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CASE REPORT

Patient with neonatal-onset chronic hepatitis presenting with mevalonate kinase deficiency with a novel MVK gene mutation

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Abstract A Japanese girl with neonatal-onset chronic hepatitis and systemic inflammation was diagnosed with hyper-immunoglobulinemia D and periodic fever syndrome (HIDS). However, she lacked the typical HIDS features until the age of 32 months. She had compound heterozygous MVK mutations, H380R and A262P, the latter of which was novel. These findings suggest that HIDS patients could lack typical episodes of recurrent fever at the onset and that HIDS should be considered as a possible cause of neonatal-onset chronic hepatitis.

Keywords Autoimmune hepatitis · Hyper-IgD syndrome · Liver biopsy · MVK gene · Neonatal-onset chronic hepatitis

Abbreviations

HIDS	Hyper-immunoglobulinemia D and periodic fever syndrome
IgD	Immunoglobulin D
MVK	Mevalonate kinase
FMF	Familial Mediterranean fever
MEFV	Familial Mediterranean fever gene
AIH	Autoimmune hepatitis
CRP	C-reactive protein

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Introduction

Mevalonate kinase deficiency is an autosomal recessive metabolic disorder caused by mevalonate kinase (MVK) gene mutations. The disorder presents as a phenotypic spectrum in which mevalonic aciduria is the more severe form, with neurological complications, and hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) is the milder form. HIDS is characterized by recurrent febrile attacks, with lymphadenopathy, abdominal symptoms, skin eruptions, and joint involvement [1]. In this report, we describe a patient with a severe form of HIDS caused by a novel MVK mutation; the patient had presented with neonatal-onset chronic hepatitis that was temporarily diagnosed as autoimmune hepatitis (AIH). The lack of typical recurrent fever and rashes at the onset of the disease delayed the diagnosis of HIDS, which alerted the clinicians that HIDS could exist in patients with continuous inflammatory episodes even with atypical clinical courses.

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Case report

A Japanese girl was born at 36 weeks' gestation with a weight of 2,240 g. Her parents were non-consanguineous and the family history was unremarkable. At birth she had no symptoms, but physical examination revealed hepatomegaly (1.5 cm below the right costal margin) without splenomegaly. No jaundice, ascites, or coagulation abnormalities were present. Laboratory examinations showed increased white blood cell count (45,700/mm^3) and serum C-reactive protein (CRP) (15.8 mg/dl), as well as increased transaminase levels (aspartate aminotransferase [AST] 207 IU/l, alanine aminotransferase [ALT] 96 IU/l), lactate dehydrogenase (LDH) (6,575 IU/l), and biliary enzyme levels (γ-guanosine triphosphate [GTP] 61 IU/l). An increased immunoglobulin M level (53.0 mg/dl) caused us to work on congenital infections, with bacterial cultures of blood, cerebrospinal fluid (CSF), and gastric fluid, and determination of serum β D-glucan, and measurements of serum antibodies against cytomegalovirus, toxoplasmosis, syphilis, rubella, herpes simplex type I and type II, listeriosis, Epstein-Barr virus, adenovirus, hepatitis A and B and C viruses, *Chlamydia trachomatis*, and mycoplasma, all of which were negative. Radiographic work-up with computed tomography (CT), magnetic resonance imaging (MRI), and gallium scintigraphy, as well as bone-marrow aspirate examination, did not reveal any congenital neoplasm. A search for metabolic disorders by measurements of blood amino acids and urinary organic acids was negative.

Without any specific treatments, the hepatomegaly gradually increased, although abdominal MRI revealed diffuse inflammation of the liver. To explore further the cause of the hepatomegaly, a needle liver biopsy was performed at the age of 6 months. The biopsied liver specimen showed the presence of mild lymphocytic infiltration and fibrosing lesions in the portal area, and short septa extending from a slightly enlarged portal tract (Fig. 1a, b), which indicated a diagnosis of chronic hepatitis without specific causes.

At the age of 14 months, splenomegaly appeared, and elevated serum IgG (2,299 mg/dl) as well as anti-smooth muscle antibodies (1:160) were detected, which led us to diagnose the patient as having AIH [2]. The patient received methylprednisolone pulse therapy, followed by prednisolone and azathioprine for the presumed AIH [3]. Serum transaminase levels normalized in response to the treatment, although cervical lymphadenopathy, hepatosplenomegaly, and elevated serum CRP persisted.

The continuous elevation of CRP prompted us to consider autoinflammatory diseases; thus, we performed genetic analysis for familial Mediterranean fever (FMF), tumor necrosis factor (TNF) receptor-associated periodic syndrome, and cryopyrin-associated periodic syndrome, at the age of 26 months. After obtaining written informed consent from the parents and approval from the Institutional Review Board of Kyoto University, peripheral blood samples were collected from the patient and her parents for genetic analysis. The analysis was done by sequencing all the exons, including exon–intron junctions, which showed heterozygous L110P and E148Q missense mutations on the familial Mediterranean fever (MEFV) gene (Fig. 2a) without any mutations of the TNFRSF1A and NLRP3

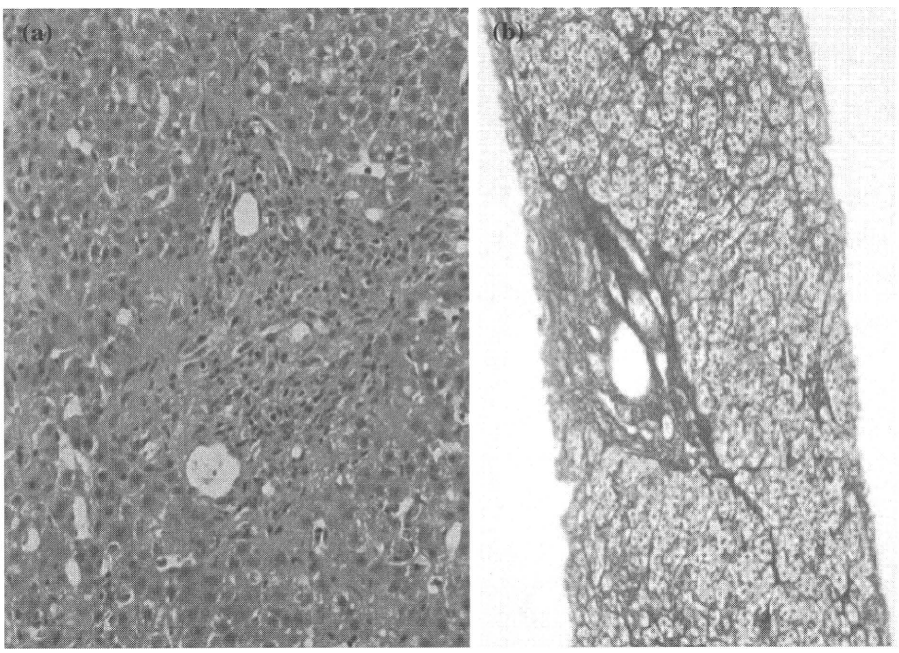

Fig. 1 Liver biopsy specimen showing chronic hepatitis. **a** The portal tract is infiltrated with lymphocytes (H&E, ×200). **b** Short septa extend from the slightly enlarged portal tract (reticulum, ×100)

genes. The L110P and E148Q mutations on MEFV were considered to be single-nucleotide polymorphisms (SNPs), based upon the prevalence of the mutations, as well as their weak association with FMF in Japan. Because none of periodic fever, rashes, gastrointestinal symptoms, or elevated serum IgD was observed at that time, the MVK gene was not examined.

The patient continued to show a good response to the AIH treatments, although tapering off the prednisone induced periodic fever with maculopapular rashes approximately once a month, shown for the first time at the age of 32 months. The fever episodes persisted for 3–5 days and the duration of the fever was reduced to 1–2 days by temporarily increasing the dose of prednisone. Serum CRP levels were around 20 mg/dl at the onset of fever, and 1–4 mg/dl in the asymptomatic period. The newly emerged clinical symptoms and the good response to the systemic steroid prompted us to consider HIDS. Full examination for HIDS showed: (1) elevated serum IgD (19.2 mg/dl) (control 0–9 mg/dl); (2) increased urinary mevalonic acid (49.1 μg/mg creatinine) (control 0.091 ± 0.028 μg/mg creatinine); and (3) a significant decrease in the mevalonate kinase activity of peripheral blood mononuclear cells (PBMCs; below the detection limit). Genetic analysis of the MVK gene revealed compound heterozygous mutations, A262P and H380R, the former of which was a novel mutation (Fig. 2b). The MVK mutations were not identified in 100 healthy Japanese controls. Finally we diagnosed the patient with HIDS, at the age of 6 years. We treated the patient with simvastatin (0.07 mg/kg/day), which was partially effective in reducing the frequency of the periodic fever. Although no decline in urinary mevalonic acid has been produced by simvastatin (33.7–107.8 μg/mg creatinine), the patient has had a benign course, without mental retardation or neurological impairments (Fig. 3).

To see if the patient's liver abnormalities were due to either AIH or HIDS, we performed an immunohistological analysis of the biopsied liver specimen. It was stained for CD68, and unstained for CD3 and CD79 (Fig. 4). These

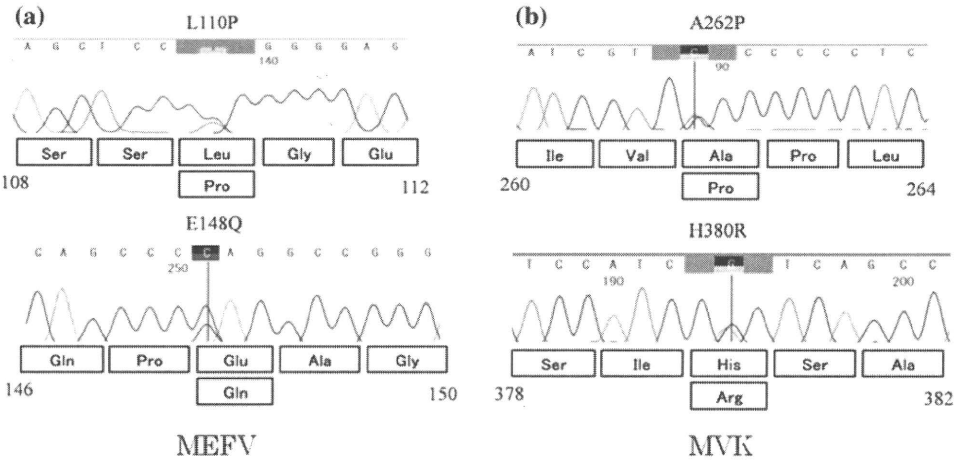

Fig. 2 Genetic analysis. **a** Genetic analysis of the MEFV gene. The patient had heterozygous amino acid changes of L110P and E148Q. **b** Genetic analysis of the MVK gene. The patient had heterozygous mutations of A262P and H380R

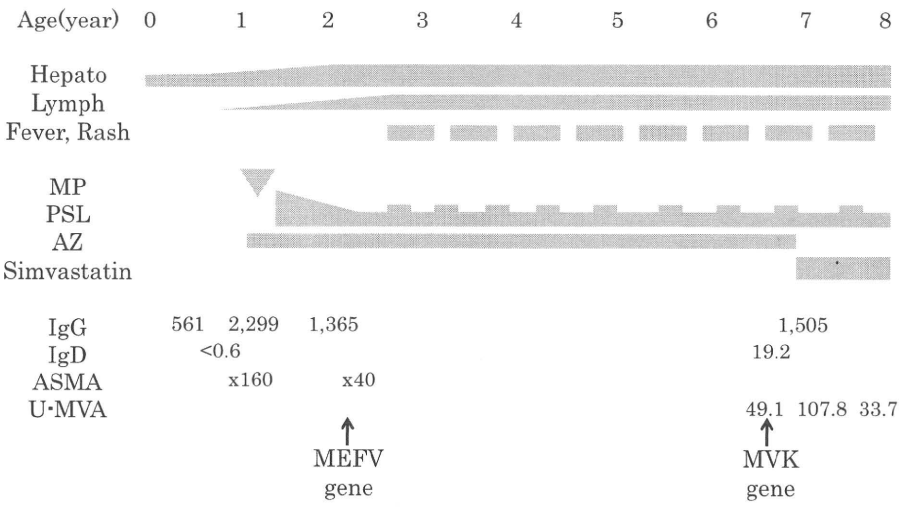

Fig. 3 Clinical course. *Hepato* Hepatosplenomegaly, *Lymph* cervical lymphadenopathy, *MP* methylprednisolone, *PSL* prednisolone, *AZ* azathioprine, *ASMA* anti-smooth muscle antibody, *U-MVA* urinary mevalonic acid (μg/mg creatinine)

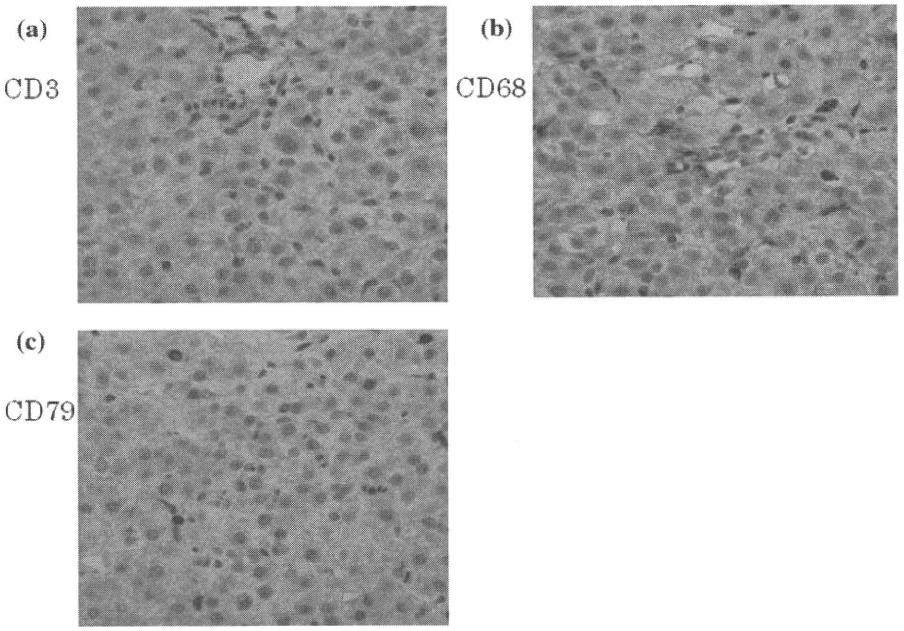

Fig. 4 Immunohistochemical analysis of the biopsied liver tissues. **a** CD3 (×400), **b** CD68 (×400), **c** CD79 (×400)

data led us to conclude that the hepatitis seemed to be a manifestation of HIDS, rather than resulting from an autoimmune response.

Discussion

We have reported here a Japanese girl who was diagnosed with HIDS by genetic analysis, as well as by laboratory examinations such as mevalonate kinase activity and urinary excretion of mevalonate. According to the report of the Japanese HIDS registry, the 4 most prevalent MVK mutations (V377I, I268T, H20P/N, and P167L) accounted for 71.5% of the mutations found. Our patient had a very rare genotype among the patients on the HIDS registry, as the H380R mutation was identified in 1.5% of the patients and A262P was a novel mutation. Because mevalonate kinase activity was below the detection limit, mevalonic aciduria could have been considered as the diagnosis in our patient. However, the mevalonic acid level in the urine was not as high as that reported for patients with mevalonic aciduria [4] and the clinical features of our patient lacked the neurological and developmental abnormalities that are distinctive signs of mevalonic aciduria. Thus, we concluded that the patient suffered from a severe form of HIDS, although we note that mevalonate kinase deficiency presents as a phenotypic continuum in which disease severity ranges from mevalonic aciduria to HIDS [5].

Serum transaminase levels in our patient were elevated since birth, which is relatively rare for HIDS, and liver biopsy showed chronic non-specific hepatitis [6]. Although the serum transaminase levels were improved by the treatment for AIH, the histological and immunohistochemical findings were not typical of AIH [6], which is a generally unresolving inflammation of the liver of unknown cause [7]. There have been some reports of HIDS patients with liver abnormalities. Topaloglu et al. [8] reported a similar case of HIDS in a patient who had neonatal hepatosplenomegaly without fever at the beginning, and they performed liver biopsy which showed portal fibrosis. Hinson et al. [9] reported two patients with mevalonate kinase deficiency who had neonatal hepatosplenomegaly and elevated transaminase levels; liver biopsy showed chronic active cholestatic hepatitis and portal fibrosis, respectively.

Neonatal hepatitis is a syndrome associated with a history that includes any type of infectious, genetic, toxic, or metabolic causation. Neonatal hepatitis is characterized by clinical and laboratory findings of liver dysfunction, particularly conjugated hyperbilirubinemia. In our patient, the clinical course in early childhood was not typical of neonatal hepatitis. But the clinical course in our patient suggests that it is important to include HIDS in the differential diagnosis of neonatal hepatitis or neonatal-onset chronic hepatitis.

Genetic analysis of autoinflammatory disease genes in our patient revealed 2 heterozygous amino acid changes, L110P and E148Q, in the MEFV gene, which were shared with the patient's asymptomatic mother. It has been reported that the allele frequency of E148Q in the Japanese population was high (16.38%), and both E148Q and L110P are considered as SNPs [10]. On the other hand, Touitou et al. [11] demonstrated that E148Q may have an exacerbating effect on FMF when it is part of complex alleles. In addition, there are other reports that mutations in 2 autoinflammatory

genes cause more severe diseases [8, 12]. Thus, the heterozygous E148Q and L110P amino acid changes in the MEFV gene may cause a more severe form of HIDS.

The name 'HIDS' was given to the disorder because of the observed elevation in serum IgD, before the identification of the disease-causing mutations in the MVK gene. In a study of 103 HIDS patients, 22% had normal serum IgD, particularly during infancy [13], which indicates that serum IgD is not sensitive enough for diagnosing HIDS. In Asian countries like Japan, HIDS is so rare that clinicians do not know the clinical relevance of IgD in relation to the diagnosis of HIDS. Therefore, it is very important to let clinicians know that more specific and more sensitive diagnostic tests; namely, measurement of urinary mevalonic acid and/or genetic analysis of the MVK gene are necessary to diagnose HIDS. It should also be pointed out that both the measurements of urinary mevalonic acid and the genetic tests of the MVK gene require special laboratory equipment, which makes it difficult to access such diagnostic tests.

In conclusion, we have reported a patient with a severe form of HIDS who presented with neonatal-onset chronic hepatitis with a novel MVK mutation. HIDS should be included in the differential diagnosis of neonatal-onset chronic hepatitis, even if the serum IgD is within the normal range and typical recurrent fever is not identified.

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Conflict of interest The authors have no conflicts of interest to declare.

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TABLE 2 Details of response to sequential treatments where applicable ($n = 10$)

No.	Severity of disease	First treatment		Second treatment		Third treatment	
1	Severe	Amlodopine	×	Nifedipine	√	–	–
2	Moderate	Amlodopine	×	GTN	×	–	–
3	Moderate	Amlodopine	×	GTN	×	–	–
4	Severe	Nifedipine	×	Amlodopine	×	–	–
5	Severe	Nifedipine	×	Amlodopine	×	GTN	√
6	Moderate	Nifedipine	×	GTN	×	–	
7	Severe	GTN	×	Amlodopine	×	Nifedipine	√
8	Moderate	Nifedipine	×	GTN	√	–	–
9	Severe	Amlodopine	×	Nifedipine	×	GTN	×
10	Moderate	Amlodopine	√	GTN	√	–	–

×: no response/inadequate response; √: response.

Overall, GTN patches were effective in 55% of the treated patients. Efficacy was better than that of nifedipine and amlodipine (33 *vs* 25% response rate, respectively), but small numbers and retrospective analysis does not allow statistical comparison. Response was similar in primary and secondary RP. Children with severe RP had a better response to nifedipine and amlodopine than children with moderate disease. The sub-group with severe disease was more likely to be using a disease-modifying drug, which may have had an impact. However, numbers are too small for any conclusion to be drawn from this.

Application of GTN patches allows removal if adverse events occur. Together with absence of tablets, this may make treatment with GTN attractive in paediatric practice. All patients received Deponit GTN patches. Alternative brands may not have adequate skin adhesion when cut into quarters for this off-license use.

GTN patches, nifedipine and amlodipine offer symptomatic relief for patients with moderate primary/secondary RP. Further studies, including head-to-head trials, are needed to determine if one agent is superior. Meanwhile, GTN patches offer an alternative to oral calcium channel blockers for symptomatic relief of paediatric RP.

Rheumatology key message

- GTN patches are an efficacious treatment option in paediatric RP.

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A case of early-onset sarcoidosis with a six-base deletion in the *NOD2* gene

SIR, We present the first case of early-onset sarcoidosis (EOS, MIM no. 609464) with a six-base deletion in the *NOD2* gene, resulting in the replacement of one amino acid and the deletion of two additional amino acids. All previous mutations reported for EOS and Blau syndrome (BS, MIM no. 186580) were single-base substitutions that resulted in the replacement of a single amino acid [1–3].

The patient was a Japanese male born after an uncomplicated pregnancy and delivery. His family had no symptoms of skin lesions, arthritis or uveitis. At 5 years of age, he was diagnosed with bilateral severe uveitis. He became blind in both eyes during adolescence. He had swollen ankles without pain during childhood,

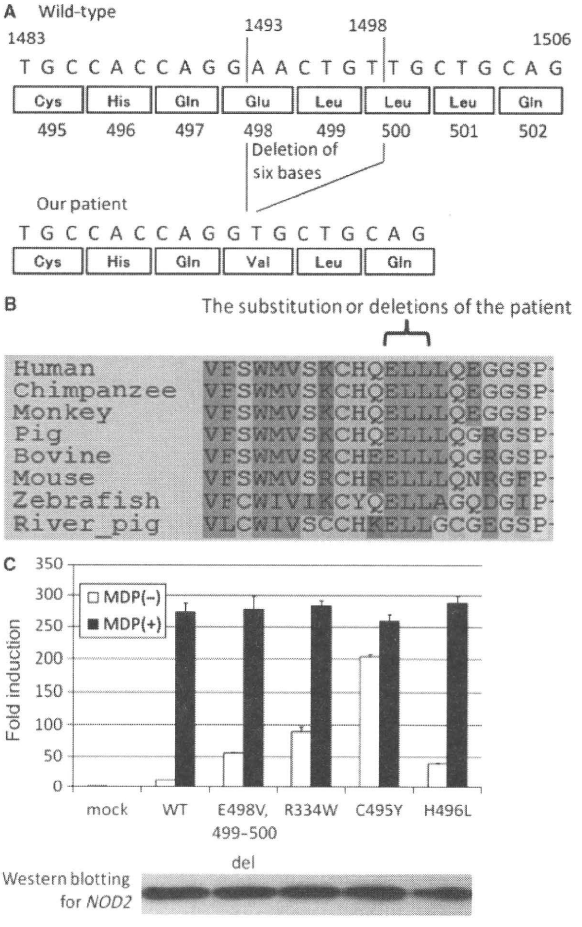
and developed arthritis in his both knees and ankles at 15 years of age. At 30 years, a skin rash had developed on his extremities after his first BCG vaccination. The skin lesions were scaly erythematous plaques with multiple lichenoid papules and some pigmentation. At the same age, camptodactyly without obvious synovial cysts of the hands was observed, and the deformity in all fingers developed by 35 years. At 41 years, he had low-grade fever for 1 year. He had no pulmonary lesions. His laboratory investigations showed normal white blood cell count, mildly elevated CRP (1.0 mg/dl) and ESR (20 mm/h). A skin biopsy from his left forearm revealed non-caseating granulomas without lymphocyte infiltration. There were no indications of infection by *Mycobacterium*.

The clinical symptoms and pathological findings on the biopsied skin indicated that the patient suffered from EOS. It has been reported that EOS and BS have a common genetic aetiology due to mutations in the *NOD2* gene that cause constitutive Nuclear Factor (NF)-κB activation [4, 5]. Thus we analysed the *NOD2* gene from the patient to look for mutations that might correlate with the pathology of EOS. A written informed consent was obtained from the patient and his families, according to the protocol of the institutional review board of Kyoto University Hospital and in accordance with the Declaration of Helsinki. Genomic sequencing analysis of the patient's *NOD2* gene showed the presence of a heterozygous deletion of six bases in exon 4, which resulted in c.1493_1498delAACTGT, p.E498V, 499–500del (Fig. 1A). The mutation was novel and was not identified in 100 normal controls. A genome alignment of *NOD2* among several species showed that E498, L499 and L500 are conserved from zebrafish to human (Fig. 1B). These data strongly suggested that the identified deletion of six bases in the *NOD2* gene is not a single nucleotide polymorphism (SNP), but is probably responsible for EOS in the patient.

Previous studies report that *NOD2* mutations causing EOS/BS show constitutive activation of NF-κB [6–8]. Therefore, we investigated the level of NF-κB activity associated with the new mutation identified here. First, we confirmed the level of mRNA expression of the mutated allele by subcloning analysis of *NOD2*-cDNA, which showed that the mutated allele was expressed as well as the wild type allele (data not shown). We then evaluated the ability of the *NOD2* mutant to constitutively activate NF-κB by using an *in vitro* reporter system in HEK293T cells transfected with both *NOD2* mutants and NF-κB reporter plasmids (Fig. 1C). The deletion mutant demonstrated almost five times more NF-κB activity than wild type without muramyl dipeptide (MDP) stimulation. Western blot analysis confirmed that NOD2 mutant protein expression was similar to that of wild type (Fig. 1C). Thus, like other mutations of *NOD2* identified previously, the deletion mutant identified here also showed constitutive activation of NF-κB.

The mechanism underlying EOS/BS has not been totally understood, although two pathways downstream from *NOD2* have been identified: NF-κB activation through receptor-interacting protein (RIP) like interacting caspase-like apoptosis regulatory protein kinase (RICK) and MAP kinase activation through the caspase recruitment domain 9 (CARD9) [9]. We previously tested 10 *NOD2* missense mutations that have been identified in our cohort of EOS/BS patients in Japan, and all of them demonstrated constitutive activation of NF-κB [3]. By analysing this newly identified deletion mutant, we have further confirmed the importance of constitutive activation of NF-κB by mutated *NOD2* for the pathogenesis of EOS/BS. We would like to emphasize the

FIG. 1 (**A**) Summary of the mutations identified in our patient. (**B**) NOD2 protein alignment among different species on the mutated amino acids. (**C**) NF-κB reporter assay using the *NOD2* deletion mutant. *In vitro* NF-κB reporter assays were performed as previously described [1, 3, 6, 7]. Mock vector, wild type *NOD2* (WT) and three *NOD2* variants (R334W, C495Y, H496L) derived from EOS/BS patients, were used as controls. Values represent the mean of normalized data (mock without MDP = 1) of triplicate cultures, and error bars indicate S.D. Shown is one representative result of three independent experiments. Protein expression levels of *NOD2* mutants analysed by western blotting are shown in the bottom panel.



usefulness of the NF-κB reporter assay with mutant *NOD2* for observing its role in EOS/BS, although the MAP kinase activation pathway and other possible pathways need to be evaluated to more completely understand the pathogenesis of the *NOD2* mutation in EOS/BS.

We have identified the first deletion mutation in the *NOD2* gene responsible for EOS/BS, and the mutant showed constitutive activation of NF-κB, which is one of the key features that lead to the pathogenesis of EOS/BS.

Rheumatology key message

- A six-base deletion in *NOD2* gene causes EOS.

Acknowledgement

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Comment on: Hepatotoxicity rates do not differ in patients with rheumatoid arthritis and psoriasis treated with methotrexate

Sir, We read with interest the recent article by Amital *et al*. [1] that compared hepatotoxicity rates in PsA and RA patients treated with MTX based on the evaluation of standard liver function tests. The authors conclude that the incidence of hepatotoxicity does not differ between the two disease groups after adjusting for the cumulative dose of MTX.

Several studies in MTX-treated psoriasis patients have reported that isolated abnormalities of liver enzymes (i.e. alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase) were poor predictors of the severity of liver histopathology. The authors state that the combined sensitivity of aspartate aminotransferase, alanine aminotransferase and bilirubin for detecting an abnormal liver biopsy has been rated at 0.86 based on a previous study [2]. This figure implies that 14% of those with normal liver function tests will have undetected hepatic disease. Larger studies have suggested that 30–50% of the psoriasis patients on MTX have normal standard liver function test results despite histology showing fibrosis and cirrhosis [3]. The lack of correlation between liver enzymes and hepatic fibrosis and cirrhosis has been the major factor leading to the recommendation that liver biopsies be done to monitor potential hepatotoxicity. In this study, the liver function tests were performed with varying frequency which could allow abnormal liver function tests to be missed. The authors acknowledge that the rates of other hepatotoxic agents such as alcohol use and the occurrence of other hepatic comorbidities were not known. We believe that these are significant confounding variables, which make the interpretation of the results of this study difficult. The British Association of Dermatologists recommends serial monitoring

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

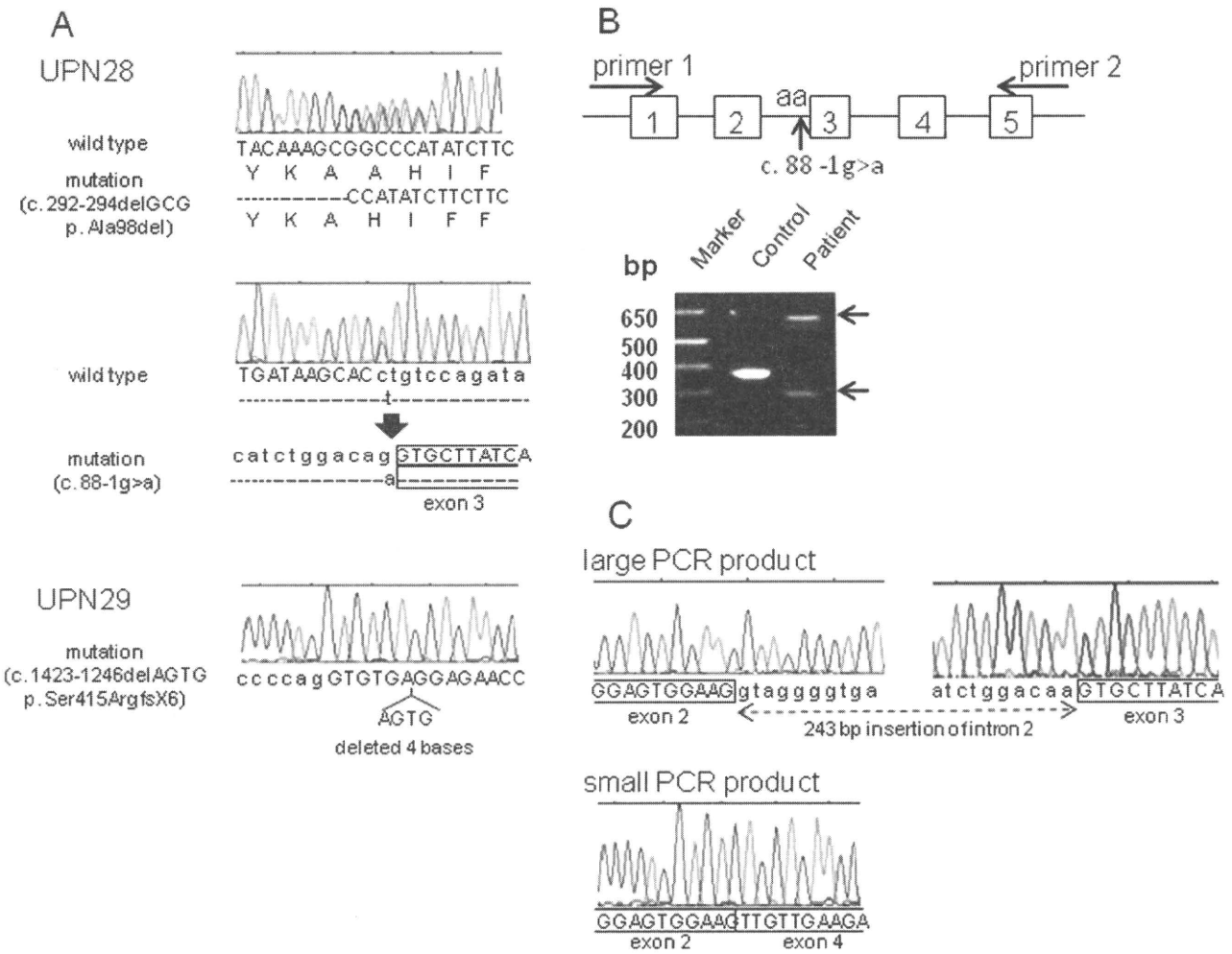

Figure 1. Identification of *STXBP2* mutations. (A) Sequencing analysis of 4 patients with non-FHL2/3/4 and detection of 3 novel mutations in 2 of them: a compound heterozygous mutation of 292_294delGCG resulting in Ala98del at exon 5 (upper panel) and 88-1g>a in intron 2 (lower panel) in one patient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

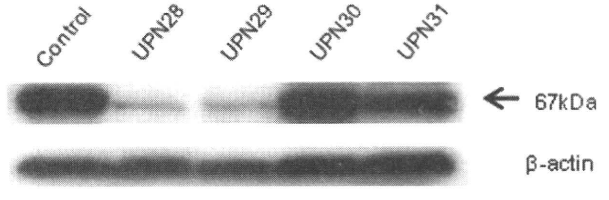

Figure 2. Western blot analysis of Munc18-2 protein expression. Expression of Munc18-2 protein in each $CD8^+$ T-cell line that had been stimulated with allogeneic B-LCL cells was analyzed by Western blotting using anti-Munc18-2 antibody. Munc18-2 protein was abundantly detected at 67 kDa in CTL lines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

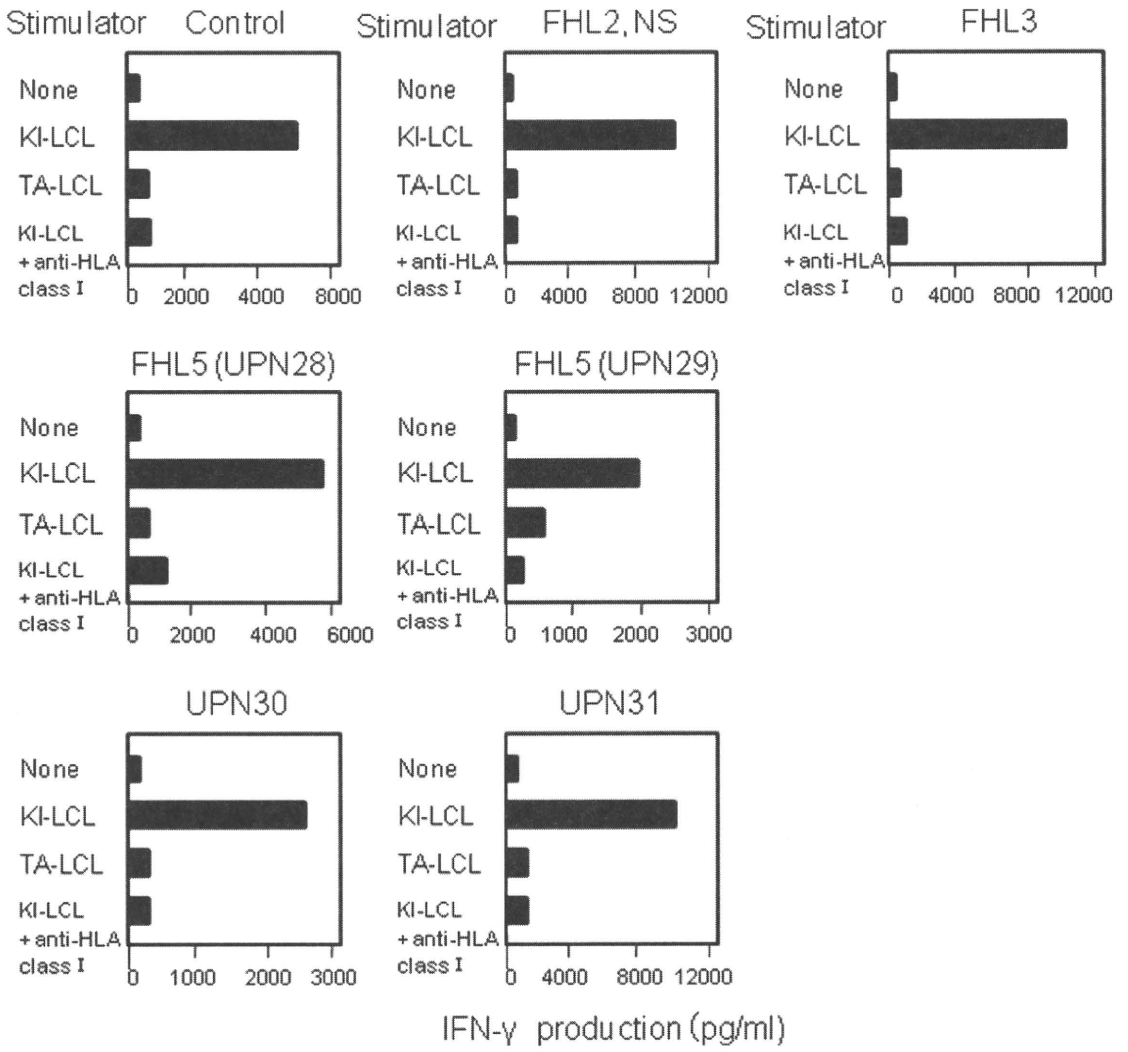

Figure 3. IFN-γ production by alloantigen-specific CD8$^+$ T cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Responder cells were co-cultured with or without KI-LCL or TA-LCL,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

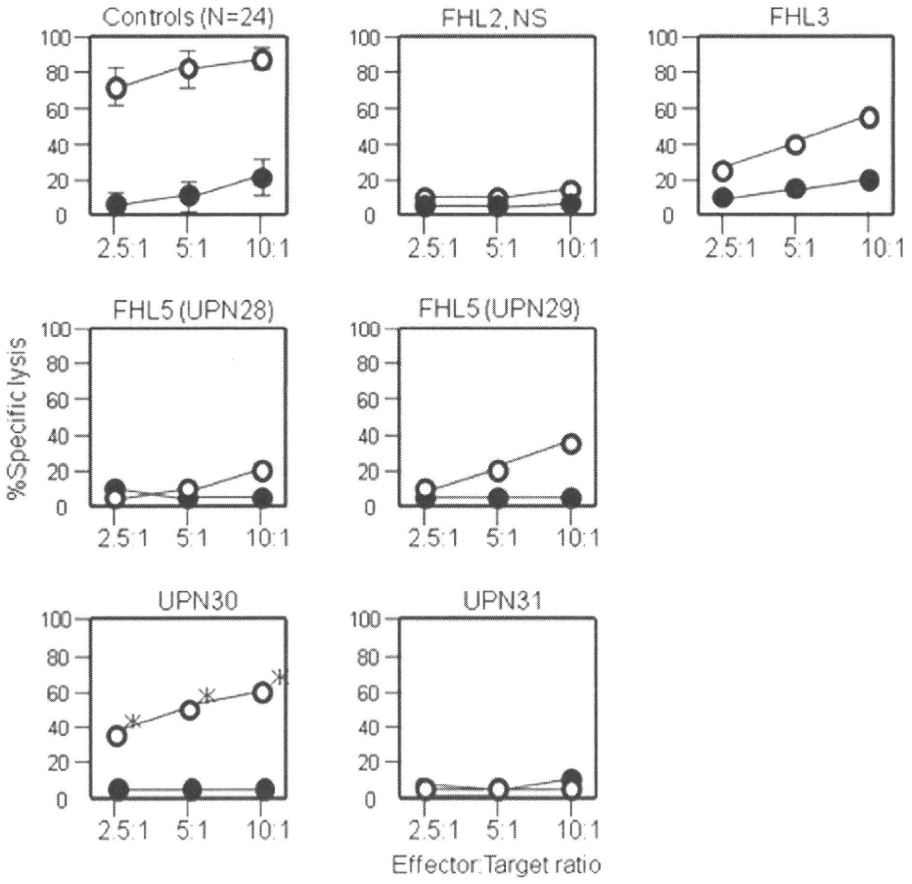

Figure 4. Cytotoxicity of alloantigen-specific CD8$^+$ T-cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and 24 healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Their cytotoxicity was determined against allogeneic KI-LCL (clear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

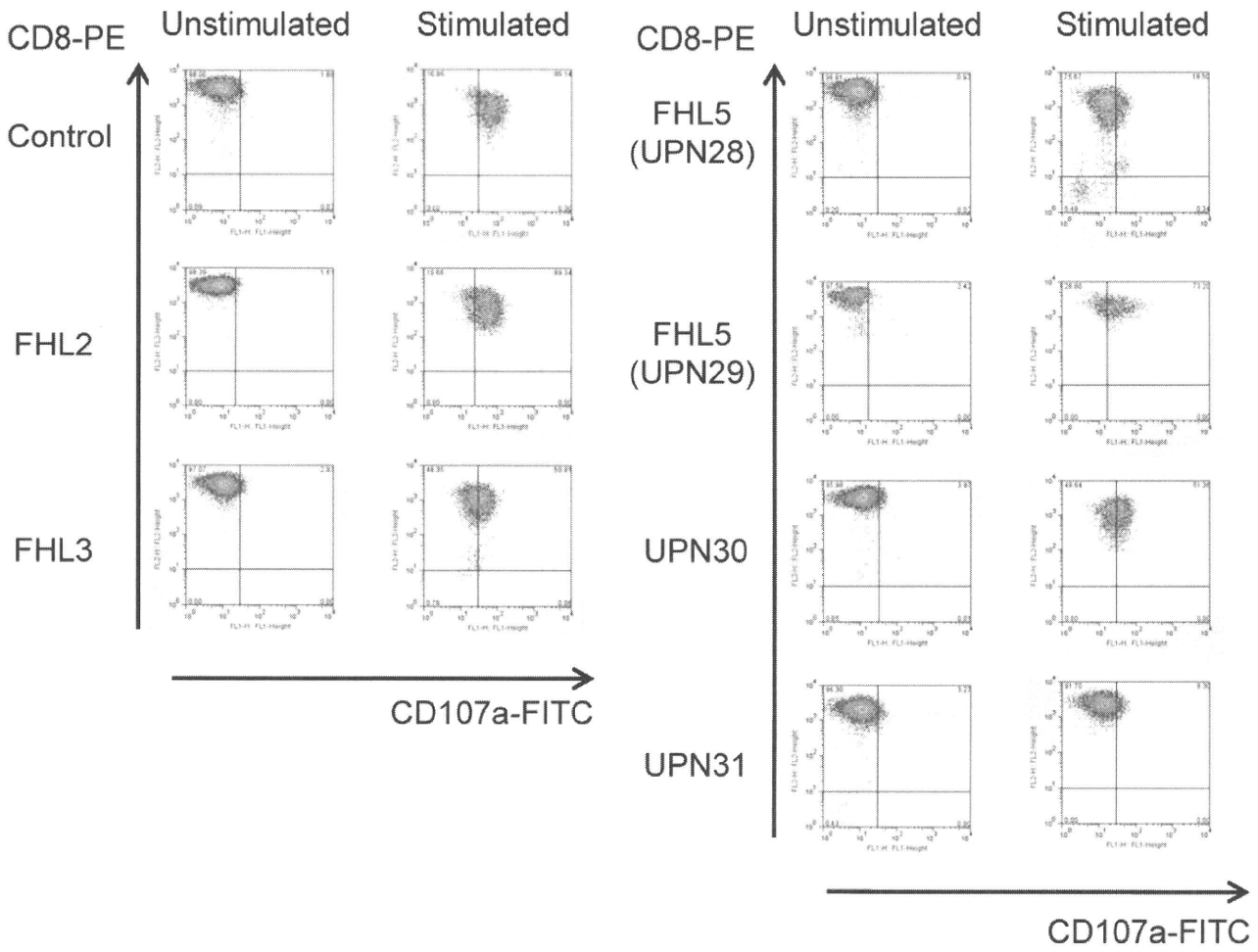

Figure 5. CD107a expression of alloantigen-specific CD8$^+$ T-cell lines. Flow cytometric analysis of CD107a expression was performed using CD8$^+$ T-cell lines generated from a healthy individual and FHL patients, as detailed in the text. Left panel of each column shows CD107a expression in CD8$^+$ 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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