significantly influenced by the type of mutation or intravenous immunoglobulin or antibiotic prophylaxis. Splenectomy resulted in increased risk of severe infections. This analysis of the clinical outcome and molecular basis of patients with XLT shows excellent long-term survival but also a high probability of severe disease-related complications. These observations will allow better decision making when considering treatment options for individual patients with XLT. (*Blood*. 2010;115(16): 3231-3238)

Introduction

In 1937 Wiskott described a clinical entity characterized by thrombocytopenia, eczema, bloody diarrhea, and recurrent otitis media in male infants. After rediscovery in 1954 by Aldrich as an X-linked recessive disorder, it was designated the Wiskott-Aldrich syndrome (WAS).[1-3] X-linked thrombocytopenia (XLT), sometimes associated with mild eczema and/or infections, was recognized in the 1960s and was suspected to be a variant of WAS.[4-6] This was confirmed when patients with XLT were shown to have mutations in the Wiskott-Aldrich syndrome protein gene (*WAS*).[7-9]

WAS gene mutations result in 3 distinct clinical phenotypes: classic WAS, XLT, and X-linked neutropenia,[10,11] and a strong genotype phenotype correlation has been suggested.[12-15] Mutations completely averting WAS protein (WASP) expression typically lead to the classic phenotype. Missense mutations resulting in expression of defective WASP, often in reduced quantity, most often result in the XLT phenotype, sometimes with only intermittent thrombocytopenia.[16] X-linked neutropenia is caused by gain of function mutations resulting in constitutively activated WASP.[17-19] There are however exceptions to these rules, making it difficult to predict the clinical course of a male infant solely based on the type of *WAS* gene mutation and its effect on WASP expression.

The classic WAS phenotype with microthrombocytopenia, severe eczema, increased susceptibility to pyogenic and opportunistic infections, and increased risk of autoimmune disease and cancer usually leads to death in early childhood or adolescence if left untreated.[10,20,21] Curative treatment by allogeneic hematopoietic stem cell transplantation (HSCT) should be offered to all such patients. The outcome is excellent if performed early in life from a human leukocyte antigen–matched related or unrelated donor.[10,22-24] Hematopoietic stem cell gene therapy might in the future offer an alternative approach in patients lacking a suitable donor.[25-27]

Generally accepted treatment policies do not exist for patients exhibiting the XLT phenotype, in whom HSCT would seem like an excessively risky procedure if they have thrombocytopenia and

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*B.H.B. and H.D.O. contributed equally to this study.

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eczema only. Although it has been assumed that patients with XLT have a lower risk of cancer or autoimmunity than patients with WAS, this has never been formally examined. Therefore, the risk–benefit ratio for HSCT is not known in XLT.

In this multicenter study we assessed retrospectively the spectrum of clinical phenotypes, the associated genotypes, and the long-term outcome of the largest cohort of patients with XLT studied so far.

Methods

Data accrual

Questionnaires were sent worldwide to major centers treating patients with primary immunodeficiency diseases (PIDs), asking to enroll their patients with the XLT phenotype and to provide data on the following disease parameters: infections, eczema, thrombocytopenia, bleeding, malignancy, autoimmunity, *WAS* gene mutation, WASP expression, and type and extent of therapy. An alternative possibility was documentation online with the same questionnaire in the European Society for Immunodeficiencies registry (www.esid.org). Patient information was made anonymous by the submitting physician. The study was approved by the ethics committee of the University of Munich, Germany.

Patients

All submitted patient data were evaluated, and patients were included as study patients by consensual decision of a central review board (M.H.A., T.C.B., B.H.B., H.D.O.). To be enrolled into the final study, patients had to fulfill all of the following criteria: (1) confirmed mutation within the *WAS* gene; (2) classified by their treating physician as having XLT; (3) with or without mild-to-moderate eczema or mild, infrequent infections not resulting in sequelae; (4) age older than 2 years; and (5) no severe infection, autoimmunity, or malignancy within the first 2 years of life.

Bleeding events before the age of 2 years were no reason for exclusion from the study. Older than 2 years, severe infections, the development of autoimmunity, or malignancy was recorded and included in the analysis, but it was no reason for exclusion from the study.

If patients underwent allogeneic HSCT, the transplantation was recorded as the last date of follow-up; the resulting events/outcome were not part of this analysis.

Definitions

Life-threatening infections were defined as requiring hospitalization such as sepsis, meningitis, or pneumonia needing oxygen supply or mechanical ventilation. Serious bleeding was defined as a fatal or life-threatening bleeding episode resulting in hospitalization or red blood cell transfusion. Other serious complications were a diagnosis of autoimmunity, malignancy, or death. If a patient experienced more than 1 serious event, only the first event was registered for the analysis of event-free survival. Severity of thrombocytopenia was defined as follows: less than 20.0×10^9/L (20 000/μL) was severe, 20.0 to 50.0×10^9/L (20 000 to 50 000/μL) was moderate, and greater than 50.0×10^9/L (50 000/μL) or cyclic was mild. All patients with normal or reduced levels of WASP detectable by Western blot or fluorescence-activated cell sorting were designated as WASP positive; those with truncated (by Western blot) or undetectable protein were categorized as WASP negative. Intravenous immunoglobulin (IVIG) or antibiotic (AB) prophylaxes were defined as having had IVIG or prophylactic ABs more than once for any period of time.

Mutations are reported according to the current nomenclature of the Human Genome Variation Society (www.hgvs.org).[28]

Statistical analysis

Kaplan-Meier survival estimates and cumulative incidence rates were compared with the use of the log-rank test (Prism; GraphPad Software Inc). Cumulative incidence for different events adjusting for competing risks was estimated with the use of the statistics language R[29] with the cmprsk package that used the method by Gray.[30] Other analyses used the χ^2 or Fisher exact test and were accepted as significantly different at a level of P less than .05.

Results

Study cohort

A total of 69 centers known to treat patients with PID were contacted and 50 responded (72%). Of 213 completed forms, representing 12 countries from 4 continents, 173 (171 male, 2 female) patients from 128 families and 21 centers with a median age of 11.5 years (range, 2.0-74.6 years) fulfilled the inclusion criteria, covering 2830 patient-years. The 2 female patients of our XLT cohort had been reported previously, 1 with a homozygous missense mutation and 1 with a heterozygous missense mutation and skewed X-inactivation in favor of the mutated allele.[31,32]

Mutations in patients with XLT

We identified 62 unique mutations (Table 1), including 3 mutational hotspots, defined as affecting 10 or more nonrelated families with either the identical mutation or a missense mutation affecting the same amino acid. Two hotspots were located in exon 2 affecting either a valine at position 75 (p.Val75Met or p.Val75Leu; 23 patients) or an arginine at position 86 (p.Arg86Gly, p.Arg86Cys, p.Arg86His, or p.Arg86Leu; 33 patients). The third hotspot mutation, located in intron 6 (c.559 + 5G>A) was found in 15 patients. Thus 41% of all patients had a hotspot mutation.

The majority of mutations was located in exon 1 (10% of all patients) and exon 2 (54%). Most mutations were missense (69% of all patients), followed by splice site mutations (19%), deletions (5%), insertions (3%), nonsense mutations (2%), and no-stop mutations (1%; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). With few exceptions, patients with missense and splice site mutations expressed WASP in reduced quantity or in truncated form (Table 1).

Survival

Without curative treatment classic WAS results in premature death, often during childhood.[21,33] Patients with XLT are expected to have a better prognosis. To verify this perception, we defined the probability of survival in our cohort of patients with XLT.

Overall survival was excellent with 97% (95% confidence interval [95% CI], 95%-100%), 96% (95% CI, 91%-100%), 81% (95% CI, 66%-97%), and 81% (95% CI, 66%-97%) at 15, 30, 45, and 60 years, respectively, and only slightly reduced compared with the survival curve of the normal male German population[34] (Figure 1A). However, survival probability without having experienced a severe disease-related event was less favorable with 74% (95% CI, 65%-82%), 56% (95% CI, 43%-70%), 36% (95% CI, 20%-53%), and 27% (95% CI, 10%-44%) at 15, 30, 45, and 60 years, respectively (Figure 1B).

Thus the excellent survival in patients with XLT is associated with a high rate of severe disease-related events throughout life.

Incidence of severe disease-related events

To better define the nature and occurrence of severe disease-related events, we analyzed the cumulative incidence rate of these events separately.

Table 1. *WAS* gene mutations in patients with XLT

Exon	Coding DNA mutation	Predicted protein change	Mutation type	Pt*	Fam†	Origin	WASP expression (no. of pt)	Score (no. of pt)
1	c.G5C	p.Ser2Thr	Missense	1	1	Fr	ND	2
1	c.G18A	p.Met6Ile	Missense	2	1	JPN	Reduced (2)	1, 2→5M
1	c.C71T	p.Ser24Phe	Missense	2	2	US (1), JPN (1)	Reduced (1), ND (1)	1, 2→5A
1	c.C79T	p.Leu27Phe	Missense	1	1	US	Reduced	1
1	c.88_90delCAC	p.His30del	Deletion	5	2	UK (4), Ger (1)	Reduced (3), ND (2)	1(4), 2
1	c.G91A	p.Glu31Lys	Missense	1	1	Italy	Absent	2→5A
1	c.T116C	p.Leu39Pro	Missense	6	4	US (3), Italy (2), Ger (1)	Reduced (5), absent (1)	1, 1→5A/M, 2(4)
2	c.C134T	p.Thr45Met	Missense	13	8	JPN (4), US (2), Ger (1), UK (1), Sw (5)	Reduced (6), absent (1), ND (6)	1(6), 1→5A, 2(4), 2→5A /B (2)
2	c.C140A	p.Ala47Asp	Missense	1	1	US	Reduced	2
2	c.A142G	p.Thr48Ala	Missense	1	1	JPN	Reduced	2
2	c.C143T	p.Thr48Ile	Missense	1	1	US	Reduced	1→5M
2	c.C167T	p.Ala56Val	Missense	5	4	US (3), Italy (1), JPN (1)	Reduced (4), ND (1)	1(3), 1→5A, 2
2	c.C172A	p.Pro58Thr	Missense	2	1	US	Normal (2)	1, 2
2	c.C172G	p.Asp58Ala	Missense	1	1	US	Reduced	2→5A/M
2	c.C173G	p.Pro58Arg	Missense	3	1	Italy	Reduced (2), ND (1)	1, 1→5M, 2
2	c.G199A	p.Glu67Lys	Missense	1	1	Fr	Reduced	2
2	c.G223A	p.Val75Met	Missense	22	16	Fr (6), UK (5), US (5), Ger (2), JPN (2), Sp (1), Italy (1)	Normal (1), reduced (10), absent (3), ND (8)	1(6), 1→5A, 2(14), 2→5A
2	c.G223T	p.Val75Leu	Missense	1	1	US	ND	2
2	c.A227C	p.Lys76Thr	Missense	2	2	US	Reduced (1), ND (1)	2(2)
2	c.G229C	p.Asp77His	Missense	1	1	Italy	Reduced	1
2	c.A230G	p.Asp77Gly	Missense	2	1	Italy	Reduced (2)	1, 2
2	c.A239G	p.Gln80Arg	Missense	1	1	Rus	Reduced	2
2	c.248insA	p.Tyr83X	Insertion	1	1	Fr	ND	2
2	c.C256G	p.Arg86Gly	Missense	1	1	US	Reduced	2→5A
2	c.C256T	p.Arg86Cys	Missense	24	18	US (10), Ger (6), JPN (3), UK (3), Italy (1), Sw (1)	Normal (3), reduced (9), ND (12)	1(10), 1→5M, 2(12), 2→5A
2	c.G257A	p.Arg86His	Missense	7	7	JPN (2), Fr (1), Ger (1), Isr (1), Rus (1), US (1)	Reduced (4), absent (1), ND (2)	1→5A, 2(4), 2→5A(2)
2	c.G257T	p.Arg86Leu	Missense	1	1	US	Absent	2
2	c.A263G	p.Tyr88Cys	Missense	1	1	NL	ND	2→5A
2	c.G266A	p.Gly89Asp	Missense	1	1	UK	Normal	1
3	c.A320G	p.Tyr107Cys	Missense	1	1	US	Reduced	2
3	c.326_330insC	p.Thr111HisfsX9	Insertion	1	1	US	Absent	2
3	c.G355A	p.Gly119Arg	Missense	1	1	NL	ND	1
4	c.dup355_361	p.Asp121InsGD	Insertion	1	1	JPN	Absent	2
4	c.G399T	p.Glu133Asp	Missense	1	1	US	Reduced	2
5	c.G505T	p.Asn169X	Nonsense	1	1	JPN	Reduced	2→5M
6	c.G538A	p.His180Asn	Missense	1	1	Italy	Reduced	1
7	c.C707G	p.Ala236Gly	Missense	1	1	Italy	Absent	1
7	c.A724T	p.Ser242Cys	Missense	1	1	NL	ND	1
9	c.854_855insG	p.Thr286AspfsX1	Insertion	2	1	UK	Reduced and truncated (1), absent (1)	1(2)
9	c.A919G	p.Met307Val	Missense	1	1	Ger	ND	2
10	c.C961T	p.Arg321X	Nonsense	1	1	JPN	Absent	2→5M
10	c.983_984delC	Multiple products	Deletion	1	1	US	Reduced and truncated	2
10	c.991insA	p.Gly334X	Insertion	1	1	US	Absent	2
10	c.1073_1074delGA	p.Gly358AlafsX135	Deletion	1	1	US	Reduced and truncated	2
10	c.1079delC	p.Pro360HisfsX84	Deletion	2	2	Ger, JPN	Reduced (1), absent (1)	2(2)
10	c.C1090T	p.Arg363X	Nonsense	2	1	Fr	ND (2)	2(2)
11	c.G1430A	p.Arg477Lys	Missense	1	1	Sp	Reduced	2
11	c.T1442A	p.Ile481Asn	Missense	2	1	Italy	Normal (1), reduced (1)	1(2)
12	c.G1453A	p.Asp485Asn	Missense	1	1	US	Reduced	2→5A
12	c.A1454G	p.Asp485Gly	Missense	3	1	Sp	ND (3)	1(3)
12	c.G1508C	p.X503SerextX76	No-stop	2	1	US	Absent (1), ND (1)	2(2)
Int 3	c.360+1G>A	p.Ala92_Asp120del	Splice (donor site)	1	1	JPN	Reduced	2
Int 3	c.361-1G>A	p.fsX201	Splice (acceptor site)	1	1	US	Reduced	2
Int 4	c.[463+1_463+8del; 464-3_464-2insG]	p.fsX178/fsX251	Splice (donor +acceptor site)	1	1	JPN	Reduced	2
Int 6	c.559+5G>A	70% fsX190/30% normal	Splice (donor site)	15	11	US (9), Ger (2), JPN (3), UK (1)	Reduced (12), absent (1), ND (2)	1(6), 1→5M, 2(6), 2→5A(2)
Int 7	c.734+5G>A	ND	Splice (donor site)	4	1	Ger	ND (4)	2 (3), 2→5A
Int 7	c.735-25A>C	ND	Splice (acceptor site)	3	1	UK	Reduced (3)	1(3)
Int 8	c.777+1G>A	p.fsX246	Splice (donor site)	2	2	Australia, US	Absent (1), ND (1)	1, 2
Int 8	c.777+3insT	ND	Splice (donor site)	2	1	Italy	Reduced (2)	1, 2
Int 8	c.778-6G>A	ND	Splice (acceptor site)	1	1	UK	Reduced	1
Int 9	c.(931_932)ins250	ND	Splice site	1	1	JPN	Reduced	1
Int 11	c.(1484_1485)ins118	Normal and abnormal splice products	Splice site	2	1	JPN	Reduced (2)	2→5A(2)

Pt indicates number of patients with the respective mutation; Fam, number of families with the respective mutation; 1→5, WAS score progressing from 1 to 5 because of either A, autoimmunity, or M, malignancy; Fr, France; ND, not done; JPN, Japan; US, United States of America; UK, United Kingdom; Ger, Germany; Sw, Sweden; Sp, Spain; Rus, Russia; Isr, Israel; and NL, The Netherlands.

*There was a total of 173 patients.

†There was a total of 128 families.

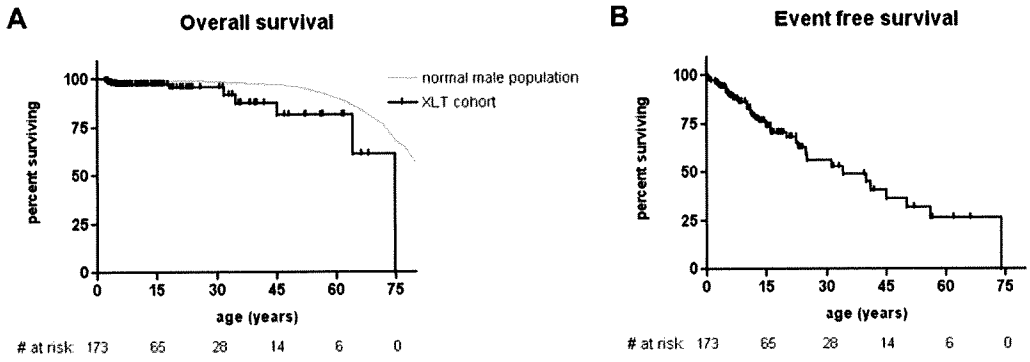

Figure 1. Overall and event-free survival. (A) Kaplan-Meier estimate of overall survival probability of all study patients compared with survival of the normal German male population 2006.[34] (B) Event-free survival probability. Event was defined as a severe or fatal infection, severe or fatal bleeding, autoimmunity, malignancy, or death. Each hash mark on a graph line indicates a censored event; # at risk, number of patients at risk at indicated time point.

Median event-free survival was 10.2 years (range, 0.1-73.9 years). A total of 86 events in 47 patients were reported, some of them occurring in different event categories in the same patient (detailed in Table 2). Cumulative incidences for each event category are detailed separately in Figure 2. If events were analyzed honoring other events as competing, the cumulative incidences were slightly lower because later events in the same patient were ignored (data not shown).

Table 2. Disease-related events

	Total events	Fatal events
Infections*		
Pneumonia	6	0
Bacterial meningitis	4	0
Sepsis	4	2
Gastrointestinal (salmonellosis)	1	1
Orchitis	1	0
Tuberculosis	1	0
No. of events	17†	3‡
No. of patients	12	3
Bleeding§		
ICH‖	18	3
Gastrointestinal	6	1
Ear/nose/throat	4	0
Pulmonary	2	1
Traumatic, not ICH	2	0
Retinal	1	0
No. of events	33	5
No. of patients	24	5
Autoimmunity¶		
Nephropathy	9	0
AIHA	6	0
Vasculitis	3	0
ITP	4	0
Arthritis	3	0
Colitis	1	0
No. of events	26	0
No. of patients	21	0
Malignancy#		
Lymphoma/EBV-LPD	4	1
MDS	1	0
Spinalioma	2	0
Seminoma	1	0
ALL	1	0
Pancreatic cancer	1	1
No. of events	10	2
No. of patients	9	2

ICH indicates intracranial hemorrhage; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenic purpura; ALL, acute lymphoblastic leukemia; EBV-LPD, Epstein-Barr virus–associated lymphoproliferative disease; and MDS, myelodysplastic syndrome.

*Three patients had more than 1 infectious event.

†Eight events were in patients who had undergone a previous splenectomy.

‡Two events were in patients who had undergone a previous splenectomy.

§Four patients had more than 1 bleeding episode.

‖Fifteen were spontaneous, 3 were traumatic.

¶Three patients had more than 1 autoimmune disease.

#One patient had 2 malignancies.

Life-threatening infections occurred at a median age of 24.8 years (range, 2.0-73.9 years), 3 of which were fatal. There was no discernible effect of patient age on the incidence of infectious events (Figure 2A). In contrast, all but 1 serious hemorrhage occurred before the age of 30 years, at a median age of 5.7 years (range, 0.1-74.6 years; Figure 2B). Most serious bleeding events (18 of 33) were intracranial hemorrhages. Five bleeding episodes were fatal at a median age of 4.9 years (range, 2.0-74.6 years). There was no correlation between the recorded platelet counts and the incidence of severe or fatal bleeding, which was 12.5% in mild, 9.7% in moderate, and 18.4% in severe thrombocytopenia ($P = .31$). Autoimmune nephropathy and hemolytic anemia were the most frequent autoimmune manifestations; the former occurring more frequently in Japanese patients than in patients from other countries (5 of 28 vs 4 of 145; $P = .006$). In general, autoimmune diseases were not significantly more frequent in Japanese patients (5 of 28 vs 16 of /145; $P = .34$). Autoimmunity was not restricted to adult patients but occurred at all ages with a median of 12.2 years (range, 4.9-56.0 years; Figure 2C). Malignancies developed at a median age of 34.0 years (range, 7.8-74.0 years; Figure 2D), half (5 of 10) of which were of lymphoid origin. Two patients died of their malignancies, 2 more went on to have HSCT and died of transplantation-related causes and 2 died of other complications.

In conclusion, with the exception of severe bleeding, which seems to be limited to the first 3 decades of life, a relatively high rate of life-threatening or fatal disease-related events was observed in XLT at all ages.

Influence of WAS gene mutation, protein expression, IVIG, or AB prophylaxis on overall and event-free survival

Because some patients with XLT have a largely uneventful course of disease and a normal life expectancy and others have severe or even fatal complications at any age, we asked whether individual *WAS* gene mutations, the presence or absence of WASP, or the prophylaxis with ABs and intravenous immunoglobulin had any influence on outcome.

WASP expression, if assessed, was detectable in 98 patients and absent in 21. Presence or absence of WASP had no influence on overall and event-free survival in patients with the XLT phenotype (Figure 3A). Similarly, there was no significant effect on the incidence of disease-related events (data not shown). The same was true when the influence of IVIG prophylaxis (n = 39) was analyzed in comparison to patients having never received IVIG (n = 134; Figure 3B). AB prophylaxis had no positive influence on

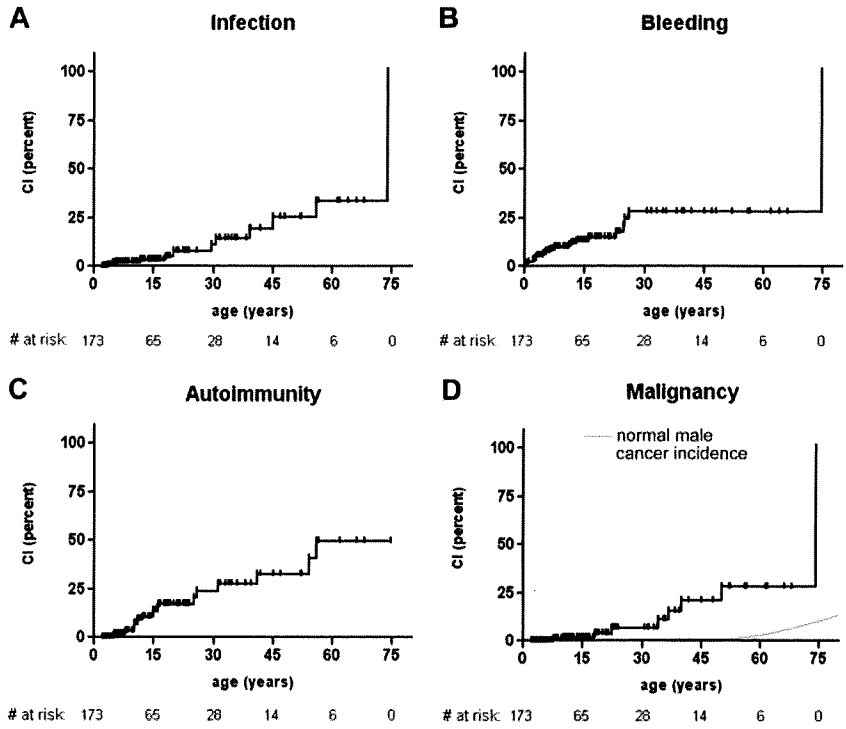

Figure 2. Cumulative incidence rate of severe events. Cumulative incidence of (A) severe or fatal infectious episodes in the study cohort, (B) severe or fatal bleeding episodes, (C) autoimmune disease, and (D) malignancy, compared with cancer incidence in the US male population.[35] Each hash mark on a graph line indicates a censored event, # at risk, number of patients at risk at indicated time point.

outcome (Figure 3C). Patients with hotspot mutations had no different overall and event-free survival and event incidences compared with others (data not shown).

In summary none of the tested outcome variables were of significance in this cohort of patients with XLT selected on the basis of their mild phenotype.

Influence of splenectomy on infections and bleeding episodes

Splenectomy in patients with XLT/WAS usually leads to a sustained increase in platelet counts and is considered an effective measure to control the bleeding predisposition. Therefore, splenectomy has been recommended by some investigators for patients with WAS and patients with XLT.[36,37]

A total of 41 patients (23.7%) underwent splenectomy at a median age of 7.02 years (range, 0.8-43.0 years). The indication for splenectomy was not reported, but 7 of these 41 patients had experienced a severe bleeding episode before splenectomy, and 28 of 41 patients had had severe thrombocytopenia. All 13 patients in whom postsplenectomy platelet counts were available had experienced an increase in platelet numbers, 7 having counts greater than 100.0×10^9/L (100 000/μL). In the 2 patients who experienced a severe bleeding event after splenectomy, platelet counts were not reported. Therefore, it cannot be excluded that these 2 patients may have had low counts despite splenectomy. The overall cumulative incidence rate of serious bleeding events in these patients after splenectomy compared with before splenectomy was reduced although not significantly ($P = .15$). However, there was a significantly higher incidence of severe infectious events after splenectomy than before ($P = .005$). This might possibly be due to negligent AB prophylaxis in some patients. Of the 9 patients who did not receive AB prophylaxis, 3 had a severe (1 fatal) infection up to 53 years after splenectomy. This compared unfavorably, however not statistically significant, to patients who underwent splenectomy with AB prophylaxis in whom only 5 of 32 (1 fatal) had such an event ($P = .34$). Overall survival in patients who underwent splenectomy was not significantly different from patients not undergoing splenectomy (data not shown).

These data indicate that patients with XLT who underwent splenectomy are at significant risk of severe infections and require life-long AB prophylaxis.

Discussion

WAS is a multifaceted disorder with a wide spectrum of disease severity. In contrast to classic WAS, patients with a mild clinical phenotype, termed XLT, require comprehensive assessment in deciding on the strategy to provide optimal treatment. This is true for children who often present with selective microthrombocytopenia and have an uncertain long-term prognosis at a time when they are excellent candidates for allogeneic HSCT.[23,24] Similarly, adult patients with XLT who often are wrongly categorized as having chronic immune thrombocytopenic purpura and who may already have developed complications such as autoimmunity pose unique therapeutic challenges. This retrospective study was designed to better define the type of mutations and the clinical course of patients with XLT and to collect supportive evidence for optimal treatment choices.

The design of such a study requires a stringent definition of inclusion and exclusion criteria. The WAS scoring system has been used successfully in categorizing patients according to their disease severity.[10,11] However, an individual patient is not expected to keep the same score throughout his or her life. Progression from a score of 1 to 4 to a score of 5 by developing cancer or autoimmunity can occur at any age, and patients with classic WAS often present with a relatively mild phenotype during infancy. We, therefore, chose inclusion criteria that best reflect the situation when patients with XLT/WAS present in an immunodeficiency clinic. In addition to the classification as XLT by physicians experienced in treating patients with PIDs, we deliberately chose stringent criteria to prevent the

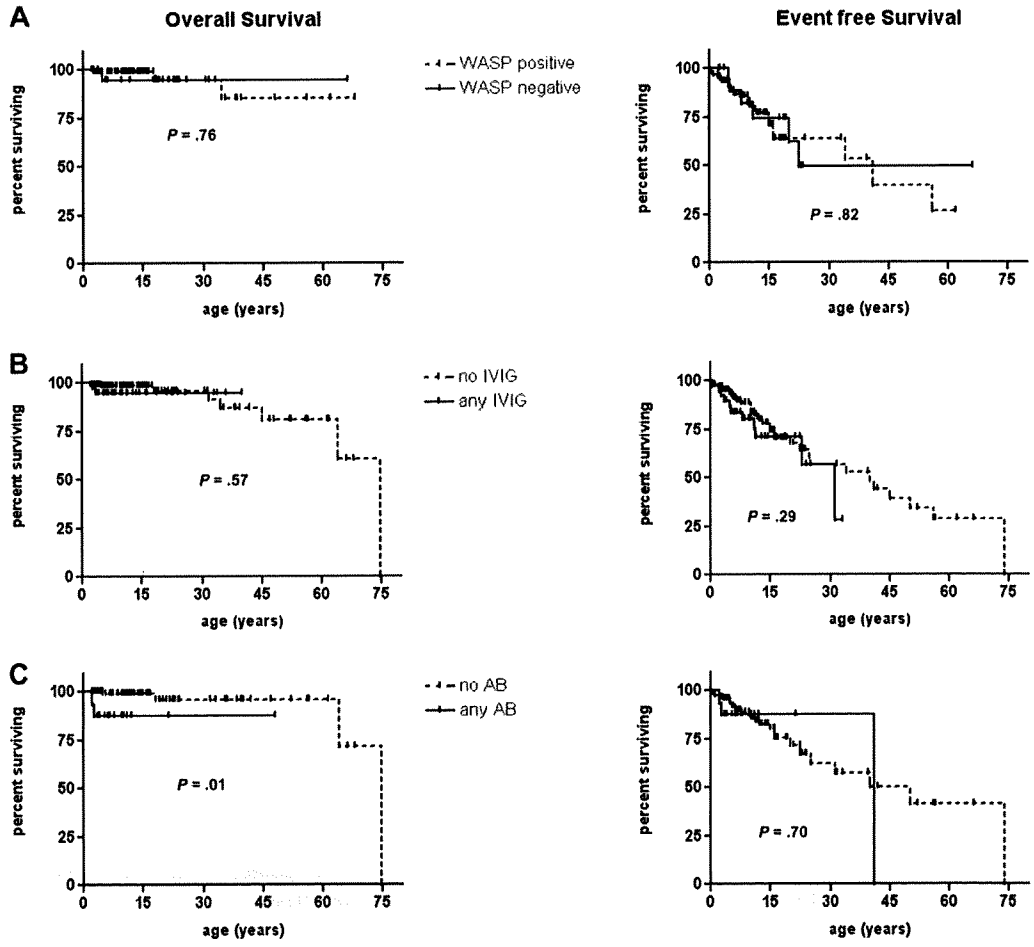

Figure 3. Influence of WASP expression, IVIG, or AB prophylaxis on overall and event-free survival. Kaplan-Meier estimate of overall survival and event-free survival probability of (A) WASP-positive (n = 98, dotted line) and WASP-negative (n = 21, solid line) patients. (B) Patients receiving any IVIG prophylaxis (n = 39, solid line) or no IVIG prophylaxis (n = 134, dotted line) and (C) patients receiving any AB prophylaxis (n = 16, solid line) or no AB prophylaxis (n = 116, dotted line). Patients who underwent splenectomy were excluded from the analysis in panel C. Each hash mark on a graph line indicates a censored event.

inclusion of patients with classic WAS with few disease symptoms as may be the case during the first 2 years of life. One possible drawback of this study could be its retrospective, cross-sectional design. It is probable that some events took place when medical care differed from that of today. Naturally, the study design might encompass a bias by some confounding factors such as patient compliance, physician preference, choice of prophylactic measures, and availability of HSCT. We can also not exclude some selection bias, missing very mild cases that are undiagnosed or misdiagnosed and not referred to an immunology center. But some older patients in this study had lived an uneventful life, before being diagnosed as XLT because their brothers, nephews, or grandsons were discovered to have a *WAS* gene mutation. Of note, the outcome of these older relatives did not differ from that of the rest of the cohort (data not shown). At this time the retrospective study design seems to be the only possible means to assess the clinical characteristics of a large cohort of patients with XLT. Having established this database of patients with XLT, we now have the opportunity to prospectively follow their course of disease.

Only 17.6% of evaluable patients with XLT from this cohort lacked WASP expression. In contrast, the proportion of WASP-negative patients from a multinational cohort of patients with WAS/XLT with known *WAS* mutations was 57% (104 of 184).[15] Some patients may in fact express WASP because the methods used to assess expression, such as Western blot analysis, might not be sensitive enough to detect low protein levels. This possibility is supported by the fact that 10 patients who were WASP negative had mutations (missense and invariant splice site) expected to result in WASP expression. In this selected cohort of patients with XLT, the clinical outcome of patients who did not express WASP was not different from patients who expressed WASP. Similarly, we did not find any beneficial effect of IVIG or AB prophylaxis on overall and event-free survival or on the incidence of life-threatening infectious events. These results have to be interpreted with caution, and a possible beneficial effect of these measures cannot be ruled out because data on AB and IVIG prophylaxis were very heterogeneous about dose and duration of treatment. They might solely reflect the fact that, by definition, most patients with XLT can mount effective antibody responses and therefore do not need IVIG or AB prophylaxis. It is possible that the initiation of these prophylactic measures might have been triggered by slightly more severe disease symptoms.[33]

In this cohort of 173 patients, 108 (62%) had missense mutations in the first 4 *WAS* exons; the remaining 38% (including 11 patients with missense mutations in exons 6-12) were spread over the entire gene, including 19% in noncoding regions. This is in line with previous reports of XLT.[13-15,33] We could not detect any

influence of the type of mutation on survival or on the incidence of specific disease-related events. A mild phenotype despite a deleterious mutation might be due to other disease-modifying genes, pathogen exposure, or somatic mosaicism caused by in vivo reversion, leading to some WASP expression and thus a milder phenotype. Reversion is an event quite frequent in WAS,[38,39] but it was not specifically analyzed in this cohort.

Forty-one patients (23.7%) had undergone splenectomy, reflecting the acceptance of splenectomy by some health care providers to reduce the risk of bleeding and thus improve quality of life in patients with XLT.[37,40] Interestingly, there was only a nonsignificant reduction of severe bleeding episodes after splenectomy, possibly because of the low overall incidence that decreased with age. However, the incidence of severe infections was significantly increased, especially in patients not receiving AB prophylaxis. These data suggest that, before splenectomy in patients with XLT, one needs to carefully weigh the pros and cons of this procedure. If performed, that is, in patients with recurrent episodes of serious bleeding, the family must understand the risk of infections and be willing to accept the need for AB prophylaxis. In addition, vaccination against pneumococci and meningococci has to be considered, given the fact that most patients with XLT can be effectively immunized.[33] The high incidence of severe infectious complications after splenectomy, including adult patients, highlights the importance of lifelong AB prophylaxis in patients with XLT who have undergone splenectomy.

The excellent overall survival rate that is close to that of the normal male population supports the perception that XLT is a mild, chronic disease and that, as a rule, patients with XLT do not require standard prophylactic interventions. Declining immune function has been observed in XLT, and defective antibody responses may require prophylactic measures such as IVIG in some patients. However, the reduced event-free survival shows substantial risks of severe, life-threatening or potentially debilitating disease-related complications. The cumulative incidence rate analysis of events showed that serious bleeding episodes were generally restricted to the first 30 years of life. In contrast, the risk of developing autoimmune disease, developing malignancy, or having a life-threatening infectious episode was rather constant throughout the patients' lifetime. The prevalence of autoimmunity is 12% in our cohort, suggesting that this complication is less common than in classic WAS whereby it was reported to be as high as 40% to 72%.[20,41,42] Interestingly, we found a significantly higher incidence of autoimmune nephropathy in Japanese patients. Similarly, the prevalence of malignancy was less in our XLT cohort (5%) than in classic WAS (13%).[20,43] Considering the higher mean age of patients with XLT compared with patients with classic WAS who have not received a transplant, these differences are even more significant.

The persistent morbidity associated with XLT might argue for HSCT as a treatment option for these patients. Given the excellent success in young children with classic WAS,[23,24] HSCT might be considered a viable option for patients with XLT if an human leukocyte antigen–identical donor can be identified. However, when discussing HSCT, which requires full conditioning in patients with WAS and patients with XLT, one needs to carefully weigh the advantage of a possible cure against the acute risks and long-term consequences of this procedure, such as risk of secondary malignancy and infertility. Thus, HSCT in XLT has to be decided on an individual patient basis. In our cohort 25 of 173 patients underwent HSCT at a median age of 7.3 years (range, 2.1-38.0 years) and 22 (88%) are alive after a median follow-up of 2.2 years (range, 0.0-12.1 years). Of note, more than half of the patients received their transplant at an age older than 5 years, when matched unrelated transplants in WAS may have a less favorable outcome.[23] Long-term studies of HSCT in patients with XLT, not available at present, are urgently needed.

Because patients with XLT may present to different medical specialists, it seems vital to raise awareness of this probably underdiagnosed or misdiagnosed condition. Although this study showed a high overall survival rate of patients with XLT, it also showed that they are at risk of life-threatening complications. By defining the natural course of XLT and recognizing the life-long medical problems that affect the prognosis and quality of life of these patients, it has become possible to select safe and effective individualized therapies for this unique set of patients with mutations of the *WAS* gene that are generally expected to be less devastating.

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Authorship

Contribution: M.H.A., B.H.B., and H.D.O. designed the study; all authors except P.P. contributed data; M.H.A., T.C.B., P.P., and H.D.O. analyzed the data; and M.H.A., T.CB., B.H.B., and H.D.O. wrote the paper.

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Correspondence: Michael H. Albert, Dr von Haunersches Kinderspital der LMU, Lindwurmstr 4, 80337 Munich, Germany; e-mail: michael.albert@med.lmu.de.

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ORIGINAL PAPER

Successful cord blood transplantation for a CHARGE syndrome with *CHD7* mutation showing DiGeorge sequence including hypoparathyroidism

Hirosuke Inoue · Hidetoshi Takada · Takeshi Kusuda · Takako Goto · Masayuki Ochiai · Tadamune Kinjo · Jun Muneuchi · Yasushi Takahata · Naomi Takahashi · Tomohiro Morio · Kenjiro Kosaki · Toshiro Hara

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Abstract It is rare that coloboma, heart anomalies, choanal atresia, retarded growth and development, and genital and ear anomalies (CHARGE) syndrome patients have DiGeorge sequence showing severe immunodeficiency due to the defect of the thymus. Although the only treatment to achieve immunological recovery for these patients in countries where thymic transplantation is not ethically approved would be hematopoietic cell transplantation, long-term survival has not been obtained in most patients. On the other hand, it is still not clarified whether hypoparathyroidism is one of the manifestations of CHARGE syndrome. We observed a CHARGE syndrome patient with chromodomain helicase DNA-binding protein 7 mutation showing DiGeorge sequence including the defect of T cells accompanied with the aplasia of the thymus, severe hypoparathyroidism, and conotruncal cardiac anomaly. He received unrelated cord blood transplantation without conditioning at 4 months of age. Recovery of T cell number and of proliferative response against mitogens was achieved by peripheral expansion of mature T cells in cord blood without thymic output. Although he is still suffering from severe hypoparathyroidism, he is alive without serious infections for 10 months.

Keywords CHARGE syndrome · DiGeorge sequence · *CHD7* mutation · Hypoparathyroidism · Cord blood transplantation

Abbreviations

CHD7	Chromodomain helicase DNA-binding protein 7
CBT	Cord blood transplantation
TCR	T cell receptor
PHA	Phytohemagglutinin
Con A	Concanavalin A
ABR	Auditory brainstem response
GVHD	Graft versus host disease

Introduction

Coloboma, heart anomalies, choanal atresia, retarded growth and development, and genital and ear anomalies (CHARGE) syndrome is a distinctive clinical entity with multiple congenital anomalies [12]. Mutations in the gene chromodomain helicase DNA-binding protein 7 (*CHD7*) were identified as a cause of CHARGE syndrome [25]. *CHD7* on chromosome 8 (8q12.1) is a member of the chromodomain helicase DNA binding domain family [25]. Chromatin remodeling is a recognized mechanism of gene expression regulation, and the *CHD7* gene is likely to play a significant role in embryonic development and cell cycle regulation [29]. CHD7 is expressed throughout the neural crest containing mesenchyme of the pharyngeal arches. Mouse embryo at 10.5 days postcoitum expressed *Chd7* in

H. Inoue · H. Takada (✉) · T. Kusuda · T. Goto · M. Ochiai · T. Kinjo · J. Muneuchi · Y. Takahata · T. Hara
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University,
3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan
e-mail: takadah@pediatr.med.kyushu-u.ac.jp

N. Takahashi · T. Morio
Department of Pediatrics and Developmental Biology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University,
Tokyo, Japan

K. Kosaki
Department of Pediatrics, School of Medicine, Keio University,
Tokyo, Japan

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the cardiac outflow tract, truncus arteriosus, facio-acoustic preganglion complex, hindbrain, forebrain, mandibular component of the first branchial arch, otic vesicle, optic stalk/optic vesicle, and olfactory pit [12]. Thus, CHARGE syndrome has the potential of multiple presentations.

Cellular immunodeficiency due to the lack of the thymus is not widely recognized as a manifestation of CHARGE syndrome. Recently, severe hypoparathyroidism and conotruncal cardiac anomaly were reported in patients with CHARGE syndrome caused by *CHD7* mutations having DiGeorge sequence characterized by the defect of T cells accompanied by thymus aplasia [21, 28, 30]. Although thymus hypoplasia or agenesis is rare in postnatal CHARGE syndrome cases [3], Sanlaville et al. reported that it was observed in seven of ten CHARGE syndrome fetuses [22]. Recently, Jyonouchi et al. reported that 8% (two of 25) of CHARGE syndrome patients had a phenotype of severe combined immunodeficiency with defect of T cells [10]. On the other hand, it is still not clarified whether hypoparathyroidism is one of the manifestations of CHARGE syndrome since only three CHARGE syndrome patients with *CHD7* mutation were reported to have hypoparathyroidism [21, 28, 30]. It is suggested that neural crest defect underlies the clinical overlap of both chromosome 22q11 deletion and CHARGE syndrome [22]. Accordingly, a case manifesting the CHARGE syndrome with deletion in chromosome 22q11 was reported [7].

Here, we report a patient with CHARGE syndrome with a *CHD7* mutation, who had severe T cell immune deficiencies due to thymic aplasia, severe limb anomalies, and congenital hypoparathyroidism. He was successfully treated with cord blood transplantation (CBT).

Case report

The patient was born at 39 weeks of gestational age. His birth weight was 2,488 g. Cardiac anomaly and polyhydramnion were detected by fetal ultrasound examination during his late prenatal period. Karyotype analysis of amniotic fluid showed 46,XY. His family members were healthy without having even minor anomalies.

Shortly after birth, he was admitted to the neonatal intensive care unit (NICU) in Kyushu University Hospital. He showed the characteristic facial features such as a hypertelorism and unilateral facial palsy (Fig. 1a), asymmetry of ears with protruding, helix hypoplasia, low-set and square-shaped right ear, absent anthelix, low-set left ear (Fig. 1b, c), and bilateral coloboma of the choroid. In addition, thumb polydactyly and cleft of the right hand and cleft and cutaneous syndactyly of the bilateral feet were observed (Fig. 1d–g). He had no genital abnormalities. Hematological examinations revealed white blood cell count of 4,330/μl with severe lymphopenia (neutrophils 68.5%, lymphocytes 7%, monocytes 18%). Serum calcium, phosphorus, and parathyroid hormone levels were 7.8 mg/dl, 8.4 mg/dl, and 4.5 pg/ml, respectively, showing hypoparathyroidism. Serum thyroid hormone levels were normal. Lymphocyte surface marker analysis by a flow cytometer revealed a marked decrease of T lymphocytes: $CD3^+$ 2.8% (8 cells/μl), $CD4^+$ 2.3% (7 cells/μl), and $CD8^+$ 15.3% (46 cells/μl; Table 1). T cell receptor (TCR) $\gamma\delta^+$ cells, $CD16^+/CD56^+$ cells, and $CD19^+$ cells were 0.1%, 35.9%, and 52.9%, respectively. Proliferative response of mononuclear cells against phytohemagglutinin (PHA) and concanavalin A (Con A) was 123 %S.I. (normal controls; 254–388) and 2,530 cpm (20,300–65,700), respectively. Analysis of the TCRVβ repertoire showed an abnormal pattern with overexpansion of $V\beta21.3^+$ cells (20.9%; Fig. 2a). Serum IgG, IgA, and IgM concentrations were 899, 5, and 19 mg/dl, respectively. Fluorescent in situ hybridization analysis revealed a lack of maternal cell engraftment in peripheral blood and no deletion at 22q11.2.

Computed tomography and fiberoptic laryngoscope examination revealed left choanal atresia with posterior choanal stenosis and laryngomalacia, respectively. Auditory brainstem response revealed bilateral severe sensorineuronal hearing loss. An echocardiogram and chest computed tomography scan revealed truncus arteriosus (Van Praagh type A4) and interruption of aortic arch (type B) with aberrant right subclavian artery. At 14 days of age, he underwent bilateral pulmonary artery banding operation because he was too small to receive the radical correction of truncus arteriosus and interruption of aortic arch at that time. Thymus was not detected at the time of operation.

Thus, we made the clinical diagnosis of CHARGE syndrome with manifestations of complete-type athymic DiGeorge sequence. The *CHD7* gene of the patient was analyzed according to the method described previously [2], and heterozygous c.1036A > T (R346X) mutation was observed in exon 2. He received unrelated CBT without conditioning at 4 months of age (Fig. 2b). Human leukocyte antigen full-matched female cord blood cells (28.03×10^7 cells/kg) were infused. FK506 and short-term methotrexate were used for graft versus host disease (GVHD) prophylaxis. He had only mild skin manifestation of GVHD, which resolved by prednisolone (1 mg/kg/day). On day 25 after CBT, $CD3^+$ cells increased to 60.1% of lymphocytes (1,471 cells/μl), 93.8% of which were positive for CD45RO. Analysis of the TCRVβ repertoire on day 27 showed an abnormal pattern with overexpansion of $V\beta16^+$ cells (7.3%) and $V\beta17^+$ cells (9.7%), and a different profile was observed between pre-CBT and post-CBT (Fig. 2a). Proliferative response to Con A and PHA normalized on day 50 (20,500 cpm) and on day 174 (284 %S.I.), respectively. Chimerism analysis on day 173 showed that most of the $CD3^+$

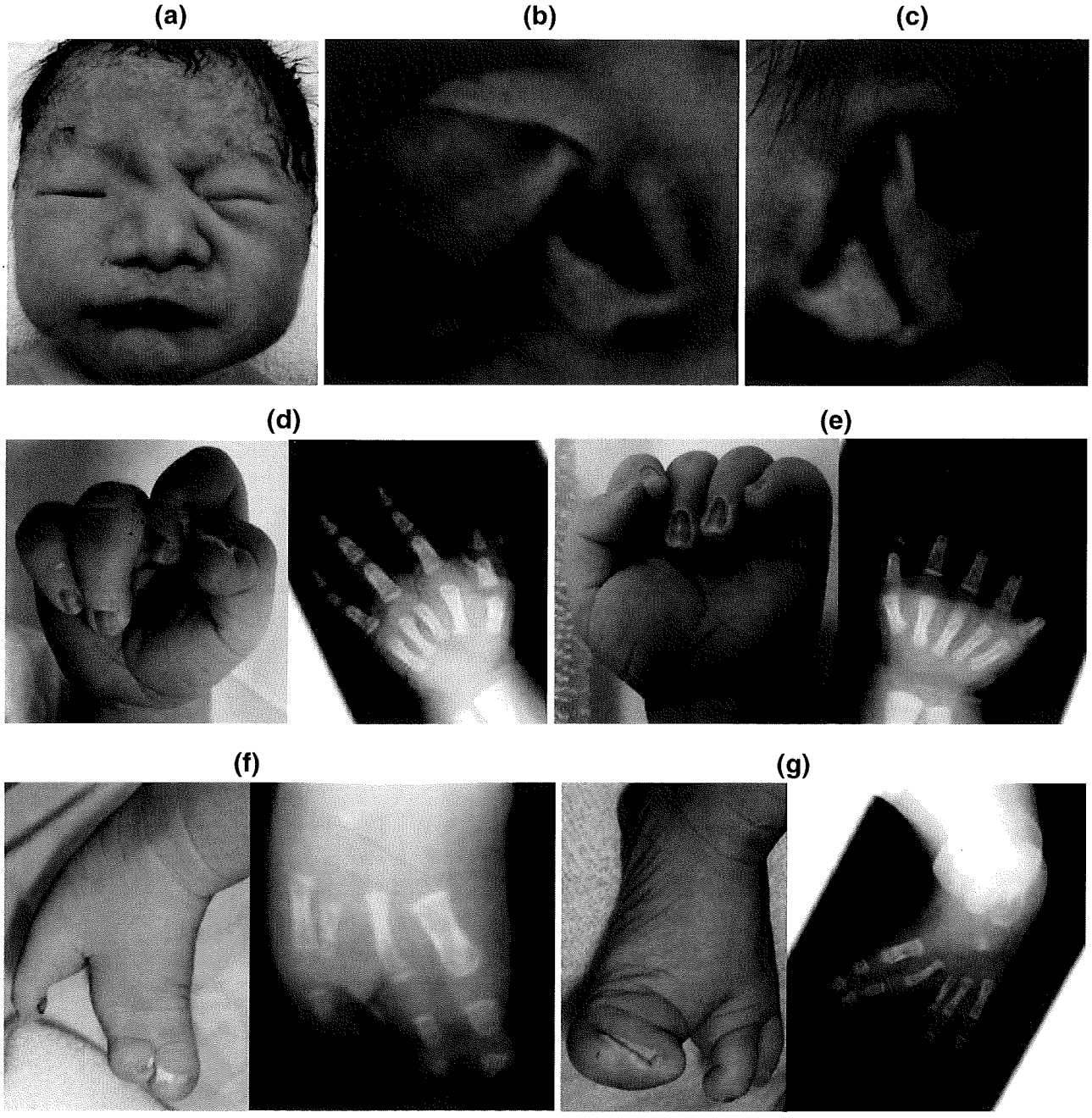

Fig. 1 Clinical manifestations of the patients. **a** Frontal view of the face showing hypertelorism and right facial palsy. **b** Lateral view of the right ear showing protruding, helix hypoplasia, and low-set ear. **c** Lateral view of the left ear showing square-shaped, absent anthelix, and low-set ear. Note asymmetry of ears. **d** Thumb polydactyly and cleft of the right hand. **e** Normal left hand. **f**, **g** Cleft and cutaneous syndactyly of the bilateral foots. Written consent was obtained for publication of these pictures

cells were of donor origin (94.5% of $CD3^+$ cells were XX, 5.5% were XY). At 10 months of age (day 169 after CBT), $CD3^+$ cells were 36.3% of lymphocytes (973 cells/μl), and 86.2% of T cells were positive for CD45RO. T cell receptor excision circles were below the detection limit before CBT, confirming the lack of thymic output (data not shown).

He is alive without serious infections with regular administration of immunoglobulin and prophylactic antibiotics. At 10 months of age, serum calcium, phosphorus, and parathyroid hormone levels are 7.2 mg/dl, 6.1 mg/dl, and 3.0 pg/ml, respectively. He is still receiving calcium preparation and alfacalcidol.

Table 1 Immunological studies

	Pretransplantation	Posttransplantation
$CD3^+$ cells (% lymphocytes)	2.8	36.3
$CD3^+$ cells (cells/μl)	8	973
$CD45RO^+/CD3^+$ (%)	87.7	86.2
$CD45RO^-/CD3^+$ (%)	12.4	10.9
$CD4^+$ cells (% lymphocytes)	2.3	24.2
$CD4^+$ cells (cells/μl)	7	648
$CD8^+$ cells (% lymphocytes)	15.3	12.1
$CD8^+$ cells (cells/μl)	46	324
TCR $\gamma\delta^+$ (%)	0.1	0.2
$CD19^+$ (%)	52.9	33.3
$CD16^+/CD56^+$ (%)	35.9	29.7
Proliferative response		
Against PHA (%S.I.)	123	284
Against Con A (cpm)	2,530	20,500
IgG (mg/dl)	899	425
IgM (mg/dl)	19	83
IgA (mg/dl)	5	66
Karyotype of $CD3^+$ cells	99.5% of 46,XY	5.5% of 46,XY
	0.5% of 46,XX	94.5% of 46,XX

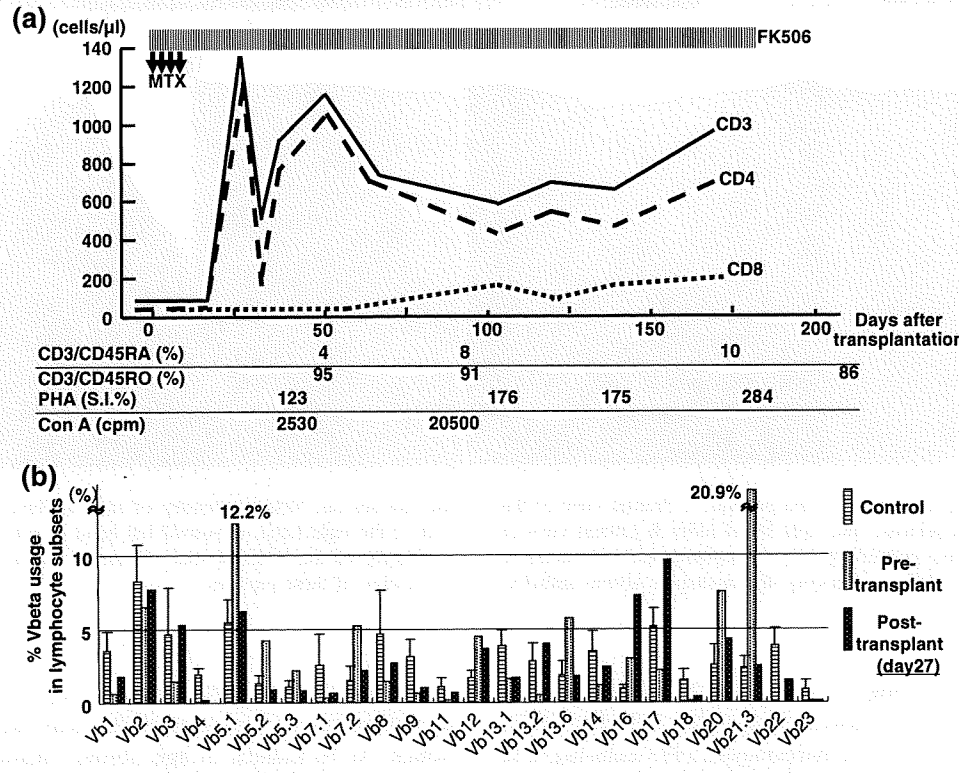

Fig. 2 Clinical course and immunological recovery after the cord blood transplantation. **a** Clinical course of the cord blood transplantation. *MTX* methotrexate, *PHA* phytohemagglutinin, *Con A* concanavalin A. **b** TCRVβ repertoire profile on the patient and control subjects. Note the skewing in the TCR repertoire before and after transplantation. *TCR* T cell receptor

Discussion

Our patient showed absence of T lymphocytes accompanied with aplasia of the thymus manifesting complete-type DiGeorge sequence, a rare complication of CHARGE syndrome [1, 30]. T cell number of the patient was recovered by CBT, although most of the T cells showed memory phenotype reflecting peripheral expansion of donor cord blood-derived mature T cells and the lack of the thymic output. He presented with additional rare manifestations, severe limb anomalies, and congenital hypoparathyroidism. DiGeorge sequence is associated with a deletion of chromosome 22q11.2 in approximately 80% of patients [23]. Interestingly, Markert et al. reported that only 52% of 54 patients with DiGeorge sequence had a deletion of 22q11, and 26% had CHARGE phenotype without the deletion [15]. A number of genes have been identified in the 22q11.2 region [31], including *TBX1* that is a major genetic determinant of del22q11.2 syndrome. As TBX1 is a transcription factor that contains a DNA binding domain, it is possible that TBX1 is a functional target for CHD7.

Thymic hypo/agenesis was observed in 70% of fetuses with CHARGE syndrome [22]. The high frequency of thymic defect in fetuses suggests that accompanying immune deficiency may be more common in this disease than previously reported. It is possible that many of athymic patients were counted on DiGeorge syndrome, rather than CHARGE syndrome. Otherwise, CHARGE syndrome patients with thymic defect may more often die during perinatal period because of the immunodeficiency or other accompanying anomalies such as severe cardiac defect. Although there have been a few reports of stem cell transplantation for the treatment of T cell deficiency in complete-type DiGeorge sequence, this is the first case of CBT for the treatment of CHARGE syndrome with *CHD7* mutation manifesting T cell defect [8, 14, 18]. The optimal treatment for patients with complete-type DiGeorge sequence has not been established. In the absence of treatment, patients usually die in the first 2 years of life [16]. Therefore, prompt reconstitution of the immune function is required to prevent fatal infectious complications. The common treatments for immunological reconstitution in complete-type DiGeorge sequence are thymic and bone marrow transplantation [13, 15]. Although thymic transplantation would be more reasonable from the physiological point of view, it is not ethically approved in Japan. We selected CBT without conditioning regimen for our patient because of the following reasons: (1) lack of sibling donors, (2) more noninvasive procurement and more rapid availability than the matched unrelated donors, (3) lower risk of GVHD or viral transmission in CBT compared with bone marrow or peripheral blood stem cells [5], and (4) higher frequency of naïve T cells in cord blood [6], which have a longer lifespan than their memory counterparts [26]. Because of the lack of thymic output after the transplantation in this disease, the high frequency of naïve T cells in the donor cells may be an important factor to avoid early immune senescence. On the other hand, it may take more time for the recovery of neutrophils in CBT leaving a higher risk of infection compared with bone marrow or peripheral stem cell transplantation [5]. In addition, naïve T cells in cord blood may require a longer time to mature into effecter memory cells and thus do not provide immediate defense against microbial agents [11]. Our patient received CBT in the NICU and has been bred in a closed infant incubator since birth. This might in part contribute to the decrease of the risk of infections and to the success of CBT.

Ryan et al. [20] reported that only a few patients (1–4%) had mild limb abnormalities in 548 patients with chromosome 22q11 deletions. Limb anomalies were not initially described in CHARGE syndrome [4]. Recently, limb anomalies have been reported as a rare manifestation in CHARGE syndrome [3, 17, 19]. On the other hand, Brock et al. [4] reported that limb anomalies occurred in about 30% of patients with definite or probable CHARGE syndrome. It is interesting that limb anomalies with DiGeorge sequence are more frequently observed in male (P value <0.034), and limb anomalies were observed in 70.0% of male DiGeorge sequence with definite CHARGE syndrome [4]. Williams proposed that CHARGE syndrome is caused by a disruption of mesenchymal–epithelial (including ectoderm and endoderm) interaction [27]. Sanlaville et al. [22] showed that the *CHD7* gene is also expressed in the limb bud mesenchyme during embryogenesis. Van de Laar et al. [24] reported that three CHARGE syndrome patients with CHD7 mutation had severe limb anomalies. Therefore, it is possible that *CHD7* mutation itself is responsible for limb defects, and limb anomalies are more strongly associated with *CHD7* mutation than 22q11 deletion.

In patients with 22q11 deletion, 203 of 340 (60%) had hypoparathyroidism and hypocalcaemia, and the hypocalcaemia resolved in 70% [20]. On the other hand, only three CHARGE syndrome patients with *CHD7* mutations had hypoparathyroidism [21, 28, 30]. It is interesting that the three patients had severe T cell deficiency [21, 28, 30]. As TBX1 might be a functional target of CHD7, it is possible that hypoparathyroidism may be more common in CHARGE syndrome than previously recognized. Günther et al. showed by using *glial cells missing2*-deficient mice that thymus had a backup mechanism of parathyroid gland and thymus itself secreted parathyroid hormone when parathyroid glands was absent [9]. It is possible that intractable hypocalcemia continues when both parathyroid gland and thymus are absent.