observed in urban areas [5, 7, 16]. This may be because many PID patients were treated or followed by PID specialists distributed nationwide in Japan; this is assumed by the location of hospitals with which they were affiliated.

The distribution ratios of BTK deficiency (14.7%) and CGD (11.9%) in Japan were higher than those in a previous report from Europe (5.87% and 4.33%, respectively), while those of CIDs and other well-defined immunodeficiency syndromes were comparable [17]. The prevalence of BTK deficiency was previously reported to be 1/900,000–1,400,000 in a European cohort study [18]. In contrast, this value was estimated to be 1/300,000 in Japan in our study. BTK deficiency appears to be common in Japan, although this may be partially because more patients, including those showing atypical clinical manifestations, were diagnosed more accurately by the recently established genetic diagnostic network in Japan [19]. This is supported by the highest proportion of Japanese patients in the international mutation database for X-linked agammaglobulinemia (BTKbase) [20]. The reason for the low number of registered CGD patients in Europe in a recent report (1/620,000) [17] is unknown; the prevalence of CGD was 1 in 250,000 in a previous European survey [21], which was similar to our results (1 in 380,000 in this study and 1 in 280,000 in our previous study [22]). The percentage of BTK deficiency and CGD would be lower if more adult cases were registered because the prevalence of these disorders is low in adults. CVID was the most commonly reported PID (20.7%) in Europe, and the onset of symptoms was observed most commonly in the third decade of life in these patients [17, 23]. In this study, CVID constituted 11.0% (136 cases) of PID cases, and only 29 cases were reported from internal medicine departments (Table II). A lower number of registered CVID patients may have led to a lower number of reported patients with antibody deficiency and a lower prevalence of PID, although it is still possible that CVID is not as common in Japan as in European countries. There was no significant difference in the distribution rate of SIgAD between Japanese and Europeans, although SIgAD is rare in Japanese (1/18,500) compared with Caucasians (1/330–2,200) according to seroepidemiologic studies [24]. This may be because most SIgAD patients lack clinical manifestations. The distribution ratio of autoinflammatory disorders in Japan (9%) was much higher than that in Europe (1.02%) [17] (Table II). Considering the disease type of the autoinflammatory disorders was not specified in 22 cases (20%), it is possible that many other patients with autoinflammatory disorders remain undiagnosed in Japan as well as in other countries.

The percentage of men (69.7%) with PID is higher in Japan than in Europe (60.8%) or Kuwait (61.8%), but is equivalent to that in Taiwan (70.2%) [6, 13, 17]. The higher ratio of men, particularly in younger generation (<15 years), appears to be due to the larger number of X-linked PID patients (BTK deficiency, X-CGD, γc deficiency, etc.) in this study compared to that in Europe or Kuwait. Adolescents or adults (≥15 years) constituted 42.8% of the patients in this study, which is equivalent to the number in the European study (≥16 years: 46.6%), while those >16 years constituted only 10.9% in the previous survey [3, 17]. In this study, it was found that CVID and SIgAD are common in adults (Table II) and that antibody deficiencies are more common with increasing age (Fig. 2b). A reason for the increased number of adult PID patients may be long-term survival of PID patients due to improved treatments such as immunoglobulin replacement therapy. In addition, an increased likelihood of patients being diagnosed by internists as having late-onset PID, e.g., CVID and SIgAD, may have contributed to these values [17, 25, 26]. Therefore, it is important for internists to be well-informed regarding PID. In contrast, CIDs are fatal during infancy without hematopoietic stem cell transplantation or gene therapy. Because hematopoietic stem cell transplantation has been widely performed in Japan since the 1990s, surviving patients with CID are limited to the younger generation, similar to French patients (Fig. 2b) [5, 27, 28].

It has been reported that PID patients are at increased risk of developing malignant diseases, in particular, non-Hodgkin lymphoma, leukemia, and stomach cancer [29]. Although lymphoma and leukemia were relatively common, stomach cancer was not observed in our study. In the previous survey in Japan, eight of nine PID patients with malignant disorders (including one gastric cancer patient) died [3]. It is possible that some PID patients with malignant disorders were not registered because they were deceased. PID is also associated with immune-related diseases because of a defect in the mechanisms to control self-reactive B and T cells. The frequency of immune-related manifestations varied among individual PID patients, as reported previously [30, 31]. Four PID patients who had developed Kawasaki disease, one patient with WHIM syndrome and type 1 diabetes mellitus, and one patient with TRAPS and SLE in our study may provide new pathophysiological insights of these diseases and the association between PID and autoimmune diseases.

Conclusions

We report the prevalence and clinical characteristics of PIDs in Japan. Although the advances in diagnostic technologies and treatments have improved the prognoses of PID, many patients continue to experience severe complications such as malignancy and immune-related diseases as well as infections. To improve the quality of life of PID patients, it is necessary to pay attention to

complications and treat them appropriately. Web-based PID databases and consultation systems have been created in Japan (Primary Immunodeficiency Database in Japan [4] and Resource of Asian Primary Immunodeficiency Diseases in Asian countries [32]) to reveal precise information regarding PID and to promote cooperation between doctors and researchers [19].

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of departure from Hardy–Weinberg equilibrium ($P=0.06$), because the variant G allele is significantly more prevalent among Whites than non-Whites with the allele frequency of 0.24 versus 0.073, respectively ($P=0.0003$). Still, the observed associations retained significance in analyses restricted only to Whites. The G allele was associated with better EFS and OS in univariate analyses ($P=0.0173$ and 0.035, respectively, data not shown) and in multivariable analyses ($P=0.023$ and 0.005, respectively, data not shown).

We also observed that the variant A allele of FKBP5 SNP rs7755289 (T>A; intron 8) was significantly associated with worse EFS ($P=0.014$, hazard ratio = 3.193, 95% CI = 1.258–8.104, Figure 1c) and OS ($P=0.0036$, hazard ratio = 4.846, 95% CI = 1.68–14, Figure 1d). In addition, A allele was associated with increased day 22 MRD ($P=0.017$), increased cumulative incidence of relapse ($P=0.045$, hazard ratio = 3.4, 95% CI = 1.03–11.22) and an increased cumulative incidence of treatment-related mortality ($P=0.012$, hazard ratio = 5.57, 95% CI = 1.44–21.47). However, as this SNP occurred with the allele frequency of only ~0.2, the low sample size restricted us from performing further analysis. Although the above mentioned SNPs were the most interesting SNPs, we also observed association of SNP rs16878591 ($P=0.011$) with day 22 MRD levels and SNPs within LD block-2 with *in vitro* ara-C LC_{50} values ($P=0.03$; Table 1).

In previous reports, FKBP5 expression has been shown to positively influence response to cytarabine and gemcitabine. More recently, FKBP5 has been identified as scaffolding protein that facilitates PHLPP-mediated dephosphorylation of AKT-Ser473, thus indicating that higher expression of FKBP5 might contribute to enhanced chemosensitivity.[3–5] siRNA-mediated FKBP5 knockdown increases the resistance to cytarabine and other agents as etoposide, paclitaxel and doxetaxel.[1,3–5] Thus, FKBP5 SNPs may also be associated with response to other agents used in combination with cytarabine in AML patients. In conclusion, our preliminary results suggest that the FKBP5 polymorphisms mentioned above may also be relevant for AML treatment response. These results should be confirmed with functional studies and independent clinical studies. Identification of pharmacogenetic markers of response, such as FKBP5 SNP such as rs3798346, might help in further understanding inter-patient variation in response to chemotherapy.

Conflict of interest

The authors declare no conflict of interest.

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AK Mitra[1], K Crews[2], S Pounds[3], X Cao[3], JR Downing[4], S Raimondi[4], D Campana[5], RC Ribeiro[5], JE Rubnitz[5] and JK Lamba[1]

[1]*Department of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN, USA;*
[2]*Department of Pharmaceutical Sciences, St Jude Children's Research Hospital, Memphis, TN, USA;*
[3]*Department of Biostatistics, St Jude Children's Research Hospital, Memphis, TN, USA;*
[4]*Department of Pathology, St Jude Children's Research Hospital, Memphis, TN, USA and*
[5]*Department of Oncology, St Jude Children's Research Hospital, Memphis, TN, USA*
E-mail: lamba004@umn.edu

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CBL mutation in childhood therapy-related leukemia

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Therapy-related leukemia and myelodysplastic syndrome (t-Leuk/MDS) are mainly caused by topoisomerase II inhibitors that cause acute myeloid leukemia (AML) with an 11q23 translocation or by alkylating agents that induce MDS/AML with an *AML1* mutation and monosomy 7.[1,2] Two types of t-Leuk/MDS can be distinguished, one of which has a long latency (⩾5–7 years) and is seen following alkylating agents, frequently with an preleukemic phase.[1] The other has a short latency period (1–3 years), no preleukemic phase, and is strongly associated with the administration of topoisomerase II inhibitors and chromosomal abnormalities involving 11q23 translocation/*MLL* rearrangement (*MLL*-R).[2] Repair of etoposide (VP-16)-stabilized DNA topoisomerase II covalent complexes may initiate *MLL*-R observed in patients.[3]

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because

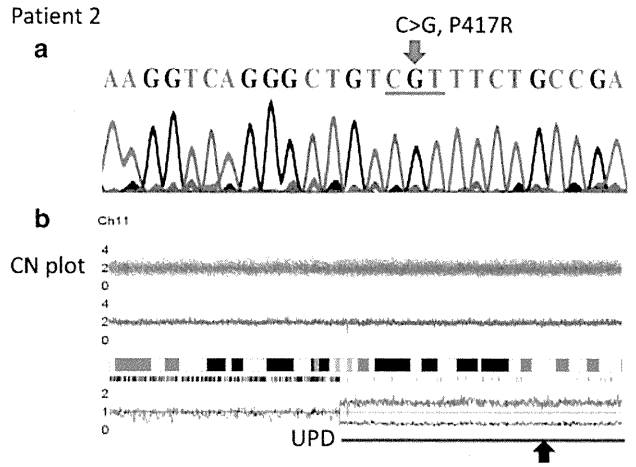

Figure 1 Identification of acquired isodisomy of 11q and *CBL* mutation in therapy-related leukemia. (**a**) Homozygous mutation of the *CBL* gene was identified in patient 2. (**b**) Copy number (CN) analysis for the gene chip output for therapy-related leukemia in patient 2. Total CNs (red plot) are shown above the cytoband, and the result of allele-specific CN analysis with anonymous references plots are shown below the cytoband. Larger allele is presented in red line, and smaller allele is presented in green line. Allele-specific analysis showed 11q-aUPD (blue line), which contained *CBL* region (black arrow).

these *CBL* mutations were shown to result in aberrant tyrosine kinase signaling, which would lead also to activation of RAS signaling pathways. We and others reported that *CBL* mutations occurred in a variety of myeloid neoplasms, including *de novo* AML,[4] MDS[4] and myeloproliferative neoplasm,[4,5] especially in chronic myelomonocytic leukemia[5] and juvenile myelomonocytic leukemia.[6] The importance of *CBL* mutations concerning about leukemogenesis is substantially increased. This prompted us to search for possible *CBL* mutations in pediatric t-Leuk/MDS.

Analysis of *CBL* gene was carried out in 20 pediatric t-Leuk/MDSs, including 15 AMLs (range: 1 year and 10 months to 17 years; 8 males and 7 females), 4 MDSs (range: 7 years to 14 years; 4 males) and 1 acute lymphoblastic leukemia (4 years and 2 months; 1 male). Median age at diagnosis was 8 years and 1 months (range: 1 year and 10 months to 17 years; 13 males and 7 females). Rearrangements of *MLL* gene were found in 17 patients (85%), including 15 of 16 who received VP-16 (Sugita *et al.*[7]), and 2 of 4 who did not receive it. An initial diagnosis was made as non-Hodgkin's lymphoma in seven patients, neuroblastoma in five, acute lymphoblastic leukemia in five, AML in two and juvenile myelomonocytic leukemia in one.

Because *CBL* mutations thus far reported almost exclusively involved exons 8–9 that encode linker/RING finger domains,[4–6] we confined our mutation analysis to these exons, in which PCR-amplified exons 8–9 were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA). The study adhered to the principles of the Helsinki declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

Homozygous mutation of the *CBL* gene was identified in 1 out of the 20 t-Leuk/MDS cases (5%), which were located in the RING finger domain (P417R in patient 2). As the frequency of 11q-acquired uniparental disomy (11q-aUPD) was reported ~85–90% in *CBL* mutations,[4–6] we analyzed his sample using Affymetrix GeneChip 250K *Nsp*I array (Affymetrix, Santa Clara, CA, USA), and found the presence of 11q-aUPD, which was the sole abnormality seen by single-nucleotide polymorphism array (Figure 1), confirming a strong association of *CBL* mutations with 11q-aUPD as previously described.[4–6] Furthermore, we examined *NRAS* and *KRAS* mutations in these patients whose samples were available and found *KRAS* mutation in one patient with t-Leuk (acute monocytic leukemia having t(9;11)(p21;q23) after B-cell precursor acute lymphoblastic leukemia having 6p−, 7q+, 9q+ and 12q−).

CBL mutation was detected in MDS cells from the patient with t-MDS after malignant lymphoma. The patient was initially diagnosed as having diffuse large T-cell type malignant lymphoma, whose biopsied specimen of the buccal lymph node showed MT1(+), MB1(−) and UCHL1(+), when he was 5 years old. He subsequently was treated with chemotherapy according to T-8801 protocol including VP-16 (200 mg/m^2) given twice weekly,[7] and obtained a complete remission. However, at 7 months after diagnosis, tumor appeared in the right maxilla, and was diagnosed as the relapsed lymphoma, then, he received local irradiation (30 Gy) and chemotherapy including ifosfamide, vincristine, THP-adriamycin and L-asparaginase. At 4 months later, enlarged spleen was resected, and the infiltrated tumor cells were microscopically seen in the tumor sections. At 6 months later, 19 months after initial diagnosis, blast cells appeared in peripheral blood. His laboratory data revealed leukocytosis (14 700/μl with 18% blast cells) and an elevated serum lactate dehydrogenase level (1458 U/l). Bone marrow aspiration revealed 9.8% blasts, which were positive for cytoplasmic myeloperoxidase, suggesting MDS. Surface marker analysis showed that the leukemic blasts in the bone marrow were positive for CD33. Chromosomal analysis of bone marrow cells revealed t(5;11)(q21;q23) in 11 of 20 cells. Rearrangement of *MLL* gene of these cells was identified by Southern blotting, however, no known chimeric mRNA with *MLL*, such as *MLL-AF5q31* and *MLL-GRAF* in t(5;11)(q31;q23), could be detected. These suggested that the gene at 5q21 was a novel partner gene of *MLL*. Although another chemotherapy for AML was performed, his blast cells increased >30% blasts in bone marrow at 25 months after initial diagnosis. Therefore, he was diagnosed as having t-Leuk resembling acute monoblastic leukemia due to VP-16. He died of mycotic infection at 35 months after initial diagnosis.

No *CBL* mutations were found in his lymphoma sample at diagnosis and in tumor cells in the enlarged spleen. We also performed tissue-fluorescence *in situ* hybridization analysis with *MLL* probe on paraffin-embedded tissue sections of the tumor cells in the enlarged spleen, however, no evaluable results could be detected because of poor quality of samples. No initial samples for tissue-fluorescence *in situ* hybridization analysis could be obtained.

The 11q23 translocation/*MLL*-R in t-Leuk/MDS was considered to be induced by VP-16,[3] however, gene alterations in addition to *MLL*-R have rarely reported. Recently, *CBL* mutations were found in a variety of myeloid neoplasms.[4–6] Among 2000 samples from the patients with myeloid neoplasms, *CBL* mutations have been found in ~5% samples, including AML transformed from MDS, but not *de novo* or therapy-related acute leukemia with 11q23 translocation/*MLL*-R. To our knowledge, this is the first t-Leuk/MDS patient with 11q23 translocation/*MLL*-R and *CBL* mutation. Interestingly, a *de novo* AML case with *MLL–CBL* fusion gene has also been reported.[8] These findings suggest that alterations of *CBL* gene and 11q23 translocation/*MLL*-R may cooperate in the pathogenesis of a subtype of t-Leuk/MDS and *de novo* leukemia.

Conflict of interest

The authors declare no conflict of interest.

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N Shiba[1,2], T Taki[3], M-j Park[1], M Nagasawa[4], T Kanazawa[2], J Takita[5], H Ohnishi[6], M Sotomatsu[1], H Arakawa[2] and Y Hayashi[1]
[1]*Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Japan;*
[2]*Department of Pediatrics, Gunma University Graduate School of Medicine, Maebashi, Japan;*
[3]*Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan;*
[4]*Department of Developmental Biology, Post Graduate School, Tokyo Medical and Dental University, Tokyo, Japan;*
[5]*Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan and*
[6]*Department of Laboratory Medicine, Kyorin University School of Medicine, Tokyo, Japan*
E-mail: hayashiy-tky@umin.ac.jp

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SNP array analysis of leukemic relapse samples after allogeneic hematopoietic stem cell transplantation with a sibling donor identifies meiotic recombination spots and reveals possible correlation with the breakpoints of acquired genetic aberrations

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Allogeneic hematopoietic stem cell transplantation (HSCT) with a sibling donor is commonly used for treating hematologic malignancies.[1] Although this procedure is frequently curative, a proportion of the patients eventually suffers a relapse of the original malignancy.[1] Leukemogenesis is associated with acquired genetic aberrations caused by various mechanisms including induction of double-stranded DNA breaks by DNA toposiomerase II poisons followed by non-homologous end joining, recombination between homologous sequences and illegitimate V(D)J recombination.[2] It has been hypothesized that neoplasia-associated breakpoints may correlate with the breakpoints of meiotic events, that is, some parts of the genome are more prone to both meiotic and somatic rearrangements; however, this remains controversial.[3–5]

During the last five years, numerous studies have used single-nucleotide polymorphism (SNP) array analysis to investigate genetic abnormalities in hematologic malignancies, including paired diagnostic and relapse samples.[6] To the best of our knowledge, however, the particular scenario of a relapse occurring after allogeneic HSCT with a sibling donor has not been addressed with this technique. In such cases, the bone marrow consists of a mixture of the patient-derived leukemic cells and the donor-derived normal hematopoietic cells, displaying different degrees of chimerism depending on the proportion of leukemic cells. In the present study, we have investigated hematologic malignancies that relapsed after allogeneic HSCT with a sibling donor, and we here provide examples and discuss the particular properties of these samples in terms of SNP array analysis. Furthermore, we have, for the first time, investigated whether the breakpoints of acquired leukemia-associated genetic abnormalities and meiotic recombination events are correlated in a single individual genome.

The study included six cases of relapsed hematologic malignancies after HSCT with a sibling donor, comprising one acute myeloid leukemia M0, two acute myeloid leukemia M5, two myelodysplastic syndromes and one chronic myeloid leukemia. DNA was extracted according to standard methods from bone marrow samples obtained at relapse. In addition, a dilution series of a mixture of peripheral blood samples from two unrelated healthy individuals was prepared in ratios of 1:9, 2:8, 3:7, 4:6 and 5:5. SNP array analysis was performed using the Illumina 1M-duo bead Infinium BD BeadChip platform (Illumina, San Diego, CA, USA) as previously described.[7] Expected B-allele frequency (BAF) values for each combination of genotypes in two mixed cell populations were calculated using the formula $BAF_{exp} = [B_1p + B_2(1-p)]/[L_1p + L_2(1-p)]$, where B is the number of B alleles in the respective cell population, p is the frequency of cell population 1, and L is the

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Exonic deletion of *CASP10* in a patient presenting with systemic juvenile idiopathic arthritis, but not with autoimmune lymphoproliferative syndrome type IIa

H. Tadaki*,†, H. Saitsu*, H. Kaneganeǂ, N. Miyake*, T. Imagawa†, M. Kikuchi†, R. Hara†, U. Kaneko†, T. Kishi†, T. Miyamae†, A. Nishimura*, H. Doi*, Y. Tsurusaki*, H. Sakai*, S. Yokota† & N. Matsumoto*

Summary

Systemic juvenile idiopathic arthritis (s-JIA) is a rare inflammatory disease classified as a subtype of chronic childhood arthritis, manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly. The genetic background underlying s-JIA remains poorly defined. To detect copy number variations, we performed single nucleotide polymorphism (SNP) array analysis in 50 patients with s-JIA. We found a 13-kb intragenic deletion of *CASP10* in one patient. RT-PCR of the mRNA extracted from the patient's lymphoblastoid cells revealed that *CASP10* mRNA was truncated. Sequencing the mRNA revealed that this deletion resulted in a frame shift with an early stop codon. *CASP10* is known as a causative gene for autoimmune lymphoproliferative syndrome (ALPS) type IIa, another childhood syndrome of lymphadenopathy and splenomegaly associated with autoimmune haemolytic anaemia and thrombocytopenia. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in the peripheral blood as a diagnostic marker of ALPS were not high in this patient and lymphocyte apoptosis induced by anti-Fas antibody was normal, denying ALPS in the patient. The father and a sister of the patient showing no symptoms of ALPS or s-JIA, also had the same deletion. Furthermore, we found no other mutations of *CASP10* in the other 49 s-JIA patients. These data suggest that the pathogenic significance of *CASP10* mutations should be carefully evaluated in s-JIA or even ALPS type IIa in further studies.

* Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan, † Department of Pediatrics, Yokohama City University Graduate School of Medicine, Yokohama, Japan and ǂ Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan

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Correspondence: Professor Naomichi Matsumoto, Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan.
Tel: +81 45 787 2604; Fax: +81 45 786 5219;
E-mail: naomat@yokohama-cu.ac.jp

Introduction

Systemic juvenile idiopathic arthritis (s-JIA) (OMIM #604302) is a rare inflammatory disease classified as a subtype of chronic childhood arthritis. The annual UK incidence of JIA is ten cases per 100 000 children under 16 years of age (Symmons *et al.*, 1996), and approximately 11% of patients with JIA suffer from s-JIA (Symmons *et al.*, 1996). s-JIA is a clinically heterogenous febrile disease, manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly. Abnormalities in the innate immunity [cytokines such as interleukin (IL)-1, IL-6 and neutrophils and monocytes/macrophages] play a major role in the pathogenesis of s-JIA, being distinguished from other JIA subtypes. One of the major features of s-JIA is its progression to macrophage activation syndrome. On the basis of these features, consensus is emerging that s-JIA should be classified as an autoinflammatory syndrome rather than a classic autoimmune disease (Vastert *et al.*, 2009).

To date, two genetic factors, *HLA* and *PTPN22*, have been confirmed as JIA susceptibility genes in multiple populations (Hinks *et al.*, 2009). For example, HLA-DR1 and HLA-DR4 have been reported to increase risk for polyarticular JIA in many populations (Glass & Giannini, 1999). However, as seen in these reports, the associations are mainly seen in polyarticular JIA but not in s-JIA. There is some evidence which show other genes, such as *MIF*, *IL6*, *IL10*, *TNF*, *MUNC13-4* and *PRF1* being associated with s-JIA in different populations and subtypes (Fishman *et al.*, 1998; Donn *et al.*, 2001, 2002; Thomson & Donn, 2002; De Benedetti *et al.*, 2003; Zhang *et al.*, 2008; Vastert *et al.*, 2010). However, these genes account for only a small part of the total genetic contribution to JIA. Therefore, the genetic background underlying the s-JIA remains poorly defined.

Autoimmune lymphoproliferative syndrome (ALPS) is a rare childhood syndrome characterized by chronic massive, nonmalignant lymphadenopathy and splenomegaly, expansion of TCR $\alpha\beta^+$ double-negative T cells

and an *in vitro* lymphocyte apoptotic defect (Su & Anderson, 2009). ALPS is classified into several groups, according to the genetic defects. ALPS type 0 is caused by homozygous mutations of *FAS* (Rieux-Laucat *et al.*, 1995; Kasahara *et al.*, 1998; van der Burg *et al.*, 2000), type Ia by heterozygous mutations of *FAS* (Jackson *et al.*, 1999; Rieux-Laucat *et al.*, 1999; Vaishnaw *et al.*, 1999) and type Ib by heterozygous mutations in the Fas ligand (*FasL*) gene (Wu *et al.*, 1996). Heterozygous *CASP10* mutants are classified as ALPS type IIa, and homozygous *CASP8* mutations cause ALPS type IIb. In ALPS type III, the genetic defect is unknown.

Recently, genomic structural variations such as copy number variations (CNVs) are recognized as important causes for many human diseases including autoimmune diseases (Stankiewicz & Lupski, 2010). In this study, we performed genome-wide SNP array analysis to detect CNVs for the first time in s-JIA patients. In this process, we found an intragenic deletion of *CASP10* in one patient, a causative gene for ALPS type IIa, raising a question of the pathogenic significance of *CASP10* mutation in s-JIA.

Materials and method

Subjects

A total of 50 patients with s-JIA who had disease refractory to conventional treatment and were given tocilizumab were enrolled with informed consent in IRB-approved protocols at Yokohama City University Hospital. There were no family histories in each patient. Genomic DNA of peripheral blood leucocytes from all patients were isolated using DNA isolation systems (Quick Gene-800; Fujifilm, Tokyo, Japan). DNA of nail tissues and buccal cells from the patient with the *CASP10* deletion was isolated using ISOHAIR (Wako, Tokyo, Japan) and Puregene Kit C (Quiagen, MD, USA), respectively, according to each manufacturer's protocol.

SNP array

To detect CNVs, two different commercially available SNP array platforms, the Genechip Human Mapping 250K array (Affymetrix Inc., Santa Clara, CA, USA) (23 patients) and the Genome-wide Human SNP array 6.0 (Affymetrix Inc.) (27 patients) were used following the manufacture's protocols. In brief, for the Genome-wide Human SNP array 6.0, 500-ng DNA was digested with *Nsp* I and *Sty* I (only *Nsp* I was used for 250K array). The adaptors were ligated to the digested DNA, and the ligation-mediated PCR with singleprimer was performed. PCR products were purified by magnetic beads (Ampure; Beckman Coulter Company, Beverly, MA, USA). Microcon YM-100 (Millipore Corporation, Bedford, MA, USA) was used for purification for the 250K array. The product was fragmented, end labelled and hybridized to an array. CNAG3.0 (Nannya *et al.*, 2005), Genotyping Console (Copy Number Analyser for GeneChip, Affymetix Inc.) and Partek Genomic Suite (Partek Inc., St. Louis, MO, USA) were used to validate copy number alterations. The qualities of the results were high in every sample [250K array: SNP call rate >95%, MDR >99%, (MDR–MCR) <5%, SNP array 6.0: Contrast QC >2, QC call rate >93%, MAPD <0.4].

Quantitative real-time PCR

The deletion breakpoints were analysed using genomic DNAs by quantitative real-time polymerase chain reaction (qPCR) with Quantifast SYBR Green PCR kit on Rotor-Gene™ 6200 HRM (Corbett Life Science, Sydney, Australia). The delta–delta Ct relative quantitative method was employed according to the manufacturer's protocol. Averages of duplicates were calculated by ROTOR-GENE 6000 SERIES software (Corbett Life Science).

Direct sequencing of a deletion junction

Fragments containing the deletion break point were amplified by PCR for direct sequencing. Long PCR primers adjacent to presumed deleted regions by qPCR were generated. PCR was cycled once at 94°C for 2 min, 35 times at 98°C for 10 s, and at 68°C for 3 min in 20-μL mixture using KODFX (Toyobo, Osaka, Japan). PCR products were purified with ExoSAP™ (USB Co., Cleveland, OH, USA) and sequenced using BigDye™ terminator (Applied Biosystems, Foster City, CA, USA) on the ABI 3100 automatic DNA sequencer (Applied Biosystems).

RT-PCR analysis

Total RNA was extracted from lymphoblastoid cell line (LCL) of all patients using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with 3 μg of total RNA using PrimeScript™ first-strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. PCR was cycled once at 94°C for 2 min, 35 times at 94°C for 30 s, at 64°C for 30 s, and at 68°C for 2 min in 20-μL mixture using KODFX. Pre treatment of cells with cycloheximide (protein synthesis inhibitor, 150 μg/1.0 × 10^6 cells) for 4 h was done to examine the influence of nonsense-mediated mRNA decay (NMD). Primers are listed below: *CASP10*-forward, 5′-CCTGTAGACAAGGAAGCCGAGTCGT-3′ and *CASP10*-reverse, 5′-TTCGACTCACATCATCGTTGACAGC-3′.

Mutation search for *CASP10* and *CASP8*

Mutation of *CASP10* and *CASP8* was screened by high-resolution melt analysis. As *CASP10* and *CASP8*

are both causative for ALPS, showing similarity at the nucleotide level, we also looked for *CASP8* mutations. PCR and HRM were performed on Rotor-Gene™ 6200 HRM. PCR was cycled 35–40 times with denaturation for 10 s at 95°C, annealing for 20 s at 60°C, and extension for 30 s at 72°C in 12-μL mixture using ExTaq (Takara Bio Inc.) and SYTO™ 9 green fluorescent (Invitrogen). The annealing temperature varied according to the amplicon. Variants were selected for sequencing when the melting profile deviated from control samples. PCR products showing variant melting profiles were sequenced using BigDye terminators by standard methods with the same primers used in HRM-PCR.

As *CASP8* mutations in ALPS were reported to be homozygous mutations (Chun *et al.*, 2002), we performed HRM with samples which were spiked with 10% control DNA to detect homozygous mutations.

T-cell apoptosis assay

Peripheral blood mononuclear cells from the patient were activated with phytohemagglutinin and IL-2 for 10 days, and Fas-mediated apoptosis in these activated T cells was evaluated by a flow cytometric method after their incubation with anti-Fas monoclonal antibody (CH-11; MBL, Nagoya, Japan) for 24 h as previously described (Kasahara *et al.*, 1998).

Results

CASP10 intragenic deletion

A 13.4-kb intragenic deletion was detected in a patient with s-JIA who is unlikely to be affected with ALPS using Genome-wide Human SNP array 6.0 (Fig. 1a). We also confirmed the deletion by qPCR (Table 1, Fig. 1b). Sequencing a deletion junction successfully amplified by long PCR revealed that the deleted region contained exons 6–9 of *CASP10* (Figs 1a & 2). Proximal and distal breakpoints were located in two directly oriented *Alu*Y and *Alu*Sx elements. Identity between these *Alu* elements was 97% and the possible crossing-over region was 36 bp in length (Fig. 2). *Alu*-mediated nonallelic homologous recombination was the likely mechanism of this microdeletion. The father and sister, who had no symptoms of ALPS or s-JIA, also had the same deletion (Fig. 1d,e). This deletion was seen in the DNA extracted from blood, buccal cells and nails of the proband (Fig. 1e), suggesting that it was indeed a germline change (not somatic). RT-PCR of the mRNA extracted from the patient's LCL revealed that *CASP10* mRNA was truncated (Fig. 3). This was seen in both samples pretreated with or without cycloheximide (data not shown), indicating that the truncated *CASP10* mRNA does not suffer from NMD. We further sequenced the mRNA, and found that this deletion resulted in a frame shift with an early stop codon (the termination codon appeared at the second amino acid in exon 10). No deletions containing *CASP10* were observed in 54 patients (108 alleles) with other diseases (29 patients with autism, 21 patients with mental retardation and multiple congenital disorders, and four patients with premature ovarian failure) by Genome-wide Human SNP array 6.0 or Nimblegen 385K array.

Mutation search for *CASP10* and *CASP8*

We could not detect any mutations in *CASP10* as well as *CASP8* in the other 49 s-JIA patients. Furthermore, to search for abnormal *CASP10* transcripts, we performed RT-PCR using mRNA extracted from LCLs of s-JIA patients, but no truncated mRNAs were found.

Clinical features of the proband and her family members with *CASP10* deletion

The proband is a 9-year-old girl who developed s-JIA with high fever, liver damage and enlargement of lymph nodes at 4 years of age. Although she recovered after symptomatic treatment, she suffered a recurrence of spiking fever, erythematous skin rash, pain and swelling of the knee and foot and was diagnosed as s-JIA at 6 years of age. The patient recovered after being given methylprednisolone pulse therapy twice. However, when the oral administration of prednisolone 15 mg day^{-1} was reduced to 13 mg day^{-1}, the swelling of her knee worsened. Therefore, she was admitted to Yokohama City University Hospital. As she was resistant to conventional therapies, she received tocilizumab (anti-IL-6 receptor antibody) therapy. Her condition got stable since tocilizumab was medicated. The level of IgG was normal, and rheumatoid factor and antinuclear antibodies were not detected (Table 2). The levels of IL-10, IL-5, IL-4 and TNF-α were normal (Table 2), showing no shift to a Th2 cytokine production pattern. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in the peripheral blood were not high in this patient (Fig. 4a), and lymphocyte apoptosis induced by anti-Fas antibody was normal (Fig. 4b). The patient's father and her sister, both having the same partial *CASP10* deletion, are totally healthy.

Discussion

In our study, we detected an intragenic deletion of *CASP10*, a causative gene for ALPS (OMIM #601859) type IIa. Although the exonic deletion may produce a truncated protein (if translated) in this patient lacking the entire CASc domain where all the reported missense mutations harboured (Wang *et al.*, 1999; Zhu *et al.*, 2006) (Fig. 1c), she had no symptoms for ALPS. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in her peripheral blood were not high, and lymphocyte apoptosis induced by anti-Fas antibody was normal (Fig. 4), denying ALPS.

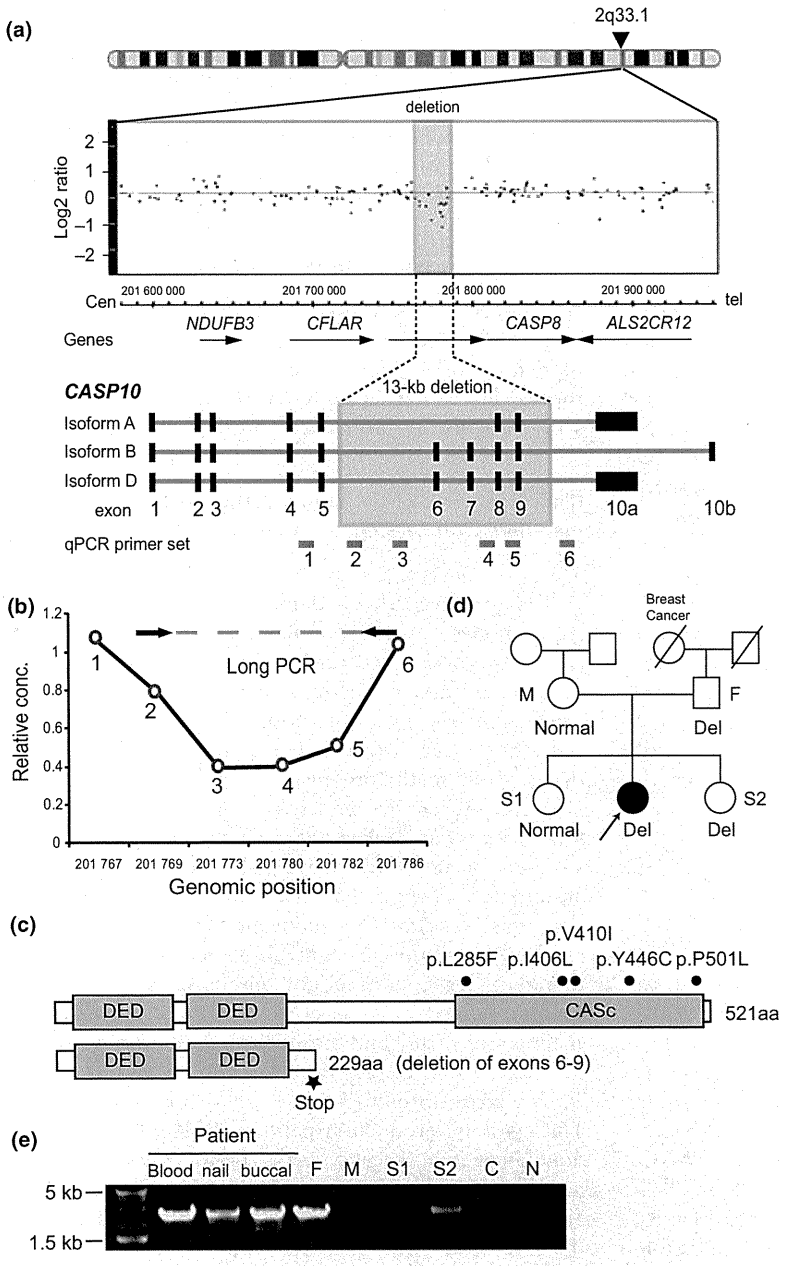


Figure 1. Characterization of the 2q33.1 microdeletion. (a) Result of Genome-wide human SNP array 6.0. The position (*x*-axis matching the genomic location of chromosome 2) and log2 ratio (*y*-axis) of each SNP probes are indicated. The 13-kb deletion is within *CASP10* encompassing exons 6–9. (b) Breakpoint analysis of the s-JIA patient. Result of quantitative RT-PCR is shown. Heterozygous deletion of positions 3, 4 and 5 was implied. Arrows show the primer positions for long PCR. (c) Protein structure of caspase 10. All the reported mutations clustered at the CASc domain. The deletion of exons 6–9 results in protein truncation lacking the entire CASc domain. (d). Family pedigree of the patient. Patient is indicated by arrow. Normal: no deletion, del: caspase 10 intragenic deletion. F: father, M: mother, S1: older sister, S2: younger sister. (e) Result of long PCR using DNAs of the patient's blood leucocytes, nails and buccal cells. Long PCR could successfully amplify 3.3-kb fragments from the patient and the patient's father (F) and sister (S2) respectively. DNA from father, mother and control was extracted from blood leucocytes, and DNA from the two sisters was extracted from their nails. F: father, M: mother, S1: older sister, S2: younger sister, C: control, N: negative control.

Table 1. Primers for quantitative real-time PCR and long PCR

Position (kb)		Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Common primer			
1	2017671–2017674	AGTCAAACCTGGCTGCCTTA	TGCTCCTCAACTCATTCTGTG
2	2017694–2017695	GCAAGGGTTTCTGGTTTCTG	CCAAGTCTGCTGGAAGAACC
3	2017734–2017737	ACGCCCACCTGAAGACTATG	AGGCGGAGGTGTTACCATTT
4	2017809–2017811	GATCCATTGGAGTGGTTGGT	TCAGGGAGGTAAAGCTGTGG
5	2017822–2017824	AGTGCCCTAGACTGGCTGAA	GTGGCCAGACCAAGTAGGAA
6	2017859–2017861	GAAAGTGCATGCGACAGCTA	ATGCCTCCATGCCTAACAAC
Long PCR primer	2017695–2017855	GGGATTTGTGGTTCTTCCAGCAGAC	GACATGGCCAAGCAGATGCTAACAC

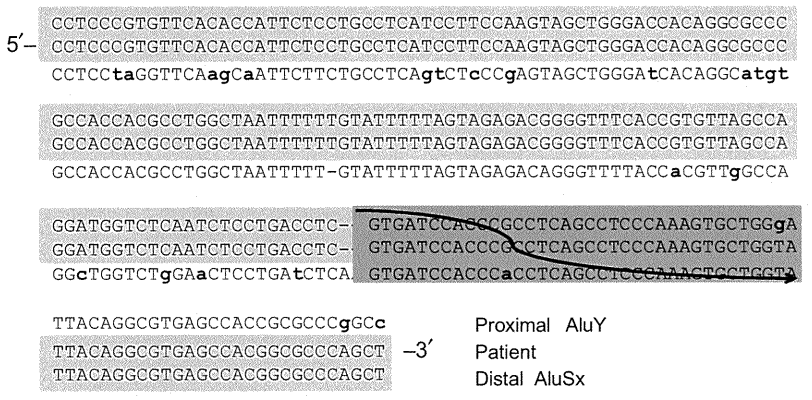


Figure 2. Result of deletion breakpoint sequence. The top, middle and bottom nucleotide strands show the proximal, recombined and distal sequences respectively. Matched sequences are shown as uppercase letters and unmatched ones as lowercase letters. Pale grey boxes show the same sequences and darker grey ones indicate a possible crossing-over region. Curved arrow shows recombination.

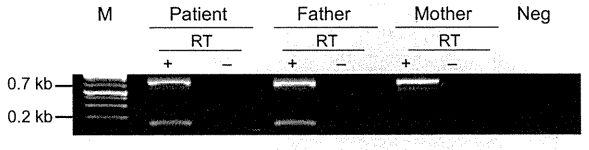
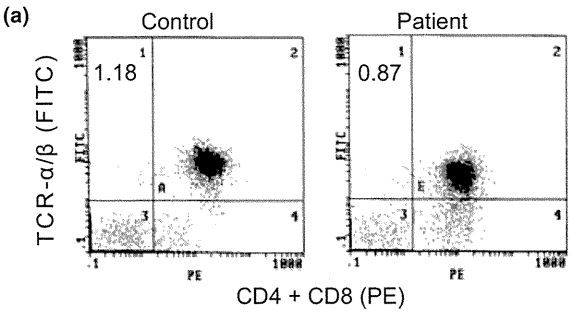


Figure 3. Result of *CASP10* RT-PCR. The lymphoblastoid cell lines which were not treated with cycloheximide were used. Forward primer was designed at exon 5 and reverse primer was designed at exon 10. The predicted size of the PCR product was 742 bp for normal cDNA and 145 bp for the deletion product. M: marker, Neg: negative control.



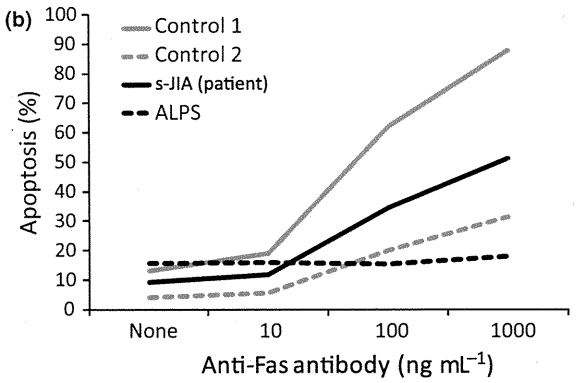


Figure 4. (a) Fluorescence-activated cell sorting (FACS) analysis of TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in the peripheral blood were not high in this patient. (b) Fas-induced T-cell apoptosis assay. Apoptosis of activated T cells were induced by anti-Fas monoclonal antibody for 24 h and percentage of apoptotic cells was analysed as previously described (Kasahara *et al.*, 1998).

Table 2. Laboratory findings for the patient

Subject	Data	Normal range
IL-6	24.3 pg mL^{-1}	<2.0 pg mL^{-1}
sIL-6-R	37.5 pg mL^{-1}	14–46 pg mL^{-1}
IFNr	<0.1 IU mL^{-1}	<0.1 IU mL^{-1}
IL-5	<7.8 pg mL^{-1}	<10 pg mL^{-1}
IL-4	7.3 pg mL^{-1}	<6.0 pg mL^{-1}
IL-10	<2 pg mL^{-1}	<5 pg mL^{-1}
TNF-α	0.8 pg mL^{-1}	0.6–2.8 pg mL^{-1}
sTNF-RI	828 pg mL^{-1}	749–1966 pg mL^{-1}
sTNF-R2	1720 pg mL^{-1}	1003–3170 pg mL^{-1}
IgG	1492 mg dL^{-1}	870–1700 pg mL^{-1}
RF	–	–
Antinuclear antibody	–	–

IL, interleukin.

The *CASP10* mutations are characterized by resistance to Fas-mediated apoptosis despite the presence of normal FasL and Fas. The reported mutations for *CASP10* are missense mutations within the CASc domain (Wang *et al.*, 1999; Zhu *et al.*, 2006). Only two previous studies show ALPS patients having *CASP10* mutation so far, and both of them are reported to be inherited from nonaffected parents (Wang *et al.*, 1999; Zhu *et al.*, 2006). Although both mutations decreased caspase 10 activity and exerted a dominant negative effect on the wild-type protein, neither report was sufficient enough to prove that the mutations consistently induced the overt disease, as several mutated familial members were healthy, and some showed multiple autoantibodies and defective lymphocyte apoptosis. Moreover, in one previous report, two ALPS patients carried double heterozygous mutations in the *CASP10* and *FAS* genes, showing that mild *CASP10* mutations alone were not enough to exert a dominant

negative effect to the wild protein, and the concurrent effect of mutations hitting different genes involved in Fas function causes ALPS (Cerutti *et al.*, 2007). In our study, we detected a truncation mutation of *CASP10* in one s-JIA patient, which was inherited from the healthy father, and also was seen in the healthy sister. Although the *CASP10* mRNA extracted from the patient's LCL results in an early stop codon, the patient had no evidence of ALPS. As both previous studies and ours show mutations sharing with nonaffected parents and siblings, we need further evidence for supporting the pathogenic significance of *CASP10* mutations.

Approximately 24% of ALPS patients are classified as ALPS type III, in which no gene defects are found (Puck & Straus, 2004). In ALPS type III patients, somatic mutations of *Fas* in isolated double-negative T cells have been reported (Holzelova *et al.*, 2004). These mutations were found in a fraction of $CD4^+$ and $CD8^+$ T cells, monocytes, and $CD34^+$ hematopoietic precursors, but not in hair or mucosal epithelial cells (Holzelova *et al.*, 2004). Therefore, in our study, we investigated whether the *CASP10* deletion is somatic by examining not only blood leucocyte DNA but also nail and buccal cell DNAs, but no evidence of somatic changes was obtained.

As the phenotype and laboratory data of the patient with *CASP10* intragenic deletion were different from those of ALPS, we hypothesized that *CASP10* could be responsible for s-JIA. However, *CASP10* was not mutated at the level of genomic DNA and transcripts in other s-JIA patients. Furthermore, we searched for *CASP8* mutations, but no mutations were found. In conclusion, a 13.4-kb intragenic deletion of *CASP10* was detected in the s-JIA patient using genome-wide human SNP array. Our report provides a new insight into the pathogenic significance of caspase 10 in relation to apoptosis and human diseases. Further investigation is absolutely necessary.

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Conflict of Interest

Authors declare no conflict of interest in this study.

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ORIGINAL ARTICLE

Autoimmune lymphoproliferative syndrome mimicking chronic active Epstein–Barr virus infection

Keiko Nomura · Hirokazu Kanegane · Keisuke Otsubo · Hiroshi Wakiguchi · Yukihiro Noda · Yoshihito Kasahara · Toshio Miyawaki

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Abstract Chronic active Epstein–Barr virus infection (CAEBV) is defined as a systemic EBV-associated lymphoproliferative disease characterized by fever, lymphadenopathy, and splenomegaly in apparently immunocompetent persons. Recent studies have revealed that EBV infects T or natural killer cells in most patients with CAEBV; the etiology of CAEBV, however, remains unknown. Autoimmune lymphoproliferative disorder (ALPS) is an inherited disorder associated with defects in apoptosis, and clinically characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, and autoimmune disease. ALPS is most often associated with mutations in the *FAS* gene, which is an apoptosis-signaling receptor important for homeostasis of the immune system. Based on the clinical similarity between ALPS and CAEBV with respect to lymphoproliferation, we have examined the possibility of the co-occurrence of ALPS in patients with a diagnosis of CAEBV. In this study, we have identified *FAS* gene mutations in three Japanese patients with lymphadenopathy, hepatosplenomegaly, and unusual EBV infection, who were diagnosed with CAEBV. These observations, which indicate that the clinical development of ALPS may be associated with EBV infection, alert us to a potential diagnostic pitfall of CAEBV.

Keywords Autoimmune lymphoproliferative syndrome · Chronic active Epstein–Barr virus · FAS · IL-10

K. Nomura · H. Kanegane (✉) · K. Otsubo · T. Miyawaki
Department of Pediatrics, Graduate School of Medicine, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan
e-mail: kanegane@med.u-toyama.ac.jp

H. Wakiguchi
Department of Pediatrics, Medical Science Cluster, Research and Education Faculty, Kochi University, Nankoku, Japan

Y. Noda
Department of Pediatrics, Kansai Medical University, Hirakata, Japan

Y. Kasahara
Department of Pediatrics, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

1 Introduction

Autoimmune lymphoproliferative syndrome (ALPS, MIM 601859) is a disorder characterized by nonmalignant lymphoproliferation, autoimmunity, and an increase in TCR-α/β^+ $CD4^-$ $CD8^-$ double negative T (DNT) cells [1, 2]. Most patients with ALPS display inherited heterozygous mutations in the *FAS* gene, which encodes a cell surface molecule involved in programmed cell death (apoptosis). Several patients with ALPS display mutations in the *FASLG* or *CASP10* genes [3, 4], and the remaining patients likely display genetic defects that have not been defined. Genetic and immunologic studies have demonstrated that some unaffected family members show elevations of DNT cells and carry the same mutations in the *FAS* gene, suggesting that the occurrence of ALPS may require not only *FAS* deficiency but also other genetic or environmental factors.

Chronic active Epstein–Barr virus (EBV) infection (CAEBV) is characterized by chronic or recurrent infectious mononucleosis-like symptoms and unusual patterns of anti-EBV antibodies [5]. The etiology of CAEBV remains to be elucidated. However, recent studies have demonstrated that patients with CAEBV have a high viral

load in their peripheral blood and have a clonal expansion of EBV-infected T cells and natural killer (NK) cells [6]. Lymphadenopathy, hepatosplenomegaly, thrombocytopenia, and hypergammaglobulinemia, which are usually present in patients with CAEBV, are also found in patients with ALPS. We have identified germline *FAS* mutations in three patients diagnosed with CAEBV.

2 Patients and methods

2.1 Patients

Patient 1 was born to nonconsanguineous healthy parents. He had hepatosplenomegaly since the age of 3 months and was admitted to the hospital at the age of 9 months. At admission, he demonstrated systemic lymphadenopathy and mild thrombocytopenia (platelets of 110×10^{-9}/L). The EBV serology included an anti-viral capsid antigen (VCA) IgG antibody titer of 1:640 and an anti-early antigen (EA) IgG antibody titer of 1:80. Anti-VCA IgM antibody and anti-EBV nuclear antigen (EBNA) antibody were negative. The presence of persistent hepatosplenomegaly, lymphadenopathy, and a persistent positive anti-EA IgG antibody suggested that the patient might have CAEBV. He received anti-viral drugs and interferon without improvement. He suffered from aseptic meningitis twice at the age of 13 years. However, he displayed stable hepatosplenomegaly, thrombocytopenia, and elevated titer of EA antibody at the age of 15 years.

Patient 2 was born to healthy, unrelated parents and was admitted to the hospital at the age of 2 years because of hepatosplenomegaly for 6 months. At admission, he had cervical lymphadenopathy. He showed an unusual pattern of anti-EBV antibodies: an anti-VCA IgG antibody titer of 1:640, an anti-EA antibody titer of 1:80, and an anti-EBNA antibody titer of 1:160. At the age of 3 years, he complained of abdominal pain and had severe anemia (hemoglobin level of 5.8 g/dL) and jaundice. Laboratory data showed elevated levels of indirect bilirubin and reticulocytes, and decreased levels of haptoglobin, a pattern which is indicative of hemolytic anemia. The Coombs test, however, was negative. The anemia and hepatosplenomegaly improved gradually without treatment, but the EBV serology persistently showed an unusual pattern: an anti-VCA IgG antibody titer of 1:2,560 and an anti-EA antibody titer of 1:2,560. The patient had thrombocytopenia (platelets of 9×10^{-9}/L), and responded to pulse steroid therapy at the age of 13 years. He had been followed as a patient with CAEBV associated with hemolytic anemia until the age of 14 years. EBV had mainly infected his $CD19^{+}$ B cells (1,210 copies/μg DNA).

Patient 3 was born to healthy, unrelated parents. He had presented with hepatosplenomegaly and thrombocytopenia since early infancy. At the age of 2 years, the patient contracted a primary EBV infection and has had cervical lymphadenopathy since then. He showed high titer of anti-VCA IgG (1:1,280) and increased copies of EBV-DNA (3,000 copies/μg DNA) in the peripheral blood. His sister also developed lymphadenopathy and an urticarial rash since a primary EBV infection.

Clinical symptoms, including fever, persistent hepatitis, and fatigue, were not observed in these patients. Patients 1 and 3 had persistent thrombocytopenia, but anti-platelet antibodies were not measured.

2.2 Analysis of lymphocyte subpopulations and Fas-mediated apoptosis

Heparinized venous blood samples were obtained from patients and healthy donors after informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient centrifugation. Lymphocyte subpopulations were analyzed by flow cytometry (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan).

Peripheral blood mononuclear cells were stimulated with 0.1% phytohemagglutinin (Sigma-Aldrich, Inc., St. Louis, MO, USA) in the presence of 100 U/mL recombinant human IL-2 (Takeda, Osaka, Japan) in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 200 U/mL penicillin G, 10 μg/mL gentamicin, and 25 mM HEPES. After 4 days, the culture medium was supplemented with 50 U/mL of IL-2 for 7–10 days. Activated T cells were treated with different concentrations of anti-Fas mAb (CH11, MBL, Nagoya, Japan) for 12 h. Apoptosis was evaluated by a flow cytometric method previously described [7].

2.3 Mutational analysis of the *FAS* gene

Total RNA was extracted from the patients' PBMC by the Trizol reagent (Invitrogen), and single-stranded cDNA was synthesized using Superscript II transcriptase (Invitrogen). Oligonucleotide primer sets were used for PCR amplification of the four overlapping regions, which included all nine exons of *FAS* mRNA previously described [7]. PCR was performed by conventional methods. The nucleotide sequence was determined using each amplified PCR product with the BigDye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) with an automated ABI PRISM 310 DNA sequencer (Applied Biosystems). Genomic DNA was isolated from whole blood leukocytes by Qiagen Blood Mini Kit (Qiagen, Hilden, Germany). The primer pairs, 5′-GCTTAGTTTCTGGCAAGGCCG-3′ and

5′-GCTTAGTTTCTGGCAAGGCCG-3′ were used for amplifying the exon 8 boundaries of the *FAS* gene.

2.4 Measurement of IL-10

Serum IL-10 concentrations were evaluated by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendation.

3 Results

Three patients with hepatosplenomegaly, lymphadenopathy, cytopenia, hypergammaglobulinemia and an unusual pattern of EBV infection received a tentative diagnosis of CAEBV (Table 1). Monoclonal or oligoclonal EBV infection of T or NK cells is one of the characteristics of CAEBV [6]. Because the clinical findings of hepatosplenomegaly, lymphadenopathy, cytopenia, and hypergammaglobulinemia are also found in patients with ALPS, we suspected the diagnosis of ALPS. The percentage of DNT cells was increased (Table 1; Fig. 1). Fas-mediated apoptosis assays demonstrated that the cells of these patients displayed no apoptosis after stimulation with an anti-Fas monoclonal antibody (Fig. 2). The patients were therefore tentatively diagnosed with ALPS. *FAS* gene analysis was performed in these patients. Patient 1 had a mutation in intron 8 characterized by a splicing donor site (IVS8+5G>T) resulting in no transcription of exon 8. Patients 2 and 3 had the same nonsense mutation, Q226X,

Table 1 Clinical and laboratory features of patients at the time of diagnosis of CAEBV

	Patient 1	Patient 2	Patient 3
Present age	27 years	27 years	12 years
Age at onset	3 months	1 year	2 years
Sex	Male	Male	Male
Splenomegaly	+	+	+
Hepatomegaly	+	+	+
Lymphadenopathy	+	+	+
Anemia	−	+	−
Thrombocytopenia	+	−	+
Hypergammaglobulinemia	+	+	+
VCA-IgG	1:640	1:640	1:1,280
VCA-IgM	<1:10	<1:10	<1:10
EA-IgG	1:80	1:80	<1:10
EBNA	<1:10	1:160	1:10
EBV-DNA	+	+	+
TCRα/β^+ $CD4^-$ $CD8^-$ (%)	10.4	20.3	12.5
Serum IL-10 (pg/mL)	173	NE	296

NE not examined

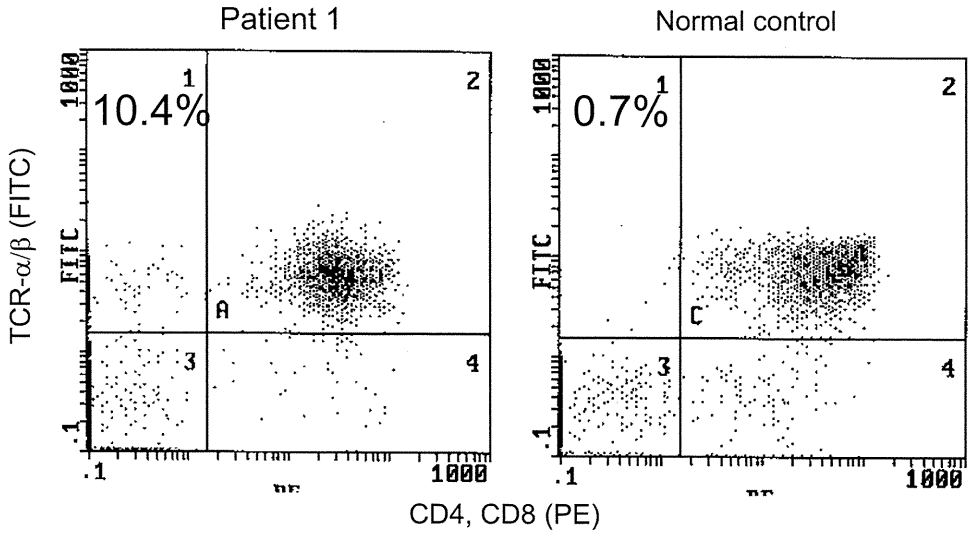

Fig. 1 Analysis of DNT cells. PBMC from Patient 1 and from a normal control were analyzed by flow cytometry with a monoclonal antibody specific for TCR-α/β, CD4, and CD8. The percentages of DNT cells are indicated in the *upper left* quadrants

caused by a single base substitution (1020C>T). The mutations of Patients 1 and 2 were previously reported [8]. All the mutations produced a truncated protein of a defective intracellular domain, causing a defect in Fas-mediated apoptosis. Patients with ALPS frequently have markedly elevated levels of IL-10, vitamin B12 and soluble Fas ligand [9]. In this study, serum IL-10 levels were markedly elevated (normal value <15 pg/mL) in Patients 1 and 3.

4 Discussion

Hepatosplenomegaly, lymphadenopathy, cytopenia, hypergammaglobulinemia, and autoimmunity are detected in patients with both CAEBV and ALPS [1, 2, 5, 6]. Hematologic complications, including hemolytic anemia and thrombocytopenia, may be due to autoimmunity. The current results suggest that patients with ALPS displaying unusual patterns of EBV antibodies may be misdiagnosed as having CAEBV. The EBV might mainly infect B cells in these patients, whereas the EBV infects T or NK cells in most patients with CAEBV. The cell type of EBV infection may be a critical point for differentiating ALPS from CAEBV. Some patients with *FAS* mutations are asymptomatic, and the onset of ALPS may be associated with additional environmental or genetic factors. Arkwright et al. [10] described two boys with ALPS who were infected with cytomegalovirus (CMV) during infancy. CMV infection may trigger the onset of ALPS in these patients. In our patients, EBV infection may have triggered the onset of ALPS. Therefore, we believe that ALPS should be differentiated from EBV-associated lymphoproliferative disorders (LPD) (Table 2). X-linked lymphoproliferative syndrome type 1 (XLP-1), a genetic immunodeficiency caused by mutations in the *SH2D1A* gene, is characterized clinically not only by fulminant infectious mononucleosis or severe EBV-associated hemophagocytic lymphohistiocytosis (HLH), but also by dysgammaglobulinemia and malignant lymphoma [11–13]. Patients with XLP do not present with autoimmunity. Posttransplant LPD (PTLPD) is a form of EBV-LPD. Patients with PTLPD can show hypogammaglobulinemia, but no hypergammaglobulinemia. PTLPD is not associated with HLH or autoimmunity.

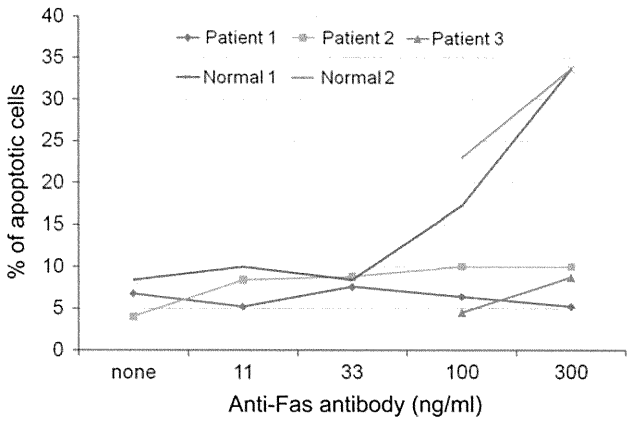

Fig. 2 Fas-mediated apoptosis of activated T cells from the patients and normal controls. PBMC were activated by incubation with PHA and IL-2 for 2 weeks and then tested for Fas-mediated apoptosis

IL-10 is a pleiotropic cytokine that is produced mainly by Th2 lymphocytes and monocytes. Serum or plasma IL-10 is increased in patients with ALPS and CAEBV. Cytokine imbalance and Th1 to Th2 shift may play a pivotal role in the pathogenesis of ALPS, and IL-10 may be a key cytokine [14]. In ALPS, IL-10 is mainly produced by DNT cells [8]. More than half of the patients with CAEBV demonstrated elevated levels of plasma IL-10 [15], and IL-10 may be mainly produced by EBV-infected T cells [16]. IL-10 may be associated with lymphoproliferation in CAEBV. Therefore, IL-10 may be a key cytokine in the pathogenesis of EBV-associated LPD.

Clinical and immunological findings of hepatosplenomegaly, lymphadenopathy, cytopenia, and hypergammaglobulinemia overlap in ALPS and CAEBV, and ALPS may be misdiagnosed as CAEBV. In addition, EBV infection may trigger the onset of ALPS. Serum or plasma IL-10 is increased in both diseases, and IL-10 may not only play a key role in the pathogenesis of this disease due to its anti-apoptotic function but may also account for

Table 2 ALPS-FAS and EBV-associated lymphoproliferative diseases

Disease	ALPS-FAS	XLP-1	CAEBV	PTLPD
Inheritance	Autosomal	X-linked	?	?
Sex	Both	Male only	Both	Both
Gene locus	10q25	Xq25	?	?
Gene	*FAS*	*SH2D1A*	?	?
HLH	Rarely	Frequent	Sometimes	Rarely
Malignant lymphoma	Sometimes	Frequent	Sometimes	Sometimes
Immunoglobulin	Hyper	Hypo or Hyper	Hyper	Hypo ~ normal
Autoimmunity	++	−	+	−

autoimmunity. Although a genetic defect in CAEBV has not been identified, a gene related to the apoptosis pathway may be associated with CAEBV. Further studies should address this question.

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Clinical similarities and differences of patients with X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) versus type 2 (XLP-2/XIAP deficiency)

Jana Pachlopnik Schmid,[1-3] Danielle Canioni,[4] Despina Moshous,[1-3] Fabien Touzot,[3] Nizar Mahlaoui,[3] Fabian Hauck,[1,2] Hirokazu Kanegane,[5] Eduardo Lopez-Granados,[6] Ester Mejstrikova,[7] Isabelle Pellier,[8,9] Lionel Galicier,[10] Claire Galambrun,[11] Vincent Barlogis,[11] Pierre Bordigoni,[12] Alain Fourmaintraux,[13] Mohamed Hamidou,[14] Alain Dabadie,[15] Françoise Le Deist,[16] Filomeen Haerynck,[17] Marie Ouachée-Chardin,[18] Pierre Rohrlich,[19-21] Jean-Louis Stephan,[22] Christelle Lenoir,[1] Stéphanie Rigaud,[1,2] Nathalie Lambert,[1,23] Michèle Milili,[24] Claudin Schiff,[24] Helen Chapel,[6] Capucine Picard,[2,23,25] Geneviève de Saint Basile,[1-3] Stéphane Blanche,[3] Alain Fischer,[1-3] and Sylvain Latour[1,2]

[1]Inserm Unité 768, Laboratoire du Développement Normal et Pathologique du Système Immunitaire, Hôpital Necker-Enfants Malades, Paris, France; [2]Université Paris Descartes, Institut Fédératif de Recherche Necker Enfants-Malades (IFR94), Paris, France; [3]Unité d'Immunologie et Hématologie Pédiatrique, Assistance Publique–Hôpitaux de Paris (AP-HP), Hôpital Necker Enfants-Malades, Paris, France; [4]Service d'Anatomie et de Cytologie Pathologiques, AP-HP, Hôpital Necker Enfants-Malades, Paris, France; [5]Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; [6]Department of Clinical Immunology, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom; [7]Department of Pediatric Hematology and Oncology, Teaching Hospital Motol and 2nd Medical School, Charles University, Prague, Czech Republic; [8]Unité d'Hématologie-Immunologie-Oncologie Pédiatrique, Centre Hospitalier Universitaire (CHU) Angers, Angers, France; [9]Inserm Unité 892, Centre de Recherche en Cancérologie Nantes-Angers, Nantes, France; [10]Service Immuno-Hématologie, AP-HP, Hôpital Saint-Louis, Paris, France; [11]Service d'Hématologie Pédiatrique, Hôpital Timone Enfants, Marseille, France; [12]Departement d'Hémato-Oncologie Pediatrique et de Transplantation Medullaire, CHU Nancy, Vandoeuvre, France; [13]Service de Néonatologie, Groupe Hospitalier Sud Réunion, Saint Pierre, La Réunion, France; [14]Médecine Interne, CHU Nantes, Nantes, France; [15]Service de Hépato-Gastro-Entérologie, CHU Rennes, Rennes, France; [16]Département de Microbiologie et d'Immunologie, et Département de Pédiatrie, Université de Montréal, CHU Sainte-Justine, Montréal, QC; [17]Département of Child Immunology, Ghent University Hospital, Ghent, Belgium; [18]Service d'Hématologie, AP-HP, CHU Hôpital Robert Debré, Paris, France; [19]Inserm UMR645, Besançon, France; [20]Université Franche-Comté, Besançon, France; [21]Service de Pédiatrie, CHU Besançon, Besançon, France; [22]Unite d'Hématologie et Oncologie Pédiatrique, Hôpital Nord, CHU Saint-Etienne, Saint-Etienne, France; [23]Centre d'Etude des Déficits Immunitaires, Hôpital Necker-Enfants Malades, Paris, France; [24]Centre d'é3[1]Centre d'Immunologie de Marseille-Luminy, Parc Scientifique de Luminy-Case 906, Marseille, France; and [25]Inserm Unité 980, GHMI, Hôpital Necker Enfants-Malades, Paris, France

X-linked lymphoproliferative syndromes (XLP) are primary immunodeficiencies characterized by a particular vulnerability toward Epstein-Barr virus infection, frequently resulting in hemophagocytic lymphohistiocytosis (HLH). XLP type 1 (XLP-1) is caused by mutations in the gene *SH2D1A* (also named *SAP*), whereas mutations in the gene *XIAP* underlie XLP type 2 (XLP-2). Here, a comparison of the clinical phenotypes associated with XLP-1 and XLP-2 was performed in cohorts of 33 and 30 patients, respectively. HLH (XLP-1, 55%; XLP-2, 76%) and hypogammaglobulinemia (XLP-1, 67%; XLP-2, 33%) occurred in both groups. Epstein-Barr virus infection in XLP-1 and XLP-2 was the common trigger of HLH (XLP-1, 92%; XLP-2, 83%). Survival rates and mean ages at the first HLH episode did not differ for both groups, but HLH was more severe with lethal outcome in XLP-1 (XLP-1, 61%; XLP-2, 23%). Although only XLP-1 patients developed lymphomas (30%), XLP-2 patients (17%) had chronic hemorrhagic colitis as documented by histopathology. Recurrent splenomegaly often associated with cytopenia and fever was preferentially observed in XLP-2 (XLP-1, 7%; XLP-2, 87%) and probably represents minimal forms of HLH as documented by histopathology. This first phenotypic comparison of XLP subtypes should help to improve the diagnosis and the care of patients with XLP conditions. (*Blood*. 2011;117(5):1522-1529)

Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare immunodeficiency condition characterized by an extreme vulnerability to Epstein-Barr virus (EBV) infection, frequently resulting in hemophagocytic lymphohistiocytosis (HLH) or virus-associated hemophagocytic syndrome (VAHS).[1-3] HLH is caused by overwhelming T-cell and macrophage activation, leading to fever, splenomegaly, cytopenia, hypofibrinogenemia, or hypertriglyceridemia, hyperferritinemia, and hemophagocytosis.[4]

XLP belongs to the group of familial hemophagocytic lymphohistiocytosis (FHL) as originally proposed by Purtilo et al.[1] In the original description, the term "lymphoproliferative disease" in the Duncan kindred[1] was used for benign or malignant lymphoproliferation but also for the diffuse organ "infiltrates composed of lymphocytes, plasma cells, and histiocytes, some containing erythrocytes," describing histologic features of HLH. Thus, the term "X-linked lymphoproliferative disease or syndrome" used thereafter to name this condition refers not only to malignant lymphomas but also to HLH. Two genetic causes are responsible for XLP. XLP type 1 (XLP-1) is caused by hemizygous mutations in the gene *SH2D1A* encoding the signaling lymphocyte activation molecule (SLAM)–associated protein (SAP) (MIM no. 308240).[5,6] Hemizygous mutations in the gene encoding the X-linked inhibitor of

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apoptosis protein (XIAP; also termed *BIRC4*; MIM no. 300635) have been discovered in a cohort of patients with clinical XLP without any identified mutations in *SH2D1A* and normal SAP protein expression.[7] Thus, mutations in *XIAP* define the XLP type 2 (XLP-2). These findings were confirmed by the identification of additional patients with XIAP deficiency.[8,9] After EBV infection in most (but not all) cases, patients bearing mutations in *SH2D1A* (hereafter denoted SAP-deficient patients) may experience variable manifestations such as fulminant infectious mononucleosis corresponding pathophysiologically to HLH, malignant lymphoma, and hypogammaglobulinemia.[2,10,11] Less common findings are dysgammaglobulinemia, bone marrow hypoplasia, especially aplastic anemia, and lymphocytic vasculitis.[12,13] However, although HLH is almost always triggered by EBV, the other manifestations can be present even in SAP-deficient patients who have never encountered EBV.[2,3,10,11] The clinical features of the 12 patients with mutations in *XIAP* (hereafter denoted XIAP-deficient patients) initially described, slightly differed from the features described above. In some XIAP-deficient patients, splenomegaly was noticed as the first clinical symptom, and chronic colitis occurred during the disease course in 2 patients.[7]

The gene product affected in XLP-1 patients, SAP, is a small SH2-containing adaptor protein that is expressed in T, natural killer (NK), and invariant NKT (iNKT) cells.[5,14] SAP binds with high affinity and specificity to tyrosine-based motifs located in the cytoplasmic domains of the transmembrane receptors of the SLAM family. SAP couples SLAM family receptors to downstream signaling pathways and thereby enables SLAM receptors to mediate an array of activating or regulatory signals. In SAP-deficient humans and mice, multiple cellular defects have been documented, including altered $CD8^+$ T- and NK-cell cytotoxicity responses, $CD4^+$ T helper cell cytokine production and function, block of CD1d-restricted iNKT-cell development, defective antibody production associated with reduced numbers of switched memory B cells and defects in germinal center formation.[11,14] Studies of SAP-deficient humans and mice support the notion that the immune dysfunctions seen in SAP-deficiency are mostly caused by alterations in the signal transduction of SLAM family receptors.

The XLP-2 gene product, XIAP, belongs to the family of inhibitor of apoptosis proteins and is well known to be a potent physiologic inhibitor of caspases 3, 7, and 9.[15] XIAP is ubiquitously expressed.[7] In addition to its antiapoptotic role, XIAP is also involved in multiple signaling pathways, including copper metabolism, activation of the nuclear factor κB and the mitogen-activated protein kinases pathways and the transforming growth factor-β–receptor and bone morphogenetic protein–receptor signal transduction.[16] In XIAP-deficient patients, lymphocytes are characterized by an increased susceptibility to apoptosis in response to CD95 and tumor necrosis factor receptor–related apoptosis-inducing ligand receptor stimulation as well as enhanced activation-induced cell death.[7] XIAP-deficient patients also display low but detectable numbers of iNKT cells in blood although a recent study indicated that they can have normal numbers of iNKT cells.[9] NK cell–mediated cytotoxicity is apparently normal in XIAP-deficient patients.[7,9]

Our knowledge of the immune dysfunctions underlying the clinical manifestations in SAP-deficient patients has been largely improved in the past decade. However, this is not the case for XIAP-deficient patients. A better characterization of the clinical similarities and the differences between XLP-1 and XLP-2 could provide hints for a better understanding of the pathogenesis of these conditions and, furthermore, improve diagnostic and therapeutic procedures for these patients. Therefore, we performed a retrospective analysis of the clinical features observed in cohorts of 33 SAP- and 30 XIAP-deficient patients.

Methods

Patients and diagnosis

We performed a retrospective analysis of the clinical and laboratory features of SAP- and XIAP-deficient patients in whom confirmative molecular diagnosis had been performed at the Necker Children's Hospital. Patient conditions were diagnosed as XLP-1 and XLP-2 on the basis of molecular results or on the basis of clinical features when disease had been molecularly proven in male relatives on the mother's side (supplemental Methods and Results, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Patients and families provided informed consent for genetic and immunologic studies in accordance to the 1975 Declaration of Helsinki, and the study was approved by the local ethics regulations (Necker-Enfants Malades Ethical Board Committee).

Protein expression

Expression of SAP and XIAP was analyzed by Western blotting or flow cytometry or both after intracellular staining in phytohemagglutinin-induced T-cell blasts or peripheral blood mononuclear cells or both as described.[7] The monoclonal antibody (mAb) anti-SAP was kindly provided by Dr A. Veillette, IRCM, Montréal. Intracellular SAP was stained by fluorescein isothiocyanate– or phycoerythrin-coupled anti-SAP mAb and XIAP detected with noncoupled anti-XIAP mouse mAb (clone 48; BD Biosciences) revealed with fluorescein isothiocyanate–coupled anti–mouse antibodies (Jackson ImmunoResearch Laboratories Inc) after cell permeabilization with Perm 2 (BD Biosciences).

Histology and immunohistochemistry

All diagnostic specimens were fixed in 10% buffered formalin and stained with hematoxylin and eosin, Giemsa, or trichrome dyes (for the liver). Immunohistochemistry was performed on fixed tissues with a peroxidase-based method (Dako). Antibodies used were raised against CD20, CD3, CD8, and latent membrane protein 1 (LMP-1) (Dako); CD25 (Novocastra); and T-cell intracellular antigen-1 (Immunotech). EVB-encoded RNA (EBER) was probed on some specimen with the use of in situ hybridization technique. Slides were observed using a Leica DM LB microscope with ×20, ×40, and ×100 objectives and a 10× eyepiece. Acquisition of images was with IM50 software (Leica Microsystems). All slides were analyzed by the same pathologist (D.C.), and an independent review was also performed (F.H.).

Clinical assessment

The patients' clinical events and laboratory features were assessed retrospectively by retrieval of data from medical records.

Statistical analysis

The statistical analyses were performed with Fisher exact tests or log-rank tests (for comparison of survival curves) with the use of the PRISM software (GraphPad Software Inc).

Results

XLP-1 was diagnosed in 33 patients from 19 families, and mutations of *SH2D1A* were found in 18 families, and XLP-2 was

Table 1. Characteristics of patients with mutations in *SH2D1A/SAP* (XLP-1)

Patient ID*	*SH2D1A/SAP* mutation	SAP protein	HLH (age in years at diagnosis)	EBV at first HLH	HLH relapses (age in years at relapse)	SM (age in years at diagnosis)	Hypo-γ (age in years at diagnosis)	Lymphoma (age in years at diagnosis)	Other manifestations (age in years at diagnosis)	Outcome (age in years)
S1.1	E67G		–	NA		–	–	13	–	Alive, well (19)
S1.2	E67G	–	3	+	+ (25)	–	+ (26)	34	–	Alive, under lymphoma treatment (34)
S1.3	E67G		15	?	–	–	–	7, 30	–	Alive, under lymphoma treatment (30)
S1.4	E67G		–	NA		–	+ (4)†	–	–	Alive, well, IVIG (10)
S2.1	I96X	–	4	?	–	?	?	–	–	Died (4, HLH)
S3.1	del. of exons 1-4		–	NA		–	+†	–	Chronic gastritis,	Alive, well, IVIG (20)
S3.2	del. of exons 1-4		–	NA		–	+†	–	IM (2), chronic gastritis	Alive, well, IVIG (20)
S4.1	R55X		–	NA		–	–	40	–	Alive, well (42)
S4.2	ND		6	+	–	–	–	–	–	Died (6, HLH)
S5.1	del. of exon 2		3.7	+	–	?	?	–	–	Died (3.7, HLH)
S5.2	ND		–	NA		–	?	5	–	Died (5, lymphoma)
S6.1	del. of exon 1		2.2	+	–	–	?	–	–	Died (2.2, HLH)
S7.1	R55X	–	2.5	+	–	–	?	–	Recurrent infections	HSCT (2.7), alive (11)
S8.1	X129RfsX141	–	2.4	+	+ (9)	–	+ (3)†	–	–	First HSCT (9); second HSCT (10); died (10.2)
S8.2	ND		2	+	–	–	?	–	–	Died (2, HLH)
S9.1	C42Y	+/–	–	NA		–	–	2	–	Alive (18)
S9.2	C42Y		–	NA		–	+ (1)†	–	–	Alive, well, IVIG (16)
S10.1	R55Q		14	?	–	?	?	–	–	Died (14, HLH)
S11.1	X129R fsX141	–	–	NA		–	+	–	–	Alive, well, N+T, IVIG (22)
S11.2	X129R fsX141		–	NA		?	?	–	Recurrent pneumonia	Alive, well (66)
S11.3	X129R fsX141		–	NA		–	+	–	–	Alive, well, IVIG (15)
S11.4	X129R fsX141		–	NA		–	+ (9)	7	–	Alive, well, IVIG (19)
S12.1	del. of exon 3	–	19	+	–	–	+ (10)†	11	T (22)	Alive, T, IVIG (23)
S12.2	del. of exon 3		19	?	–	–	+ (19)†	20	–	Died (21, lymphoma)
S13.1	N82FfsX103	ND	10§	–	+ (12, EBV+)	+ (9)‡	?	–	–	Died (12, HLH)
S14.1	del. of exons 1-4		3.5	+	–	–	–	–	HUS (3.5)	Died (3.6, HLH)
S15.1	A22P	–	–	NA		–	+ (13)†	–	–	Alive, well, IVIG (25)
S15.2	ND		3.6	?	–	–	?	–	–	Died (3.6, HLH)
S15.3	ND		–	NA		+ (45)‡	?	–	–	Died (69, myelodysplasia)
S16.1	del. of exons 2-4	–	3.1	+	–	–	?	–	–	Died (3.1, HLH)
S17.1	M1T	–	–	NA	–	–	+ (4)†	–	IM (2.4)	Alive, N+T, IVIG (20)
S18.1	No mutation	–	16§	?	–	–	+ (15)†	9	–	Died (17, HLH)
S19.1	del. of exons 1-4	–	3.3	+		–	–	–	Hypopigmented hair	HSCT (3.7), died (3.8)

SM indicates recurrent splenomegaly or hepatosplenomegaly; Hypo-γ, hypogammaglobulinemia; NA, not applicable; del., deletion; ?, unknown; IM, infectious mononucleosis; ND, not done; HSCT, hematopoietic stem cell transplantation; N, neutropenia; T, thrombocytopenia; and HUS, hemolytic uremic syndrome.

*Patient identification: S indicates SAP-deficiency, the first number corresponds to the family and the second to the individual patient.

†With recurrent respiratory infections; + indicates yes or positive; –, no or negative.

‡Recurrent splenomegaly or hepatosplenomegaly associated with intermittent fever, anemia, and cytopenia.

§Diagnosed as incomplete HLH.

diagnosed in 30 patients from 11 families (Tables 1 and 2). In one patient (PS18.1), no mutation in *SH2D1A* was found; however, no SAP protein expression was detected.[17] Six and 7 mutations in *SH2D1A* and *XIAP* were novel and not reported, respectively (supplemental Methods and Results).

Clinical manifestations included HLH, splenomegaly and incomplete forms of HLH, lymphoma, dysgammaglobulinemia, colitis, and rare clinical manifestations.

HLH

The mean age at first episode of HLH was 7.35 years (range, 2.0-19.0 years) in SAP-deficient and 6.5 years (range, 0.1-23.0 years) in XIAP-deficient patients ($P = .89$). The occurrence of HLH in SAP-deficient (18 of 33, 55%) and in XIAP-deficient (22 of 29, 76%, one unknown) patients did not differ significantly ($P = .112$) (Figure 1A; Table 3). XIAP-deficient patients with null mutations (families X1 to X7 and X11) more frequently developed HLH (19 of 20, 95%) compared with XIAP-deficient patients expressing non-null mutations (families X8, X9, and X10; 3 of 9, 33%; $^{**}P = .0011$; supplemental Figure 1A).

Overall, 11 of the 33 SAP-deficient patients (33%) and 5 of 30 the XIAP-deficient patients (17%) succumbed to HLH ($P = .1563$). Among patients with HLH, HLH-associated lethality was significantly higher in SAP-deficient patients (11 of 18, 61%) than in XIAP-deficient patients (5 of 22, 23%) ($^{*}P = .0230$). HLH