KI-LCL cells or TA-LCL cells for 5 hours at an effector:target cell ratio (E/T) of 2.5:1, 5:1, and 10:1. Target cells were also added to wells containing medium alone and to wells containing 0.2% Triton X-100 to determine the spontaneous and maximal levels of ^{51}Cr release, respectively. After 5 hours, 0.1 ml of supernatant was collected from each well. The percentage of specific ^{51}Cr release was calculated as (cpm experimental release - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release) ×100, where cpm indicates counts per minute.

Degranulation analysis by flow cytometry

Degranulation activity was analyzed by flow cytometry using anti-CD107a antibody (BioLegend, San Diego, CA) as described previously [16,17]. Briefly, 1×10^5 alloantigen-specific CTLs were co-cultured with or without 1×10^5 KI-LCL cells in 0.2 ml of RPMI 1640 medium supplemented with 10% FCS, and then FITC-conjugated anti-CD107a antibody was added to each well. After 3 hours, incubated cells were collected and analyzed by flow cytometry using PE-conjugated anti-CD8 antibody (BD Biosciences, Franklin Lakes, NJ). For analysis of degranulation, the relative log fluorescence of live cells was measured using a FACS flow cytometer (BD Biosciences).

The immunofluorescence intensities of CTLs cultured with and without alloantigen stimulation were measured, and the mean fluorescence index (MFI) was calculated as (mean value for stimulated sample – mean value for non-stimulated sample)/mean value for non-stimulated sample.

Results

Genetic subtypes of FHL patients

Among the 31 patients with FHL, 17 appeared to have *PRF1* mutation and lacked expression of perforin protein as measured by flow cytometry and Western blotting, whereas 10 patients appeared to have *UNC13D* mutation and lacked Munc13-4 protein expression as measured by Western blotting. No *STX11*

Table 1. Genetic mutations of *PRF1*, *UNC13D*, *STX11*, and *STXBP2* identified in 31 patients.

UPN	Age/Sex	*PRF1*	*UNC13D*	*STX11*	*STXBP2*
1	3 mo/F	1090.91delCT/1090.91delCT	-	-	-
2	2 mo/F	1090.91delCT/207delC	-	-	-
3	1 mo/F	1090.91delCT/207delC	-	-	-
4	11 y/F	949G>A (M)/1A>G (N)	-	-	-
5	1 mo/F	1083delG/1491T>A (N)	-	-	-
6	4 mo/F	1289insG/1289insG	-	-	-
7	1 mo/F	1349C>T (M)/1349C>T	-	-	-
8	2 mo/F	1090.91delCT/1246C>T (N)	-	-	-
9	12 y/F	1090.91delCT/1228C>T (M)	-	-	-
10	7 y/F	1349C>T (M)/1349C>T	-	-	-
11	2 mo/M	207delC/1122G>A (M)	-	-	-
12	1 mo/M	1090.91delCT/NT	-	-	-
13	4 mo/F	757G>A (M), 253G>A (M)/853-855delAAG	-	-	-
14	1 mo/F	160C>T (M), 272C>T (M)/853-855delAAG	-	-	-
15	3 mo/F	853-855delAAG/1491T>A (N)	-	-	-
16	5 mo/M	1090-1091delCT/1168C>T (N)	-	-	-
17	1 y/M	1090-1091delCT/1349C>T (M)	-	-	-
18	1 mo/M	-	640C>T (M)/-	-	-
19	6 mo/F	-	1596+1g>c (S)/1596+1g>c (S)	-	-
20	4 mo/F	-	766C>T (M)/1545-2a>g (S)	-	-
21	2 mo/M	-	640C>T (M)/1596+1g>c (S)	-	-
22	5 mo/M	-	1596+1g>c (S)/1723insA	-	-
23	5 mo/M	-	1596+1g>c (S)/754-1g>c (S)	-	-
24	6 mo/M	-	754-1g>c (S)/754-1g>c (S)	-	-
25	11 mo/M	-	1596+1g>c (S)/322-1g>a (S)	-	-
26	1 mo/M	-	754-1g>c (S)/2163G>A (N)	-	-
27	2 mo/F	-	322-1g>a (S)/754-1g>c (S)	-	-
28	2 mo/M	-	-	-	292-294delGCG/88-1g>a
29	2 mo/M	-	-	-	1243-1246delAGTG/1243-1246delAGTG
30	0 day/M	-	-	-	-
31	0 day/F	-	-	-	-

UPN, unique patient number; M, male; F, female; -, not detected, NT, not tested.
In parenthesis, M means missense mutation, N means nonsense mutation, and S means splicing abnormality.
doi:10.1371/journal.pone.0014173.t001

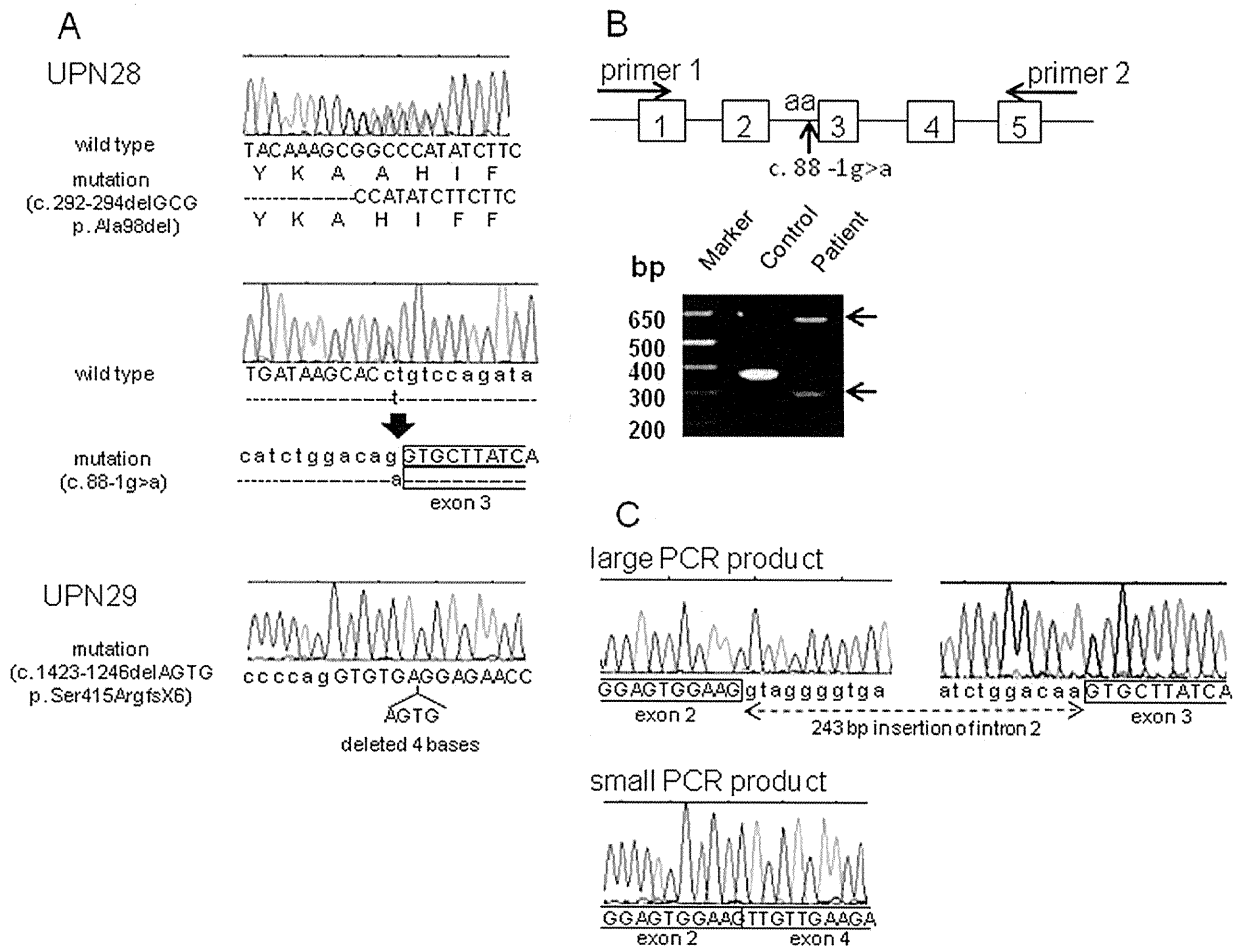

Figure 1. Identification of *STXBP2* mutations. (A) Sequencing analysis of 4 patients with non-FHL2/3/4 and detection of 3 novel mutations in 2 of them: a compound heterozygous mutation of 292_294delGCG resulting in Ala98del at exon 5 (upper panel) and 88-1g>a in intron 2 (lower panel) in one patient (UPN28), and a homozygous mutation of 1243-1246AGTG resulting in Ser415ArgfsX6 at exon 15 in the other (UPN29). (B) Expression of *STXBP2* cDNA in UPN28 with 88-1g>a mutation. Schematic representation of positions of the primers for RT-PCR and 88-1g>a mutation is shown in the upper panel, and for RT-PCR products from 88-1G>A mutation of *STXBP2* in the lower panel. The expected 350-bp product of *STXBP2* exons 1–5 was detected in a healthy control individual, whereas extra larger- and smaller-sized products were detected in UPN28 (arrow). (C) Sequence analysis revealed that the 88-1g>a mutation retained the entire intron 2 (243 bp) in the cDNA. This insertion is predicted to cause addition of 81 amino acids to the N-terminal region of the large Sec1 domain of the Munc18-2 protein (upper panel). Sequence analysis of the smaller fragment revealed that the mutation caused skipping of exon 3 (82 bp), resulting in a frame shift and translational arrest after an additional 20 amino acids (lower panel).
doi:10.1371/journal.pone.0014173.g001

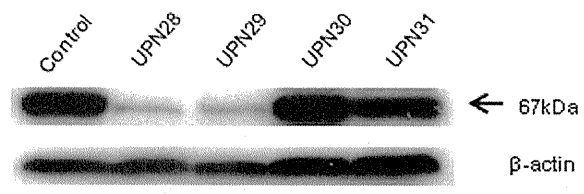

Figure 2. Western blot analysis of Munc18-2 protein expression. Expression of Munc18-2 protein in each $CD8^+$ T-cell line that had been stimulated with allogeneic B-LCL cells was analyzed by Western blotting using anti-Munc18-2 antibody. Munc18-2 protein was abundantly detected at 67 kDa in CTL lines established from healthy control individuals and 2 non-FHL2/3/4/5 patients (UPN30, and UPN31).
doi:10.1371/journal.pone.0014173.g002

mutations were detected in any of the patients (Table 1). Most of the data have been reported elsewhere [11,12,14,21,22,26]. For the remaining 4 patients (UPN28-31), *STXBP2* mutation and CTL function were further analyzed.

STXBP2 analysis and Munc18-2 expression in 4 patients with non-FHL2/3/4

Genetic analysis of *STXBP2* was performed in 4 patients with non-FHL2/3/4 (UPN28-31). As shown in Fig. 1A, a compound heterozygous *STXBP2* mutation with 292_294delGCG and 88-1g>a was detected in UPN28, and a homozygous mutation with 1243_1246delAGTG appeared to be present in UPN29. These 3 mutations of *STXBP2* are all novel. RT-PCR analysis showed that 2 aberrant cDNAs were produced in UPN28 (Fig. 1B). Sequence analysis revealed that the large fragment 88-1g>a mutation caused insertion of the entire intron 2 (243 bp) into the cDNA,

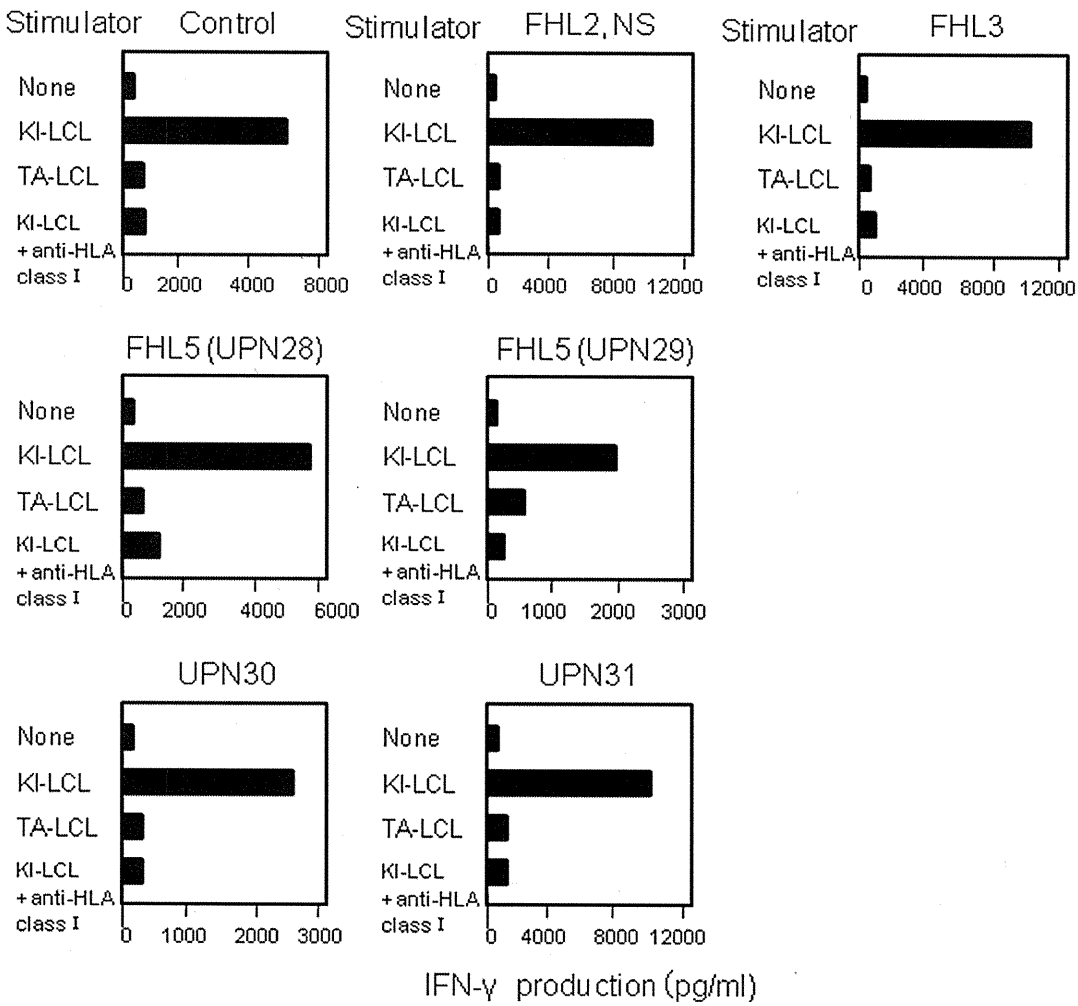

Figure 3. IFN-γ production by alloantigen-specific CD8$^+$ T cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Responder cells were co-cultured with or without KI-LCL or TA-LCL, which shared no HLA antigens with KI-LCL, in the presence or absence of anti-HLA class I monoclonal antibody for 24 hours. IFN-γ production was measured by ELISA. All FHL patients showed normal production of IFN-γ. The HLA type of KI-LCL is HLA-A01/30, B13/17, Cw6/-, DRB1*0701/*0701, and that of TA-LCL is HLA-A24/26, B62/-, Cw4/w9, DRB1*0405/*0901. NS indicates *PRF1* nonsense mutation.
doi:10.1371/journal.pone.0014173.g003

while in the small fragment the mutation caused skipping of exon 3 (82 bp), resulting in a frame shift and translational arrest after an additional 20 amino acids (Fig. 1C).

We analyzed the expression of Munc18-2 protein in CTLs of these 4 patients using Western blotting. As shown in Fig. 2, the Munc18-2 protein band at approximately 67 kDa was scarcely detectable in 2 FHL patients with *STXBP2* mutation (UPN28, UPN29). On the basis of these data, these 2 were diagnosed as having FHL5. On the other hand, Munc18-2 protein expression was clearly detected in CTL lines established from the remaining 2 patients (UPN30, UPN31); therefore, these patients were considered to have FHL with unknown genetic mutations.

Functional analysis of CTL lines established from FHL patients

Alloantigen-specific CD8$^+$ CTL lines were generated from healthy individuals, and from patients with FHL2 (UPN8), FHL3 (UPN23), and non-FHL2/3/4 (UPN28-31). The antigen specificities of the T-cell lines were examined by measuring their IFN-γ production in response to stimulation with allogeneic LCL cells. As shown in Fig. 3, all alloantigen-specific CD8$^+$ T-cell lines generated by stimulation with allogeneic KI-LCL produced large amounts of IFN-γ in response to stimulation with KI-LCL cells, but not with TA-LCL cells, which share no HLA antigens with KI-LCL. These results indicated that T lymphocytes of FHL patients can respond normally to antigen stimulation and produce inflammatory cytokines. Their IFN-γ production was significantly abrogated by anti-HLA class I antibody, indicating that the responses of these T-cell lines were alloantigen-specific and HLA class I-restricted.

Cytotoxic activity mediated by CD8$^+$ alloantigen-specific T-cell lines generated from healthy individuals (n = 24) and FHL patients are measured, and the representative data are shown in Fig. 4. Antigen-specific cytotoxicity mediated by CTLs from FHL2 patients with *PRF1* nonsense mutation was entirely deficient, whereas that of CTLs from FHL3 patients with *UNC13D* splicing abnormality was low but still detectable, as we have reported

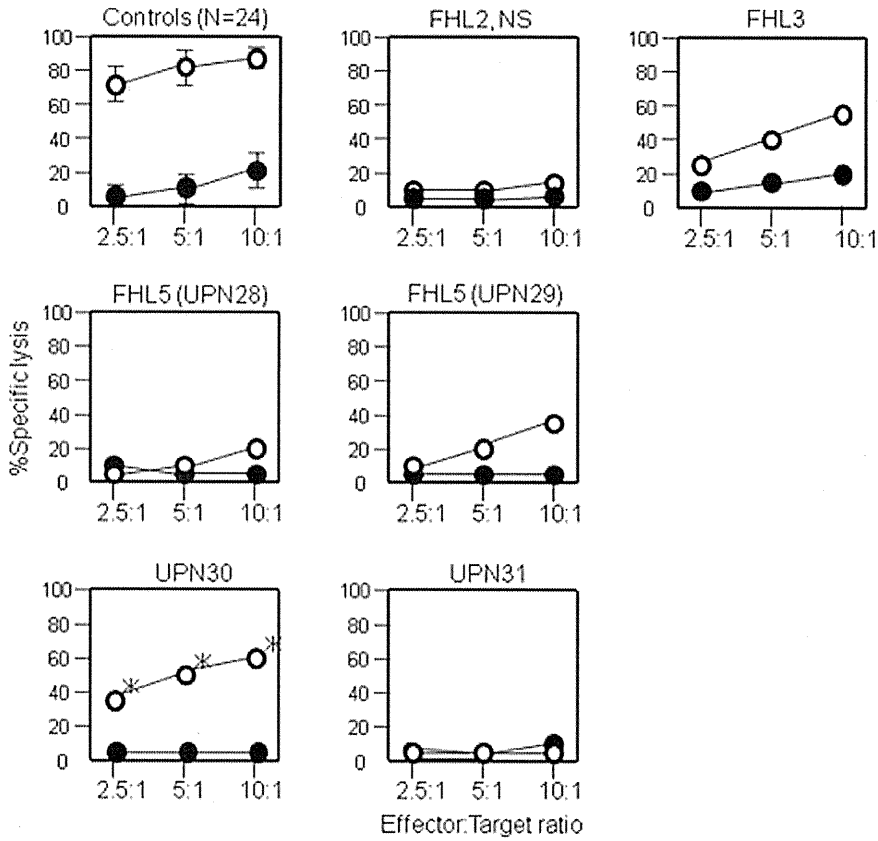

Figure 4. Cytotoxicity of alloantigen-specific CD8$^+$ T-cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and 24 healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Their cytotoxicity was determined against allogeneic KI-LCL (clear circles) and against allogeneic TA-LCL (solid circles). All FHL patients showed various degrees of impairment of CTL-mediated cytotoxicity against allogeneic B-LCLs. NS indicates *PRF1* nonsense mutation.
doi:10.1371/journal.pone.0014173.g004

previously [14,21]. Cytotoxicity mediated by CTLs generated from 2 FHL5 patients also appeared to be low but still detectable. However, the cytotoxicity from 2 patients with unknown genetic mutations was variable; moderately impaired in one (UPN30), and deficient in the other (UPN31).

Degranulation analysis of CTL lines established from FHL patients

Degranulation activity mediated by CTLs established from healthy individuals and FHL patients are measured, and the representative data are shown in Fig. 5. The fluorescence intensities of CTLs cultured with and without alloantigen stimulation were compared by calculating MFI. Both control CTLs generated from healthy individuals and perforin-deficient (FHL2) CTLs showed a marked increase of fluorescence intensity following alloantigen stimulation, indicating that CTLs with perforin deficiency had no impairment of degranulation activity; MFI of CTLs generated from healthy individuals (n = 4) and the patient with perforin deficiency was 4.19±1.15 (mean ± SD) and 5.90, respectively. On the other hand, the increase of fluorescence intensity in Munc13-4-deficient (FHL3) CTLs following alloantigen stimulation was relatively slight; i.e. MFI was 1.81. In repeated experiments, similar degrees of degranulation were detected using CTLs established from other FHL2 or FHL3 patients. CTLs established from 2 FHL5 patients also showed a slight but significant change in fluorescence intensity (MFIs was 1.35). Notably, the increase of fluorescence intensity by CTLs established from 2 patients with unknown genetic mutations was also variable; a slight but significant change in UPN30 (MFI was 1.53), while completely undetectable even after alloantigen stimulation in UPN31 (MFI was 0.16).

Clinical and laboratory findings of 2 FHL patients with unknown genetic mutations

Clinical and laboratory findings of 2 FHL patients with unknown genetic mutations were analyzed. Both had splenomegaly, deficient NK cell activity and hemophagocytosis in bone marrow, and had shown onset of the disease at birth. One patient (UPN30) also showed hydrocephalus as CNS involvement. They had a positive family history of HLH, i.e. their sibling had shown severe hemophagocytosis and died in infancy. Both received immunochemotherapy with or without stem cell transplantation, but three subsequently died due to disease progression or complications related to the treatment.

Discussion

We have been performing a continuous nationwide survey of HLH in Japan [27]. Among 87 young patients with HLH registered so far, 31 were diagnosed as having FHL. Among these

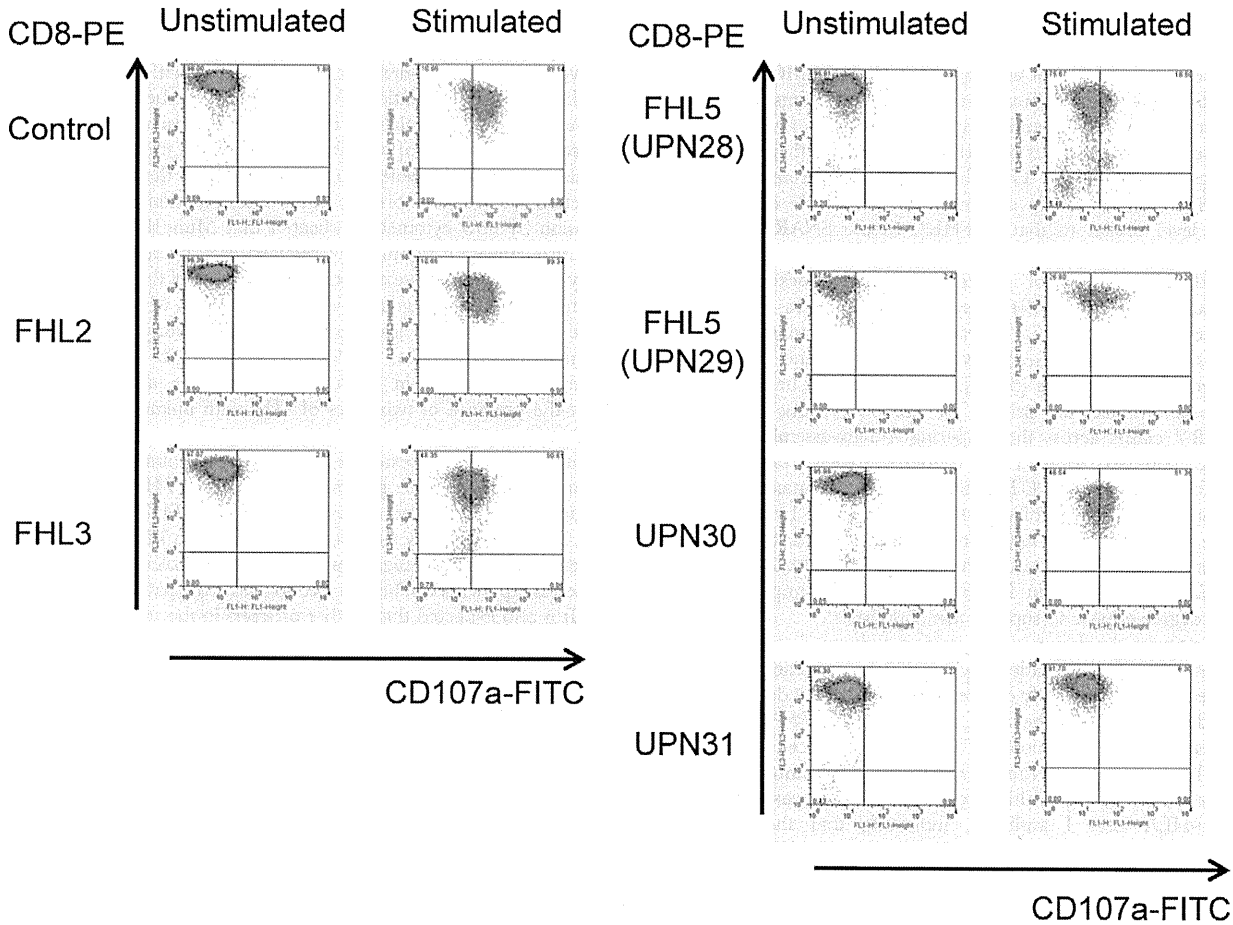

Figure 5. CD107a expression of alloantigen-specific CD8$^+$ T-cell lines. Flow cytometric analysis of CD107a expression was performed using CD8$^+$ T-cell lines generated from a healthy individual and FHL patients, as detailed in the text. Left panel of each column shows CD107a expression in CD8$^+$ T cells without any stimulation. Right panel of each column shows CD107a expression in CD8$^+$ T cells stimulated with KI-LCL cells.
doi:10.1371/journal.pone.0014173.g005

31 patients, 17 and 10 patients appeared to have FHL2 and FHL3, respectively, while no FHL4 patient was detected. In the present study, we carried out precise genetic characterizations of 4 non-FHL2/3/4 patients. Among these patients, 2 showed *STXBP2* mutations and were diagnosed as having FHL5. These findings demonstrate that the actual incidence of FHL2 and FHL3 in Japan is approximately 55% and 32%, respectively. FHL5 with *STXBP2* mutation accounts for only 6%, and no FHL4 patients have yet been found in Japan. Since more than 80% of FHL patients in Japan have been registered by our laboratory, these findings reflect the actual epidemiology of FHL in Japan. In a cohort study using samples from West Asian countries, mutations of 3 known genes (*PRF1*, *UNC13D*, *STX11*) were identified in 80% of FHL patients, while *STXPB2* mutation accounted for 10% and the causes remained unknown for the remaining 10% of FHL cases [17]. These data suggest the presence of other gene deficiencies responsible for FHL in various ethnic groups.

STXBP2 is a newly identified causative gene for FHL5. zur Stadt et al. reported 12 patients with 9 kinds of *STXBP2* mutations from Turkey, Saudi Arabia, and Central Europe [16]. Cote et al. also reported 9 patients from Turkey, Saudi Arabia and Palestine [17]. Among *STXBP2* mutations in FHL5, 1430C>T resulting in P477L and 1247-1g>c resulting in a splicing effect are the most frequent mutations in these countries [16,17]. The association between phenotype and genotype in FHL5 is still obscure. The former report described that patients with mildly impaired CD107 expression or residual CTL activity showed late onset [16]. The latter report mentioned that most of the FHL5 patients with 1430C>T showed very early onset and rapid death, whereas all of the patients with splice site mutation developed their symptoms several years later [17]. In the present study, 3 novel mutations of *STXBP2* were identified in 2 Japanese patients. Both of these patients showed onset in early infancy and the cytotoxic activities of their CTLs and NK cells were low. Further accumulation of FHL5 patients should make it possible to clarify the relationship between phenotype and genotype in this disease.

Bryceson et al. [28] demonstrated that syntaxin11 deficiency is predominantly manifested in the context of NK, rather than CD8$^+$ CTLs. Two recent studies [16,17] have shown that Munc18-2 deficiency is strongly manifested at the level of naive NK cells, whereas relatively milder defects are evident in CD8$^+$ CTLs. These studies suggest that NK deficiency is the likely trigger for at least two types of FHL (FHL4 and FHL5), while perforin and Munc13-4 deficiencies affect both cell types and thus the trigger cannot be discriminated. However, the number and cytotoxic function of NK cells vary depending on a number of factors,

including the nature of the disease, infections, and type of treatment, as indicated by Bryceson et al. [28]. Therefore measurements of NK cell activity using whole PBMCs may not accurately reflect the immune status of the patients [21]. We therefore established alloantigen-specific CTL lines from patients with the different subtypes of FHL and compared their cytotoxic activities. Consequently, CTL lines generated from 2 FHL5 patients showed markedly decreased but detectable cytotoxicity with a level similar to that in FHL3. In the SNARE systems, perforin is critical for granzyme delivery, and Munc13-4 is essential for priming of cytotoxic granules docked at the immunologic synapse, whereas syntaxin11 regulates membrane fusion events [29,30]. Via interaction with syntaxins, Munc18 proteins are required for secretory vesicle docking and fusion with the immunologic synapse [31,32]. A recent report has indicated that docked vesicles are primed for fusion by Munc13-4 when Munc18-2 clasps across the zippering 4-helix-assembled trans-SNARE complex [33]. These findings suggest that at the immunologic synapse of CTLs, the Munc18-2/syntaxin11 complex could play a role similar to that of Munc13-4 by regulating granule docking and the initiation of SNARE formation prior to the priming step. Our data indicating that the cytotoxic activities of CTLs and NK cells in FHL3 and FHL5 are impaired to a similar degree appear to support this hypothesis.

Interestingly, the degrees of cytotoxic activity mediated by CTL lines generated from 2 patients with unknown genetic mutations appeared to be significantly different, i.e. moderately decreased in one and undetectable in the other, as is the case for *PRF1* nonsense mutation [21]. A large amount of IFN-γ was produced by both of the CTL lines generated from these patients after stimulation with allogeneic LCL cells, and this cytokine production was abrogated by anti-HLA class I antibody, indicating that the antigen-recognition system mediated via the T-cell receptor/CD3 complex was intact in both cases. These data also indicate that immunological synapses are normally formed between CTLs from these FHL patients and target cells.

A recent study has indicated that CD107a expression mediated by antigen stimulation is a good candidate marker for the cytotoxic activity of CTLs and NK cells [34]. The lysosome-associated membrane protein-1, also known as CD107a, is usually located in cytotoxic granules in CTLs and NK cells. During the cytotoxic activity of CTLs and NK cells, these molecules are transported to the cell surface. Therefore, the level of CD107a expression is well correlated with degranulation activity in CTLs and NK cells. Indeed, activated NK cells derived from patients with FHL3 showed a sharply lower frequency and MFI of CD107a staining compared with healthy control subjects [35]. CD107a assay is effective tool for rapid identification of patients with FHL3 and other impaired degranulation. Furthermore, it has been reported previously that degranulation in Munc18-2-deficient CTLs is significantly impaired [16], and that transfection of these cells with the wild-type *STXBP2* gene results in recovery of the degranulation activity [17]. In our present study, determination of CD107a expression by flow cytometry indicated that Munc18-2-deficient CTLs also showed a significantly reduced level of degranulation activity. Similarly to cytotoxic activity, the degree of degranulation mediated by CTL lines generated from 2 patients with unknown genetic mutations appeared to differ significantly. That is, degranulation activity was moderately impaired in one patient and severely impaired in the other. These data also strongly suggest the presence of two types of FHL with unknown genetic mutation.

In summary, we have examined the genetic and immunological abnormalities in Japanese patients with different FHL subtypes, and our data have clarified the frequency of each FHL subtype in Japan, as well as strongly suggesting that unknown FHL subtypes are present. Further investigations to identify the molecular defects in these FHL patients will be required to clarify the pathogenesis of FHL. It is also expected that further progress in the study of FHL may clarify the detailed mechanisms of CTL- and NK cell-mediated cytotoxicity.

Supporting Information

Table S1 Primer sets for mutation screening of *STXBP2*. Found at: doi:10.1371/journal.pone.0014173.s001 (0.06 MB DOC)

Acknowledgments

We thank all the patients and their family members who participated in this study. We also wish to thank all members of the Japan FHL study group. This work was performed as part of the Cooperative Research project program of the Medical Institute of Bioregulation, Kyushu University.

Author Contributions

Conceived and designed the experiments: HF EI MY. Performed the experiments: KN KY HF JA TO KS TY MY. Analyzed the data: KN KY HF JA TO KS HT TH EI MY. Contributed reagents/materials/analysis tools: KN KY HT KK MS AM EI. Wrote the paper: KN KY EI MY.

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Pediatr Blood Cancer 2010;54:98–102

Nationwide Survey of Single-System Single Site Langerhans Cell Histiocytosis in Japan

Akira Morimoto, MD,[1]* Yasushi Ishida, MD,[2] Nobuhiro Suzuki, MD,[3] Shouichi Ohga, MD,[4] Yoko Shioda, MD,[5] Yuri Okimoto, MD,[6] Kazuko Kudo, MD,[7] Eiichi Ishii, MD[8] and HLH/LCH Committee of the Japanese Society of Pediatric Hematology

Background. Since neither a standard treatment nor a protocol study for single-system single site (SS-s)-type Langerhans cell histiocytosis (LCH) exists, we conducted a nationwide survey in Japan to clarify the epidemiology and clinical outcome of this subtype. ***Procedure.*** Questionnaires regarding the clinical course of children with SS-s-type LCH diagnosed between 1995 and 2006 were sent to all members of the Japanese Society of Pediatric Hematology. ***Results.*** One hundred forty-six children with histologically proven SS-s LCH were evaluable. The most frequently affected organ was bone (82%), followed by skin (12%). Few patients (14%) had a CNS-RISK lesion defined by the Histiocyte Society. Patients with a skin lesion were diagnosed at a significantly younger age than patients with a bone lesion (median: 6 months vs. 5 years 11 months, $P < 0.001$). The treatment regimen varied, but one-third of the patients in total and 71% of patients with a CNS-RISK lesion received chemotherapy that did not include etoposide. All but one patient attained remission. Ten patients (7%) showed reactivation. Of these, all eight with an initial bone lesion only exhibited reactivation in the bone(s). One patient with an initial skin lesion exhibited reactivation in the thymus. None of the patients died from disease progression or treatment complications. ***Conclusions.*** Our retrospective study, in which a relatively large proportion of the patients received chemotherapy, reveals that patients with SS-s LCH have a good prognosis. A prospective study should be conducted to confirm this and to identify the most effective and least toxic therapy for SS-s LCH. Pediatr Blood Cancer 2010;54:98–102.

Key words: chemotherapy; epidemiology; Langerhans' cell histiocytosis; single system

INTRODUCTION

Langerhans cell histiocytosis (LCH) is the most common histiocytic disorder characterized by the uncontrolled clonal proliferation of Langerhans cells. Its clinical manifestations and course are highly variable, and range from a self-healing solitary lesion to fatal multiorgan involvement [1]. LCH is classified into three distinct forms: single-system single site (SS-s), single-system multisites (SS-m), and multisystem (MS) type. An epidemiological study in Japan [2] has reported that the SS-s, SS-m, and MS types of LCH are diagnosed at a ratio of almost 1:1:1.

Several clinical studies have been performed to improve the outcome of LCH. These include international clinical trials run by the Histiocyte Society [3,4] and a Japanese clinical study performed by the Japan LCH Study Group (JLSG) [5]. These studies have improved the outcome of SS-m and MS-type LCH. However, in terms of SS-s-type LCH, a standard treatment or a protocol study for it is lacking [6]. To date, only one study has examined a large number of patients with single-system LCH, namely, the prospective observational study denoted as DAL-HX 83/90 [7]. Because it appears that the prognosis of patients with SS-s-type LCH is generally good, it is less common that chemotherapy is applied to them [6]. However, the patients with the craniofacial bone(s) (orbital, temporal, mastoid, sphenoidal, zygomatical, ethomoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa) with intracranial soft tissue extension (the so-called CNS-RISK lesion(s)) had higher risk for the development of diabetes insipidus (DI) [8], and the LCH-III protocol study conducted by the Histiocyte Society suggests that chemotherapy should be offered to these patients, even if there is only a single lesion [9].

To further clarify the epidemiology, clinical outcome of SS-s-type LCH, we conducted a nationwide survey of LCH in Japan. We found that the rates of reactivation and sequelae were remarkably low in our cohort of SS-s LCH, in which a relatively large proportion of the patients received chemotherapy.

DOI 10.1002/pbc.22224
Published online 2 September 2009 in Wiley InterScience (www.interscience.wiley.com)

MATERIALS AND METHODS

Data Collection

To compile the clinical data of new pediatric patients (age younger than 18 years at the time of diagnosis) with SS-s-type LCH who were diagnosed and treated between 1995 and 2006, the HLH/LCH Committee of the Japanese Society of Pediatric Hematology (JSPH) sent questionnaires to all the hospitals in Japan in which pediatric hematologists (JSPH members) worked. The SS-s type of LCH was defined as the infiltration of LCH cells in one site of one affected organ, as confirmed by histology. The questionnaire asked about the diagnostic procedure, the age at diagnosis, the sex, the site of the lesion, the treatment, the occurrence of complications, and the outcome. We received replies from 294 of 320 hospitals (92%). Eventually, the details of 174 patients from 81 hospitals were

[1]Department of Pediatrics, Jichi Medical University School of Medicine, Shimotsuke, Japan; [2]Division of Pediatrics, St. Luke's International Hospital, Tokyo, Japan; [3]Department of Pediatrics, Sapporo Medical University School of Medicine, Sapporo, Japan; [4]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [5]Division of Pediatric Oncology, National Center for Child Health and Development, Tokyo, Japan; [6]Division of Hematology Oncology, Chiba Children's Hospital, Chiba, Japan; [7]Department of Pediatrics, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan; [8]Department of Pediatrics, Graduate School of Medicine, Ehime University, Toon, Japan

The authors all state that there is no potential conflicts of interest.

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

*Correspondence to: Akira Morimoto, Department of Pediatrics, Jichi Medical University School of Medicine, 3311-1, Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. E-mail: akira@jichi.ac.jp

Received 19 January 2009; Accepted 2 July 2009

complied. Of these, 28 patients were excluded from this study for following reasons: 5 because they had multisystem-type disease, 7 because they had multifocal bone type disease, and 16 because the diagnosis was not confirmed by biopsy and histology.

Statistical Analysis

The age of diagnosis of the patients was compared by using the Mann–Whitney *U*-test. In patients with bone lesion, the therapeutic modality and the factors affecting reactivation including gender, age at diagnosis, the region affected at onset, and type of initial treatment were analyzed by using the chi-square test. *P*-values less than 0.05 were considered significant.

RESULTS

One hundred forty-six patients with SS-s LCH from 71 hospitals were evaluable. The median observation time was 3.3 years. The diagnosis was based on the presence in the lesional cells of CD1a antigen and/or Birbeck granules (98 patients), langerin antigen (1 patient), and S100 protein (31 patients), or the hematoxylin–eosin staining findings (16 patients). There were 77 males and 69 females (Table I). The median age at diagnosis was 4.8 years, ranging from 0.0 to 16.8 years. The most frequently affected organ was bone (120 patients, 82%), followed by skin (18 patients, 12%). The site of the bone lesion was a CNS-RISK in 21 patients, the skull or facial bone other than a CNS-RISK lesion in 49, the vertebra in 8, the extremities in 26, the pelvis in 5, and the thorax in 11. The age of diagnosis of the patients with a CNS-RISK lesion was significantly lower than that with other bone lesions (median age: 3 years 7 months vs. 6 years 3 months, $P = 0.021$). Of the patients with a skin lesion, 61% were less than 1 year old and were significantly younger than those with a bone lesion (median age: 6 months vs. 5 years 11 months, $P < 0.001$). The patients with a bone lesion were more frequently male (male/female ratio: 1.22), especially in those with a lesion on an extremity (ratio: 2.25). In contrast, neither gender was more likely to have a skin lesion.

Of the patients with a bone lesion, 33% were treated with chemotherapy, 35% were treated with curettage, and 23% received a biopsy only. More than 70% in the patients with a CNS-RISK lesion and nearly two-third of patients with vertebral bone lesion received chemotherapy. The frequency of receiving chemotherapy in patients with a CNS-RISK lesion was significantly high compared to in patients with other bone lesions (15/21 vs. 24/99, $P < 0.001$).

Of the patients with a skin lesion, 28% were treated with chemotherapy, while 56% were treated with biopsy only and remaining patients received surgical treatment or corticosteroid therapy (Table II). Although the chemotherapy regimen used varied, none of the patients received etoposide. All but 1 patient (99%) attained remission, but 10 patients (7%) subsequently suffered a reactivation. None of the patients died of disease progression or treatment complications. At last follow-up, 144 of 146 (99%) did not have active disease (Table II).

All eight patients with reactivated disease and an initial bone lesion exhibited a skeletal reactivation only (two in the same site at onset, one in another site, and five in multiple sites). Of the two reactivated patients with an initial skin lesion, the reactivation occurred in the skin in one and in the thymus in the other. The median duration from diagnosis to reactivation was 4 months (range, 0.1–2.5 years) (Table III). Any factors including gender, age at diagnosis, the region affected at onset, and the type of initial treatment were not associated with reactivation of LCH involving a single bone in this analysis (Table IV).

Six patients (4%) had late sequelae. Four with an initial bone lesion had orthopedic sequelae. Two patients suffered developmental impairments: one patient with a thymus lesion had a developmental impairment due to hypoxia arising from airway obstruction, while the other patient, who had a lesion on the intracranial mass, had a developmental impairment because of damage during surgery. None of the patients had DI. There was no correlation between reactivation and the sequelae (Table III).

TABLE I. Characteristics of Patients With SS-s LCH

Site involved	n (%)	Gender (M/F)	Age at diagnosis (median)
Bone	120 (82)	66/54	5m to 16y 9m (5y 11m)
CNS-RISK lesion[a]	21 (14)	14/7	6m to 14y0m (3y7m)*
Non CNS-RISK lesion[b]	49 (34)	26/23	10m to 16y0m (7y4m)
Extremities	26 (18)	18/8	5m to 15y3m (4y5m)
Thorax/shoulder	11 (8)	1/10	1y7m to 9y8m (5y0m)
Vertebra	8 (5)	5/3	11m to 16y9m (11y2m)
Pelvis	5 (3)	2/3	2y6m to 13y2m (7y0m)
Skin	18 (12)	9/9	0m to 14y1m (6m)[#]
Soft tissue	2 (1)	1/1	3m and 4y3m
Oral mucosa	2 (1)	1/1	1m and 6y7m
Thymus	2 (1)	0/2	5m and 3y0m
Lymph node	1 (1)	0/1	1y6m
Intra cranial mass	1 (1)	0/1	1m
Total	146 (100)	77/69	0m to 16y9m (4y10m)

m, months; y, years. [a]Combined lesions in the orbital, temporal, mastoid, sphenoidal, zygomatical, ethomoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa, with intracranial soft tissue extension; [b]Skull or facial bone lesion other than CNS-RISK lesion; *Significantly young compared to patients with other bone lesion ($P = 0.021$); [#]Significantly young compared to patients with the bone lesion ($P < 0.001$).

TABLE II. Initial Treatment and Outcome of SS-s LCH (n (%))

		Initial treatment						Outcome			
				Corticosteroid						Status at last follow-up		
Site involved		None	Curettage / resection	Local	Systemic	Radiation	Chemotherapy	Attained remission	Subsequent reactivation	NAD	AD	Sequelae
Bone	120	27 (23)	42 (35)	7 (6)	4 (3)	1 (1)	39 (33)	120 (100)	8 (7)	119 (99)	1 (1)	4 (3)
CSN-RISK lesion[a]	21	2 (10)	3 (14)	0	1 (5)	0	15 (71[#])	21 (100)	1 (5)	21 (100)	0	0
Non CNS-RISK lesion[b]	49	8 (16)	28 (57)	0	1 (2)	1 (2)	11 (22)	49 (100)	3 (6)	49 (100)	0	2 (4)
Extremities	26	11 (42)	5 (19)	3 (12)	1 (4)	0	6[c] (23)	26 (100)	2 (8)	26 (100)	0	1 (4)
Thorax/shoulder	11	3 (27)	4 (36)	2 (18)	1 (9)	0	1 (9)	11 (100)	2 (18)	10 (91)	1 (9)	0
Vertebra	8	1 (13)	0	2 (25)	0	0	5[c] (63)	8 (100)	0	8 (100)	0	1 (13)
Pelvis	5	2 (40)	2 (40)	0	0	0	1 (20)	5 (100)	0	5 (100)	0	0
Skin	18	10 (56)	1 (6)	1 (6)	1 (6)	0	5 (28)	17 (94)	2 (11)	17 (94)	1 (6)	0
Other	8	0	4 (50)	0	0	0	4 (50)	8 (100)	0	8 (100)	0	2 (25)
Total	146	37 (25)	47 (32)	8 (5)	5 (3)	1 (1)	48 (33)	145 (99)	10 (7)	144 (99)	2 (1)	6 (4)

NAD, no active disease; AD, active disease. [a]Combined lesions in the orbital, temporal, mastoid, sphenoidal, zygomatical, ethomoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa, with intracranial soft tissue extension; [b]Skull or facial bone lesion other than CNS-RISK lesion; [c]Including one patient received treatment combined chemotherapy and radiation; [#]Significantly high incidence compared to patients with the other bone lesion ($P < 0.001$).

DISCUSSION

In this study, we retrospectively analyzed 146 patients with SS-s LCH. Although the pediatric hematologists in over 90% of the hospitals in Japan answered the questionnaire we sent, it remains possible that some patients were excluded because they were under the care of an orthopedist or dermatologist.

In our cohort, the organ that was most frequently affected was bone (over 80% of the patients had a lesion in bone), followed by skin. The patients with a skin lesion were younger than those with a bone lesion, while males developed SS-s LCH more frequently than women. These features were quite similar to those of the cohort studied by the DAL-HX study [7]. They were also consistent with the results of an epidemiological study that found, of unifocal LCH patients, 70% had a bone lesion, 77% of the patients with a skin lesion were less than 1 year old, and males were more often affected by the disease than females (male/female ratio: 1.3) [10].

The involvement of CNS-RISK lesion(s) carry an about threefold risk for the development of DI which is the hallmark of central nervous system involvement in LCH [8]. Of patients enrolled onto DAL-HX83/90, LCH-I, and LCH-II, majority of whom were MS or SS-m-type LCH, 43% had CNS-RISK lesion(s) [8]. In our SS-s cohort, only 14% of patients had a CNS-RISK lesion, who were significantly younger than patients with other bone lesion. The frequency of the CNS-RISK lesion might rise as SS-s, SS-m, MS, and the disease stage progress.

We found one-third of the patients with a bone lesion were treated with chemotherapy. In particular, more than 70% of patients with a CNS-RISK lesion and nearly two-thirds of patients with a vertebral bone lesion received chemotherapy. A considerable proportion of the patients with a skin lesion (28%) also received chemotherapy. In the DAL-HX study [7], only 8% of patients with a single bone lesion were given systemic treatment. In the LCH-III protocol study chemotherapy is offered to patients with vertebral lesion(s) as well as CNS-RISK lesion(s), even if only a single lesion is present [9]. However, in general, few patients with unifocal bone lesion are treated with chemotherapy. Indeed, in one report from a neurosurgeon, only 3 of 27 (11%) patients with unifocal LCH in a craniospinal site were treated [11].

Regardless of the type of treatment, almost all patients attained remission, and none of the patients died of disease progression or treatment complications. Some patients suffered from reactivation, mostly within a year after diagnosis. In patients exhibiting reactivation, all with only an initial bone lesion showed reactivation in bone(s), whereas some patients with a skin lesion suffered a reactivation in areas other than skin and progressed to multisystem-type LCH. These features were also similar to those of the cohort described by the DAL-HX study [7]. As previously reported [12], isolated cutaneous LCH in infants may be an aggressive disorder that can progress to multiorgan involvement.

The rates of both reactivation and sequelae of LCH involving a single bone in our study were low compared to the rates reported in the DAL-HX study (8/120 vs. 22/121; $P = 0.007$, and 3/120 vs. 25/121; $P < 0.001$, respectively) [7]. Four of the 120 patients (3%) with a bone lesion suffered from orthopedic consequences and two patients with lesions in special areas other than the skin or bone suffered from developmental impairment. In contrast, the DAL-HX study reported that sequelae were already present at diagnosis in 10% of patients with a bone lesion, and that more than half of the sequelae involved orthopedic disabilities, followed by neurologic

TABLE III. Characteristics of the Patients Who Suffered a Reactivation or Sequelae

Initial site	Gender	Age at diagnosis	Initial treatment	Reactivation		Sequelae
				Site	Interval[a]	
Bone						
CNS-RISK lesion[b]	M	1y3m	Chemotherapy	Multiple bone	1y0m	None
Non-CNS-RISK lesion[c]	M	6y1m	Curettage	Multiple bone	3m	None
Non CNS-RISK lesion[c]	F	12y10m	Curettage	Single same bone	1y0m	None
Non-CNS-RISK lesion[c]	F	12y7m	Systemic steroid	Single same bone	1m	None
Upper limb	M	4y7m	None	Multiple bone	3m	None
Lower limb	M	2y5m	None	Multiple bone	2m	None
Thorax	F	7y1m	Curettage	Multiple bone	3m	None
Shoulder	F	7y5m	Chemotherapy	Single other bone	2y6m	None
Skin	M	5m	None	Thymus	5m	None
	F	6m	Chemotherapy	Skin	9m	None
Bone						
Non-CNS-RISK lesion[c]	M	12y5m	Curettage	None		Bone defect
Non-CNS-RISK lesion[c]	F	8y5m	Curettage	None		Bone defect
Lower limb	M	2y0m	Chemotherapy	None		Bone fracture
Vertebra	M	16y9m	Chemotherapy	None		Flat bone
Thymus	F	5m	Chemotherapy	None		DD
Cranial mass	F	1m	Resection	None		DD

DD, developmental disorder; m, months; y, years. [a]Interval from diagnosis; [b]Combined lesions in the orbital, temporal, mastoid, sphenoidal, zygomatical, ethomoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa, with intracranial soft tissue extension; [c]Skull or facial bone lesion other than CNS-RISK lesion.

consequences, and DI and/or anterior pituitary dysfunction. A retrospective study from Argentina had similar results as the DAL-HX study: of 161 patients with single-system unifocal LCH, reactivation occurred in 17.4%, and sequelae, mainly orthopedic problems, developed in 19.1% (the mean follow-up time was 4.8 years) [13]. However, this study did not include information on the type of treatment which these patients received [13].

No factor associated with reactivation of LCH involving a single bone was found in this analysis. We speculate that the low rate of patients with a CNS-RISK lesion, who have intrinsically high risk of DI, and the high rate of applying chemotherapy to these patients in our cohort could be responsible for this as well as the low rates of reactivation and sequelae in our cohort. Most reactivations occurred within 1 year from diagnosis in our study, which suggests that the observation time (median 3.3 years) is sufficient for determining the reactivation rate of our cohort. However, the observation time in our study is too short to draw conclusions with regard to the sequelae rate, because while DI usually developed within 3 years after diagnosis, the rates of neurological consequences increased rapidly 10 years after diagnosis, and the incidence of orthopedic abnormalities and growth retardation accrued with each passing year after diagnosis [14].

In conclusion, we conducted a retrospective study of patients with SS-s LCH in Japan and found that a relatively large proportion received chemotherapy and that the prognosis was generally good. A prospective study should be conducted to confirm these results and to identify the most effective and least toxic therapy for SS-s LCH.

TABLE IV. Factors Affecting Reactivation in Patients With a Bone Lesion

Variables	Reactivation	*P*-value
Gender		
Male	4/66	
Female	4/54	0.769
Age at diagnosis[a]		
<6 years old	3/59	
>6 years old	5/59	0.464
Region		
CNS-RISK lesion[b]	1/21	
Other than CNS-RSK lesion	7/99	0.700
Treatment		
Chemotherapy	2/39	
Other than chemotherapy	6/81	0.639

[a]Data of age at diagnosis were missing in two patients; [b]Combined lesions in the orbital, temporal, mastoid, sphenoidal, zygomatical, ethomoidal bones, the maxilla, paranasal sinuses, or anterior, or middle cranial fossa, with intracranial soft tissue extension.

ACKNOWLEDGMENT

The authors thank the physicians who participated in this study. This work was supported by a Grant for Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare, Japan.

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Pediatr Blood Cancer 2010;54:299–306

Hematopoietic Stem Cell Transplantation for Familial Hemophagocytic Lymphohistiocytosis and Epstein–Barr Virus-Associated Hemophagocytic Lymphohistiocytosis in Japan

Shouichi Ohga, MD,[1,2]* Kazuko Kudo, MD,[2,3] Eiichi Ishii, MD,[2,4] Satoshi Honjo, MD,[1] Akira Morimoto, MD,[2,5] Yuko Osugi, MD,[6] Akihisa Sawada, MD,[7] Masami Inoue, MD,[7] Ken Tabuchi, MD,[8] Nobuhiro Suzuki, MD,[2,9] Yasushi Ishida, MD,[2,10] Shinsaku Imashuku, MD,[2] Shunichi Kato, MD,[2,11] and Toshiro Hara, MD[1]

Background. Post-transplant outcomes of hemophagocytic lymphohistiocytosis (HLH) patients were analyzed in Japan where Epstein–Barr virus (EBV)-associated severe forms are problematic. ***Methods.*** Fifty-seven patients (43 familial HLH [12 FHL2, 11 FHL3, 20 undefined], 14 EBV-HLH) who underwent stem cell transplantation (SCT) between 1995 and 2005 were enrolled based on the nationwide registration. ***Results.*** Fifty-seven patients underwent 61 SCTs, including 4 consecutive SCTs. SCTs were employed using allogeneic donors in 93% of cases (allo 53, twin 1, auto 3). Unrelated donor cord blood transplantation (UCBT) was employed in half of cases (21 FHL, 7 EBV-HLH). Reduced intensity conditioning was used in 26% of cases. The 10-year overall survival rates (median ± SE%) were 65.0 ± 7.9% in FHL and 85.7 ± 9.4% in EBV-HLH patients, respectively. The survival of UCBT recipients was >65% in both FHL and EBV-HLH patients. Three out of four patients were alive with successful engraftment after second UCBT. FHL patients showed a poorer outcome due to early treatment-related deaths (<100 days, seven patients) and a higher incidence of sequelae than EBV-HLH patients ($P = 0.02$). The risk of death for FHL patients having received an unrelated donor bone marrow transplant was marginally higher than that for a related donor SCT ($P = 0.05$) and that for UCBT ($P = 0.07$). ***Conclusions.*** EBV-HLH patients had a better prognosis after SCT than FHL patients. FHL patients showed either an equal or better outcome even after UCBT compared with the recent reports. UCB might therefore be acceptable as an alternate SCT source for HLH patients, although the optimal conditioning remains to be determined. Pediatr Blood Cancer 2010;54:299–306. © 2009 Wiley-Liss, Inc.

Key words: central nervous system disease; Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; familial hemophagocytic lymphohistiocytosis; hematopoietic stem cell transplantation; reduced intensity conditioning; umbilical cord blood transplantation

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is an immunohematologic emergency, characterized by fever, cytopenias, hepatosplenomegaly, hyperferritinemia, and disseminated intravascular coagulopathy (DIC) [1,2]. HLH comprises primary form of familial hemophagocytic lymphohistiocytosis (FHL) and secondary form occurring in association with infections, malignancies, and rheumatic diseases. FHL has currently been classified into FHL1 linked to chromosome 9, FHL2 with *PRF1* mutation, FHL3 with *UNC13D* mutation, and FHL4 with *STX11* mutation, although more than half of patients have no mutations of these genes [1]. HLH could also be a presenting symptom in patients with the other inherited disorders including X-linked lymphoproliferative disease (XLP), Griscelli syndrome, Hermansky–Pudlak syndrome, Chediak–Higashi syndrome and primary immunodeficiency diseases. HLH accounts for the common basis of hypercytokinemia arising from excessive immune activation, in which activated lymphocytes and hemophagocytosing-macrophages without malignant morphology infiltrate into systemic organs, including the bone

Additional Supporting Information may be found in the online version of this article.

Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; CB, cord blood; CBT, cord blood transplantation; CNS, central nervous system; CT, computed tomography; EBV-HLH, Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; EEG, electroencephalography; FHL, familial hemophagocytic lymphohistiocytosis; HLH, hemophagocytic lymphohistiocytosis; PB, peripheral blood; SCT, hematopoietic stem cell transplantation; MRI, magnetic resonance imaging; OS, overall survival; SCT, hematopoietic stem cell transplantation; TRM, treatment-related mortality; RIC, reduced intensity conditioning; VOD, venoocclusive disease; XLP, X-linked lymphoproliferative disease/syndrome.

[1]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]The HLH/LCH and SCT Committees in the Japanese Society of Pediatric Hematology, Tokyo, Japan; [3]Division of Hematology and Oncology, Shizuoka Children's Hospital, Shizuoka, Japan; [4]Department of Pediatrics, Ehime University Graduate School of Medicine, Toon, Japan; [5]Department of Pediatrics, Jichi Medical University, Tochigi, Japan; [6]Division of Pediatrics, Osaka Municipal Medical Center, Osaka, Japan; [7]Department of Hematology/Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan; [8]Division of Hematology, Kanagawa Children's Medical Center, Yokohama, Japan; [9]Department of Pediatrics, Sapporo Medical University School of Medicine, Sapporo, Japan; [10]Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan; [11]Department of Cell transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant number: 19591255; Grant sponsor: HLH/LCH Committee in the Japanese Society of Pediatric Hematology.

*Correspondence to: Shouichi Ohga, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: ohgas@pediatr.med.kyushu-u.ac.jp

Received 12 May 2009; Accepted 31 August 2009

DOI 10.1002/pbc.22310
Published online 13 October 2009 in Wiley InterScience (www.interscience.wiley.com)

marrow (BM), liver, spleen, lymph nodes, skin, and central nervous system (CNS) [3,4]. FHL is a fatal disease if allogeneic hematopoietic stem cell transplantation (SCT) has not been successfully performed.

Epstein–Barr virus (EBV)-associated HLH (EBV-HLH) is a severe form of secondary HLH more frequently occurring in Asian children [5–7]. Activated EBV-infected $CD8^+$ T cells account for the disease process of EBV-HLH [8], however no predisposing factors have yet been clarified. EBV-HLH patients mostly respond to immunochemotherapy, but a small fraction of patients experience a fatal course without SCT. Therefore, although numbers were still small, SCT has been included in the salvage for refractory EBV-HLH cases [9–11]. The optimal timing of SCT, the source of donor cells and the conditioning are critical, particularly for young HLH patients. In this setting, the appropriate SCT for HLH patients needs to be established.

This study analyzed the outcomes of patients with FHL or EBV-HLH who underwent SCT in Japan over the past 10 years, in order to address the issues in the transplant-related problems including engraftment, late sequelae as well as to find out if there are distinct transplant strategies for FHL and EBV-HLH patients.

PATIENTS AND METHODS

Data Collection

The HLH/LCH Committee in the Japanese Society of Pediatric Hematology (JSPH) sent the first questionnaires to the hospitals administered by JSPH members based on the SCT registry in JSPH, asking if SCT was performed for any HLH patients between 1995 and 2005. The second questionnaires were sent to 57 hospitals with SCT cases, asking the patients' characteristics, treatment prior to SCT, donor sources, conditioning regimens, complications, and outcome. Of the 47 responses (recover rate 82%), 61 definite SCT cases from 33 hospitals were eligible for the study (mean 1.7 case/hospital, Supplemental Table). Forty-three FHL patients underwent 46 SCT, while 14 EBV-HLH patients underwent a total of 15 SCT. The majority of SCT (EBV-HLH 87%, FHL 89%) were performed between 2000 and 2005.

Diagnosis and Classification

All 57 patients fulfilled the diagnostic criteria of HLH [12]. FHL was diagnosed when the patient had a genetic abnormality, positive family history, and/or other evidence such as impaired natural killer cell activity [13]. The genetic study of FHL 2, 3, and 4, approved by the ethics committee of Kyushu University, Japan (No. 45), was partly completed postmortem according to our methods [14–17]. FHL2 and FHL3 determined by *PRF1* or *UNC13D* mutations accounted for 28% (n = 12), and 26% (n = 11), respectively, in this group. In addition, a total of eight patients were found with siblings diagnosed as having HLH. EBV infection might be associated with the development of HLH in four FHL patients (one FHL2, one FHL3, and two familial). These cases were classified as FHL, not as EBV-HLH. Other types of primary HLH such as XLP were excluded in this study.

EBV-HLH was diagnosed when a non-FHL patient had a primary infection or reactivation of EBV at the onset of HLH. EBV infection was assessed by the detection of EBV DNA and/or the pattern of serum EBV-specific antibody titers [18]. Cases with secondary HLH occurring in a chronic active EBV infection [19], and/or a histologically confirmed EBV-related lymphoma were excluded in this study. CNS involvement was determined when patients showed neurological manifestations, clinically as well as with any evidence of abnormality in the cerebrospinal fluids (CSF), neuroimagings (CT/MRI), and/or electroencephalography (EEG).

Prior Treatment to SCT

Treatment was based on the HLH-94 protocol using a combination of corticosteroid, cyclosporine-A (CSA), and etoposide (VP16) for both groups [20,21]. As the multidrug chemotherapy, CHOP-VP16-based regimen (VP16, vincristine, cyclophosphamide [CY], doxorubicin, and prednisolone) was chiefly employed. SCT was performed for all FHL patients, but limited for EBV-HLH patients who were resistant to any other treatments.

SCT

Allogeneic SCT was performed in 53 of the 57 patients (93%). Autologous SCT and identical-twin donor SCT were performed in three and one sporadic patients, respectively, because the molecular diagnosis was not available at the time of SCT. Donor sources, infused cell doses, conditioning regimens, and other SCT-related data are summarized in Table I. Allogeneic donor sources for EBV-HLH were HLA-matched sibling peripheral blood (PB) 1, haploidentical parent BM/PB 2, HLA-matched unrelated BM 1, HLA-matched unrelated cord blood (UCB) 2, and HLA-mismatched UCB 5, and those for FHL were HLA-matched related BM 7 (sibling 6), haploidentical parent BM/PB 2, HLA-matched unrelated BM 12, HLA-matched UCB 9, and HLA-mismatched UCB 12. All CBs were obtained from unrelated donors registered in the Japanese Cord Blood Bank Network. All unrelated donor BMs were obtained from the Japanese Marrow Donor Program. Myeloablative conditioning for EBV-HLH included VP16/busulfan (BU)/CY in 8 patients (4 in UCB transplantation [UCBT]) and other regimens in 3 patients, while those for FHL were VP16/BU/CY plus or minus anti-thymocyte globulin (ATG) in 23 patients (10 in UCBT) and others in 8 patients. Reduced intensity conditioning (RIC) for EBV-HLH included melphalan (MEL)/fludarabine (FLU) plus or minus thoracoabdominal irradiation in three patients (two in UCBT), and those for FHL were MEL/FLU plus or minus low-dose total body irradiation plus or minus ATG in eight patients (four in UCBT) and others in three patients. Donor chimerism was assessed by using short tandem repeats or sex chromosome analyses.

Evaluation of Late Sequelae

Long-term survivors were further questioned concerning their physical growth, endocrinological status, and neurological deficits. Neurological development including cognitive functions was assessed by Karnofsky score, developmental quotient and/or school performance.

Statistical Analysis

The 10-year overall survival (OS) rate with 95% confidence intervals were estimated by the Kaplan–Meier method. The OS was calculated for the period from the day of SCT until the death of any cause or the final observation. All results were updated to May 31,

TABLE I. Profiles of Patients Who Underwent Hematopoietic Stem Cell Transplantation

	EBV-HLH	FHL	*P*-value
Number, male:female	14, 4:10	43, 23:20	0.37
Age at onset (median, range)	5.5y, 6m–18y	0.5y, 6d–12y	<0.0001
Age at SCT (median, range)	5.9y, 1.4–18y	1.2y, 0.4–15y	0.0002
Observation period (median, range)	5.5y, 0.3–16y	4.8y, 0.2–19y	0.94
Manifestation at diagnosis (%)			
Fever	100	95	>0.99
Hepatosplenomegaly	86	86	>0.99
Lymphadenopathy	36	21	0.30
Skin eruption	7	14	0.67
Respiratory failure	36	14	0.12
DIC	50	33	0.26
Treatment prior to SCT (%)			
HLH94 only	36 (5/14)	60 (25/42)	0.14
Multidrug chemotherapy	57 (8/14)	19 (8/42)	0.017
Diagnosis to SCT (median, range)	5.8m, 1.8–24m	7.5m, 1.6–84m	0.18
SCT (n)			
Allogeneic	11	42	
Auto/Identical twin	3	1	
Nucleated cell doses ($\times 10^8$/kg)	1.3 (0.2–6.6)	2.5 (0.1–12.7)	0.14
Donor			
UCB	7	21	0.94
Others	7	22	
HLA disparity no	4	28	0.09
HLA disparity yes (>1 locus[a])	7	14	
Conditioning			
Myeloablative[b]	11	31	>0.99
RIC[c]	3	11	
Irradiation yes	4	11	0.73
Irradiation no	9	31	
ATG yes	0	8	0.18
ATG no	14	34	
CNS abnormality (%)			
At diagnosis	29[d] (4/14)	21[d] (9/42)	0.72
Before SCT	57 (8/14)	67 (28/42)	0.52
CSF pleocytosis	25 (2/8)	32 (7/22)	>0.99
MRI abnormality	36 (5/14)	51 (20/39)	0.36
Convulsion	43 (6/14)	41 (17/41)	0.93
Disturbed consciousness	36 (5/14)	24 (10/41)	0.49
Post-transplant state (n)			
Early death (<100 days)	2	7	0.48
Alive	12	29	0.31
Neurological deficit (%)	8[d] (1/12)	29[d] (7/24)	0.22
Late sequelae[e] (%)	8 (1/12)	52 (11/21)	0.022

ATG, anti-thymocyte globulin; BU, busulfan; CNS, central nervous system; CSF, cerebrospinal fluid; CY, cyclophosphamide; DIC, disseminated intravascular coagulopathy; EBV, Epstein–Barr virus; FHL, familial hemophagocytic lymphohistiocytosis; FLU, fludarabine; HLH, hemophagocytic lymphohistiocytosis; MEL, melphalan; MRI, magnetic resonance imaging; SCT, hematopoietic stem cell transplantation; TAI, thoracoabdominal irradiation; TBI, total body irradiation; UCBT, unrelated donor cord blood transplantation; VP16, etoposide. Parenthesis means the positive number of patients per the evaluable number of patients. The observation period means the time from the onset to the last visit or death. [a]Human leukocyte antigen (HLA) disparity was assessed by the serotyping data of HLA-A, -B, and -DR; [b]Myeloablative conditionings for EBV-HLH were VP16/BU/CY 8 (4 in UCBT) and others 3, and those for FHL were VP16/BU/CY + ATG 23 (10 in UCBT) and others 8; [c]Reduced intensity conditionings (RIC) for EBV-HLH were MEL/FLU + TAI 3 (2 in UCBT), and those for FHL were MEL/FLU + low dose TBI + ATG 8 (4 in UCBT) and others 3; [d]The proportion of patients having neurological abnormality was lower in survived patients with EBV-HLH ($P = 0.0015$). Survived patients were neurodevelopmentally assessed at the last visit to the hospital; [e]Late sequela(e) in EBV-HLH was hemiparesis (n = 1), and those in FHL were short stature (n = 5), endocrinological abnormality (n = 1), psychomotor retardation with or without seizure (n = 5), brain atrophy (n = 1), and hearing difficulty (n = 1).

2008. An analysis of the risk factors for SCT outcome was possible for FHL, but not for EBV-HLH because of the small number of subjects. Age at onset of HLH or at the SCT, duration from the onset to SCT, CNS disease before SCT, donor sources, and the type of conditioning were tested using the log-rank method. Cox proportional-hazard model was employed to examine the association between selected clinical variables and the risk for death. A logistic regression model was used to investigate factors associated with neurological sequelae. Chi-square test or Fisher's exact test were employed in other comparisons. P values less than 0.05 were considered to be significant.

RESULTS

Profiles of EBV-HLH and FHL Patients

A comparison of the clinical profiles (Table I) revealed that the ages at disease onset and at the time of SCT were each higher in EBV-HLH than in FHL patients ($P < 0.0001$, $P = 0.0002$, respectively). No clinical manifestations differed between the two groups during the disease course, including respiratory failure as well as CNS abnormalities at diagnosis. The proportion of patients who failed VP16 and CSA therapy including HLH94 protocol and needed combination chemotherapy such as CHOP-VP16 before planning SCT was higher in EBV-HLH patients than FHL patients (57% vs. 19%, $P = 0.0168$).

Outcomes of SCT

Engraftment and survival. Post-transplant outcomes of 43 FHL patients and 14 EBV-HLH patients are summarized in Figures 1 and 2. The 10-year OS rates (median ± SE%) of FHL and EBV-HLH patients were 65.0 ± 7.9% and 85.7 ± 9.4%, respectively ($P = 0.24$; Fig. 3). In the allogeneic SCT cases with FHL (Fig. 1), 29 attained engraftment, 6 had rejection or graft failure, and 7 were undetermined. On the other hand, in EBV-HLH (Fig. 2), seven were engrafted, three were rejected, and one was undetermined. Of all 29 FHL patients engrafted after the first SCT, 26 were alive with no HLH relapse, but 3 died of treatment-related mortality (TRM). Seven engrafted patients with EBV-HLH were alive and well at the final follow-up. Among the nine rejection/graft failure patients (six FHL, three EBV-HLH), a second UCBT was successful in three of the four patients (three FHL, one EBV-HLH). Twelve of the UCBT recipients for FHL that received a graft with the first UCBT and two that received a second UCBT were alive at the last follow-up; while seven died; six were due to TRM and one was due to active HLH disease. Six of the seven UCBT recipients for EBV-HLH were alive and well at the last follow-up, while only one died of active HLH disease on day 18 post-transplant. A total of 29 FHL survivors after allogeneic SCT(s) had 17 complete donor chimera (2 patients after second UCBTs), 3 mixed chimera (1 had 42% donor chimera in remission 18 months after SCT, 2 attained >90% donor chimera until 6 months after SCT), 8 undefined, and 1 graft failure with CNS disease. Ten EBV-HLH survivors after allogeneic SCT attained eight complete donor chimera (seven patients after the first SCT and one patient after second SCT [UCBT]), and two with autologous recovery. Two of three EBV-HLH patients who rejected allogeneic cells were alive and disease free more than 6 years post-transplant. One of two EBV-HLH patients who underwent autologous SCT was alive and well 13 years post-transplant [22]. One EBV-HLH patient was alive and well 10 years after the identical twin donor BMT.

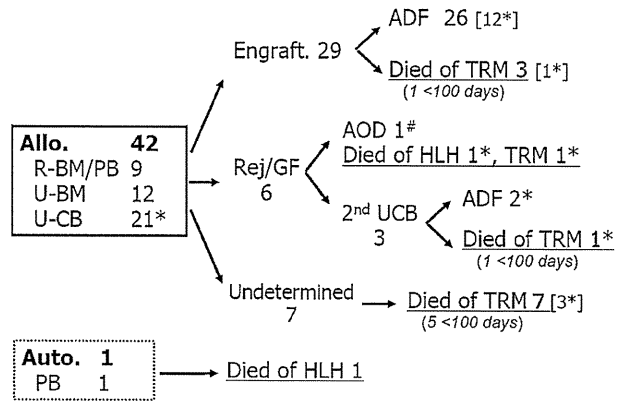

Fig. 1. Cohort diagram for the clinical outcome of 43 patients with familial hemophagocytic lymphohistiocytosis (FHL) who underwent stem cell transplantation (SCT). Of 42 patients after allogeneic SCT, 29 achieved engraftment (18 complete, 3 mixed) and 6 failed to engraft. One (#) with graft failure was alive with central nervous system disease 12 years after SCT. A total of 29 patients (67%) were alive after SCT. The underlined data indicate the number of deceased patients. Seven patients died within 100 days post-SCT (parenthesis). Asterisk (*) means UCB. R, related; U, unrelated; BM, bone marrow; PB, peripheral blood; CB, cord blood; ADF, alive with the disease free state; AOD, alive on disease; Rej/GF, rejection or graft failure; TRM, treatment-related mortality.

Causes of death. Of 14 deceased FHL patients, 12 died of TRM, including 3 chronic GVHD while 2 died of recurrent HLH. Seven patients experienced early death from TRM within 100 days after SCT (Fig. 1). One patient, later diagnosed with FHL2, died of CNS disease 5 years after autologous SCT [14]. Two EBV-HLH patients died of recurrent HLH within 50 days after SCT (Fig. 1). No TRM-related deaths were noted among the EBV-HLH patients.

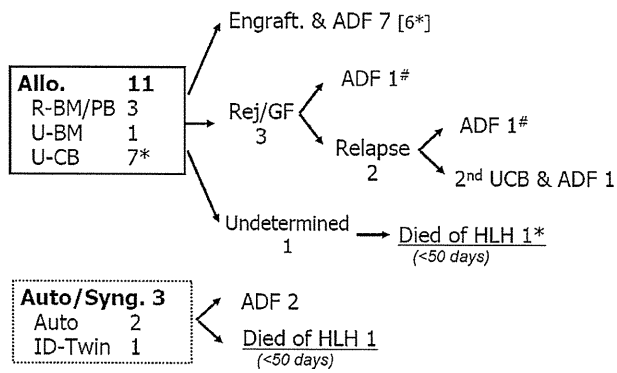

Fig. 2. Cohort diagram for the clinical outcome of 14 patients with Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) who underwent SCT. Among 11 patients after the first allogeneic SCT, 7 achieved successful engraftment and 3 failed to engraft. A total of 12 patients (86%) were alive after SCT. Two patients (#) were alive and well more than 6 years after SCT failure. The underlined data indicate the number of deceased patients. Two patients died within 50 days post-SCT (parenthesis). Asterisk (*) means UCB. Auto/Syng: autologous/syngeneic, ID: identical.

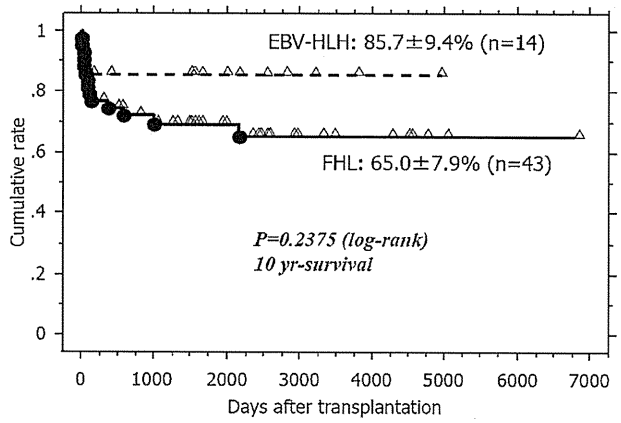

Fig. 3. Cumulative probability of post-transplant overall survival of FHL (*solid line*) and EBV-HLH patients (*dashed line*) who underwent SCT. Closed circle and open triangle represent deceased and alive patients, respectively. Each value indicates the 10-year overall survival rate plus or minus standard error assessed by the log-rank test.

Analysis of Prognostic Factors in FHL

A log-rank test on the OS rate did not show any significant difference in terms of age at SCT (<2 years vs. ≥2 years), time of SCT from HLH treatment (<6 months vs. ≥6 months), conditioning regimens (myeloablative vs. RIC) and various donor sources (R-PB/BM vs. UCBT vs. UBM; Table II). The Cox hazard model with adjustment for gender and age at engraftment indicated that the risk of death for UBM might be higher than that for R-PB/BM (adjusted hazard ratio = 0.07, 95% confidence interval [CI] = 0.01–1.02, $P = 0.05$) and that for UCB (0.27, 95% CI = 0.07–1.09, $P = 0.07$; Table II). No significant variables were found to predict the risk of early death within 100 days post-transplant, or the risk of neurological sequelae.

CNS Abnormalities and Late Sequelae

Table I shows that the frequency of CNS abnormalities at onset and the time of SCT did not differ between the EBV-HLH and FHL patients. Whereas, post-transplant CNS abnormalities were significantly higher in the FHL patients ($P = 0.0015$). Eleven FHL patients (52%) have had late sequelae including neurological as well as endocrinological problems, in comparison to only one EBV-HLH patient with left hemiparesis ($P = 0.022$). Late sequelae of FHL

TABLE II. Association Variables Influencing on the Risk of Mortality in FHL Patients

(A) Log-rank analysis Variables	No.	Survival (OS %)		*P*-value
Age				
<2 years	30	66.2 ± 8.7		0.56
≥2 years	12	75.0 ± 12.5		
Time from HLH treatment				
<6 months	14	62.9 ± 13.3		0.65
≥6 months	28	71.4 ± 8.5		
Conditioning				
Myeloablative	31	71.0 ± 8.2		0.50
RIC	11	60.6 ± 15.7		
Donor sources				
R-PB/BM, a	9	88.9 ± 10.5	a vs. b	0.22
UCB, b	21	65.6 ± 10.6	a vs c	0.15
UBM, c	12	58.3 ± 14.2	b vs c	0.61

(B) Cox's model analysis Variables	No.	Adjusted hazard ratio	95% CI lower–upper limit	*P*-value
Stem cell source				
Unrelated BM	12	1.00	Reference	
Unrelated CB	21	0.27	0.07–1.09	0.07
Related PB/BM	9	0.07	0.01–1.02	0.05
Conditioning				
Reduced intensity	11	1.00	Reference	
Myeloablative	31	0.48	0.09–2.47	0.38
Radiation				
No	31	1.00	Reference	
Yes	11	0.52	0.11–2.52	0.41
Use of ATG				
No	34	1.00	Reference	
Yes	8	0.91	0.18–4.70	0.91
HLA disparity				
No	28	1.00	Reference	
Yes (>1 locus)	14	2.79	0.75–10.38	0.13

Both analyses (A, B) were performed for 42 FHL patients who underwent the first allogeneic SCT. The Cox model analysis was performed with adjustment for selected variables including sex and age at engraftment.

included psychomotor retardation with or without seizures (n = 5), brain atrophy (n = 1), hearing difficulty (n = 1), short stature (n = 5), and impaired sexual development (n = 1).

DISCUSSION

No underlying immunodeficiency has yet been identified for idiopathic EBV-HLH, which has been recognized to be distinct from familial or inherited disease-related HLH like FHL. However, EBV also acts as a trigger in the development of HLH episodes in FHL patients. Therefore, caution must be exercised in the differentiation of the two types of HLH disease. Strict use of the renewed diagnostic criteria for the registered cases in Japan enabled an analysis of the SCT results of 43 FHL and 14 EBV-HLH patients. The data first revealed a high survival rate in UCBT recipients in either type of HLH, indicating that CB could be preferable BM as the unrelated donor source in SCT for pediatric patients with refractory HLH. In addition, SCT in FHL patients was more problematic than that in EBV-HLH, where it was associated with a high incidence of post-transplant early death rate as well as late sequelae including neurological deficits. The EBV-HLH patients showed no apparent sequelae even if they had CNS involvement at diagnosis.

Information concerning SCT for HLH patients has been accumulated mostly in FHL, but little has been published in EBV-HLH except for sporadic case reports [10,11]. Previously published major studies on SCT in FHL patients are summarized in Table III. Because of the historical changes in the available genetic analyses, supportive care practices, donor sources and conditioning, the pre-2000 studies [23–27] might not be comparable to the current data. Henter et al. [21] showed the improved survival of patients treated with HLH-94 followed by BMT, in which the 3-year post-BMT survival was 62%. Horne et al. [28] noted significant TRM due to venoocclusive disease (VOD) after myeloablative conditioning, and that an active disease status at SCT was associated with a poor prognosis. Ouachee-Chardin et al. [29] reported 59% of OS in a series of 48 patients including 60% of haploidentical SCT, and indicated a high TRM due to VOD associated with young age. Recently, Baker et al. [30] reported that BU/CY/VP16 plus or minus ATG-conditioning provided a cure in 53% of patients after unrelated donor BMT, but a high mortality rate at day 100 (32 of 50 [64%] deceased patients). The present study showed a comparably high OS rate (69%) and similarly high incidence of early death until day 100 (7 of 13 [54%] deaths after allogeneic SCT) in Japan. Probably, the major distinction of the current study from the other reports is a higher usage of UCBT (50%) and RIC (26%). Unfortunately, the combined usage of RIC-UCBT was applied only in eight cases (14%) in this study, which was insufficient to fully evaluate its effectiveness. With regard to RIC-SCT with or without UCBT for FHL, Cooper et al. [31] reported a high disease free survival (75%) in 12 HLH patients (including 5 FHL) who underwent RIC-SCT from matched family/unrelated or haploidentical donor, in which 3 of 9 survivors had mixed chimerism but remain free of disease. The most recent report by Cesaro et al. [32] analyzed 61 cases including an appreciable number of RIC (18%) and UCBT (10%), but did not document the superiority of RIC-UCBT. In the present study, UCBT had a tendency to yield a more favorable outcome than UBMT, although the difference was not statistically significant. FHL infants received SCT early; however the fact that survival of FHL patients who underwent SCT at <2 years of age was not better than later SCT might reflect the difficulty in determining the optimal timing of SCT

TABLE III. Reports on the Clinical Outcome of Patients With HLH Who Underwent Allogeneic Hematopoietic Stem Cell Transplantation

No. pts	Median age at SCT (months)	FH (%)	Major conditioning regimen	Donor	Source	OS (%)	Engraft. (%)	Causes of death	Refs.
9	13	45	Myeloab	VP16/BU/CY ± anti-LFA1	MRD/MMRD/haplo	BM	44.0	100	TR, HLH	[24]
29	NR	48	Myeloab	NR	MRD/MUD/haplo	BM	66.0	72	TR, HLH	[25]
20	9	30	Myeloab	VP16/BU/CY ± ATG	MSD/URD (80%)	BM	45.0	90	TR, HLH	[26]
14	14	36	Myeloab	VP16/BU/CY, ATG/BU/CY	MMRD/MUD	BM (T cell depleted)	64.3	65	TR, HLH	[27]
12	18	42	Myeloab	VP16/BU/CY	MSD/URD (67%)	BM	100	100	No	[33]
17	NR	NR	Myeloab	VP16/BU/CY ± ATG, TBI	MRD/URD/haplo	BM, CB (2), PB, CD34	58.0	94	TR, HLH, lymphoma	[8]
65[a]	13	31	Myeloab	VP16/BU/CY ± ATG	MRD/URD/haplo	BM, CB (5), PB, CD34	62.0	89	TR, HLH, AML	[21]
86[a]	13	34	Myeloab	VP16/BU/CY ± ATG, TBI	MRD/URD/haplo	BM, CB (7)	64.0	90	TR, HLH, 2nd AML	[28]
48	6	35	Myeloab	VP16/BU/CY, ATG/BU/CY	MSD/URD/haplo	BM, PB	58.5	78	HLH	[29]
12	14	17	RIC	FLU/MEL ± BUS, FLU/2GyTBI	MRD/URD/haplo	BM, CD34	75.0	100	TR	[31]
91	12	NR	Myeloab	VP16/BU/CY ± ATG	URD	BM, PB, CB (9)	45.0	83	TR, HLH	[30]
61	13	20	RIC (18%)	VP16 or MEL/BU/CY ± ATG	MRD/MMRD/URD	BM, PB, CB (6)	63.9	78	TR (68%), HLH (27%)	[32]
42	17	55	RIC (26%)	VP16/BU/CY ± ATG, TBI	MRD/MMRD/URD	BM, PB, CB (21)	69.0	78	TR (79%), HLH (21%)	Ours

AML, acute myelogeneous leukemia; BM, bone marrow; BU, busulfan; CB, cord blood; CY, cyclophosphamide; FHL, familial hemophagocytic lymphohistiocytosis; FH, family history; FLU, fludarabine; MEL, melphalan; MMRD, HLA-mismatched related donor; MRD, HLA-matched related donor; MSD, HLA-matched sibling donor; MUD, HLA-matched unrelated donor; NR, not recorded; PB, peripheral blood; RIC, reduced intensity conditioning; TBI, total body irradiation; TR, transplantation-related events; URD, unrelated donor; VP16, etoposide. [a]Sixty four of 65 patients studied by Henter et al. [21] were included in 86 patients by Horne et al. [28].

or introducing appropriate RIC regimens in young infants. In UCBT, a major obstacle was thought to be early graft failure, but once engrafted no late graft failure could not be seen [29]. We confirmed this finding in our UCBT cases.

Dürken et al. [33] reported that six HLH patients with CNS disease underwent allogeneic BMT and three of them had no persistent neurological problems after transplant. More recently, SCT is thought to be preferable for FHL patients at the early stage of CNS disease with variable presentation [34,35]. Fludarabine-based RIC has been preferred in SCT for FHL patients in order to reduce late sequelae [36,37]. Since CNS disease itself had no impact on the OS in the current study, but nearly half of the long-term survivors of FHL had late sequelae associated with growth and development, further prospective studies should be focused on how to reduce late sequelae in SCT for FHL patients.

In the treatment of refractory EBV-HLH, no consensus has yet been reached concerning the treatment of patients who fail to respond to the HLH-2004 protocol type immunochemotherapy. Several reports documented that SCT led to a complete remission in such cases [8,10,11,28,38,39]. The present study revealed that use of pre-SCT combination chemotherapy might be associated with a better therapeutic impact on subsequent SCT in patients with EBV-HLH. Furthermore, long-term survival, that is, a probable cure, could be obtained even after autologous SCT [22] or identical twin donor BMT, suggesting that a reconstitution of allogeneic hematopoietic stem cells was not essential in the successful SCT for EBV-HLH patients as described in the autologous PBSCT success for lymphoma-associated HLH [40]. In addition, long-term survival even after graft failure or post-transplant relapse in EBV-HLH patients might suggest the possibility of resetting the adaptive immune response to the virus as postulated in autologous SCT for the treatment of autoimmune diseases [41,42]. Moreover, successful syngeneic SCT may imply that EBV-HLH is not a monogenic disease, since Chen et al. [43] observed that a primary infection of EBV incited HLH in a pair of the twins, but not in the identical twin counterpart. These observations implied that the genetic influence in patients with EBV-HLH might be distinct from that in patients with FHL on precipitating the excessive immune activation. Further prospective studies should therefore be directed toward not only the optimization of UCBT-RIC to improve survival of FHL patients, but to better understanding of the pathological interaction between cytotoxic granule disorders and EBV.

ACKNOWLEDGMENT

We thank all contributors of the Japanese Society of Pediatric Hematology who participate in the treatment of HLH patients (Supplemental Table). This work was supported in part by a Grant-in-Aid for Scientific Research (C) #19591255 (O.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a fund of the HLH/LCH Committee in the Japanese Society of Pediatric Hematology. We thank Dr. Brian Thomas Quinn (Associate Professor, Department of Linguistic Environment, Faculty of Languages and Cultures, Kyushu University) for kindly correcting the manuscript.

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