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Characterization of Epstein-Barr virus (EBV)-infected cells in EBV-associated hemophagocytic lymphohistiocytosis in two patients with X-linked lymphoproliferative syndrome type 1 and type 2

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Abstract

Background: X-linked lymphoproliferative syndrome (XLP) is a rare inherited immunodeficiency by an extreme vulnerability to Epstein-Barr virus (EBV) infection, frequently resulting in hemophagocytic lymphohistiocytosis (HLH). XLP are now divided into type 1 (XLP-1) and type 2 (XLP-2), which are caused by mutations of *SH2D1A/SLAM-associated protein (SAP)* and *X-linked inhibitor of apoptosis protein (XIAP)* genes, respectively. The diagnosis of XLP in individuals with EBV-associated HLH (EBV-HLH) is generally difficult because they show basically similar symptoms to sporadic EBV-HLH. Although EBV-infected cells in sporadic EBV-HLH are known to be mainly in $CD8^+$ T cells, the cell-type of EBV-infected cells in EBV-HLH seen in XLP patients remains undetermined.

Methods: EBV-infected cells in two patients (XLP-1 and XLP-2) presenting EBV-HLH were evaluated by in EBER-1 *in situ* hybridization or quantitative PCR methods.

Results: Both XLP patients showed that the dominant population of EBV-infected cells was $CD19^+$ B cells, whereas EBV-infected $CD8^+$ T cells were very few.

Conclusions: In XLP-related EBV-HLH, EBV-infected cells appear to be predominantly B cells. B cell directed therapy such as rituximab may be a valuable option in the treatment of EBV-HLH in XLP patients.

Keywords: B cells, Epstein Barr virus, Hemophagocytic lymphohistiocytosis, X-linked lymphoproliferative syndrome

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is clinically characterized by prolonged fever, hepatosplenomegaly, hypertriglyceridemia, systemic hypercytokinemia and cytopenia [1]. HLH consists of primary (familial) and secondary (infection, lymphoma or autoimmune disease-associated) types. Approximately half of all infection-associated HLH cases involves the Epstein-Barr virus (EBV) [2]. Most cases of EBV-HLH are sporadic, but a few cases may present the first presentation of X-linked lymphoproliferative syndrome (XLP) [3]. XLP is a rare, inherited immunodeficiency that is characterized by an extreme vulnerability to EBV infection and shows variable clinical phenotypes, including severe or fatal EBV-HLH (60%), malignant B-cell lymphoma (30%), and progressive dysgammaglobulinemia (30%) [3]. The first genes that is responsible for XLP was identified as the *SH2D1A/SLAM-associated protein* (*SAP*) gene in 1998 [4-6], and mutations in the *X-linked inhibitor of apoptosis protein* (*XIAP*) gene can also lead to the clinical phenotype of XLP in 2006 [7]. XLP is now considered to comprise two distinct diseases, namely XLP-1 (SAP deficiency) and XLP-2 (XIAP deficiency).

In addition to B cells, EBV can infect other cell types, including epithelial cells, T cells and natural killer (NK) cells [8]. Studies have shown that activated T cells,

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particularly $CD8^+$ T cells, are the primary cellular target of EBV infection in sporadic EBV-HLH [9,10], which reflects the pathogenic role of EBV-infected $CD8^+$ T cells in sporadic EBV-HLH. Patients with sporadic EBV-HLH are usually treated with immunochemotherapy, including dexamethasone, cyclosporine A and etoposide, and this therapy can be curable [11]. In contrast, XLP-related EBV-HLH is usually refractory to immunochemotherapy [3]. It is possible that the poor response of XLP-related EBV-HLH to immunochemotherapy can be attributed to the type of EBV-infected cells in this disease, which may differ from the cell type that infected in sporadic EBV-HLH. We investigated the affected cell type in EBV infection of two XLP (XLP-1 and XLP-2) patients with EBV-HLH. Our results demonstrate that the predominant EBV target cells in XLP-related EBV-HLH are $CD19^+$ B cells, which appears to be distinct from sporadic EBV-HLH cases.

Patients, materials and methods

Patients

Three patients presented with clinical features of HLH, including persistent fever, hepatosplenomegaly, cytopenia, abnormal liver function, hyperferritinemia and elevated levels of soluble interleukin-2-receptor (Table 1). The clinical features of the patients fulfilled the diagnostic criteria for HLH [1], although hemophagocytosis in the bone marrow was not observed in patients 1 and 2. Patient 3 was previously reported as patient HLH3 [10]. The number of EBV-DNA copies in the peripheral blood was increased from the normal level of $\leq 1 \times 10^2$ copies/ml to 1.4×10^5, 5.7×10^3 and 1.4×10^6 copies/ml in patients 1, 2 and 3, respectively. Blood samples from the patients were obtained using standard ethical procedures with the approval of the Ethics Committee of the University of Toyama, and an analysis of the *SH2D1A* and *XIAP* genes was performed. Patient 1 showed a one-nucleotide insertion (239_240insA) in the *SH2D1A* gene that resulted in a frameshift and a premature stop codon (80KfsX22). Patient 2 carried a two-nucleotides deletion (1021_1022delAA) in the *XIAP* gene that resulted in a frameshift and a premature stop codon (N341YfsX7). Patient 3 had no mutations in the *SH2D1A* or *XIAP* gene.

Table 1 Clinical and laboratory finding of the patients in this study

	Patient 1	Patient 2	Patient 3
Family history available	No	Yes	No
Age at the time of the study	4 years	21 months	16 months
Age at onset	3 years	17 months	16 month
Fever	Yes	Yes	Yes
Hepatomegaly	4 cm	5 cm	2.5 cm
Splenomegaly	2 cm	3 cm	1 cm
White blood cells ($\times 10^9$/L)	11.6	6.36	3.03
Neutrophils ($\times 10^9$/L)	1.61	3.915	0.56
Hemoglobin (g/dL)	8.1	9.6	7.5
Platelets ($\times 10^9$/L)	95	56	30
LDH (IU/L)	449	1,693	1,698
AST (IU/L)	88	122	453
ALT (IU/L)	31	25	255
Ferritin (μg/L)	1,276	26,282	11,129
sIL-2R (U/mL)	3,162	2,880	14,334
IgG (mg/dL)	1,821	806	423
IgA (mg/dL)	302	124	32
IgM (mg/dL)	1,843	40	18
Whole blood EBV-DNA (copies/mL)	140,000	5,700	1,400,000

LDH, lactate dehydrogenase; AST, aspartate amino transferase; ALT, alanine amino transferase, sIL-2R, soluble interleukin-2 receptor; NA: not available.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from the the patients using Ficoll-Hypaque gradient centrifugation. Lymphocytes were prepared from the PBMCs by depleting the monocytes using anti-CD14 monoclonal antibody (mAb)-coated magnetic beads (Becton Dickinson, San Diego, CA) [10]. The $CD19^+$ B cells, $CD56^+$ NK cells, $CD4^+$ T cells and $CD8^+$ T cells were purified by positive selection from the lymphocytes using the respective mAb-coated magnetic beads. The purity of each isolated cell population was assessed by flow cytometriy analysis, and each sorted population was found to be higher than 85% pure.

In situ hybridization of EBVRNA

The presence of EBV was estimated by measuring the EBV-encoded small RNA 1 (EBER-1) mRNA using the *in situ* hybridization (ISH) method as described previously [10]. The sorted cells were cytocentrifuged onto silanized slides (Dako, Kyoto, Japan), and the presence of EBER-1 mRNA was determined by ISH using the alkaline phosphatase-conjugated EBER-1 antisense probe (5'-AGCAGAGTCTGGGAAGACAACCACAGA-CACCGTCCTCACC-3') or a sense probe.

Quantitative PCR for EBV DNA

Quantitative PCR was performed using AmpliTaq Gold and a real-time PCR 7300 system (Applied Biosystems, Foster City, CA) as described previously [12]. The PCR primers for detecting EBV DNA were selected from within the *BALF5* gene, which encodes the viral DNA polymerase. The primers for amplifying the *BALF5* gene sequences were as follows: forward, CGGAAGCCC TCTGGACTTC, and reverse, CCCTGTT TATCC-GATGGAATG. The TaqMan probe was FAM-TATA-CACGCACGAGAAATGCGCC-BFQ. The PCR

conditions were as follows: denaturation at 95°C for 2 minutes, annealing at 58°C for 15 seconds, and extension at 72°C for 15 seconds, and the products were subjected to 45 cycles of PCR amplification. The EBV DNA copy number was considered to be significant when more than 500 copies/μg of DNA were observed.

Flow cytometry analysis for the T cell receptor Vβ repertoire

Flow cytometry analysis of the T cell receptor (TCR) Vβ repertoire was performed as described previously [10]. In briefl, the PBMCs were incubated with the appropriate phycoerythrin-conjugated mAbs with specificity for TCR Vβ 1-23 (Immunotech, Marseille, France), fluorescein isothiocyanate-conjugated anti-CD8 (Becton Dickinson) and R-PE-Cy5-conjugated anti-CD4 (Dako) mAbs. The stained cells were analyzed using a flow cytometer. TCR Vβ expression is represented as the percentage of $CD4^+$ or $CD8^+$ cells for each receptor family.

Results

To determine the localization of EBV infection in the lymphocyte subpopulations of patient 1, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK cells were sorted using the immunomagnetic bead method and the presence of EBV was evaluated in each lymphocyte subpopulation by EBER-1 ISH (Figure 1A). EBER-1-positive cells were observed in 34.0% of the $CD19^+$ B cells, whereas the remaining lymphocyte subpopulations contained fewer than 0.1% EBER-1-positive cells. Therefore, the EBV-infected cells in patient 1 were almost exclusively $CD19^+$ B cells. In patient 3, EBER-1-positive cells constituted 75.5% of $CD8^+$ T cells, however, they were not detected among $CD4^+$ T cells and observed in a few of $CD19^+$ B cells and $CD56^+$ NK cells (2.8% andl 7.4%, respectively) (Figure 1B).

The viral loads in the $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK cells in patient 2 were determined by quantitative PCR. The number of EBV DNA genome copies in the $CD19^+$ B cells was 1.8×10^4 copies/μg, and the copy number in the $CD8^+$ T cells was 1.0×10^3 copies/μg. The EBV DNA genome could not be detected in either the $CD4^+$ T cells or the $CD56^+$ NK cells that were isolated from patient 2.

Flow cytometry analysis of the TCR Vβ repertoire revealed a polyclonal pattern in patients 1 and 2 (Figure 2), which was in contrast to the skewed pattern that is most commonly seen in the $CD8^+$ T cells of patients with sporadic EBV-HLH [10]. No clonal dominance in $CD8^+$ T cells was demonstrated by mAb in patient 3, but TCR Vβ13.3 was predominantly found in the $CD8^+$ T cells by complentarity-determining region 3 spectratyping [10].

Discussion

XLP is a severe and rare immunodeficiency disease that is characterized by an extreme vulnerability to EBV infection and frequently results in HLH [3]. XLP was first described as X-linked progressive combined immunodeficiency in 1975 by Purtilo et al. [13]. To better understand and reflect the pathophysiology of this disease, the term "X-linked lymphoproliferative disease or syndrome" has now been used. The first gene to be linked to XLP in 1998 was *SH2D1A* which is located on Xq25 and encodes the SAP [4-6]. Importantly, in 2006, a mutation in the gene that encodes the XIAP was identified as a second XLP-linked gene [7]. Thus, XLP can be divided into XLP-1 (SAP deficiency) and XLP-2 (XIAP deficiency). Most XLP patients present with EBV-HLH. Pachlopnik Schmid et al. [14] reported that the incidence of HLH in XLP-1 and XLP-2 is 55 and 76%, respectively. Currently, hematopoietic stem cell transplantation (HSCT) is the only curative therapy for XLP. Therefore, an early definitive diagnosis and immediate treatment are extremely important for both life-saving intervention and an improved prognosis for XLP patients.

EBV infects the majority of the adult population worldwide and persists in B cells throughout the lifetime of normal individuals, usually without causing disease. EBV is the most common trigger for both the XLP-1 and XLP-2 phenotypes. Prior to being exposed to EBV, most patients with XLP can tolerate infections by other agents, although *in vitro* studies have demonstrated defects of T cell-mediated and humoral immunity. During an acute EBV infection, XLP patients develop normal or high levels of anti-viral capsid antigen IgM antibodies but usually lack heterophile antibodies. Initially, these patients fail to develop EBV-specific cytotoxic T cells, and this results in a massive and overwhelming polyclonal B cell proliferation involving lymphoid and other tissues [8]. SAP binds 2B4, which is a surface molecule involved in activation of NK cell-mediated cytotoxity. Therefore, SAP-deficient patient shows that NK cell function is impaired, allowing B cell proliferation [15]. SAP has proapoptotic function, and contributes to the maintenance of T cell homeostasis and to the elimination of potentially dangerous DNA-damaged cells. Thus, the loss of this function could be responsible for the uncontrol T cell proliferation in acute EBV infection [16].

B cells are the usual cellular targets of EBV in a primary EBV infection such as infectious mononucleosis and in the sero-positive normal host [8]. After the interaction of the viral surface glycoproteins with the CD21 receptor, EBV entry into B cells is mediated by HLA class II and other co-receptors. However, in cases of

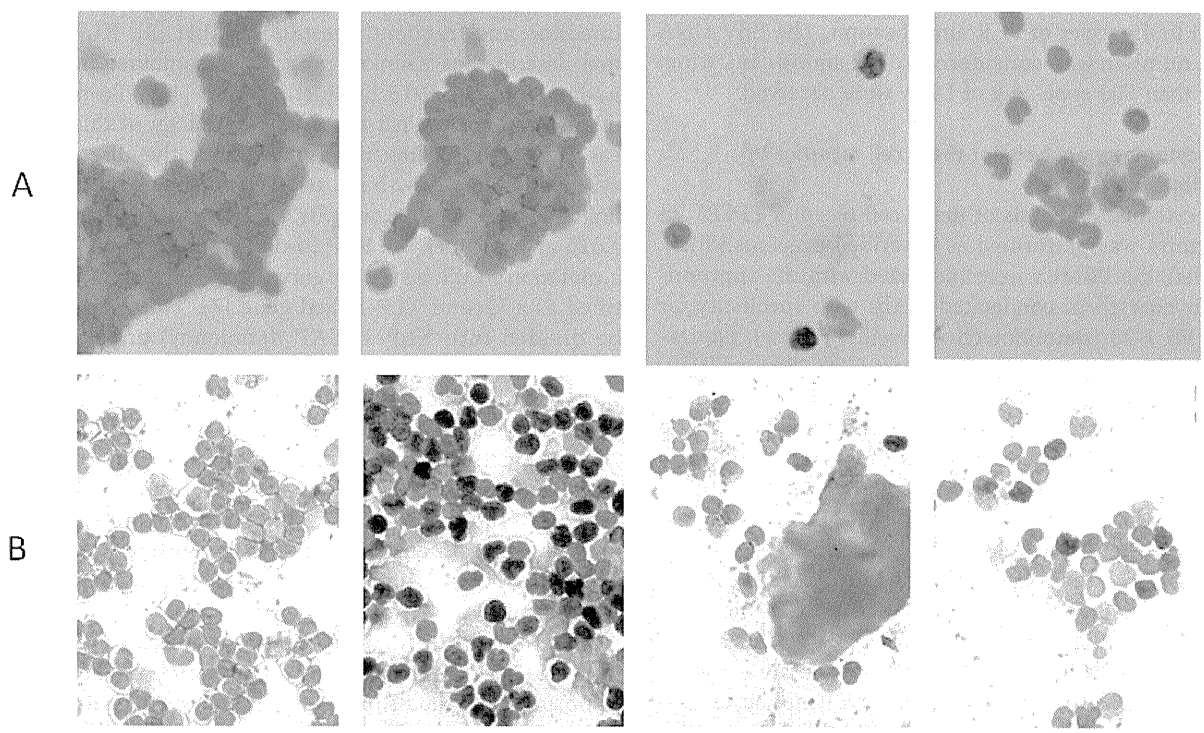

Figure 1 Cytospin preparations showing EBER-1 *in situ* hybridization of the lymphocyte subpopulations. Lymphocyte subpopulations from patients 1 and 3 were separated by magnetic bead sorting after immunostaining with anti-CD4, CD8, CD19 or CD56 mAbs. EBV infection in each subpopulation was determined using EBER-1 ISH. A, In patient 1, EBER-1-positive cells (shown by their dark nuclear staining) were detected in34.0% of the B cells but were not detected in $CD4^+$ T cells, $CD8^+$ T cells or $CD56^+$ NK cells (< 0.1% each). B, In patient 3, EBER-1-positive cells were observed in 75.5% of $CD8^+$ T cells, 2.8% of $CD19^+$ B cells, and 17.4% of $CD56^+$ NK cells, but not observed in $CD4^+$ T cells [10].

sporadic EBV-HLH, EBV infects primarily T cells and NK cells [9,10,17]. The mechanism of T cell infection by EBV in HLH is still unclear, but one hypothesis is that, in specific situations, $CD8^+$ T cells express CD21, which can mediate EBV infection. Although T cells do not express the glycoprotein, they contain mRNA for CD21 [18]. In sporadic EBV-HLH cases, EBV infection into B cells is delayed but occurs during every case of cured EBV-HLH [17]. To the best of our knowledge, this is the first report of EBV infection status in two different types of XLP patients with EBV-HLH. The present study shows that the primary EBV-infected cells in XLP-related EBV-HLH are $CD19^+$ B cells and not T cells or NK cells, which are a primary target of EBV infection in sporadic EBV-HLH.

For decades, clinicians and investigators have been puzzled by the differential diagnosis between XLP and sporadic EBV-HLH when they encountered a young boy presenting with EBV- HLH. We believe the different EBV target cells can provide additional information to help discriminate between XLP and sporadic EBV-HLH. An evaluation of specific cell type that is infected by EBV should be considered when target therapy is applied. Most patients with sporadic EBV-HLH can achieve remission by immunochemotherapy; however, patients with XLP are usually refractory to this therapy. Recently, B cell-directed therapy using an anti-CD20 mAb (rituximab) was performed in patients with XLP-1 [19]. Two XLP patients who presented with acute EBV infection were successfully treated with rituximab and were free from EBV-HLH and lymphoma for a prolonged period. In addition, rituximab combined with methylprednisolone and intravenous immunoglobulin were administered to an XLP-1 patient with EBV-HLH, and the patient achieved a remission [20]. Patient 1 was also associated with EBV-associated encephalitis and lymphoproliferative disorder. The patient's lymphoproliferative disorder was treated with rituximab, but he died of the disease. Patient 2 was successfully treated with dexamethasone

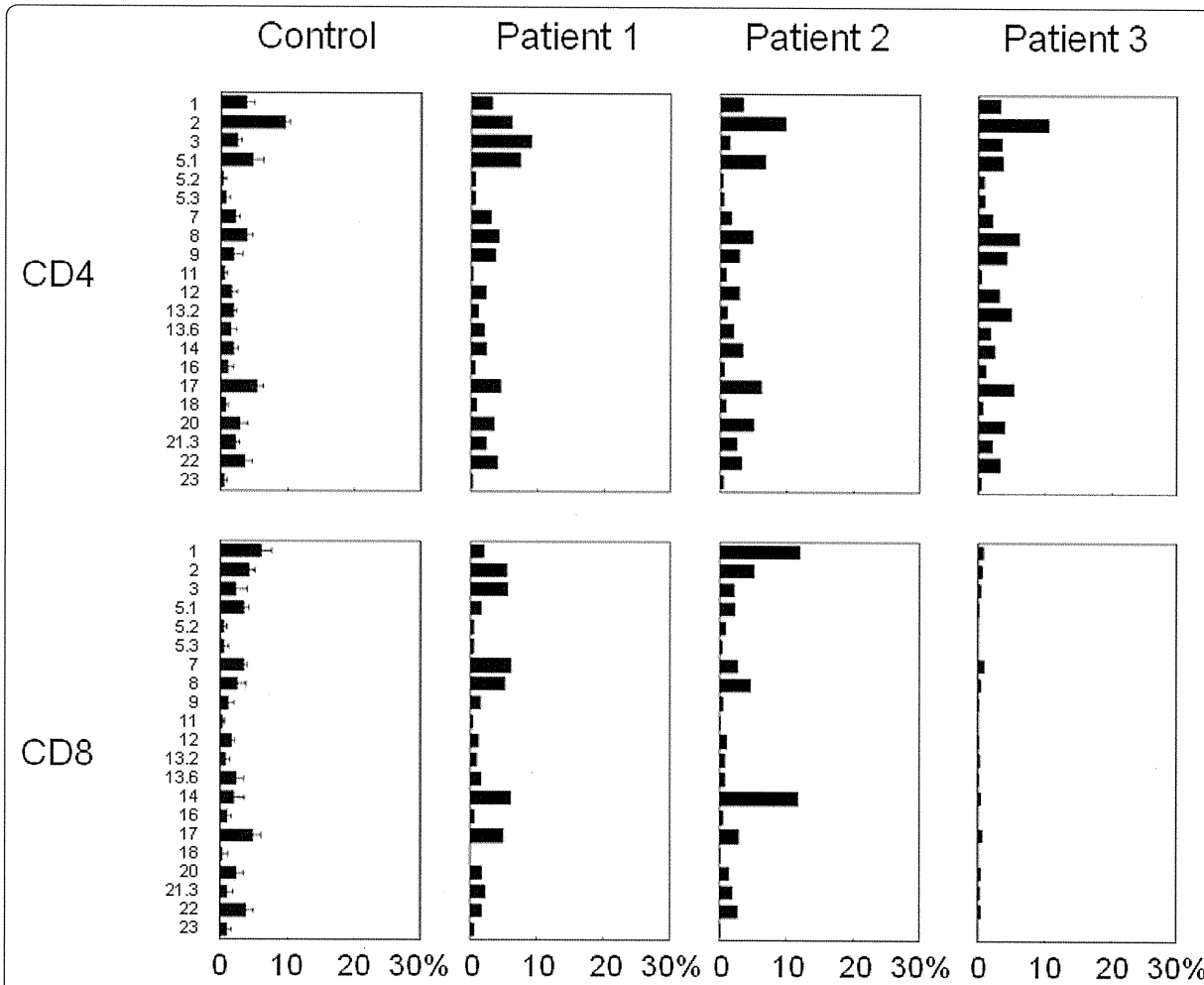

Figure 2 The results of the flow cytometric analysis of TCR Vβ. The expression profiles of the TCR Vβ subfamilies of patients 1, 2 and 3. The PBMCs were stained with mAbs for individual TCR Vβ, together with an anti-CD8 mAb. The percentage of the expression of each TCR Vβ within $CD8^+$ T cells was analyzed by flow cytometry.

and immunoglobulin. Our data suggest that B cell target therapy can be a viable therapeutic option for an initial stage of EBV-HLH in both XLP-1and XLP-2 patients.

Abbreviations

EBER: EBV-encoded small RNA; EBV: Epstein-Barr virus; HLH: Hemophagocytic lymphohistiocytosis; HSCT: Hematopoietic stem cell transplantation; ISH: *In situ* Hybridization; mAb: Monoclonal antibody; NK: Natural killer; PBMC: Peripheral blood mononuclear cells; SAP: SLAM-associated protein; TCR: T cell receptor; XIAP: X-linked inhibitor of apoptosis; XLP: X-linked lymphoproliferative syndrome.

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Authors' contributions

XY and HK wrote the manuscript. XY, TW, KI and NN performed the experimental studies. TM, MF, HK and FK managed the patients' care. SF, AY, XDZ and TM revised the manuscript. XY, TW and KI contributed equally to this study. All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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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 (l% 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.

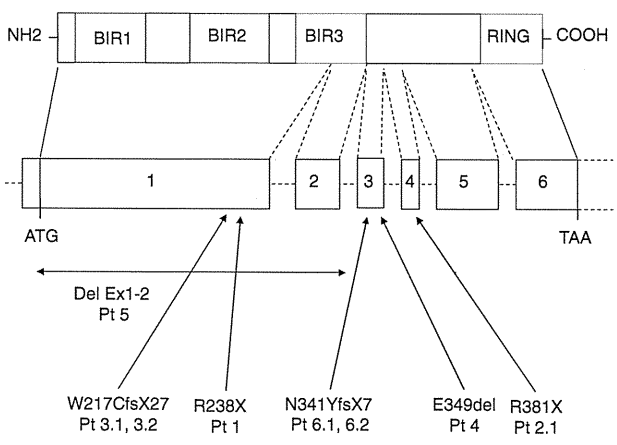

Fig. 1 *XIAP* gene mutations and their consequences for XIAP protein. *XIAP* comprises six exons and encodes the XIAP protein, which consists of 497 amino acids. XIAP contains three BIR domains and one RING domain. Mutations identified in our patients are indicated

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

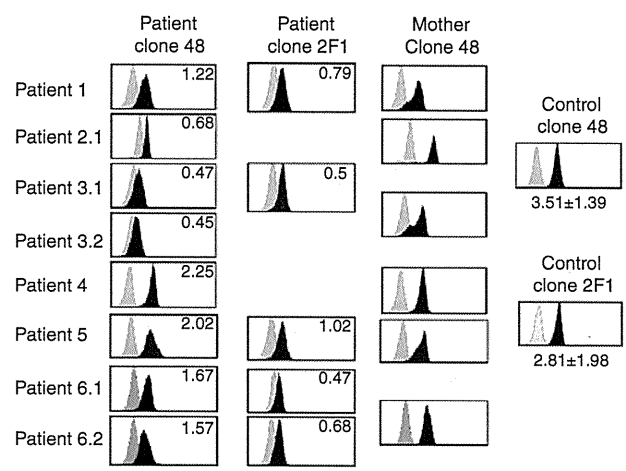

Fig. 2 XIAP protein expression in lymphocytes from the patients and their carriers. Flow cytometric detection of intracellular XIAP in lymphocytes from patients and their maternal carriers. The *gray* and *black areas* indicate the negative control and anti-XIAP staining, respectively. Anti-XIAP staining 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

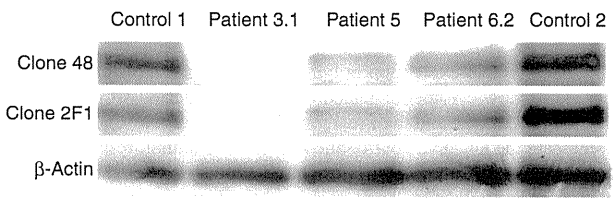

Fig. 3 XIAP expression in lymphocytes from the patients by Western blot. Analysis of XIAP expression in PBMC generated from patients with XIAP deficiency and normal controls using the antibody clone 48 (*upper panel*), the antibody clone 2 F1 (*middle panel*), and the β-actin antibody as an internal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

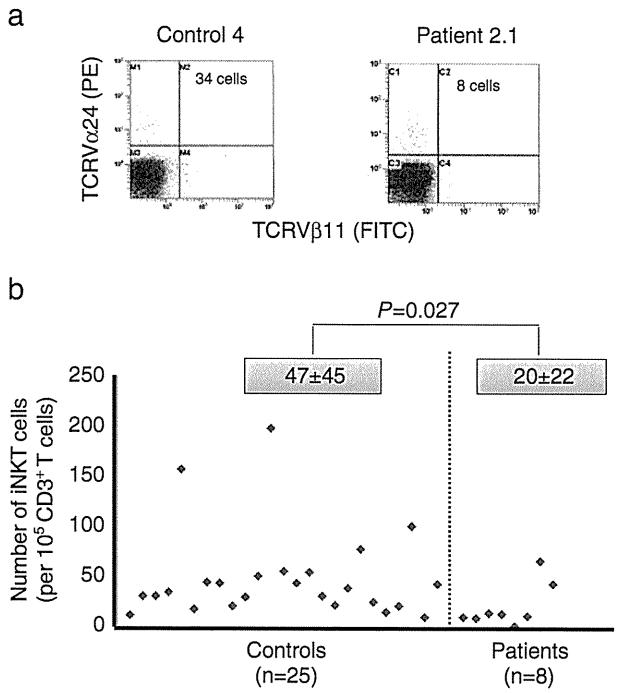

Fig. 4 iNKT cell counts in the patients and healthy controls. **a** Representative flow cytometric analysis of iNKT cells in $CD3^+$ lymphocytes from one XIAP-deficient patient and one healthy control. **b** Comparison of the number of iNKT cells in 100,000 $CD3^+$ lymphocytes between XIAP-deficient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

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.

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

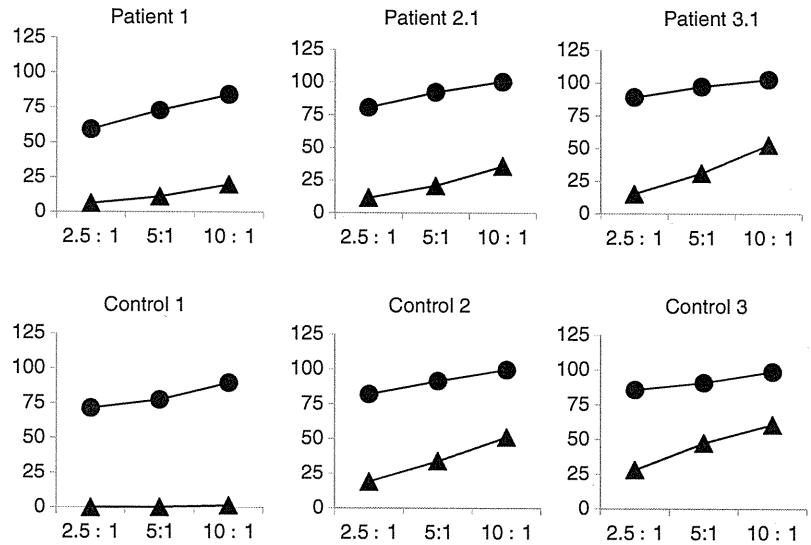

Fig. 5 Cytotoxicity of alloantigen-specific $CD8^+$ T cell lines. $CD8^+$ T cell lines were generated from PBMC of patients with XIAP deficiency and healthy controls by stimulation with allogeneic LCL (KI-LCL). Their cytotoxity was determined against allogeneic KI-LCL (*circles*) and against allogeneic 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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Nationwide Survey of Patients with Primary Immunodeficiency Diseases in Japan

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Abstract To determine the prevalence and clinical characteristics of patients with in Japan, we conducted a nationwide survey of primary immunodeficiency disease (PID) patients for the first time in 30 years. Questionnaires were sent to 1,224 pediatric departments and 1,670 internal medicine departments of Japanese hospitals. A total of 1,240 patients were registered. The estimated number of patients with PID was 2,900 with a prevalence of 2.3 per 100,000 people and homogenous regional distribution in Japan. The male-to-female ratio was 2.3:1 with a median age of 12.8 years. Adolescents or adults constituted 42.8% of the patients. A number of 25 (2.7%) and 78 (8.5%) patients developed malignant disorders and immune-related diseases, respectively, as complications of primary immunodeficiency disease. Close monitoring and appropriate management for these complications in addition to prevention of infectious diseases is important for improving the quality of life of PID patients.

Keywords Primary immunodeficiency disease · epidemiology · nationwide survey · Japan

Abbreviations

APECED	Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy
BTK	Bruton's tyrosine kinase
CGD	Chronic granulomatous disease
CID	Combined T and B cell immunodeficiency
CVID	Common variable immunodeficiency disease
FMF	Familial Mediterranean fever
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked
NEMO	Nuclear factor kappa B essential modulator
PID	Primary immunodeficiency disease
SIgAD	Selective IgA deficiency
SLE	Systemic lupus erythematosus

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TRAPS	Tumor necrosis factor receptor-associated periodic syndrome
WAS	Wiskott–Aldrich syndrome
WHIM	Warts hypogammaglobulinemia, infections, and myelokathexis

Introduction

Patients with primary immunodeficiency disease (PID) show susceptibility to infections due to congenital immune system defects. These patients are also associated with noninfectious complications including autoimmune diseases and malignant disorders. Recent studies have revealed the causes of many PIDs to be mutations in various genes encoding molecules involved in the host defense mechanisms [1]. In addition, various new PIDs including defects in innate immunity and autoinflammatory disorders were identified under the recent progress in immunology and molecular genetics [2]. PID classification has been revised according to the identification of new PIDs and on the basis of new findings in PID pathophysiology. For a more precise clinical analysis, data should be obtained in accordance with the latest PID classifications.

The first nationwide survey of patients with PID in Japan was conducted between 1974 and 1979, which included 497 registered cases [3]. By 2007, a total of 1,297 patients were cataloged by a small number of PID specialists into a registration system [4]. The approximate prevalence of PID patients in Japan in the first nationwide survey was 1.0 in 100,000 people, which was much lower than that in other countries [5–7]. This difference in PID prevalence between Japan and other countries suggested that some PID patients in Japan remained unregistered. To determine the prevalence and clinical characteristics of patients with PID in Japan on the basis of the recent international classification system for PID, we conducted a nationwide survey of PID for the first time in 30 years.

Methods

This study was performed according to the nationwide epidemiological survey manual of patients with intractable diseases (2nd edition 2006, Ministry of Health, Labour, and Welfare of Japan) as described previously [8]. PID classification was based on the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee in 2007 [2]. Patients with chronic benign neutropenia and syndrome of periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis were excluded because these were considered to be acquired diseases. The survey was conducted on PID patients who were alive on December 1, 2008 and those who were newly diagnosed and dead between December 1, 2007 and November 30, 2008 in Japan. Among the 2,291 pediatric departments and 8,026 internal medicine departments in Japan, hospitals participating in the survey were randomly selected after setting the selection ratio according to the number of beds (overall selection rate: 53.4% for pediatric departments, 20.8% for internal medicine departments; Table I). University hospitals and pediatric training hospitals, where many PID patients were considered to be treated, were stratified separately (Table I). Primary questionnaires regarding the number of patients and disease names based on PID classification were sent to the selected hospitals. Secondary questionnaires regarding age, gender, clinical manifestations, and complications of individual PID patients were sent to respondents who answered that they observed at least one PID patient with characteristics listed in the primary questionnaires.

Results

Questionnaires were distributed to 1,224 pediatric departments and 1,670 internal medicine departments of hospitals in Japan, and the response rate was 55.0% and 20.1%, respectively (Table I). A total of 1,240 patients (1,146 patients from pediatric departments and 94 patients from internal medicine departments) were registered (Table I). The estimated number of patients with PIDs in Japan was 2,900 (95% confidence interval: 2,300–3,500), and the prevalence was 2.3 per 100,000 inhabitants. We also determined the regional distribution on the basis of the patients' addresses. The estimated regional prevalence ranged from 1.7 to 4.0 per 100,000 inhabitants, and no significant differences were observed between different regions in Japan (Fig. 1). The most common form of PID was predominantly antibody deficiencies (40%), followed by congenital defects of phagocyte number, function, or both (19%) and other well-defined immunodeficiency syndromes (16%; Table II). Autoinflammatory disorders were observed in 108 cases (9%). The most common PID was Bruton's tyrosine kinase (BTK) deficiency (182 cases, 14.7%), followed by chronic granulomatous disease (CGD; 147 cases, 11.9%). However, common variable immunodeficiency disease (CVID) and selective IgA deficiency (SIgAD) were observed only in 136 (11.0%) and 49 cases (4.0%), respectively. Among patients registered from internal medicine departments, antibody deficiencies were the most common disorder (71%).

In the secondary survey, 923 cases were registered. The male-to-female ratio was 2.3:1 (n=914, unanswered: 9 cases) with a median age of 12.8 years (range: 0 to 75 years; n= 897, unanswered: 26 cases). The number of adolescent or

Table I Stratification and selection of hospitals and the survey results

	Stratification	Departments in Japan	Departments selected	Selection rate (%)	Return[a]	Response	Response rate (%)	PID Patient	Patients per department	Patients estimated
Pediatrics	University hospital	118	118	100	0	80	67.8	661	8.3	975
	Training hospital	402	402	100	4	242	60.8	376	1.6	618
	≥500 beds	92	92	100	5	48	55.2	24	0.5	44
	400–499 beds	118	118	100	3	63	54.8	42	0.7	77
	300–399 beds	287	230	80.1	4	122	54.0	31	0.3	72
	200–299 beds	289	116	40.1	4	53	47.3	6	0.1	32
	100–199 beds	486	98	20.2	0	44	44.9	4	0.1	44
	<99 beds	499	50	10.0	1	10	20.4	2	0.2	100
	Subtotal	2,291	1,224	53.4	21	662	55.0	1,146	1.7	1,961
Internal medicine	University hospital	156	156	100	1	47	30.3	37	0.8	122
	≥500 beds	374	374	100	1	86	23.1	35	0.4	152
	400–499 beds	328	263	80	1	54	20.6	6	0.1	36
	300–399 beds	692	278	40.2	6	49	18.0	10	0.2	140
	200–299 beds	1,008	202	20.0	0	36	17.8	2	0.1	56
	100–199 beds	2,460	246	10.0	1	36	14.7	1	0.0	68
	<99 beds	3,008	151	5.0	6	24	16.6	3	0.1	375
	Subtotal	8,026	1,670	20.8	16	332	20.1	94	0.3	950
Total		10,317	2,894	28.1	37	994	34.8	1,240		2,911

[a] Due to the closure of departments

adult cases (≥15 years) was 384 (42.8%; Fig. 2a). The male-to-female ratio of the younger generation (<15 years) was 2.7:1, while that of the older generation (≥15 years) was 2.0:1. Combined T and B cell immunodeficiencies (CIDs) were predominantly observed in the younger generation, while antibody deficiencies were more common with

Region	Reported PID Patients	Population (x10^5)	Estimated prevalence per 10^5 (95% C.I.)
Hokkaido	73	55.4	4.0 (0.2-7.8)
Tohoku	81	94.3	2.1 (0.9-3.3)
Kanto	387	419.8	1.8 (1.2-2.5)
Chubu	236	236.9	2.3 (1.4-3.1)
Kinki	158	208.4	2.0 (1.2-2.8)
Chugoku/Shikoku	105	116.1	1.7 (1.0-2.3)
Kyushu/Okinawa	200	146.0	2.5 (1.7-3.3)
Total	1240	1276.9	2.3 (1.8-2.7)

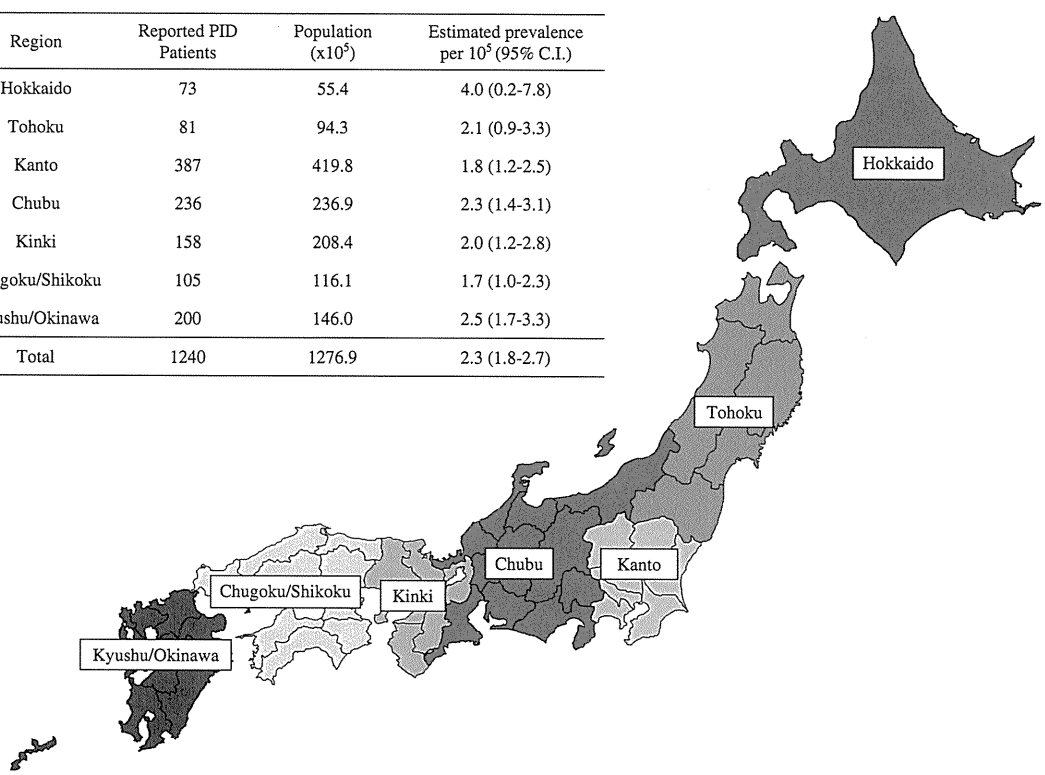

Fig. 1 Regional distribution of PID patients. *CI* Confidence interval

Table II Reported number of PID

Category	Total number	Pediatric department	Internal medicine department
I. Combined T and B cell immunodeficiencies	93 (7%)	93 (8%)	0 (0%)
γc deficiency	47	47	0
Adenosine deaminase deficiency	9	9	0
Omenn syndrome	4	4	0
Others	23	23	0
Untested or undetermined	10	10	0
II. Predominantly antibody deficiencies	501 (40%)	434 (38%)	67 (71%)
BTK deficiency	182	173	9
Common variable immunodeficiency disorders	136	107	29
Selective IgG subclass deficiency	66	58	8
Selective IgA deficiency	49	34	15
Hyper IgM syndrome	34	34	0
Transient hypogammaglobulinemia of infancy	7	7	0
Others	11	7	4
Untested or undetermined	16	14	2
III. Other well-defined immunodeficiency syndromes	194 (16%)	189 (17%)	5 (5%)
Wiskott–Aldrich syndrome	60	60	0
DNA repair defects (other than those in category I)	15	15	0
DiGeorge anomaly	38	38	0
Hyper-IgE syndrome	56	52	4
Chronic mucocutaneous candidiasis	17	16	1
Others	5	5	0
Untested or undetermined	3	3	0
IV. Diseases of immune dysregulation	49 (4%)	48 (4%)	1 (1%)
Chediak–Higashi syndrome	9	8	1
Familial hemophagocytic lymphohistiocytosis syndrome	5	5	0
X-linked lymphoproliferative syndrome	8	8	0
Autoimmune lymphoproliferative syndrome	8	8	0
APECED	4	4	0
IPEX syndrome	7	7	0
Others	2	2	0
Untested or undetermined	6	6	0
V. Congenital defects of phagocyte number, function, or both	230 (19%)	223 (19%)	7 (8%)
Severe congenital neutropenia	44	42	2
Cyclic neutropenia	19	17	2
Chronic granulomatous disease	147	144	3
Mendelian susceptibility to mycobacterial disease	5	5	0
Others	9	9	0
Untested or undetermined	6	6	0
VI. Defects in innate immunity	15 (1%)	15 (1%)	0
Anhidrotic ectodermal dysplasia with immunodeficiency	7	7	0
Interleukin-1 receptor-associated kinase 4 deficiency	2	2	0
Others	5	5	0
Untested or undetermined	1	1	0
VII. Autoinflammatory disorders	108 (9%)	101 (9%)	7 (8%)
Familial Mediterranean fever	44	40	4
TNF receptor-associated periodic syndrome	13	12	1
Hyper IgD syndrome	4	4	0
Cryopyrin-associated periodic syndrome	22	22	0