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

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

2. Materials and methods

2.1. Patients

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

Table 1 Immunological features of the patients at diagnosis.

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

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

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

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

2.2. Mutation analysis of RAG1 and RAG2

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

2.3. V(D)J recombination assay

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

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

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

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

3. Results

3.1. RAG mutations

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

Table 2 *RAG* mutations and recombination activity.

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

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

3.2. Recombination activity of RAG mutants

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

3.3. B cell analysis of patient 4

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

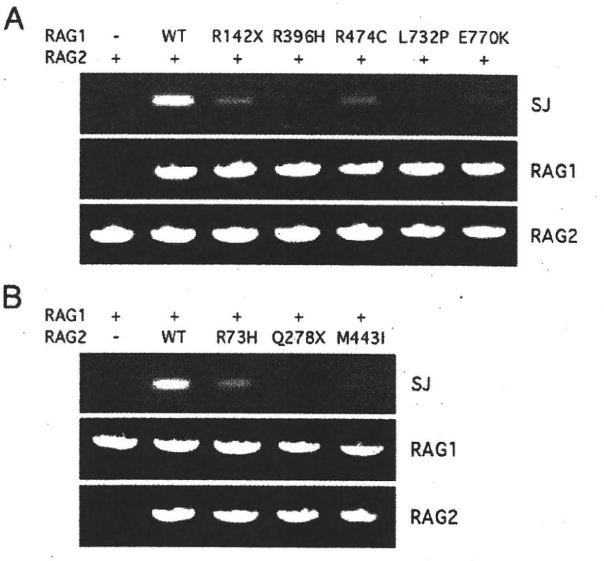
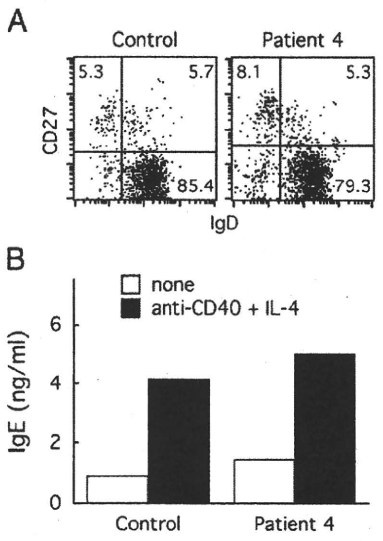


Figure 1 V(D)J recombination assay. V(D)J recombination activity was assessed by using the recombination substrate pJH200 in 293T cells that were cotransfected with mutant RAG1 and wild type RAG2 (A), or with wild type RAG1 and mutant RAG2 (B). Recombined products (signal joints, SJ) were analyzed by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



C

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

To

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

Patient 4

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

Control

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

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

4. Discussion

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

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

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

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

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

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

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

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

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LETTER TO THE EDITOR

Hematopoietic stem cell transplantation for X-linked thrombocytopenia from mild symptomatic carrier

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The prognosis of classic Wiskott–Aldrich syndrome (WAS) with complete lack of WAS protein (WASP) expression in the absence of hematopoietic stem cell transplantation (HSCT) is poor,[1,2] whereas WASP-positive patients with the X-linked thrombocytopenia (XLT) phenotype, which is characterized by thrombocytopenia and small platelets, also have a poor long-term outcome without HSCT.[3] If there is a suitable donor, HSCT is the treatment of choice for WASP-positive patients with XLT because of their high frequency of complications, including intracranial hemorrhage, autoimmune diseases and IgA nephropathy.[3] Two-thirds of WASP-positive patients with splice-site mutations exhibit XLT and show the highest incidence of lymphomas, particularly for intron 6 splice-site mutations.[4]

We reported earlier several members of a single family diagnosed with XLT.[5] Briefly, the present patient was a 9-year-old boy with a history of severe cranial hematoma at birth and purpura at the age of 2 months. He had no history of eczema, severe infection or autoimmune disease. Platelet count was 19×10^9/l with small platelets (mean platelet volume [MPV]: 4.8 fl) at age 9 years. Direct sequencing of the *WASP* gene revealed a splice-site G-to-A mutation at position +5 in intron 6. WASP expression levels in the patient were one-third those in the normal control, and the mother was a heterozygous carrier of the same mutation. His sister was diagnosed as a heterozygous symptomatic carrier of XLT at age 6 years, and her maternally derived X-chromosome was randomly inactivated.[5] Her platelet counts increased gradually from 39×10^9/l with low MPV at diagnosis to 80×10^9/l with normal MPV at age 15 years, which suggested a proliferation/survival advantage in cells with active chromosomes bearing normal *WASP* or spontaneous *in vivo* reversion of the *WASP* mutation would develop.[6]

He received HSCT with non-manipulated bone marrow from the 15-year-old sister, whose platelet count was 80×10^9/l with normal MPV and who was the only HLA-identical individual in the patient's family. The donor and recipient lymphocyte subsets and *in vitro* lymphocyte proliferation in response to mitogens (phytohemagglutinin and concanavalin A) were normal at the time of HSCT. The conditioning regimen consisted of oral busulfan 1 mg/kg × 16 doses over 4 days, followed by intravenous cyclophosphamide 50 mg/kg/day for 4 days. Busulfan achieved a steady-state concentration of 680 ng/ml. Clonazepam and mesna were used for prophylaxis against convulsion and hemorrhagic cystitis, respectively, during conditioning. GVHD prophylaxis was short-term methotrexate administered intravenously at a dose of 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6 and 11, and cyclosporine A until day +97. G-CSF was used from days +5 to +17. Hematopoietic reconstitution was rapid, with an absolute neutrophil count of $>0.5 \times 10^9$/l on day +14 and platelet engraftment was achieved on day +16. Complete donor chimerism of bone marrow cells was maintained from day 21 over the 24 months according to fluorescence *in situ* hybridization. He did not develop acute or chronic graft-versus-host disease, regimen-related complications or infection peri-HSCT. At 24 months after HSCT, lymphocyte subsets and *in vitro* lymphocyte proliferation in response to mitogens were normal, although platelet counts remained at 40×10^9/l with normal MPV; these were approximately half the platelet counts in the donor.

We used two-color flow cytometry (FCM)-WASP to serially detect WASP expression in the recipient and donor before and after HSCT (Figure 1).[7,8] We obtained approval for this study from the Dokkyo Medical University Institutional Review Board. Before HSCT, the recipient scarcely expressed intracellular WASP (WASP^{dim}) in CD3$^+$, CD19$^+$ and CD56$^+$ cells, but in the donor at HSCT, intracellular populations with both WASP (WASP^{bright}) and WASP^{dim} were noted, with the WASP^{bright} population being predominant. At 12 months after HSCT, the recipient's proportion of WASP^{bright} to WASP^{dim} among CD3$^+$ cells was comparable to that of the donor, whereas CD19$^+$ and CD56$^+$ cells were predominant among the WASP^{dim} population. At 24 months after HSCT, the proportion of WASP^{dim} in CD19$^+$ and CD56$^+$ cells was lower in the recipient, but remained present. Over time, the recipient has begun to show a similar pattern to that of the donor and now has WASP^{bright} cell populations that were absent before HSCT.

The X-chromosome inactivation patterns of CD3$^+$, CD19$^+$ and CD56$^+$ cells in the donor and recipient were investigated by methylation-specific polymerase chain reaction assay, which was performed using the human androgen receptor (HUMARA) locus involving a methylation-specific polymerase chain reaction technique, as described earlier.[9] The donor was found to be heterozygous at the *HUMARA* locus and showed a random pattern that was consistent with those reported earlier,[5] indicating a smaller allele (X1) and lager allele (X2) carrying the mutated *WASP* and normal *WASP*, respectively. The recipient at 24 months after HSCT showed a random inactive X-chromosome pattern and the ratio of inactive X1 to X2 in CD3$^+$ cells was approaching that in the donor,

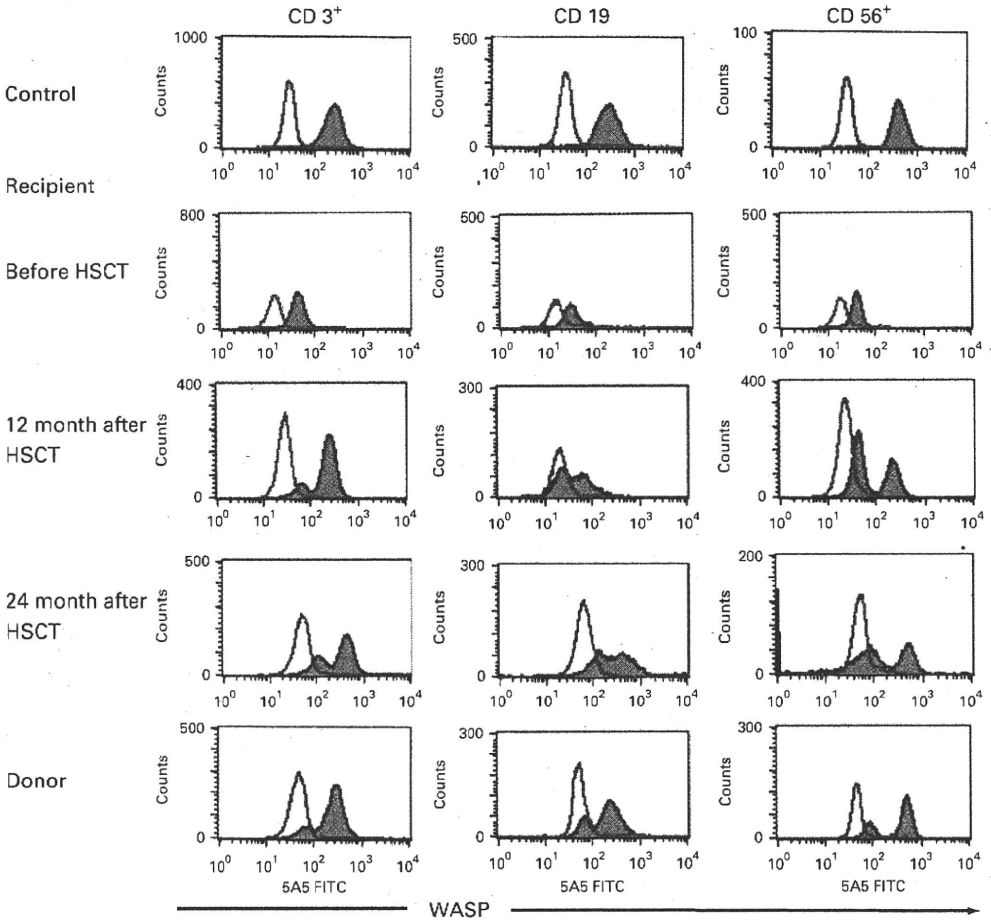

Figure 1 Results of FCM-WASP in lymphocytes before and after HSCT. The *x* axis represents WASP expression; the *y* axis represents cell numbers. The open histogram indicates isotype-control staining; the solid histogram indicates specific staining for WASP. Shown is WASP expression in subpopulations of lymphocytes from healthy control (top panel), the patient before HSCT (second panel), the patient at 12 months after HSCT (third panel), the patient at 24 months after HSCT (fourth panel) and the donor (bottom panel).

being 58:42 in the recipient and 70:30 in the donor (Figure 2, left columns). However, $CD19^+$ and $CD56^+$ cells showed suppressive inactivation (that is, preferential activation) of the mutated allele (X1) at 24 months after HSCT in the recipient, with ratios of 41:59 and 33:67, whereas $CD19^+$ and $CD56^+$ cells showed preferential inactivation of the mutated allele (X1) in the donor, with ratios of 66:34 and 53:47 (Figure 2, middle and right columns). Interestingly, these results were consistent with the recipient and donor results for $WASP^{dim}$ predominance in $CD19^+$ and $CD56^+$ cells obtained by FCM-WASP. On the basis of these results, we speculate that all lymphocyte lineages in the patient will slowly develop into the same pattern as the donor, who showed a predominantly $WASP^{bright}$ population due to the proliferation/survival advantage of the cells with normal WASP.

Long-term follow-up after HSCT is required to assess the clinical course of the patient by FCM-WASP and methylation-specific polymerase chain reaction. It is important to observe how long the acquired random X-inactivation by HSCT will be maintained in this patient.

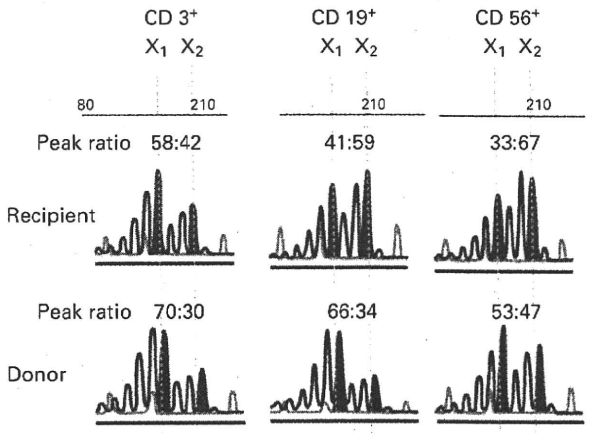

Figure 2 Results of X-chromosome inactivation ratio after HSCT. X-chromosome inactivation using *HUMARA* gene by methylation-specific polymerase chain reaction (M-PCR). Peaks on M-PCR assay of lymphocyte subpopulations show X-inactivation in the patient at 24 months after HSCT (upper panel) and the donor (lower panel). X1 allele and X2 allele refer to X-chromosomes carrying mutated *WASP* and normal *WASP*, respectively. Peak ratio numbers indicate the X-inactivation ratios of X1 and X2, respectively.

Conflict of interest

The authors declare no conflict of interest.

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

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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.

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.

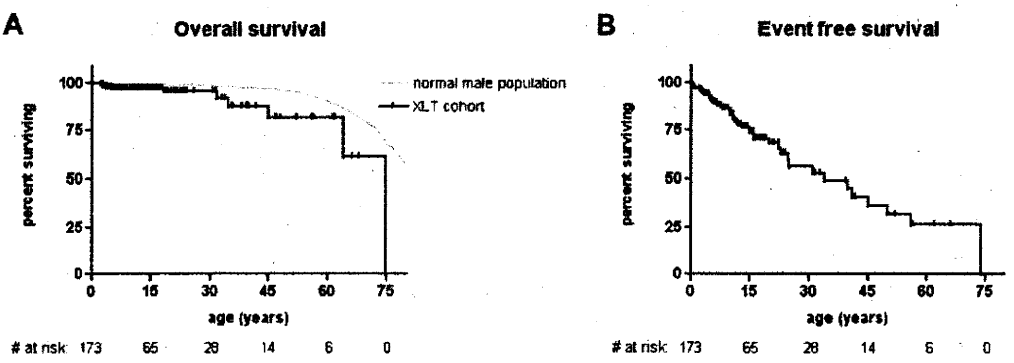

Figure 1. Overall and event-free survival. (A) Kaplan-Meier estimate of overall survival probability of all study patients compared with survival of the normal German male population 2006.[34] (B) Event-free survival probability. Event was defined as a severe or fatal infection, severe or fatal bleeding, 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

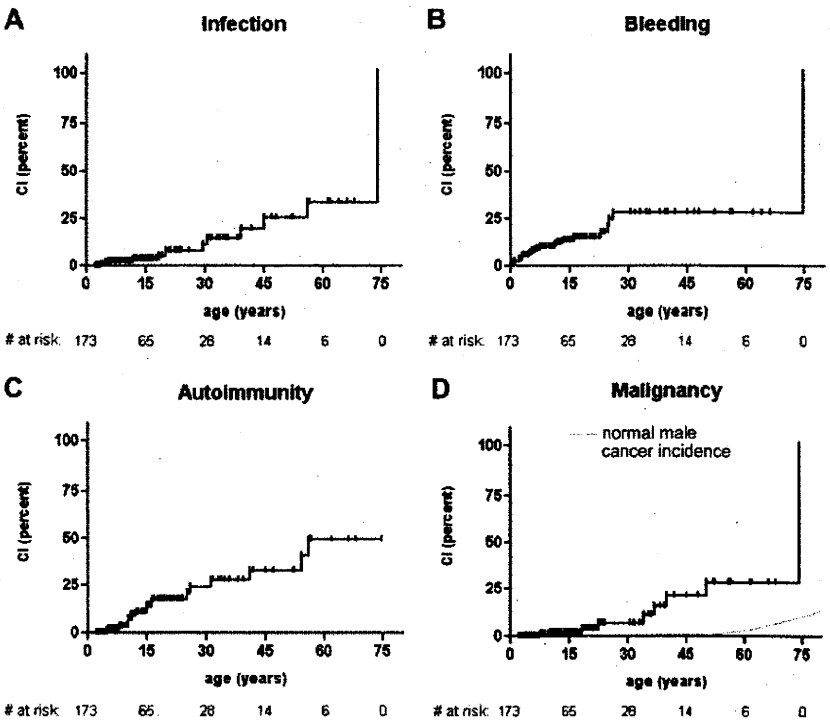

Figure 2. Cumulative incidence rate of severe events. Cumulative incidence of (A) severe or fatal infectious episodes in the study cohort, (B) severe or fatal bleeding episodes, (C) autoimmune disease, and (D) malignancy, compared with cancer incidence in the US male population.[35] Each hash mark on 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

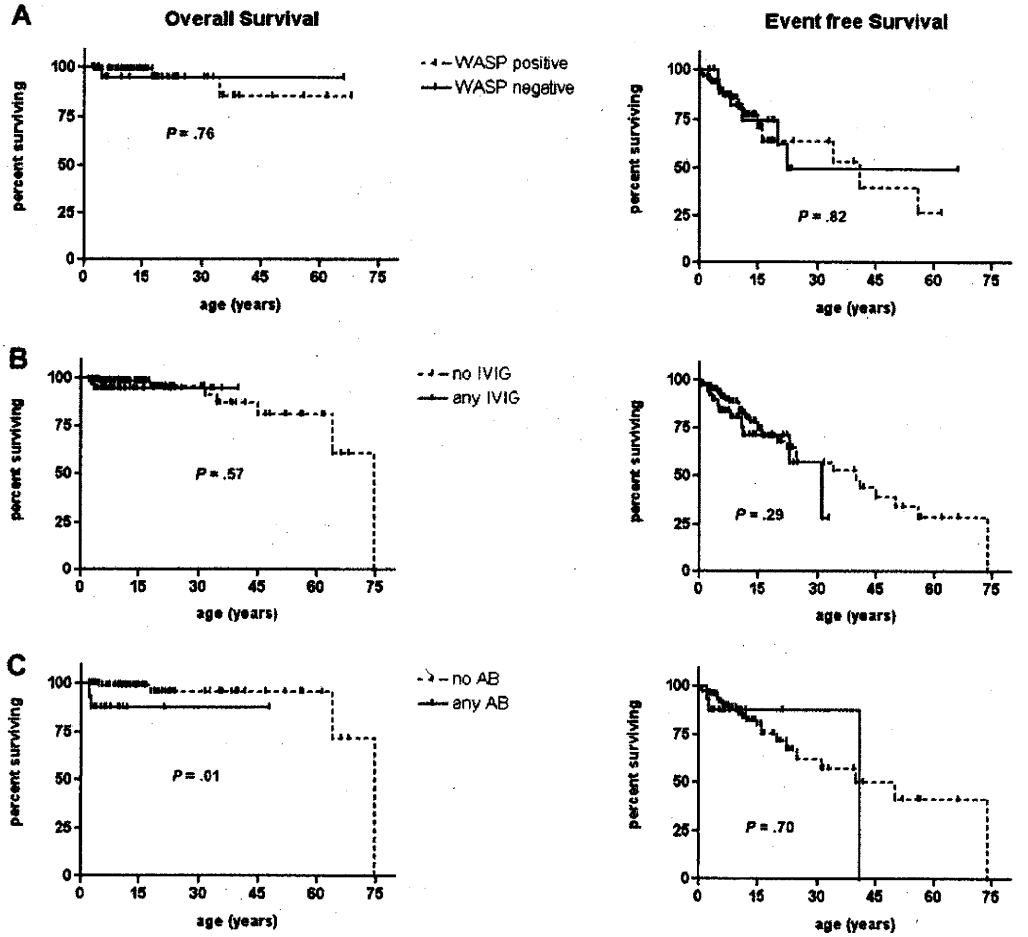

Figure 3. Influence of WASP expression, IVIG, or AB prophylaxis on overall and event-free survival. Kaplan-Meier estimate of overall survival and event-free survival probability of (A) WASP-positive (n = 98, dotted line) and WASP-negative (n = 21, solid line) patients. (B) Patients receiving any IVIG 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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Autoimmune Lymphoproliferative Syndrome Like Disease With Somatic *KRAS* Mutation

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Abstract

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 two cases with ALPS like disease with somatic *KRAS* mutation. Both of the cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia (JMML), and are likely to be defined as a new disease entity of RAS associated ALPS like disease (RALD).

Introduction

ALPS is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway[1,2], 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-γ-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[3]. 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 IL-2 depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS associated leukoproliferative disease [4].

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

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous (CFC) syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7],

among these tumors the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present two 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 CFC or Noonan syndrome.

Patients and 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 data 1 Fig. 1a, b). Blood test revealed presence of hemolytic anemia and autoimmune thrombocytopenia. hyper-γ-globulinemia with various auto-antibodies 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 1. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in results and discussion section.

Case 2

A 5-month-old girl had a fever, massive hepatosplenomegaly (Supplemental data 1 Fig. 1d). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and auto-antibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNC showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive