Five cases of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in adult

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Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) is more common in children, and is characterized by pancytopenia, liver dysfunction and coagulopathy caused by interactions between EBV-infected T cells and activated macrophages. We describe here five adults with EBV-HLH. The median age was 17 years (range 16～40). HLH developed in 4 patients within 2 months after the primary infection, and in the other one during the reactivation. All patients had a high EBV viral load in peripheral blood ($2\times10^2-3\times10^6$ copies/ml) and monoclonal proliferation of EBV-infected T cells. All patients received immunosuppressive therapy with or without etoposide, and two patients required plasmapheresis due to the severity. Three patients are alive in complete remission (follow up periods; 13, 19, 30 months), while two patients became refractory to chemo-immunotherapy and died despite multidrug chemotherapy. EBV-HLH should be more widely recognized in adults in order to achieve early diagnosis and appropriate treatment.

プライマリ・ケア医に必要な血液疾患の知識

血球貪食症候群

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プライマリ・ケアにおけるポイント

こんな所見があれば本症を疑う

- 抗菌薬に不応の高熱が持続し，血球減少，播種性血管内凝固および肝脾腫がみられたら，血球貪食症候群を疑う．
- 高フェリチン血症，低コレステロール血症，高トリグリセリド血症，血清可溶性IL-2受容体の上昇が特徴的で，骨髄に血球貪食組織球が増加する．
- まれな単一遺伝子病である原発性と，感染症，膠原病および悪性リンパ腫に合併する続発性に分けられる．
- 小児ではEBウイルス関連，成人ではリンパ腫関連が多い．

紹介する際の注意

- 播種性血管内凝固の進行が予後を左右する．
- 感染症関連では自然軽快するものもあるが，EBウイルス関連ではエトポシドを必要とする重症例が多い．
- 膠原病や自己免疫疾患に伴うものにはシクロスポリンが有用である．
- 原発性では同種造血細胞移植が根治療法となる．
- 本症を疑ったら，早急に検査を進め血液専門医へ紹介することが望ましい．

はじめに

血球貪食症候群(HPS)は，単一遺伝子病として，また感染症，膠原病や悪性腫瘍の合併症として発症する[1]．患者は高熱が持続し，急速に播種性血管内凝固(DIC)が進行するため，本症を疑ったら早期の治療介入が必要である．HPSの共通病態となる，免疫の過剰活性化をきたす疾患と誘因は多様である．続発性の臨床像は，自然軽快する軽症例から造血細胞移植の必要な重症例まで幅広いため，疫学を理解し，治療と平行しながら原疾患の診断を進めていく．

本稿では，HPSの歴史と疫学を紹介し，診断および治療方針について概説する．

I 疾患概念の変遷

歴史的には，Scottらが1939年に網内系組織球症として報告した6例にさかのぼる(表1)．家族性HPS(FHL)はFargularらの1952年の報告に始まる．Rappaportらは1960年代に，病理の立場から悪性組織球症/細網症の概念を提唱したが，成人のリンパ腫関連HPSが主体であったと推測される．1983年，RisdallらがVAHSを報告して感染症関連HPSが注目されるようになった．EBウイルス(EBV)など特異抗体価による感染症診断の進歩が背景にあったと思われる．Henterらは1991年にHLHの診断ガイドラインを，1994年に治療プロトコールを提唱した．一方，自己免疫疾患では全身性紅斑性狼瘡(SLE)の増悪などに伴うマクロファージ活性化症候群(MAS)が，HPSと同じ病態として注目されるようになった．EBV-HLHは2000年ごろにEBV DNAの定量PCRが普

及して解析が進んだ．河ら日本のグループが主に報告してきたEBV関連T/NKリンパ増殖症(LPD)の概念は，2008年のWHOリンパ腫分類で全身型EBV陽性Tリンパ増殖症とされている[2]．1999年以降，FHLの原因遺伝子が次々と同定された．現在，HPSはHLHと同義で，その概念はFHLによる**原発性**とEBVやリンパ腫関連による**続発性**に整理される．

II 基礎疾患と誘因

HPSを，基礎疾患と誘因から「原発性・遺伝性」と「続発性・後天性」に分類する(図1)．

原発性HPSは本来，原因不明のすべてのHPSを表す．以前は，原発性HPSと診断せざるを得なかった「家族歴のない第1子HPS」もより正確に鑑別されるようになった．成人発症FHLも見つかり，

表1　HPSにおける疾患概念の変遷

疾患概念	報告(報告年)
histiocytic medullary reticulosis (HMR)	Scott & Robb-Smith (1939)
familial hemophagocytic reticulosis (FHR)	Fargular & Clairreaux (1952)
malignant reticulosis, malignant histiocytosis (MH)	Merkiw 1964, Rappaport (1966)
virus-associated hemophagocytic syndrome (VAHS) cf. bacteria-(BAHS), infection-(IAHS)	Risdall (1979)
lymphoma-associated hemophagocytic syndrome (LAHS) cf. malignancy-(MAHS)	Jaffe (1983)
hemophagocytic lymphohistiocytosis (HLH)	Henter (1991)
macrophage activation syndrome (MAS)	Stephan (1993)
autoimmune-associated hemophagocytic syndrome (AAHS)	Kumakura (2001)
EBV-associated T/NK-cell lymphoproliferative disease	Kawa (2002)
systemic EBV-positive T-cell LPD (EBV-HLH, CAEBV in Asia)	Cohen (2008)
identification of FHL genes Perforin, Munc13-4, Syntaxin11, STXBP2	(1999, 2003, 2004, 2009)

EBV：Epstein-Barr virus, LPD：lymphoproliferative disease.

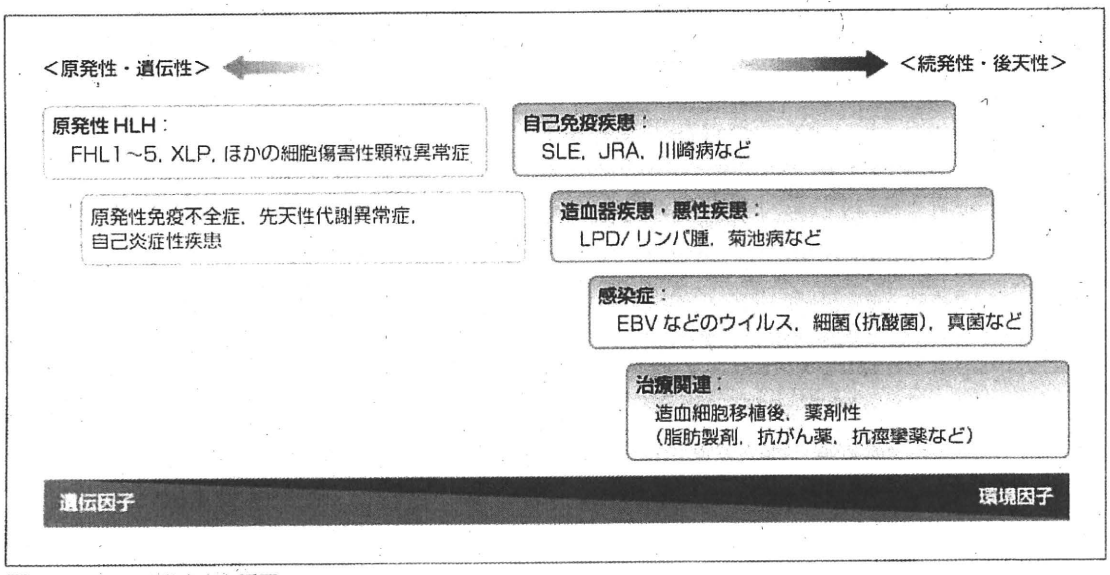

図1　HPSの基礎疾患と誘因

EBV-HLH成人例の報告も増加している．FHL責任遺伝子の機能は，perforinなど細胞傷害性顆粒代謝に関与し，ほかの顆粒異常症(Griscelli症候群など)にもHPS発症が確認され，現在，これらを包括して原発性HLHと呼ぶ[3]．HPSは，IL-2Rα鎖異常症，γC鎖異常症などの細胞性免疫不全症にも合併するが，その機序は明らかでない．また，リジン尿性タンパク不耐症など一部の先天性代謝異常症との関連も古くから知られているが，この発症機序も不明である．

続発性には，脂肪製剤・抗痙攣薬などによる薬剤性のものがある．近年，造血幹細胞移植後の生着期に発症するHPSも注目されている．感染症は後天性HPSの代表であるが，原発性であるX連鎖リンパ増殖症やChediak-Higashi症候群では，EBV感染の重症化(致死性伝染性単核球症，accelerated phase)が知られていた．FHLもウイルス感染が発症誘因となる．Perforin欠損マウスは，特異病原体のない飼育環境ではFHL2モデルとして認識されにくい．EBV-HLHの宿主因子もまだ見つかっていない．臨床報告の蓄積が，発症における宿主と感染の関係を明らかにするであろう．

Ⅲ 病　態

HPSはリンパ球とマクロファージの過剰な活性化が持続し，制御不能なサイトカイン過剰産生に陥る**高サイトカイン血症が共通病態**である．活性化した組織球は血球を貪食し，臓器に浸潤する．多彩な基礎疾患と誘因から，これに至る過程は十分明らかでないが，原発性と続発性についてFHLとEBV-HLHを例に説明する(図2)．

FHLは，現在5型(FHL1：9番染色体連鎖，FHL2：*PRF1*変異，FHL3：*UNC13D*変異，FHL4：*STX11*変異，FHL5：*STXBP2*)に細分類される．患者の10～30%の原因遺伝子はまだ不明である．細胞傷害性T細胞はperforinなどの顆粒を有し，感染細胞や腫瘍細胞を標的とする．また，役割の終わった活性化T細胞や抗原呈示細胞も標的として免疫の活性化を調節する．したがって，細胞傷害性顆粒の産生から分泌までの過程に異常がある

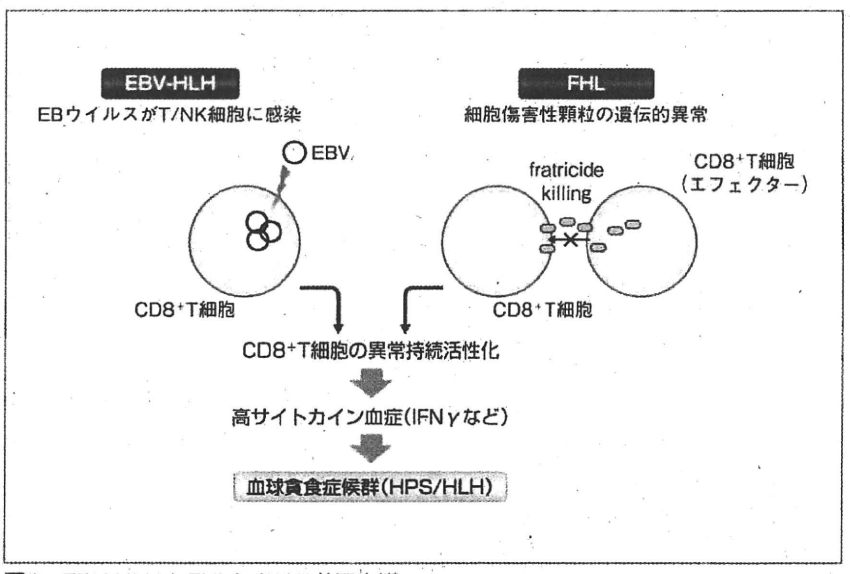

図2　EBV-HLHとFHLにおける共通病態

と，活性化した免疫細胞が制御されない．感染細胞の排除障害も活性化の持続に関与する．一方，EBV-HLHでは，EBV感染$CD8^+$T細胞がクローン増殖する．感染細胞はEBV特異的LMP1を発現する．これはNFκBを介しTNFα産生を誘導して血管内皮を傷害するとともに，TNFαR1を介して感染細胞自身のアポトーシスを抑制し，感染T細胞の持続活性化をきたす[4]．

FHLもEBV-HLHも，活性化$CD8^+$T細胞がIFNγを過剰に産生し，高サイトカイン血症を引き起こす．T細胞はIFNγやTNFαを産生し，単球・マクロファージからIL-1β，IL-6，TNFα産生を誘導する．マクロファージはIL-12/18も産生し，Th1細胞とNK細胞からIFNγ関連ケモカインを誘導する．活性化リンパ球からsIL-2RやsFas/sFasLも過剰に放出される．Th2サイトカインのIL-10も増加するが，調節性T細胞やTh17の関与はまだ明らかでない．

Ⅳ 診断ガイドラインの注意点

HPSの診断ガイドラインを示す(**表2**)[5]．❶または❷の5項目を満たせば診断する．各項目は基礎疾患を反映する．脾腫はFHLとEBV-HLHに顕著である．血球減少は2系統以上を有意とするが，川崎病，JIA・SLE，また造血細胞移植後は血球数の動態から判断する．

診断には疫学情報が重要である．**図3**にHPSの基礎疾患と発症年齢について，全国調査(n＝469)の結果を示す[6]．感染関連が64％を占め，その55％がEBV関連であった．年齢別では，15歳未満が52％，その半分はEBV-HLHでFHLはすべて小児例であった．青壮年例の1/3～半数はリンパ腫関連で，T/NK細胞性が優位であった．60歳以上は18％，その2/3はリンパ腫関連(主にB細胞由来)であった．**EBVとリンパ腫**が日本のHPSにおける主な背景因子である．EBV-HLHのT/NK細胞感染は欧米でも確認されるようになった[7]．海外ではデング熱やリーシュマニアによる例もある．

FHL患児に奇形徴候はない．ほかの原発性のうち，Griscelli症候群では白子，Hermansky-Pudlak症候群では眼皮膚白皮症と小頭症，Chediak東症候群では白血球異常顆粒が特徴である．

表2　HPS/HLHの診断ガイドライン

以下の❶または❷のいずれかを満たせばHLHと診断する

❶FHLに一致した遺伝子異常(*PRF1*，*MUNC13-4*，*STX11*，*STXBP2*)を有するか，家族歴あり

❷以下の8項目のうち5つ以上を満たす
1)発熱≧38.5℃
2)脾腫あり
3)血球減少(末梢血3系統のうち少なくとも2系統に以下の異常があること)：Hb＜9.0g/dL，血小板＜100,000/μL，好中球＜1,000/μL
4)高TG血症(空腹時≧265mg/dL)または低フィブリノーゲン血症(≦150mg/dL)
5)NK細胞活性低値または欠損
6)血清フェリチン≧500ng/mL
7)可溶性IL-2受容体≧2,400U/mL
8)骨髄，脾臓，またはリンパ節に血球貪食像あり，悪性所見なし

付記1)：診断基準には含まれないが，診断に有用な所見：(a)髄液細胞増多(単核球)かつ/または髄液蛋白増加，(b)肝で慢性持続性肝炎に類似した組織像(生検にて)．

付記2)：診断を示唆するほかの所見：髄膜刺激症状，リンパ節腫大，黄疸，浮腫，皮疹，肝酵素上昇，低蛋白血症，低Na血症，VLDL値上昇，HDL値低下．

注意1：遺伝子異常の確定には日時を要するので，実際上は蛋白発現解析で異常があれば遺伝子異常ありとみなしてよい．

注意2：症例によっては発症時に上記の基準をすべて満たすわけではなく，経過とともにいくつかを満たすことが少なくない．したがって，診断基準を満たさない場合は注意深く観察し，基準を満たした(同時期に症状・所見が揃った)時点で診断する．

注意3：FHLの発症は通常乳児期であるが，思春期や成人でも報告されている．二次性HLHはすべての年齢でみられる．

(文献9)より改変)

V 診断と治療における包括的アルゴリズム

診断と治療方針の流れを図4に示す．発熱とDICの進行が病勢を反映する．HPSを疑ったら，診断項目(表2)をよく検討し，原発性か続発性か，何による続発性かを考える．鑑別すべき疾患が多いため，可能な**検査を早急に進めながら血液専門医へ**紹介する．感染症関連では無治療軽快例もあるが，EBV-HLHは重症化することが多い．

血清フェリチン高値の特異性は高い．sIL-2R，IFNγなどが血中に増加する．高トリグリセリドかつ低コレステロール血症がみられる．IFNγ産生を示す血中および尿中のβ_2-ミクログロブリン，ネオプテリンもマーカーとなる．血清リゾチーム，アンギオテンシン転換酵素なども組織球活性化の指標である．空包化した成熟組織球と血球貪食組織球が骨髄・脾・リンパ節，末梢血や脳脊髄液に出現する．血球貪食組織球は骨髄スメア標本1枚当たり少なくとも2個以上．成熟組織球は骨髄有核細胞の3%または2,500細胞/μL以上が有意と

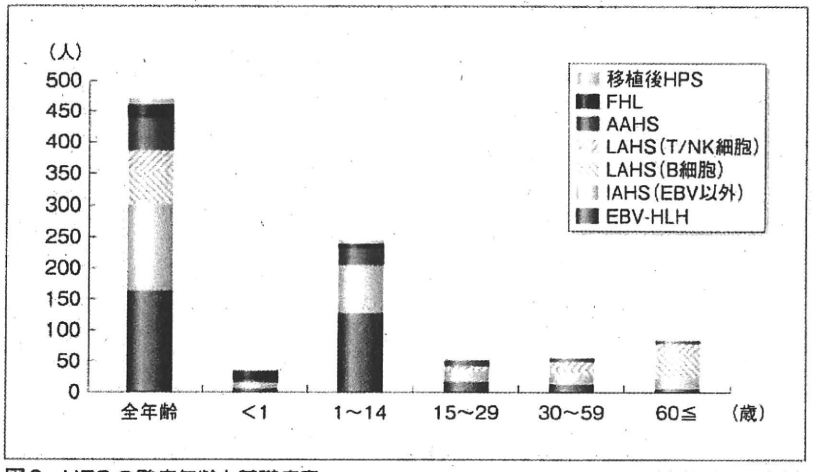

図3 HPSの発症年齢と基礎疾患 (文献6)より改変)

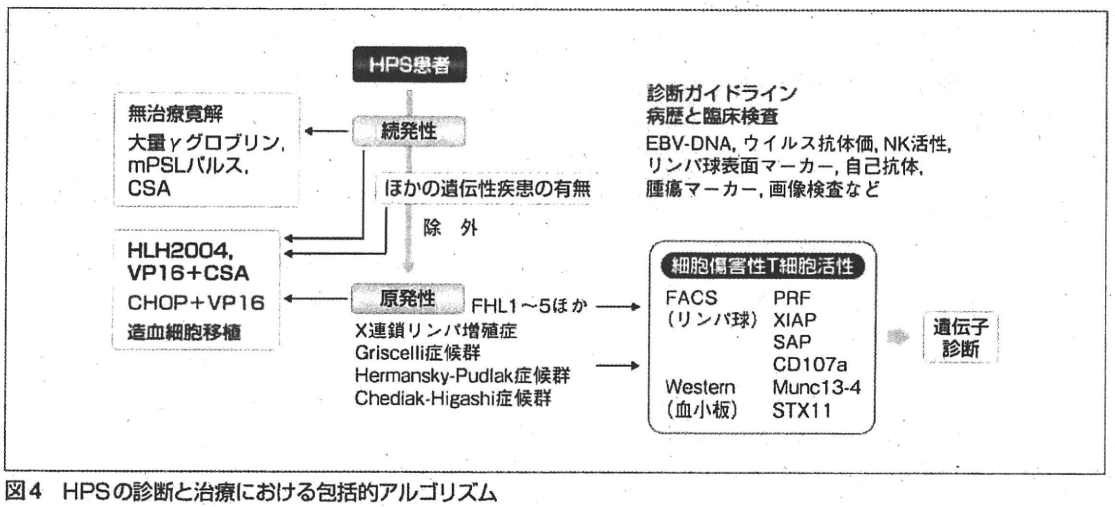

図4 HPSの診断と治療における包括的アルゴリズム

されるが，定量的評価は難しい．EBV-HLH患者には顆粒リンパ球増多をみることがある．

感染症は培養，ウイルス抗体価，遺伝子検査などから評価する．EBV-HLHの診断に，EBV DNA定量が有用である．カットオフ価が問題であるが，スクリーニングには全血法を用いる．EBV抗体価は初感染・再活性化のいずれのパターンもある．感染細胞の同定は，重症伝染性単核球症と慢性活動性EBV感染症の鑑別に有用である．EBV-HLHではCD8$^+$T細胞優位な感染とクローン増殖が確認される．原発性HPSではNK活性が著減するが，膠原病関連でもNK活性が低下する．CTL活性の検討には細胞株樹立が必要である．フローサイトメトリーにて活性化T細胞を評価する．

原発性診断の特殊検査として，フローサイトメトリーによるperforinのCD8/56細胞内発現およびWestern blotによる血小板のMunc13-4とSTX11発現解析がある．CD107a発現はSTXBP2も含めたスクリーニングとなろう．血球が保たれる時期にこれらを解析し，遺伝子診断を進める．

急性期の出血と臓器不全に注意し，適切な免疫化学療法を行う．膠原病関連にはシクロスポリンが有効である．感染が否定できない場合は大量γグロブリンから開始する．大量ステロイド療法はリンパ腫の有無を確認して使用したい．不応な場合は速やかにエトポシドを開始する．HLH-2004プロトコールに反応すれば8週以内に治療を中止する．EBV-HLHとEBV関連リンパ腫の鑑別が難しい．原発性には同種造血細胞移植を計画するが，造血細胞移植が必要なEBV-HLHはきわめてまれである．EBV-HLHとFHLのわが国の小児の移植後10年全生存率はそれぞれ86％と66％である[8]．臍帯血移植の成績が比較的良好ではあるが，さらに方法について検討が必要である．

おわりに

FHLは遺伝的な細胞傷害活性不全から，EBV-HLHは感染細胞の持続活性化から，高サイトカイン血症に至りHPSを発症する．一方，発症機序の不明なHPSも多い．新生児から高齢者まで全年齢で発症するHPSを的確に診断し，治療することは難しい．診断のための特殊検査を集約化し，造血細胞移植の可能な施設へ速やかに搬送するシステムを血液内科・小児科で整備する必要がある．

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Subtypes of Familial Hemophagocytic Lymphohistiocytosis in Japan Based on Genetic and Functional Analyses of Cytotoxic T Lymphocytes

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Abstract

Background: Familial hemophagocytic lymphohistiocytosis (FHL) is a rare disease of infancy or early childhood. To clarify the incidence and subtypes of FHL in Japan, we performed genetic and functional analyses of cytotoxic T lymphocytes (CTLs) in Japanese patients with FHL.

Design and Methods: Among the Japanese children with hemophagocytic lymphohistiocytosis (HLH) registered at our laboratory, those with more than one of the following findings were eligible for study entry under a diagnosis of FHL: positive for known genetic mutations, a family history of HLH, and impaired CTL-mediated cytotoxicity. Mutations of the newly identified causative gene for FHL5, *STXBP2*, and the cytotoxicity and degranulation activity of CTLs in FHL patients, were analyzed.

Results: Among 31 FHL patients who satisfied the above criteria, *PRF1* mutation was detected in 17 (FHL2) and *UNC13D* mutation was in 10 (FHL3). In 2 other patients, 3 novel mutations of *STXBP2* gene were confirmed (FHL5). Finally, the remaining 2 were classified as having FHL with unknown genetic mutations. In all FHL patients, CTL-mediated cytotoxicity was low or deficient, and degranulation activity was also low or absent except FHL2 patients. In 2 patients with unknown genetic mutations, the cytotoxicity and degranulation activity of CTLs appeared to be deficient in one patient and moderately impaired in the other.

Conclusions: FHL can be diagnosed and classified on the basis of CTL-mediated cytotoxicity, degranulation activity, and genetic analysis. Based on the data obtained from functional analysis of CTLs, other unknown gene(s) responsible for FHL remain to be identified.

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Introduction

Hemophagocytic lymphohistiocytosis (HLH) is characterized by fever and hepatosplenomegaly associated with pancytopenia [1–3]. Histologically, infiltration of lymphocytes and histiocytes with hemophagocytic activity is evident in the reticuloendothelial system, bone marrow, and central nervous system [4]. HLH can be classified as either primary or secondary [5]. Primary HLH, also known as familial hemophagocytic lymphohistiocytosis (FHL), is inherited as an autosomal recessive disorder that usually arises during infancy.

The pathogenesis of FHL has been considered to involve dysfunction of cytotoxic T lymphocyte (CTL) activity, leading to excessive production of inflammatory cytokines and macrophage activation [6]. The genetic mutations responsible for FHL have been identified by various methods. Linkage analysis has indicated two possible loci: FHL1 (MIM 603552) in 9q21.3-22, and FHL2 (MIM 603553) in 10q21-22 [7,8]. In 1999, a mutation in the *perforin* gene (*PRF1*) was identified as the cause of FHL2 [9–12]. Further genetic mutations of the *Munc13-4* gene (*UNC13D*) mapped to 17q25 (the cause of FHL3, MIM 608898) and the *syntaxin11* gene (*STX11*) mapped to 6q24 (the cause of FHL4, MIM 603552) were subsequently identified [13–15]. These mutations affect proteins involved in the transport and membrane fusion, or exocytosis, of perforin contained in cytoplasmic

granules. Recently, mutations of the *Munc18-2* gene (*STXBP2*), located in 19q, were detected as a cause of FHL5 [16,17]. Munc18-2 regulates intracellular trafficking and controls the soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) complex.

The molecular mechanisms underlying vesicular membrane trafficking and regulation of exocytosis have been clarified in recent years. The final step of vesicle transport is mediated by a bridge between a vesicle and its target membrane through formation of a ternary complex between a vesicle-SNARE (v-SNARE), such as a VAMP, and a target membrane-SNARE (t-SNARE), such as a syntaxin11 or a member of SNAP23/25/29 [18]. The SNARE complex is composed of three molecules: VAMP, syntaxin and SNAP23/25/29. Syntaxin11, in association with SNAP23, localizes to the endosome and trans-Golgi network [19]; however, the precise biological functions of the SNARE system are still poorly understood. Recent evidence suggests that members of the SNARE family mediate fusion of cytotoxic granules with the surface of CTLs. Syntaxin11, SNAP23 and VAMP7 are prime candidates for functioning as SNAREs in this fusion event [20].

It has been considered that clarification of the molecular abnormalities in FHL might shed light on the mechanisms of CTL-mediated cytotoxicity. Accordingly, we have been studying the functional abnormalities of CTLs in Japanese patients with FHL [21]. Our previous studies have shown that the FHL2 and FHL3 subtypes account for 20–25% of all FHL cases, respectively, whereas no FHL4 subtype exists; therefore, 45–50% of FHL cases in Japan harbor still unknown genetic mutations [21,22]. However, secondary HLH could be involved in patients with unknown genetic mutations, because both FHL and secondary HLH share similar clinical and laboratory characteristics. Therefore, in the present study aimed at clarifying the incidence and subtypes of FHL in Japanese children by genetic and functional analyses of CTLs, only patients positive for known genetic mutations, a positive family history of HLH, or impaired natural killer (NK)/CTL-mediated cytotoxicity were diagnosed definitively as having FHL.

Materials and Methods

Patients

A total of 87 Japanese children aged <15 years diagnosed as having HLH based on the diagnostic criteria of the Histiocyte Society [23] were registered at our laboratory between January 1994 and December 2009. Among them, 40 were excluded from analysis because they were diagnosed as having secondary HLH, or their parents did not provide permission for use of clinical samples. None of the patients had Chediak-Higashi syndrome, Griscelli syndrome, or Hermansky-Pudlak syndrome type 2, based on clinical and laboratory findings, including albinism or the presence of gigantic granules in lymphocytes or granulocytes. A final total of 31 patients, who met the diagnostic criteria for FHL, and for whom documented informed consent had been obtained in accordance with the Declaration of Helsinki, were entered into the study.

Genetic analysis of the *STXBP2* gene

For the detection of *STXBP2* mutations, genomic DNA was isolated from a T-cell line established from each patient. Genomic DNA (5 ng) was subjected to PCR using the primers listed in Table S1. These primer sets were designed to amplify 19 exons including the 5′-untranslated region and the coding regions with the exon-intron boundaries of *STXBP2*. The PCR products were treated with ExoSAP-IT (GE Healthcare Bio-Sciences, Little Chalfont, England) by incubation at 37°C for 15 minutes to inactivate the free primers and dNTPs, and then subjected to sequencing reactions using forward or reverse primers and BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA). The DNA fragments were purified using Magnesil (Promega, Madison, WI), and sequencing was carried out with an ABI 3730 Genetic Analyzer (Applied Biosystems). Sample sequences were aligned to reference sequences obtained from the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/index.html) using the ClustalW program in order to identify nucleotide changes. Mutations were numbered according to GenBank Reference Sequence NM_001127396.1; additionally, the A of the ATG initiator codon was defined as nucleotide +1. To identify splicing variants generated by c.88-1g>a mutation of *STXBP2*, total RNA was extracted from each patient's T-cell line and reverse transcriptase PCR (RT-PCR) was performed using the forward primer on exon 1 (5′-TTGGGACACACCCGGAAG-3′) and the reverse primer on exon 5 (5′-AAGAAGATATGGGCCGCTTT-3′). The PCR products were directly sequenced using the forward primer, as described above.

Western blot analysis of MUNC18-2 protein

Expression of Munc18-2 protein encoded by *STXBP2* in T-cell lines established from FHL patients and a healthy individual was analyzed by Western blotting. CTLs were harvested after 5 days of stimulation with allogeneic LCL cells. Cell lysates were then prepared by extraction with 1% NP-40, and the extracts (10 μg per lane) were analyzed by Western blotting with anti-Munc18-2 rabbit polyclonal antibody (LifeSpan BioSciences, Seattle, WA). Horseradish peroxidase-labeled anti-rabbit IgG polyclonal antibody was used as the secondary antibody with detection by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

Establishment of alloantigen-specific CTL lines

Alloantigen-specific $CD8^+$ CTL lines were generated as described previously [24,25]. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from FHL patients and unrelated healthy individuals. These cells were co-cultured with a mitomycin C (MMC)-treated B-lymphoblastoid cell line (B-LCL) established from an HLA-mismatched individual (KI-LCL). Using cell-isolation immunomagnetic beads (MACS beads) (Miltenyi Biotec, Auburn, CA), $CD8^+$ T lymphocytes were isolated from PBMCs that had been stimulated with KI-LCL cells for 6 days. $CD8^+$ T lymphocytes, cultured in RPMI 1640 medium supplemented with 10% human serum and 10 IU/ml interleukin-2 (Roche, Mannheim, Germany), were stimulated with MMC-treated KI-LCL cells 3 times at 1-week intervals; subsequently, these lymphocytes were used as $CD8^+$ alloantigen-specific CTL lines. The alloantigen specificity of the CTL lines was determined by assay of interferon-γ (IFN-γ) production in response to stimulation with KI-LCL cells, as described previously [24,25]. Briefly, 1×10^5 T lymphocytes were co-cultured with or without 1×10^5 MMC-treated B-LCL cells in 0.2 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a flat-bottomed 96-well plate. In some experiments, an anti-HLA class I monoclonal antibody (w6/32; American Type Culture Collection, Manassas, VA) was added to wells at an optimal concentration. After 24 hours, the supernatant was collected from each well and assayed for production of IFN-γ using an enzyme-linked immunosorbent assay (ELISA; ENDOGEN, Woburn, MA).

Analysis of CTL-mediated cytotoxicity

The cytotoxic activity of CTLs was measured by a standard ^{51}Cr-release assay, as described previously [21]. Briefly, alloantigen-specific CTLs were incubated with ^{51}Cr-labeled allogeneic

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

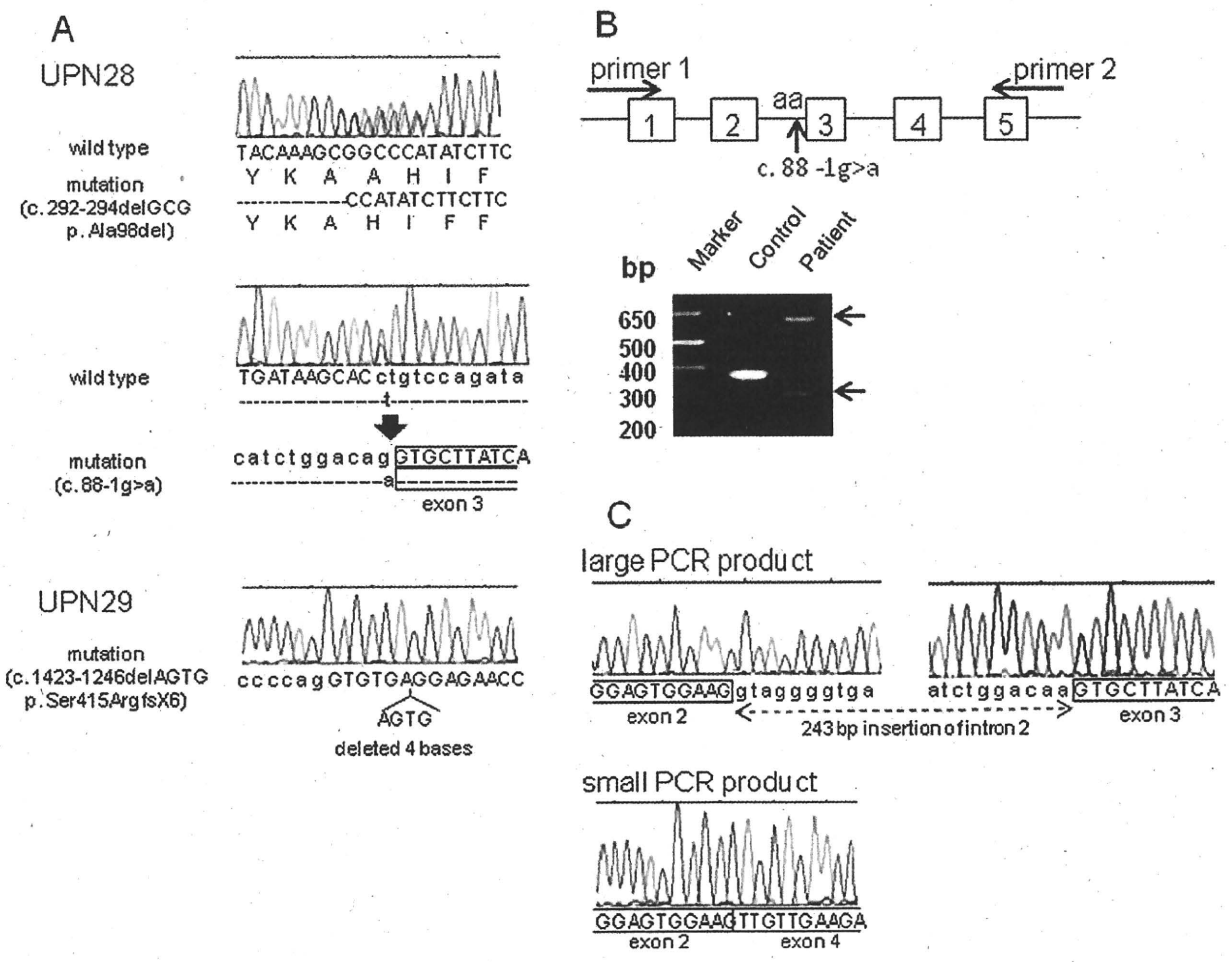

Figure 1. Identification of *STXBP2* mutations. (A) Sequencing analysis of 4 patients with non-FHL2/3/4 and detection of 3 novel mutations in 2 of them: a compound heterozygous mutation of 292_294delGCG resulting in Ala98del at exon 5 (upper panel) and 88-1g>a in intron 2 (lower panel) in one patient (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

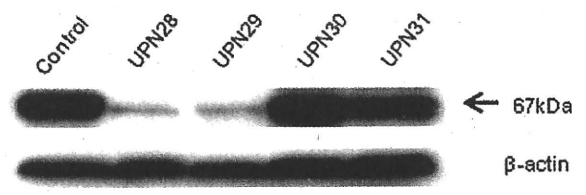

Figure 2. Western blot analysis of Munc18-2 protein expression. Expression of Munc18-2 protein in each $CD8^+$ T-cell line that had been stimulated with allogeneic B-LCL cells was analyzed by Western blotting using anti-Munc18-2 antibody. Munc18-2 protein was abundantly detected at 67 kDa in CTL lines 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,

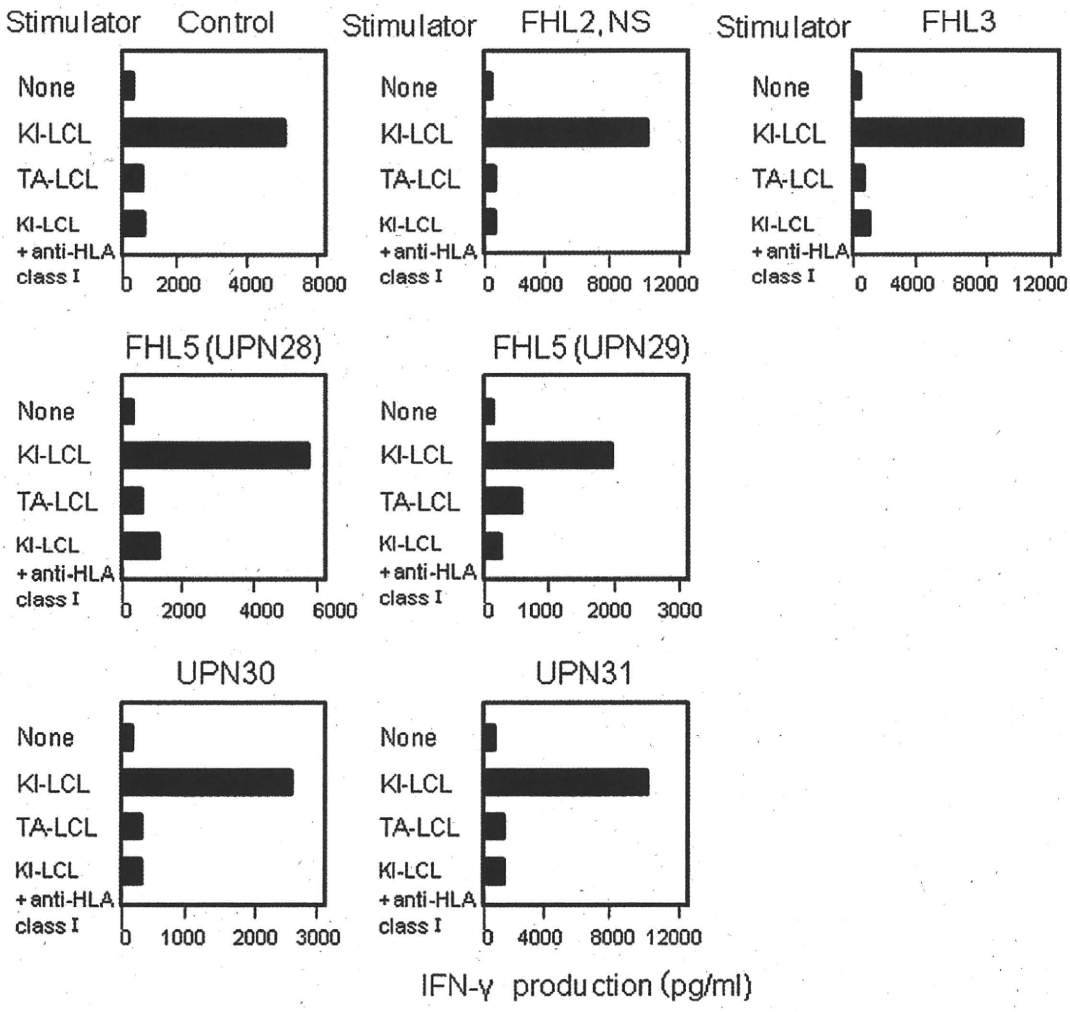

Figure 3. IFN-γ production by alloantigen-specific CD8$^+$ T cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Responder cells were co-cultured with or without KI-LCL or TA-LCL, 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.
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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

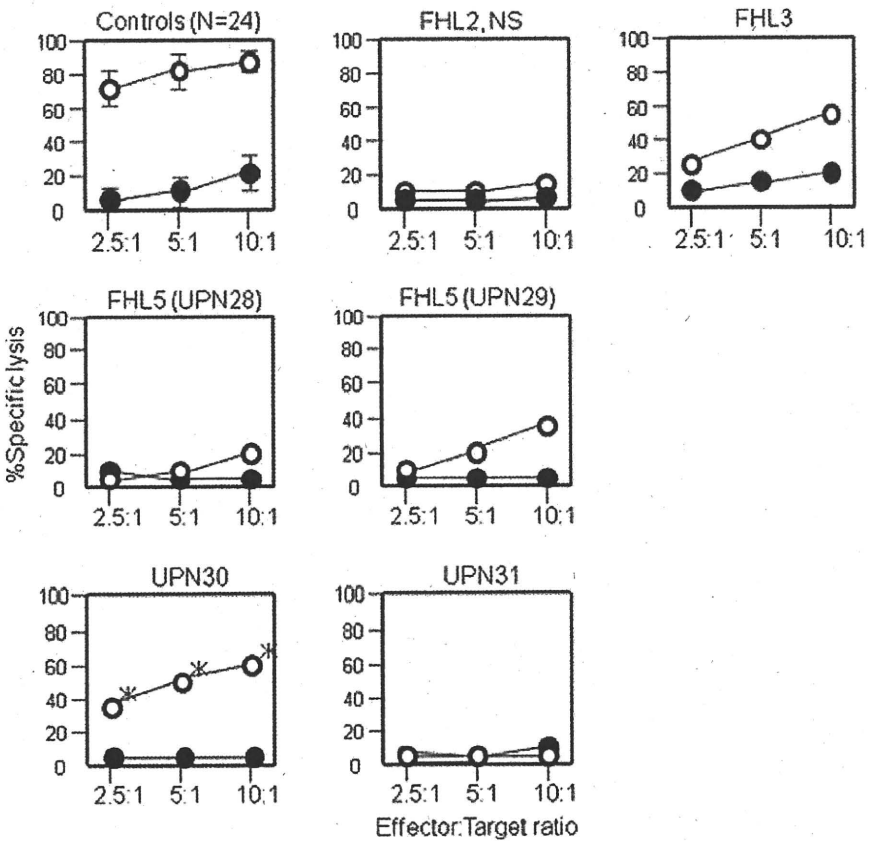

Figure 4. Cytotoxicity of alloantigen-specific CD8$^+$ T-cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and 24 healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Their cytotoxicity was determined against allogeneic KI-LCL (clear 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.
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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

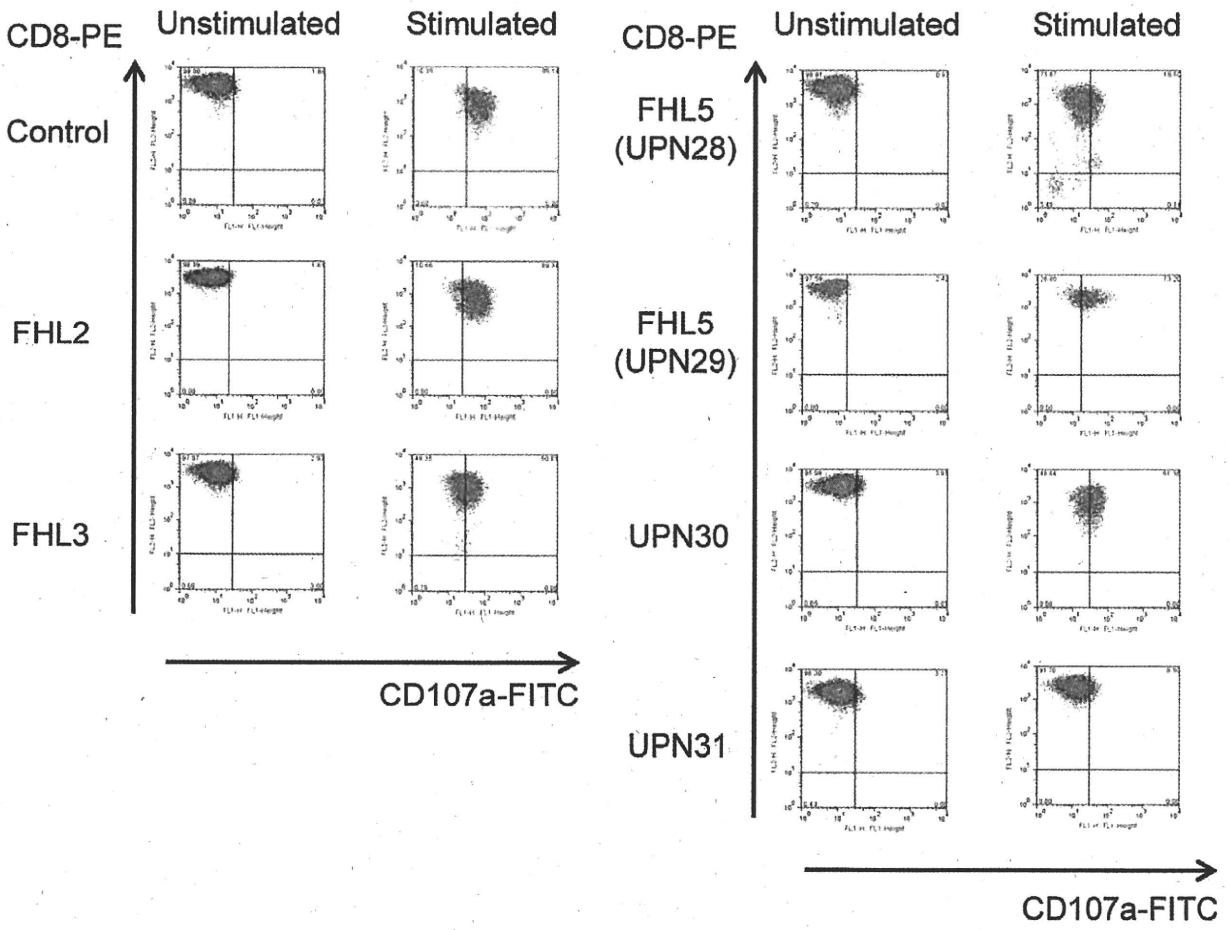

Figure 5. CD107a expression of alloantigen-specific CD8$^+$ T-cell lines. Flow cytometric analysis of CD107a expression was performed using CD8$^+$ T-cell lines generated from a healthy individual and FHL patients, as detailed in the text. Left panel of each column shows CD107a expression in CD8$^+$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

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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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