Table 1 Clinical and genetic data of patients with X-linked lymphoproliferative syndrome

Patient ID	Age at diagnosis	Family history	Clinical presentation	Epstein–Barr virus status	IVIG	Outcome	Cause of death	Age at death or presence	*SH2D1A* mutation	SAP expression
1.1	12 yr	+	Hypo-γ, LPD	+	+	Dead	GVHD	12 yr	NE	NE
1.2	7 yr	+	Hypo-γ, LPD	+	+	Alive*		21 yr	Asp33Tyr	NE
2.1	3 yr	−	FIM	+	−	Dead	FIM	3 yr	Arg55stop	NE
3.1	2 yr	+	FIM	+	−	Dead	FIM	2 yr	Arg55stop	NE
3.2	2 yr	+	FIM	+	−	Dead	FIM	2 yr	Arg55stop	NE
4.1	2 yr	+	FIM	+	−	Dead	FIM	2 yr	416C>T, fs	NE
4.2	4 yr	+	ML, vasculitis, HLH	−	−	Dead	HLH (MOF)	14 yr	416C>T, fs	Deficient
5.1	1 yr	+	FIM	+	+	Dead	FIM	1 yr	del of whole gene	NE
6.1	1 yr	−	FIM	+	−	Dead	FIM	1 yr	Gly27Ser	NE
7.1	1 yr	+	Hypo-γ, aplastic anemia	+	+	Dead	Sepsis	1 yr	NE	NE
7.2	3 yr	+	Hypo-γ, vasculitis	−	+	Alive*		30 yr	His8Asp	Deficient
8.1	1 yr	−	FIM	+	+	Dead	FIM	1 yr	584delA, fs	NE
9.1	6 yr	+	Hypo-γ	+	+	Alive*		18 yr	Arg55stop	Deficient
9.2	6 months	+	FIM	+	+	Dead*	Sepsis	6 yr	Arg55stop	Deficient
10.1	4 yr	+	ML	+	−	Alive*		15 yr	Gly49Val	Deficient
10.2	0 months	+	Healthy	−	−	Alive*		4 yr	Gly49Val	Deficient
11.1	1 yr	+	FIM	+	+	Dead	FIM (MOF)	1 yr	del of exons 3, 4	NE
11.2.	2 yr	+	FIM	+	+	Dead	FIM (MOF)	2 yr	del of exons 3, 4	Deficient
11.3	0 month	+	Healthy	−	+	Alive*		9 yr	del of exons 3, 4	Deficient
12.1	12 yr	+	Hypo-γ, ML	+	−	Dead	ML	12 yr	Ser34Gly	Deficient
12.2	10 yr	+	Hypo-γ	+	−	Unknown	Unknown	Unknown	Ser34Gly	Deficient
13.1	23 yr	−	FIM	+	−	Dead	FIM	23 yr	Tyr7Cys	Deficient
14.1	8 yr	−	Hypo-γ, ML	+	−	Alive*		16 yr	Arg55stop	Deficient
15.1	2 yr	−	FIM	+	−	Dead	FIM	2 yr	His8Asp	NE
16.1	10 yr	−	Hypo-γ, HLH	−	+	Alive*		17 yr	545insA, fs	Deficient
17.1	2 yr	+	FIM	+	−	Dead	FIM	2 yr	IVS2+1G>A	Deficient
17.2	2 yr	+	ADEM	−	−	Alive*		8 yr	IVS2+1G>A	Deficient
18.1	6 yr	−	Hypo-γ	+	+	Alive*		12 yr	312insG, fs	Deficient
19.1	10 months	+	Hypo-γ	+	+	Dead	DIC	10 months	NE	NE
19.2	1 yr	+	FIM	+	−	Dead		1 yr	NE	NE
19.3	3 yr	+	Hypo-γ, HLH, ML	+	+	Alive*		18 yr	del of exons 3, 4	Deficient
20.1	41 yr	−	FIM	+	−	Dead	FIM	42 yr	Ala3Ser	Deficient
21.1	3 yr	−	Encephalitis, LPD	+	−	Dead	Encephalitis	3 yr	538insA, fs	Deficient

Hypo-γ, hypogammaglobulinemia; LPD, lymphoproliferative disease; GVHD, graft versus host disease; FIM; fulminant infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis; MOF, multiple organ failure; ML, malignant lymphoma; ADEM, acute disseminated encephalomyelitis; DIC, disseminated intravascular coagulation; NE, not examined; fs, frameshift; del, deletion; ins, insertion.
P17.1 and 17.2 are monozygotic twins. Asterisk indicates the patients who underwent hematopoietic stem cell transplantation. P1.2, P2.1, P3.1, P3.2, P4.1, P5.1, P6.1, P7.2, P8.1, and P10.1 were described by Sumazaki et al. (14) P5.1 was described by Honda et al. (13) P9.1, P9.2, P11.1, P11.2, P11.3, P12.1, and P12.2 were described by Shinozaki et al. (11) P13.1 was described by Hoshino et al. (15) P16.1, P17.1, P17.2, P18.1, P19.3, and P20.1 were described by Zhao et al. (12). [Correction added on 10 April 2012, after first online publication: the *SH2D1A* mutation of P21.1 has been corrected.]

(Table 2) (2, 17). Lymphoid granulomatosis was not found in Japanese patients, but two patients have presented with systemic vasculitis (18). The vasculitis in these patients mainly affected the brain and was associated with encephalopathy. The mortality was different among clinical phenotypes, and the mortality of each phenotype in our study decreased from that in the XLP registry (2). However, in a recent worldwide study, the mortality associated with HLH decreased to 65%, lymphoproliferative disease to 8%, and dysgammaglobulinemia to 5% (16).

Hematopoietic stem cell transplantation is the only curative treatment for XLP-1. Twenty-one patients with XLP-1 did not undergo HSCT, and these patients died of the disease and complications. The outcome of one patient (P12.2) was unknown. Twelve patients underwent HSCT in Japan, and 11 patients survived. Most of the transplants were performed in different institutions, but the outcomes are similar to previously published data (9, 10, 17). This study revealed that unrelated donors could be used as donors as well as sibling donors. Although various types of conditioning regimen were performed, more than half included RIC regimen, and the result of RIC regimen is similar to that of myeloablative regimen. The RIC regimen should be performed for patients with XLP-1 to avoid regimen-related toxicity or morbidity (17). In

Table 2 Clinical phenotypes of patients with X-linked lymphoproliferative syndrome

	Present study (33 cases)		Seemayer (272 cases) (2)		Booth (91 cases) (17)	
Phenotype	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality
FIM or HLH	18 (55%)	16/18 (89%)	157 (58%)	127/132 (96%)	35.2%	65.6%
ML or LPD	7 (21%)	3/7 (43%)	82 (30%)	46/71 (65%)	24.2%	9.0%
Hypogammaglobulinemia	12 (36%)	4/11 (36%)	84 (31%)	34/75 (45%)	50.5%	13.0%

FIM, fulminant infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis.

Table 3 Characteristics of HSCTs

Patient ID	Age at HSCT	Donor	Sources	Conditioning regimen	GVHD prophylaxis	Acute GVHD	Chronic GVHD	Outcome
1.2	7 yr	MSD (6/6)	PBSC	TBI/CY	CsA/sMTX	Grade I	Extensive	Alive (14 yr 8 months)
7.2	24 yr	MSD (6/6)	BM	BU/CY/ATG	CsA/sMTX	Grade II	Extensive	Alive (6 yr 6 months)
9.1	8 yr	MUD (6/6)	BM	BU/VP/CY	FK/sMTX	None	None	Alive (10 yr 6 months)
9.2	6 yr	mMFD (3/6)	BM	TBI 6Gy/BU 4 mg/kg	MMF/sMTX/mPSL	NE	NE	Dead (14 days)
10.1	4 yr	mMUD (5/6)	BM	BU/CY/AraC	FK/sMTX	Grade II	Extensive	Alive (11 yr 2 months)
10.2	1 yr	MUD (6/6)	BM	BU/TAI 3Gy/Flu/CY/ATG	FK/sMTX	None	None	Alive (3 yr 3 months)
11.3	8 months	mMUD (5/6)	PBSC	Flu/Mel/ATG/TAI 6Gy	FK/sMTX/mPSL	Grade II	None	Alive (9 yr 2 months)
14.1	10 yr	MUD (6/6)	BM	BU/CY	CsA/sMTX	Grade III	Limited	Alive (8 yr 2 months)
16.1	11 yr	mMUD (5/6)	BM	BU/TAI 3Gy/Flu/CY/ATG	FK/sMTX	None	None	Alive (5 yr 6 months)
17.2	3 yr	mMFD (4/6)	BM	Flu/Mel/TBI 3 Gy	FK/sMTX	Grade I	None	Alive (8 yr 10 months)
18.1	7 yr	MUD (6/6)	BM	Flu/Mel/TBI 3 Gy	FK/sMTX	None	Extensive	Alive (4 yr 7 months)
19.3	15 yr	MUD (6/6)	BM	Flu/Mel/TBI 3 Gy	FK/sMTX	None	None	Alive (3 yr 7 months)

MSD, matched sibling donor; MUD, matched unrelated donor; mMFD, mismatched familial donor; mMUD, mismatched unrelated donor; PBSC, peripheral blood stem cells; BM, bone marrow; TBI, total body irradiation; CY, cyclophosphamide; BU, busulfan; ATG, anti-thymoglobulin; VP, etoposide; Gy, gray; AraC, cytosine arabinoside; TAI, total abdominal irradiation; Flu, fludarabine; Mel, melphalan; GVHD, graft versus host disease; CsA, cyclosporine A; sMTX, short methotraxate; FK, tacrolimus; MMF, mycophenolate mofetil; mPSL, methylprednisolone; NE, not evaluated; HSCT, hematopoietic stem cell transplantation.

this study, two patients (P10.2 and P11.3) were diagnosed because of a family history and presented no clinical features of XLP. Their parents wanted them to undergo HSCT because of the poor prognosis of the disease. Although the decision to transplant a relatively well child has been more challenging, these patients underwent transplant and were free from chronic GVHD.

In conclusion, this study verified the clinical usefulness of a flow cytometric assessment of SAP to search for XLP-1 (SAP deficiency). Flow cytometric analysis of XIAP is also useful to detect patients with XLP-2 (7, 19, 20). A male with any of the clinical phenotypes of XLP with or without EBV infection should be initially examined with a flow cytometric assay to evaluate both SAP and XIAP (21). We also identified nine Japanese patients with XIAP deficiency with a combination of flow cytometry and genetic analysis (22). Needless to say, a mutation analysis is the gold standard for confirming a definite diagnosis. The outcome of patients with XLP-1 seemed to be poor in Japan, and HSCT is the only curative treatment for patients with XLP-1.

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LETTER TO THE EDITOR

Autoimmunity and persistent RAS-mutated clones long after the spontaneous regression of JMML

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Juvenile myelomonocytic leukemia (JMML) is a chronic and aggressive myeloid leukemia in children. Patients show hepatosplenomegaly, and leukocytosis associated with monocytosis that can infiltrate the spleen, liver and lungs. About 80% of patients with JMML have a genetic abnormality in their leukemia cells, including mutations of *NF1*, *NRAS*, *KRAS*, *CBL* or *PTPN11*.[1] Occasionally JMML cases have been reported to be associated with clinical and laboratory findings compatible with autoimmune disease.[2] We and Niemela *et al.*[3] recently proposed a novel disease entity known as RALD (RAS-associated autoimmune-lymphoproliferative syndrome (ALPS)-like disease).[3,4] RALD shows ALPS-like clinical phenotypes associated with acquired *RAS* mutation at certain levels of hematopoietic stem cell differentiation involving the T, B and myeloid lineages. Given the shared genetic features of common abnormality of the RAS-MAPK signaling pathway but distinct prognostic features, an important question arises as to whether RALD and JMML can be discriminated by their clinical and biological characteristics.

In the present work, we collected and analyzed the clinical and laboratory characteristics of six Japanese patients fulfilling the clinical and laboratory criteria of JMML associated with *RAS* mutation and followed for more than 3 years without hematopoietic stem cell transplantation (HSCT).[5–8] They are phenotypically distinct from patients with Cardio-Facio-Cutaneous syndrome or Noonan syndrome. Although they fulfilled the diagnostic criteria of JMML at the initial presentation, including granulocyte-macrophage colony-stimulating factor (GM-CSF) hyper-sensitivity of bone marrow progenitors (Table 1 and Supplementary Table 1), no disease progression or recurrence was seen after regression of the disease. The follow-up periods were between 3 and 19 years. Physical examination of these patients identified one case with persistent hepatosplenomegaly (Case 6, Supplementary Table 2). Laboratory data of these six patients exhibited normal white blood cell counts, except for one case (Case 4) with persistent monocytosis (Supplementary Table 2). T/B ratio of lymphocyte population showed increased B cell population of more than 40% in all the cases. Interestingly, four of six cases showed hyper-γ-globulinemia (Figure 1a, and Table 2) and five of the six cases showed positivity for autoimmune antibodies (Table 2), mainly that for antinuclear antibody. Cases 3 and 6 presented persistent autoimmune thrombocytopenia. Case 6 also presented anemia. Thus, this patient was very likely to have had Evans syndrome. Case 2 fulfilled the diagnostic criteria for systemic lupus erythematosus. These observations were compatible with the findings in RALD. So we investigated whether these six patients continued to carry *RAS* mutation in their hematopoietic systems many years after disease regression.

Direct DNA sequencing of PCR products of the *RAS* gene using peripheral blood mononuclear cells was performed. Surprisingly, all of the six patients showed persistent *RAS* mutation-positive clones, even after 3 to 19 years of follow-up after the initial diagnosis of JMML. Then we performed direct DNA sequencing of PCR products of the *RAS* gene in isolated T cells, B cells and myelomonocytic cells. Sequencing electropherograms showed the presence of mutated *RAS* alleles in all of these hematopoietic lineages (Supplementary Figure 1). To further quantitate mutated alleles in each lineage, we subcloned PCR-amplified *RAS* genes from T cells, B cells and myelomonocytic cells into TA cloning vector and counted the colonies that carried a mutated *RAS* allele. The frequency of mutated *RAS* alleles was between about 26 and 63% in each hematopoietic cell lineage (Figure 1b). To rule out the possibility that the mutant *RAS* allele was epigenetically silenced during the long survival period, we tested mRNA expression from the mutant *RAS* allele using peripheral blood T lymphocytes. Analysis of the reverse transcription (RT) PCR product revealed that the mutant allele was expressed as much as the wild-type allele (Supplementary Figure 2).

We had a chance to analyze GM-CSF hyper-sensitivity in case 2. In this patient, 67% of CFU-GM colonies in the bone marrow carried *NRAS* G13D mutation. Intriguingly, the colonies showed no GM-CSF hyper-sensitivity, even though *NRAS* G13D mutation was present and expressed (Supplementary Table 3 and Supplementary Figure 3). To analyze whether these *RAS*-mutated cells had a neoplastic feature, we investigated the clonality of peripheral T cells in cases with *RAS* mutation by evaluating the status of T-cell receptor β rearrangement. As was expected, a polyclonal but no monoclonal or oligoclonal band was obtained, indicating that cells proliferated polyclonally in spite of the presence of *RAS* mutation (Figure 1c). Bim is the protein that accelerates apoptosis, and reduced expression of Bim was a characteristic biochemical feature in RALD.[3,4] We investigated the expression of Bim using activated T cells from these individuals and found that all of the T-cell clones established from these five cases showed reduced expression of Bim protein (Figure 1d). All these findings were compatible with those we identified in RALD previously.

It has previously been recommended that HCST be performed in most patients with JMML. JMML with somatic *PTPN11* mutation reportedly show unfavorable prognosis.[9,10] Although most of the JMML with *RAS* mutations also show an aggressive clinical course, a few of them achieve spontaneous remission. Matsuda *et al.*[6] retrospectively analyzed natural history of 8 *N*- or *KRAS*-mutated

Table 1. Characteristics of patients

ID	*Sex*	*Onset age (month)*	*Current age (years)*	*Mutation type*	*Received therapy*
1	M	11	11	KRAS G12S	13-cis RA
2	M	10	19	NRAS G12D	6-MP
3	F	4	7	KRAS G12S	None
4	F	12	7	NRAS G12S	None
5	M	10	10	NRAS G13S	None
6	M	6	3	NRAS G13S	6-MP

Cases 3, 4 and 5 were reported by Matsuda *et al.*[6,8] Case 1 was reported by Imamura *et al.*[7] Case 2 was reported by Doisaki *et al.*[9]

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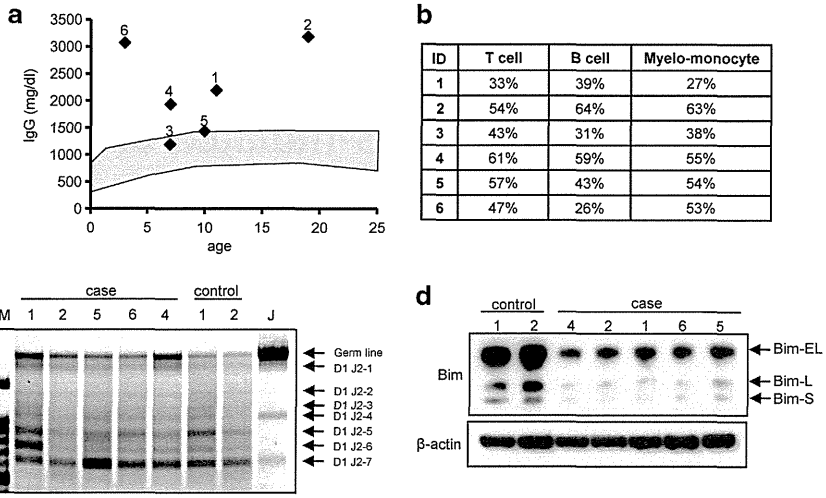

ID	T cell	B cell	Myelo-monocyte
1	33%	39%	27%
2	54%	64%	63%
3	43%	31%	38%
4	61%	59%	55%
5	57%	43%	54%
6	47%	26%	53%

Figure 1. (**a**) Serum immunoglobulin G (IgG) levels of each case; the age-dependent normal value of serum IgG is shaded. (**b**) RAS-mutated allele frequency analyzed by sequencing after TA cloning. (**c**) The TCRβ DJ recombination of each case is shown by the PCR method. J: A Jurkat cell showing monoclonal cell growth. (**d**) Western blotting analysis of Bim expression. The expression of β-actin is shown as an internal control.

Table 2. Autoimmunity of patients

ID	*IgG (mg/dl)*	*Autoimmune*
1	2187	Negative
2	3181	ANA X1280, anti-SSA Ab158 IU/ml, anti-SSB Ab 292 IU/ml, dsDNA Ab 7 IU/ml
3	1184	ANA × 80
4	1932	ANA × 40, RF 26 IU/ml
5	1432	ANA × 40
6	3060	ANA × 320

Serum IgG levels and various autoimmunity at time of re-evaluation

JMML cases who did not receive HSCT in 75 cases collected in the registry of the MDS Committee of the Japanese Society of Pediatric Hematology. Four of these patients died due to disease progression, while the remaining four patients remained alive without disease progression. They also suggested G12S *N*- or *KRAS*-mutated JMML demonstrates a milder clinical course.[5] Flotho *et al.*[11] reported six cases of *RAS*-mutated JMML who survived without HSCT in 216 cases collected in the EWOG-MDS registry, and all six patients in their group had substitutions other than G12S. Then, we tested whether there is any difference of RAS-GTPase activity depending on the mutation type and found G12S substitution of KRAS is less deleterious for RAS-GTPase activity than G to D or V substitution (Supplementary Figure 4). Thus, further study will help to demonstrate genotype and phenotype relations in JMML cases with various *RAS* gene mutation.

In the largest retrospective series of JMML patients, 65% of patients exhibited hyper-γ-globulinemia, and 14–22% of patients exhibited autoimmunity.[12] Four of the six patients evaluated in the present study exhibited hyper-γ-globulinemia and five of the six patients exhibited autoimmunity. These findings resemble RALD. Niemela *et al.*[3] reported that some of their RALD cases displayed hyperleukocytosis during their clinical courses, which is compatible with JMML.

Our study demonstrates the persistent presence of *RAS* mutation-positive clones in a substantial proportion of circulating lymphocytes and myeloid cells many years after the diagnosis of JMML. Once in remission these patients are free from the hematological findings of JMML but carry the autoimmune phenotype. We believe there is a distinct subgroup of patients with JMML who survive without HSCT, and proposed current diagnostic criteria for JMML is not sufficient to rule out those who may survive long or spontaneously resolve without HSCT. Thus the indication of HSCT as an initial treatment modality for JMML should be revised carefully for at least some cases of *RAS*-mutated JMML, as some of these cases are clinically similar to RALD. Further study is needed to identify the molecular mechanism for clinical heterogeneity of JMML with *RAS* mutation, either the aggressive type or that with spontaneous regression, the latter of which might overlap with RALD.

Details of experimental procedure are provided in Supplemental data Materials and methods.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Clinical and Genetic Characteristics of XIAP Deficiency in Japan

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Abstract Deficiency of X-linked inhibitor of apoptosis (XIAP) caused by *XIAP/BIRC4* gene mutations is an inherited immune defect recognized as X-linked lymphoproliferative syndrome type 2. This disease is mainly observed in patients with hemophagocytic lymphohistiocytosis (HLH) often associated with Epstein–Barr virus infection. We described nine Japanese patients from six unrelated families with XIAP deficiency and studied XIAP protein expression, *XIAP* gene analysis, invariant natural killer T (iNKT) cell counts, and the cytotoxic activity of $CD8^+$ alloantigen-specific cytotoxic T lymphocytes. Of the nine patients, eight patients presented with symptoms in infancy or early childhood. Five patients presented with recurrent HLH, one of whom had severe HLH and died after cord blood transplantation. One patient presented with colitis, as did another patient's maternal uncle, who died of colitis at

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4 years of age prior to diagnosis with XIAP deficiency. Interestingly, a 17-year-old patient was asymptomatic, while his younger brother suffered from recurrent HLH and EBV infection. Seven out of eight patients showed decreased XIAP protein expression. iNKT cells from patients with XIAP deficiency were significantly decreased as compared with age-matched healthy controls. These results in our Japanese cohort are compatible with previous studies, confirming the clinical characteristics of XIAP deficiency.

Keywords X-linked lymphoproliferative syndrome · X-linked inhibitor of apoptosis · Epstein–Barr virus · hemophagocytic lymphohistiocytosis · invariant natural killer T cell

Abbreviations

BIR	Baculovirus IAP repeat
CTL	Cytotoxic T lymphocyte
HSCT	Hematopoietic stem cell transplantation
HLH	Hemophagocytic lymphohistiocytosis
IAP	Inhibitor of apoptosis
LCL	Lymphoblastoid cell line
MMC	Mitomycin C
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
iNKT	Invariant natural killer T
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cells
TCR	T cell receptor
XIAP	X-linked inhibitor of apoptosis
XLP	X-linked lymphoproliferative syndrome

Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare inherited immunodeficiency estimated to affect approximately one in one million males, although it may be underdiagnosed [1]. XLP is characterized by extreme vulnerability to Epstein–Barr virus (EBV) infection, and the major clinical phenotypes of XLP include fulminant infectious mononucleosis (60%), lymphoproliferative disorder (30%), and dysgammaglobulinemia (30%) [2]. In addition, XLP is associated with a variety of additional clinical phenotypes such as vasculitis, aplastic anemia, and pulmonary lymphoid granulomatosis. Patients with XLP often develop more than one of these phenotypes. The gene responsible for XLP was identified as *SH2D1A*, located on Xq25 and encoding the SLAM-associated protein (SAP) [3–5]. However, gene analysis revealed *SH2D1A* mutations in only 50–60% of presumed XLP patients [6]. Importantly, a mutation in the gene that encodes the X-linked inhibitor of apoptosis (XIAP) called *XIAP* or *BIRC4* was identified as a second causative gene for XLP [7]. *XIAP* is located close to the *SH2D1A* gene on the X chromosome and consists of six coding exons [8–10]. XIAP produces an anti-apoptotic molecule that belongs to the inhibitor of apoptosis (IAP) family proteins. It contains three baculovirus IAP repeat (BIR) domains that, together with flanking residues, bind to caspases 3, 7, and 9, thereby inhibiting their proteolytic activity [11].

The clinical presentations of XIAP-deficient patients have been frequently reported [7,12,13]. More than 90% of patients with XIAP deficiency develop hemophagocytic lymphohistiocytosis (HLH) which is often recurrent. Therefore, it was recently suggested that the phenotype of XIAP deficiency fits better with the definition of familial HLH than with XLP disease [12]. However, familial HLH is characterized by defects in $CD8^+$ T and NK cell cytotoxicity responses, while these responses are normal in XIAP deficiency [7,12]. Other symptoms of XLP, such as splenomegaly, hypogammaglobulinema, and hemorrhagic colitis, have been reported in patients with XIAP deficiency, but lymphoma has never been noted [7,12–15].

We searched for patients with XIAP deficiency in Japan by detection of *XIAP* gene mutations and flow cytometric assessment of lymphoid XIAP expression. We previously reported the first case of XIAP deficiency in Japan [14]. Thereafter, we identified eight additional cases from five families with XIAP deficiency in our country. In this study, we describe the clinical and laboratory findings from nine patients from six unrelated families with XIAP deficiency, including previous cases, to help further the understanding of the pathogenetic features of this disease.

Materials and Methods

Patient and Family Member Samples

Patients without indentified *SH2D1A* mutations but with presumed XLP phenotypes were screened for *XIAP* mutations. Their family members were also screened for the same mutation. Upon identification of *XIAP* mutations, the patients were enrolled in this study. Patient 2.2 passed away before a genetic diagnosis of XIAP deficiency was made, but he was the maternal uncle of patient 2.1 and had presented with a XLP phenotype (Table I). In the end, nine patients from six different families were found to have XIAP deficiencies, three of whom had been reported previously [13,14]. Upon the approval of the Ethics Committee of the University of Toyama and after obtaining informed consent, 5–10 mL heparinized venous blood was collected from the patients, their mothers, and 25 age-matched healthy children (1–13 years of age). All of the samples were

transferred to our laboratory at room temperature within 24 h for analysis.

Mutation Analysis of the *XIAP* Gene

DNA was extracted from peripheral blood using the QuickGene-Mini 80 nucleic acid extraction system (FUJIFILM Co., Tokyo, Japan). The coding regions and the exon–intron boundaries of the *XIAP* gene were amplified by polymerase chain reaction (PCR) using primers flanking each of the six exons by standard methods. PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. Sequencing analysis was performed on an Applied Biosystems Prism 310 Capillary Sequencer (Applied Biosystems).

Flow Cytometric Analysis of XIAP Protein Expression in Lymphocytes

XIAP protein expression was studied by flow cytometric techniques as previously described [16,17]. Peripheral blood mononuclear cells (PBMC) from patients 1, 2.1, 3.1, 3.2, 4, 5, 6.1, 6.2, and 25 age-matched healthy children were prepared by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, Inc., St. Louis, MO, USA). The cells were first fixed in 1% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized in 0.5% saponin in washing buffer. The fixed and permeabilized cells were then incubated with an anti-XIAP monoclonal antibody (mAb) (clone 48 (BD Biosciences, Franklin Lakes, NJ, USA) or clone 2 F1 (Abcam, Cambridge, UK)) for 20 min on ice, washed, and then incubated with a FITC-labeled anti-mouse IgG1 antibody (SouthernBiotech, Birmingham, AL, USA) for 20 min on ice. The stained cells were analyzed on the FC500 flow cytometer (Beckman Coulter, Tokyo, Japan).

Western Blot Analysis of XIAP Protein Expression in Lymphocytes

PBMC from normal controls and patients 3.1, 5, and 6.2 were washed and pelleted. The cells were then lysed in 10 μL of lysing solution (1% Triton-X 100; 150 mmol/L NaCl; 10 mmol/L Tris–HCl, pH 7.6; 5 mmol/L EDTA-Na; 2 mmol/L phenylmethylsulfonyl fluoride) per 10^6 cells for 30 min on ice. The lysed cells were centrifuged for 10 min at 15,000*g* to remove nuclei, and the supernatants were diluted in the same volume of Laemmli's sample buffer. Samples were then electrophoresed in sodium dodecyl sulfate–polyarylamide 10% to 20% gradient gel and blotted on nitrocellulose filters. Blots were blocked in 5% skim milk in PBS for 1 h, treated with anti-XIAP mAb (clone 28 or clone 2F1) for 2 h, and then incubated with peroxidase-conjugated anti-mouse IgG antibody (Invitrogen, Grand Island, NY, USA) for 1 h. Immunoblots were developed by the ECL Western blotting detection system (GE Healthcare UK Ltd., Buckinghamshire, England).

Flow Cytometric Identification of Invariant Natural Killer T Cells

PBMC from eight patients (1, 2.1, 3.1, 3.2, 4, 5, 6.1, and 6.2) and 25 controls were incubated with fluorochrome-conjugated anti-CD3 (Dako Japan KK, Kyoto, Japan), anti-TCRVα24, and anti-TCRVβ11 mAbs (Beckman Coulter) to identify invariant natural killer T (iNKT) cells by flow cytometry. After the electronic gating of 100,000 $CD3^+$ T cells, iNKT cell populations were defined by the co-expression of TCRVα24 and TCRVβ11. The iNKT cell counts were evaluated at the diagnosis of XIAP deficiency.

Establishment of Alloantigen-Specific Cytotoxic T Lymphocyte Lines and Analysis of Cytotoxic T Lymphocyte-Mediated Cytotoxity

Alloantigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) lines were generated as described previously [18,19]. Briefly, PBMC were obtained from patients 1, 2.1, 3.1, and unrelated healthy individuals. These cells were co-cultured with a mitomycin C (MMC)-treated B lymphoblastoid cell line (LCL) established from an HLA-mismatched individual (KI-LCL). Using cell isolation immunomagnetic beads (MACS beads; Miltenyi Biotec, Auburn, CA, USA), $CD8^+$ T lymphocytes were isolated from PBMC that had been stimulated with KI-LCL for 6 days. $CD8^+$ T lymphocytes were cultured in RPMI 1640 medium supplemented with 10% human serum and 10 IU/mL interleukin-2 (Roche, Mannheim, Germany) and stimulated with MMC-treated KI-LCL three times at 1-week intervals. These lymphocytes were then used as $CD8^+$ alloantigen-specific CTL lines. The cytotoxic activity of CTLs was measured by a standard ^{51}Cr-release assay as described previously [20]. Briefly, alloantigen-specific CTLs were incubated with ^{51}Cr-labeled allogeneic KI-LCL or TA-LCL, which did not share HLA antigens with KI-LCL, for 5 h at effector/target cell ratios (E/T) of 2.5:1, 5:1, and 10:1. Target cells were also added to a well containing only medium and to a well containing 0.2% Triton X-100 to determine the spontaneous and maximum levels of ^{51}Cr release, respectively. After 5 h, 0.1 mL of supernatant was collected from each well. The percentage of specific ^{51}Cr release was calculated as follows: (cpm experimental release − cpm spontaneous release) / (cpm maximal release − cpm spontaneous release)×100, where cpm indicates counts per minute.

Table I Summary of our data

	Patient 1 [13]	Patient 2.1 [12]	Patient 2.2 [12]	Patient 3.1	Patient 3.2	Patient 4	Patient 5	Patient 6.1	Patient 6.2
Age at initial presentation	20 months	7 months	3 months	2 months	Asymptomatic	2 months	6 months	17 months	15 months
Current age	4 years	Deceased	Died of colitis	12 years	17 years	15 years	2 years	1 year	12 years
Family history	No	Yes	Yes	Yes	Yes	No	No	Yes	Yes
HLH	+	+	−	+	−	−	+	+	+
Recurrent HLH	+	+	−	+	−	−	+	−	+
Fever	+	+	+	+	−	−	+	+	+
Splenomegaly	+	+	ND	−	−	−	−	+	+
Cytopenia	+	+	ND	+	−	−	+	+	+
EBV	+	−	ND	+	−	−	−	+	+
Hypogammaglobulinemia	−	+	ND	−	−	+	−	−	−
Colitis	−	−	+	−	−	−	+	−	−
Treatment	PSL CsA Dex	PSL CsA Dex	ND	PSL CsA	−	IVIG	PSL, Dex CsA, IVIG Infliximab	IVIG, Dex	PSL
Allogeneic HSCT	−	+	−	−	−	−	−	−	−
Mutation	R238X	R381X	ND	W217CfsX27	W217CfsX27	E349del	Del of exons 1-2	N341YfsX7	N341YfsX7
XIAP protein expression	±	−	ND	−	−	+	±	±	±

HLH hemophagocytic lymphohistiocytosis, *ND* no data, *EBV* Epstein–Barr virus, *PSL* prednisolone, *CsA* cyclosporin A, *Dex* dexamethasone, *IVIG* intravenous immuglobulin, *HSCT* hematopoietic stem cell transplantation, + yes or positive, − no or negative, ± residual expression

Statistical Analysis

Student's *t*-test was used for statistics, with *P*-values <0.05 considered to be statistically significant.

Results

Clinical Manifestations of the Patients

Most of our patients presented with disease symptoms at very early ages; five patients presented in infancy and three patients presented in childhood (Table I). Three of the six families had family history records. Five of the nine patients had recurrent HLH, fever, splenomegaly, and cytopenia. EBV infection and hypogammaglobulinemia were also observed in multiple patients. Most patients with HLH were treated with corticosteroids with or without cyclosporin A to prevent an otherwise rapidly fatal disease course. Patients 2.2 and 5 presented with colitis, whereas patient 2.2 died; patient 5 improved with anti-TNF alpha mAb (infliximab®) treatment. Patient 2.1 underwent cord blood transplantation but died of complications. Patient 4 had a history of recurrent otitis media and pneumonia since 2 months of age, and he was found to have hypogammaglobulinemia. The patient was treated with intravenous immunoglobulin replacement therapy alone, and he is currently doing well. No patient developed lymphoma.

Detection of *XIAP* Mutations

We identified *XIAP* mutations in patients from all six unrelated families (Fig. 1) and analyzed all of the data using the US National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/SNP) to check for single-nucleotide polymorphism in the *XIAP* gene. As previously reported, patient 1 possessed a nonsense mutation, 712 C > T, resulting in an early stop codon R238X [14]. Patient 2.1 had a nonsense mutation in exon 5, 1141 C > T, resulting in R381X [13]. Patient 2.2 might have the same mutation as patient 2.1 because patient 2.2 was the maternal uncle of patient 2.1 [13]. Patients 3.1 and 3.2 were siblings and were found to have a one base pair deletion (650delG) in exon 1, resulting in a frameshift and premature stop codon (W217CfsX27). Patient 4 was found to have one amino acid deletion (1045_1047delGAG; E349del) in exon 3. Patient 5 has a large deletion, spanning exons 1 and 2. Patients 6.1 and 6.2 were brothers and had a two-nucleotide deletion (1021_1022delAA), which resulted in a frameshift and premature stop codon (N341YfsX7). All of the mothers of the patients from families 1–5 were heterozygote carriers of the mutations. Interestingly, we could not find any *XIAP* mutation in the mother of patients 6.1 and 6.2. We identified deleterious *XIAP* mutations in nine patients from six unrelated Japanese families that are likely to underlie their XLP phenotypes.

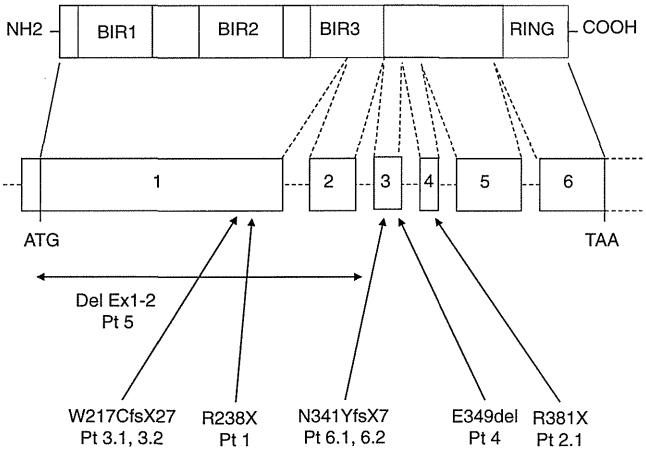

Fig. 1 *XIAP* gene mutations and their consequences for XIAP protein. *XIAP* comprises six exons and encodes the XIAP protein, which consists of 497 amino acids. XIAP contains three BIR domains and one RING domain. Mutations identified in our patients are indicated

XIAP Expression in Lymphocytes from the Patients and Carriers by Flow Cytometry

XIAP expression levels were analyzed in the lymphocytes of patients from all six families (Fig. 2). The lymphocytes of

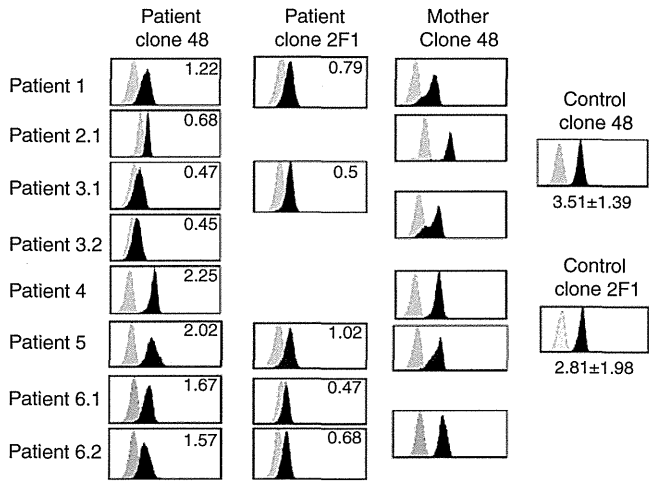

Fig. 2 XIAP protein expression in lymphocytes from the patients and their carriers. Flow cytometric detection of intracellular XIAP in lymphocytes from patients and their maternal carriers. The *gray* and *black areas* indicate the negative control and anti-XIAP staining, respectively. Anti-XIAP staining was performed using the clones 48 and 2 F1 antibodies where indicated. The *number in the box* indicates the log scale difference between the mean fluorescence intensity (ΔMFI) stained by the isotype antibody and that by the anti-XIAP antibodies. XIAP expression in 25 normal controls was also analyzed by the clone 48 and 2 F1 antibodies. The data of mean ± standard deviation of ΔMFI and each representative profile were shown

patients 1, 3.1, 5, 6.1, and 6.2 were examined by two different anti-XIAP mAbs. Using clone 48 antibody, patients 1, 2.1, 3.1, 3.2, 6.1, and 6.2 showed reduced XIAP expression, whereas XIAP was normally expressed in the lymphocytes of patients 4 and 5. In contrast to clone 48, clone 2F1 antibody showed reduced XIAP expression in patient 5. The effects of heterozygous *XIAP* mutations were studied in the lymphocytes of the patients' mothers by anti-XIAP mAb clone 48. The mothers of patients 1, 3.1, and 3.2 showed a bimodal pattern of XIAP protein (Fig. 2). The mothers of patients 2.1, 6.1, and 6.2 did not show a clear mosaic pattern, but all of these patients had reduced XIAP expression levels. Similarly to patients 4 and 5, the mothers of patients 4 and 5 demonstrated a normal XIAP expression pattern.

XIAP Expression in Lymphocytes from the Patients by Western Blot

Western blot analysis was used to evaluate the expression level of XIAP to determine the impact of patient *XIAP* mutations on protein expression and to compare this to the flow cytometric analysis. PBMCs from patients 3.1, 5.1, and 6.2 were available for Western blotting. All of these patients showed a reduction in XIAP protein expression (Fig. 3), fitting with the results obtained by flow cytometric analysis.

iNKT Cell Counts in the Patients

SAP-deficient patients had reduced numbers of NKT cells that expressed an invariantly rearranged T-cell receptor (TCR) consisting of TCRVα24 and TCRVβ11 chains [21,22]. The rare subset of iNKT cells was originally reported to be reduced in XIAP-deficient patients as well [7] but seemed to be present in normal numbers in a later study involving a larger patient cohort [23]. We analyzed the iNKT cell frequencies in 100,000 $CD3^+$ T cells in our XIAP-deficient patients and compared these with healthy controls (Fig. 4). The average frequency of iNKT cells within the $CD3^+$ T cell compartment of our XIAP patients was significantly reduced by twofold when compared with healthy controls (20 vs. 47 per 10^5 $CD3^+$ T cells). Therefore, we concluded that the number of iNKT cells was reduced in our patients with XIAP deficiency.

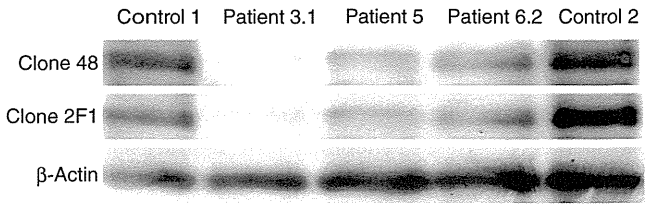

Fig. 3 XIAP expression in lymphocytes from the patients by Western blot. Analysis of XIAP expression in PBMC generated from patients with XIAP deficiency and normal controls using the antibody clone 48 (*upper panel*), the antibody clone 2 F1 (*middle panel*), and the β-actin antibody as an internal control (*lower panel*)

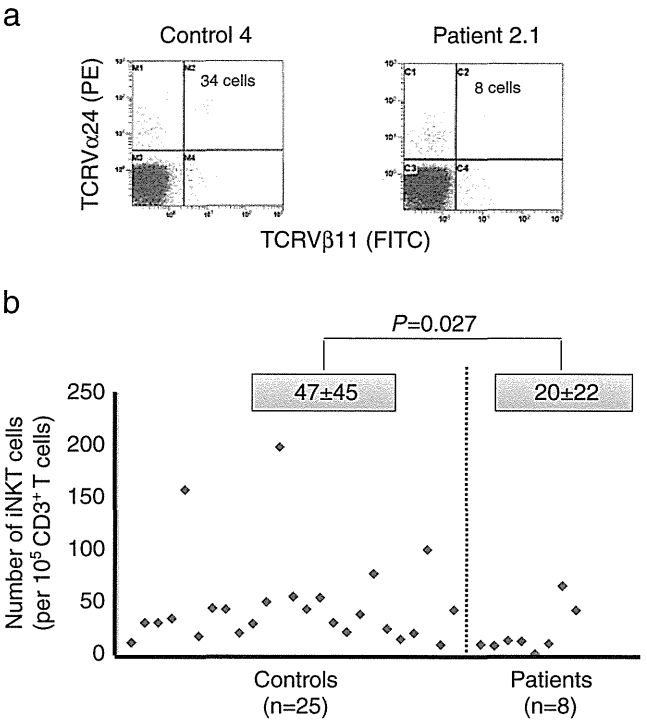

Fig. 4 iNKT cell counts in the patients and healthy controls. **a** Representative flow cytometric analysis of iNKT cells in $CD3^+$ lymphocytes from one XIAP-deficient patient and one healthy control. **b** Comparison of the number of iNKT cells in 100,000 $CD3^+$ lymphocytes between XIAP-deficient patients and control individuals. Statistical significance between patients and controls was determined with the Student's *t*-test (*p*-value=0.027)

Functional Analysis of CTL Lines Established from the Patients

To test whether our XIAP-deficient patients have similar defects in $CD8^+$ T cell cytotoxicity as described in other subtypes of familial HLH [20,38], we generated $CD8^+$ alloantigen-specific CTL from patients 1, 2.1, 3.1, and three healthy controls (Fig. 5). The cytotoxic activity of the CTL of these patients was similar to that of the healthy controls, indicating that XIAP patients clearly differ from other familial HLH patients in this aspect of the disease.

Discussion

XIAP deficiency is a rare but severe and life-threatening inherited immune deficiency [12,13]. Early diagnosis and life-saving treatment such as hematopoietic stem cell transplantation is especially important. The causative gene for

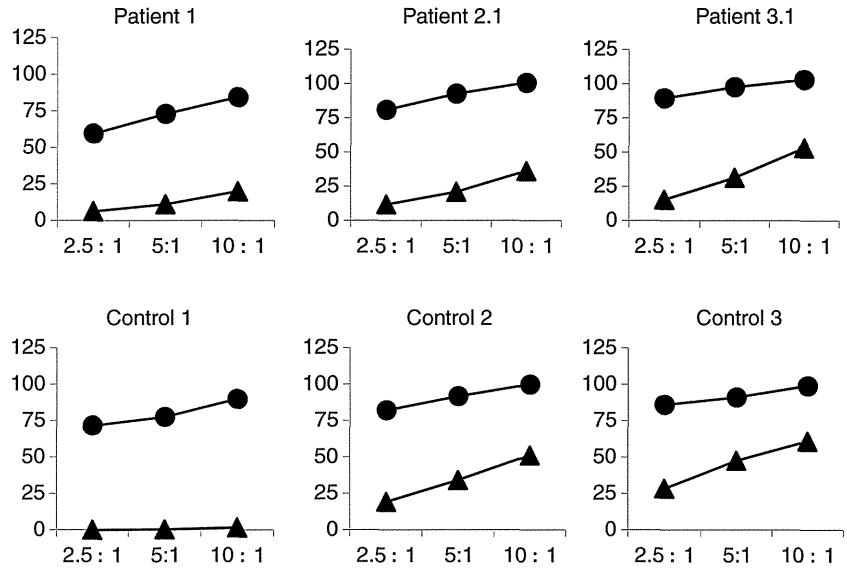

Fig. 5 Cytotoxicity of alloantigen-specific $CD8^+$ T cell lines. $CD8^+$ T cell lines were generated from PBMC of patients with XIAP deficiency and healthy controls by stimulation with allogeneic LCL (KI-LCL). Their cytotoxity was determined against allogeneic KI-LCL (*circles*) and against allogeneic TA-LCL (*triangles*), which does not share alloantigens with KI-LCL

XIAP deficiency was identified to be *XIAP/BIRC4*, and 25 mutations in the *XIAP* gene have been previously reported [7,12–14]. In the present study, we described four novel mutations (W217CfsX27, E349del, deletion of exons 1 and 2 and N341YfsX7) in the *XIAP* genes as well as previously described patients with R381X and R238X mutations [13,14]. The mother of patients 6.1 and 6.2 had no mutation in the *XIAP* gene. Because this is an X-linked inheritance, the failure to identify the same mutation in the mother suggests that the mother had a germline mosaicism for the mutation. Such mosaicism has not yet been described in XIAP deficiency, but it has been reported in Duchenne muscular dystrophy, X-linked severe combined immunodeficiency, X-linked agammaglobulinemia, and many other inherited diseases [24–26]. HLH is common in XIAP-deficient patients, and it is often recurrent [13,14]. In our study, six patients had HLH and five patients presented with recurrent HLH. Therefore, XIAP deficiency should be suspected in certain boys with HLH, especially in those with family history or recurrent HLH. The reason why XIAP deficiency increases susceptibility to HLH remains unclear. Murine studies have also failed to disclose a mechanism for the development of HLH [27]. Interestingly, Xiap-deficient mice possess normal lymphocyte apoptosis induced by a variety of means [28]. Three of our patients presented with EBV-associated HLH. EBV infection has been reported to be a trigger of the first HLH episode in patients with XIAP deficiency [13]. The excess of lymphocyte apoptosis in XIAP deficiency might account for the abnormal immune response to EBV [28]. Splenomegaly is not frequently observed in XLP type 1 or SAP deficiency but might be a common clinical feature in XIAP deficiency [12,13] as four (50%) of eight Japanese patients developed splenomegaly. Pachlopnik Schmid et al. [13] reported that recurrent splenomegaly occurring in the absence of systemic HLH was often associated with fever and cytopenia. XIAP-deficient patients are at risk for chronic colitis, which is possibly a more frequent cause of mortality than HLH [13]. Our study included two patients who developed colitis, and one of the patients died of colitis at 4 years of age. Although we did not have enough clinical information or samples from that patient because of his early death, his symptoms suggest that he had a XIAP deficiency complicated with colitis because he was the maternal uncle of patient 2.1. The other patient was 2 years old and also suffered from chronic hemorrhagic colitis.

In contrast to SAP deficiency, lymphoma has never been reported in XIAP deficiency, including our patients. Some studies indicate that the XIAP protein is a potential target for the treatment of cancer based on the anti-apoptotic function of XIAP [29]. Therefore, the absence of XIAP may protect patients from cancer, explaining why XIAP-deficient patients do not develop lymphoma. We generated a clinical summary to compare XIAP-deficient patients with the previous reports (Table II). Although our study included a relatively small number of patients, our results appear to be consistent with previous large studies [12,13] and confirm the clinical characteristics of XIAP deficiency.

Flow cytometry can be used for the rapid screening of several primary immunodeficiencies including XLP [30]. XIAP protein has been found to be expressed in various human tissues, including all hematopoietic cells [7,10]. Marsh et al. [16] described that XIAP was readily detectable in normal granulocytes, monocytes, and all lymphocyte subsets. Moreover, patients with *XIAP* mutations had decreased or absent expression of XIAP protein by flow cytometry [14,16]. We investigated XIAP expression in lymphocytes from eight patients by flow cytometry as previously described [16,17]. As demonstrated by Marsh et al. [16], clone 48 antibody provided brighter staining compared

Table II Comparison of patients with XIAP deficiency

	Marsh R et al. [12]	Pachlopnik Schmid J et al. [13]	Our study
Number of patients	10	30	9
HLH	9 (90%)	22/29 (76%)	6/9 (67%)
Recurrent HLH	6 (60%)	11/18 (61%))	5/6 (83%)
EBV-associated HLH	3 (30%)	16/19 (84%)	4/6 (67%)
Splenomegaly	9 (90%)	19/21 (90%)	4/8 (50%)
Hypogammaglobulinema	2 (20%)	8/24 (33%)	2/8 (25%)
Lymphoma	0	0	0
Colitis	0	5 (17%)	2 (22%)

to clone 2F1 antibody. In patients 5, 6.1, and 6.2, XIAP protein expression was normal when using clone 48 antibody but decreased when using clone 2F1 antibody. Western blot analysis showed XIAP expression in patients 3.1, ,5 and 6.2, and using clone 48 antibody, we found a discrepancy between flow cytometry and Western blot. Flow cytometric diagnosis may thus result in false positive results, and the gene sequencing of *XIAP* should be performed even when the patient shows normal XIAP expression levels.

All of the mothers examined in this study except for one were carriers of *XIAP* mutations. Analysis of XIAP expression in the mothers of patients 1, 3.1, and 3.2 revealed a bimodal expression pattern of XIAP in lymphocytes with cellular skewing towards expression of the wild-type XIAP allele as previously demonstrated [16]. However, the mother of patients 2.1, 6.1, and 6.2 demonstrated a normal expression pattern, possibly resulting from an extremely skewed pattern of X chromosome inactivation as shown in XIAP deficiency and other primary immunodeficiencies, and de novo mutations in *XIAP* are also observed [16,31]. The mother of patients 6.1 and 6.2 might have a germline mosaicism for the mutation, resulting in normal XIAP protein expression.

iNKT cells represent a specialized T lymphocyte subpopulation with unique features distinct from conventional T cells [32,33]. Human iNKT cells express an invariant TCR that recognizes self and microbacterial glycosphingolipid antigens presented by the major histocompatibility complex class I-like molecule CD1d [28]. The first series of XIAP-deficient patients showed decreased iNKT cell counts similar to SAP deficiency [7]. However, Xiap-deficient mice have normal numbers of iNKT cells and did not show an abnormal response to apoptotic stimuli [34]. Marsh et al. [23] reported a cohort of XIAP-deficient patients with normal numbers of iNKT cells, indicating that XIAP-deficient patients differ from SAP-deficient patients in this respect. In our cohort, we observed significantly decreased iNKT cell numbers in XIAP-deficient patients compared to healthy controls. However, we could not identify a correlation between the number of iNKT cells and the clinical disease features. Flow cytometric evaluation of iNKT cell counts can allow for the discrimination of XLP and other primary immunodeficiency diseases because patients may have normal XIAP protein expression in their lymphocytes.

CTLs kill their targets by one of two mechanisms: granule- or receptor-mediated apoptosis [35]. A recent study showed that the main pathway of cytotoxicity mediated by alloantigen-specific human $CD4^+$ and $CD8^+$ T cells is granule exocytosis and not the FAS/FAS ligand system [18]. Granzyme B is a major effector molecule of granule-mediated killing that rapidly induces cell death after entering the cytoplasm of the target cell [36]. The enzymatic activity of granzyme B is key to its ability to induce cell death. The executioner caspase-3 has been shown to be proteolytically processed and activated by granzyme B [37]. Although XIAP possesses an inhibitory effect for caspases, it is important to study the cytotoxic activities of CTLs in XIAP deficiency. Furthermore, many studies have indicated that some subtypes of patients with familial HLH show a deficiency in their cytotoxic activities [20,38]. To further investigate the function of antigen-specific CTLs, we studied $CD8^+$ alloantigen-specific CTL analysis among three XIAP-deficient patients. XIAP-deficient patients showed a normal level of cytotoxic activity, suggesting that XIAP might not play an important role in the cytotoxic responses of $CD8^+$ T cells as was previously suggested based on the normal NK cell-mediated cytotoxicity found in XIAP-deficient patients [7,12].

In this study, we have described nine Japanese patients with XIAP deficiency with clinical characteristics similar to those of patients in Europe and USA [12,13].

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Allogeneic hematopoietic cell transplantation for XIAP deficiency: an international survey reveals poor outcomes

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Key Points

- High mortality rates are observed in patients with XIAP deficiency treated with myeloablative conditioning regimens for hematopoietic cell transplantation.

There have been no studies on patient outcome after allogeneic hematopoietic cell transplantation (HCT) in patients with X-linked inhibitor of apoptosis (XIAP) deficiency. To estimate the success of HCT, we conducted an international survey of transplantation outcomes. Data were reported for 19 patients. Seven patients received busulfan-containing myeloablative conditioning (MAC) regimens. Eleven patients underwent reduced intensity conditioning (RIC) regimens predominantly consisting of alemtuzumab, fludarabine, and melphalan. One patient received an intermediate-intensity regimen. Survival was poor in the MAC group, with only 1 patient surviving (14%). Most deaths were from transplantation-related toxicities, including venoocclusive disease and pulmonary hemorrhage. Of the 11 patients who received RIC, 6 are currently surviving at a median of 570 days after HCT (55%). Preparative regimen and HLH activity affected outcomes, and of RIC patients reported to be in remission from HLH, survival is 86% ($P = .03$). We conclude that MAC regimens should not be used for patients with XIAP deficiency. It is possible that the loss of XIAP and its antiapoptotic functions contributes to the high incidence of toxicities observed with MAC regimens. RIC regimens should be pursued with caution and, if possible, efforts should be made to ensure HLH remission before HCT in these patients. (*Blood.* 2013;121(6):877-883)

Introduction

Deficiency of X-linked inhibitor of apoptosis (XIAP) is associated with X-linked lymphoproliferative disease (XLP) and familial hemophagocytic lymphohistiocytosis (FHLH) phenotypes. Traditionally, patients with inherited immune deficiencies that cause HLH have been treated with allogeneic hematopoietic cell transplantation (HCT) because of the life-threatening nature of HLH. There is extensive experience with transplantation in patients with FHLH. Over the past 10 years, survival has generally approximated 60% with myeloablative conditioning (MAC) regimens.[1-7] More recently, improvements have been made with reduced-intensity conditioning (RIC) protocols, and current survival rates are as high as 80%.[8-11] There is less experience with transplantation in patients with XLP because of SLAM-associated protein (SAP) deficiency, but survival is generally accepted to be greater than 70% regardless of the intensity of the conditioning protocol.[12-14]

To date, little has been published concerning the outcomes of HCT for patients with XIAP deficiency. XIAP deficiency was first discovered in 2006,[15] and is associated with XLP, FHLH, and colitis phenotypes.[15-18] Patients with XIAP deficiency are unique compared with patients with the other genetic forms of HLH because, as the name suggests, XIAP is an inhibitor of apoptosis that is widely expressed outside of the immune system.[19] Thymocytes from XIAP-deficient mice have been shown to have normal apoptotic responses to a variety of apoptotic stimuli,[20] but hepatocytes are more sensitive to death induced by treatment with cross-linked Fas ligand.[21] XIAP-deficient mouse embryonic fibroblasts are also more sensitive to death after infection with MHV-68.[22] In addition, there is increasing experience with the use of XIAP inhibitors in conjunction with traditional cancer treatment. In this setting, XIAP inhibitors generally increase the susceptibility

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Table 1. Patient characteristics

Patient no	Age at initial presentation	EBV HLH before HCT	Non-EBV HLH before HCT	HLH not in full remission before HCT	Colitis before HCT	Other	XIAP mutation	Protein expression
1	3 mo	−	+	−	−		1443_1449 delins 24 (P482fsX508)	NE*
2	2 mo	−	+	+	−		1443_1449 delins 24 (P482fsX508)	NE
3	2 mo	−	+	−	−		563 G → A (G188E)	Reduced
4	Asymptomatic (symptomatic brother)	−	−	−	−		563 G → A (G188E)	Reduced
5	15 mo	−	−	−	+	Recurrent enterocutaneous fistulas; multiple episodes of polymicrobial sepsis	608G → A (C203Y)	Reduced
6	9 mo	−	+	−	−		E99KfsX129	Absent
7	9 y	+	−	−	−		497G → T, R166I	NE
8	7 mo	−	+	+	−		1141C → T (R381X)	Reduced
9	Infancy	−	+	−	−		1481 T → A (I494N)	NE
10	4 mo	−	+	+	−		1445 C → G (P482R)	Reduced
11	1 y	+	−	+	−	Repeated infections: pneumonia, otitis media, history of paracentesis, mastoidectomy	1189 delA (I397fsX414)	Absent
12	1 y	+	−	+	−		387_390del (D130fsX140)	Not reported
13	3 mo	−	+	−	−		Gross Deletion Exons 1-5	Absent
14	1 y	−	−	−	+	Recurrent fevers; pneumococcal sepsis.	758 C → G (S253X)	Absent
15	3 y	−	+	−	−	Ventricular septal defect	356_359del (N119fs384)	NE
16	7 y	+	+	−	−		1141C → T (R381X)	Reduced
17	8 y	+	−	+	−		310 C → T (Q104X)	Absent
18	Infancy	−	+	−	−	Liver failure in infancy required liver transplantation; nodular lung disease; positive CMV and fungal elements	Gross deletion exon 6	Truncated (robust detection of a smaller molecular weight protein by Western blot)
19	17 y	+	−	−	−		894_898 del 5 (K299fsX307)	Absent

*Not examined.

of cancer cells to undergo apoptosis.[23,24] Because of the importance of XIAP in preventing apoptosis, patients with XIAP deficiency may be at increased risk of treatment-related toxicities because of increased sensitivity to chemotherapeutic agents.

To investigate whether deficiency of XIAP adversely affects the survival of patients undergoing allogeneic HCT, we conducted an international survey to collect information regarding the transplantation outcomes of patients confirmed to have XIAP deficiency.

Methods

Data collection

Approval for this retrospective study was granted by the Cincinnati Children's Hospital Institutional Review Board. A spreadsheet questionnaire was sent to physicians who provided treatment for patients with XIAP deficiency who underwent allogeneic HCT. Physicians were identified through contact with our center, our review of the literature regarding XIAP deficiency, or a request made to all members of the Histiocyte Society.

Patients

Only patients with a confirmed *XIAP/BIRC4* (baculoviral inhibitor of apoptosis repeat containing protein 4) mutation or with a sibling with a confirmed mutation were included in this study (Table 1), which was conducted in accordance with the Declaration of Helsinki. Supplemental lymphocyte protein analysis was performed in some patients using either Western blot or intracellular flow cytometric analysis.[15-17,25]

Transplantation procedures

Patients received transplantation at centers in the United States (n = 12), Europe (n = 6), and Japan (n = 1) between the years 2001-2011. Transplantation procedures were carried out per institutional standard practices. Conditioning regimens and graft characteristics are listed in Table 2. Conditioning regimens were classified as MAC if they contained an alkylating agent (busulfan) or total body irradiation (TBI) at a dose that would not allow autologous BM recovery.[26] Conditioning regimens were classified as RIC if they did not meet the definition of MAC regimen.[26] If there was uncertainty regarding the intensity of the regimen (n = 1, patient 8), it was classified as an intermediate-intensity regimen. Neutrophil engraftment was considered to be the day the neutrophil count reached 0.5×10^9/L. Engraftment studies were done using either XY FISH for sex-mismatched donors or variable number of tandem repeat analysis for same-sex donors. Mixed chimerism was defined as having 5% or more host-derived cells in the whole blood on more than 1 occasion. Acute and chronic GVHD were assessed by standard criteria.[27,28] Patients received GVHD prophylaxis per institutional standard practices. Other routine transplantation care, such as antimicrobial prophylaxis, IV Ig replacement, and fluid and nutrition supplementation when needed, were also provided per institutional standard practices.

Table 2. Transplantation procedures

Patient no	Age at HCT, y	Type of conditioning	Conditioning regimen	Graft HLA match*	Graft source	Relationship
1	0.42	MAC	Bu, Mel, ATG	5/6	Cord	Unrelated
2	0.58	MAC	Bu, Cy, ATG, Etop	6/6	Cord	Unrelated
3	1	MAC	Bu, Cy, ATG	7/8	BM	Unrelated
4	4	MAC	Bu, Cy, ATG	10/10	BM	Unrelated
5	5	MAC	Bu, Flu, ATG	6/6	Cord	Unrelated
6	10	MAC	Bu, Cy, ATG	6/6	BM	Unrelated
7	14	MAC	Bu, Cy, ATG, Etop	7/8	PBSCs	Unrelated
8	1	Intermediate	TBI (6 Gy), Flu, Cy, Mel (80 mg/m^2)	7/8	Cord	Unrelated
9	0.40	RIC	Alem, Flu, Mel	8/8	BM	Unrelated
10	0.98	RIC	Alem, Flu, Mel	9/10	BM	Unrelated
11	2	RIC	Alem, Flu, Mel	9/10	BM	Unrelated
12	3	RIC	Alem, Flu, Mel	9/10	Cord	Unrelated
13	3	RIC	Alem, Flu, Mel	8/8	BM	Unrelated
14	3	RIC	Alem, Flu, Mel	10/10	BM	Unrelated
15	4	RIC	Alem, Flu, Mel	8/8	PBSCs	Maternal
16	7	RIC	Alem, Flu, Treo, Thio	10/10	PBSCs	Unrelated
17	9	RIC	Alem, Flu, Mel	7/8	BM	Unrelated
18	11	RIC	Alem, Flu, Mel	8/8	BM	Unrelated
19	19	RIC	Alem, Flu, Mel	10/10	BM	Sibling

Bu indicates busulfan; Mel, melphalan; ATG, antithymocyte globulin; Cy, cyclophosphamide; Etop, etoposide; Flu, fludarabine; Alem, alemtuzumab; Treo, treosulfan; Thio, thiotepa; and PBSCs, peripheral blood stem cells.

*Six to 10 alleles (HLA-A, HLA-B, HLA-C, HLA-DRB1, or HLA-DQB1).

Statistical analysis

Survival was analyzed using Kaplan-Meier curves created with XLSTAT 2011 software (Addinsoft). Comparison of survival curves was done using the log-rank test. For multivariate analysis of survival time and the impact of preparative regimen (MAC vs RIC), donor match, (full match vs mismatch), and HLH activity (remission vs nonremission), Cox proportional hazard regression model analysis was used. The patient who received the intermediate-intensity regimen was excluded from these analyses. Statistical significance was considered as $P < .05$.

Results

Patients

Nineteen patients with XIAP deficiency underwent allogeneic HCT between 2001 and 2011 at a median age of 3 years (range, 0.4-19). Patient characteristics before HCT and *XIAP/BIRC4* mutations are listed in Table 1. Approximately one-third of patients had developed EBV-related HLH before HCT, and approximately two-thirds of patients had developed non-EBV HLH before HCT. Six of these patients were reported to have either active HLH or HLH in partial remission just before HCT. Two patients with colitis were diagnosed and treated as having Crohn disease before the diagnosis of XIAP deficiency.

Transplantation procedures

Graft characteristics and conditioning regimens are shown in Table 2. Seven patients received a MAC protocol.[26] Most patients received busulfan, cyclophosphamide, and antithymocyte globulin with or without etoposide (n = 5). The remaining 2 patients received busulfan with either fludarabine or melphalan and antithymocyte globulin. Eleven patients received a RIC protocol.[26] Ten RIC patients received alemtuzumab, fludarabine, and melphalan, and 1 patient received alemtuzumab, fludarabine, treosulfan, and thiotepa. The remaining patient (patient 8) received an intermediate protocol consisting of TBI (6 Gy), fludarabine, cyclophosphamide, and melphalan (80 mg/m^2).

Eleven patients received fully matched related (n = 2) or unrelated (n = 9) grafts based on typing of 6-10 HLA antigens (HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1). Eight patients received a single allele mismatched graft. The stem cell source was BM in 11 patients, cord blood in 5 patients, and peripheral blood stem cells in 3 patients.

Engraftment

All patients engrafted with a median of 15 days (range, 8-22) except for patient 11, who died before engraftment on day +13.

Toxicities

There was a high incidence of conditioning-related toxicities among MAC patients (Table 3). There were 3 cases of hepatic venoocclusive disease (VOD), which contributed to deaths on days +17, +50, and +144 in patients 6, 2, and 1, respectively. Two of these patients also developed pulmonary hemorrhage. One patient (patient 3) developed pulmonary hypertension of uncertain etiology with pulmonary hemorrhage after transplantation and died on day +170. This patient had received MAC after having previously undergone HCT twice with RIC.

There were no cases of hepatic VOD or pulmonary hemorrhage in patients who received RIC. However, 1 patient (patient 11) developed multiorgan failure and cardiac toxicity with asystole and died at day +13. A second patient (patient 15) suffered an unexpected death related to idiopathic pneumonitis and respiratory failure at day +125.

Patient 8, who received the intermediate preparative regimen (consisting of TBI, fludarabine, cyclophosphamide, and melphalan), suffered posttransplantation cytokine storm syndrome with acute respiratory distress syndrome and died on day +22.

GVHD

Three patients developed acute GVHD of grade 2 or greater (Table 3). One additional patient developed acute GVHD after receiving a donor lymphocyte infusion that was administered as an intervention for declining donor contribution to hematopoiesis.

Table 3. Toxicities and complications

Patient no	VOD	Pulmonary hemorrhage	Acute VHD	Pneumonitis or ARDS	Confirmed bacteremia/sepsis	Fungal infection	Viremia with EBV, CMV, adenovirus, or HHV6	BK virus hemorrhagic cystitis
1	+	+	–	NR	NR	NR	NR	NR
2	+	–	–	NR	NR	NR	NR	NR
3	–	+ (shown by autopsy, not clinically)	II	–	+ (*S marcescens*)	–	+ (EBV, adenovirus)	–
4	–	+ (related to fungal septic thrombosis of the pulmonary veins and pulmonary artery)	III	–	–	+ (fungal septic thrombosis of the pulmonary veins and pulmonary artery)	+ (EBV, adenovirus)	+
5	–	–	I	–	+ (*K oxytoca*, *Enterococcus* sp, *P aeruginosa*)	–	+ (CMV, adenovirus, HHV6)	–
6	+	+	–	–	–	–	–	–
7	–	–	III	+	–	–	–	–
8	–	–	–	+	–	–	–	–
9	–	–	–	–	–	–	+ (adenovirus)	–
10	–	–	– (+ after DLI)	–	+ (*K oxytoca*, *S maltophilia*, *P aeruginosa*)	–	–	–
11	–	–	–	+	–	–	–	–
12	–	–	–	–	–	–	–	+
13	–	–	–	–	–	–	+ (adenovirus)	–
14	–	–	–	–	–	–	+ (EBV, CMV)	–
15	–	–	–	+	–	–	–	–
16	–	–	–	–	–	–	+ (adenovirus)	–
17	–	–	I	–	+ (*S aureus*)	–	+ (adenovirus)	–
18	–	–	–	–	+ (*S aureus*)	–	+ (CMV)	–
19	–	–	–	–	–	–	–	–

ARDS indicates acute respiratory distress syndrome; NR, not reported; and DLI, donor lymphocyte infusion.

Two patients developed chronic GVHD (limited, n = 1, and extensive, n = 1).

Infections

Most patients experienced an infectious complication of HCT (Table 3). Common viral complications included EBV viremia (n = 3, all patients received rituximab), CMV viremia (n = 3, all patients received CMV-directed therapy), and adenovirus viremia (n = 7, 4 patients received adenovirus-directed therapy). Other reported viral complications included human herpesvirus 6 (HHV6) viremia and encephalitis (n = 1), varicella zoster (n = 1), and BK virus hemorrhagic cystitis (n = 2).

Reported bacterial infections included pneumonias, bacteremias and episodes of sepsis (n = 5) related to *Serratia marcescens*, *Klebsiella oxytoca*, *Stenotrophomonas maltophilia*, *Enterococcus* sp, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. One patient developed fatal fungal septic thrombosis of the pulmonary veins and pulmonary artery.

Donor contribution to hematopoiesis

Six patients were reported to develop mixed donor and recipient chimerism (< 95% donor cells detected in peripheral blood) at a median of 37 days after HCT. All of these patients had received RIC. Patient 12 was reported to lose the graft by 35 days after HCT. For the remaining 5 patients (patients 9, 10, 13, 18, and 19), the lowest observed donor contributions to hematopoiesis ranged from 13.8%-92%. Three patients received a stem cell boost and/or donor lymphocyte infusion(s). At the time of last follow-up at a median of 867 days after HCT (range, 139-1706), all 5 patients possessed greater than 90% donor contribution to hematopoiesis and remained free of disease.

Survival and outcome

Only 1 of the 7 patients who received MAC is currently surviving, 414 days after HCT (Table 4). The other 6 patients died at a median of 97 days after HCT (range, 17-247) from toxicities and complications including VOD, pulmonary hemorrhage, pulmonary hypertension, GVHD, sepsis, multiorgan failure, and fungal septic thrombosis of pulmonary veins and pulmonary artery with pulmonary hemorrhagic necrosis.

Patient 8, who received an intermediate-conditioning regimen, also died, on day +22, of posttransplantation cytokine storm syndrome with acute respiratory distress syndrome.

Of the patients who received RIC, 6 of 11 are currently alive and well at a median of 570 days after HCT (55%). All but 1 survivor were given a Lansky or Karnofsky score of 100 at the time of last follow-up. Patients 10, 11, 12, 15, and 17 died at a median of 140 days after HCT (range, 13-416). Reported causes of death were heterogeneous and included pneumonitis with respiratory failure, cardiac toxicity with asystole and multiorgan failure, encephalitis, and ongoing CNS HLH (with loss of graft), sepsis, and pneumonia with respiratory failure (Table 4).

The 1-year probabilities of survival for MAC and RIC patients are 14% and 57%, respectively (Figure 1A), with long-term probabilities of survival of 14% and 43%, respectively (Figure 1B).

Influences on survival

We examined the significance of multiple factors known to influence transplantation outcomes including preparative regimen (MAC vs RIC),[11] donor match,[29] and HLH disease status at the time of transplantation.[2-4] HLH disease status at the time of transplantation was based on the judgment of the treating/contributing physician who reported HLH to be in remission, in partial