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Novel Syntaxin 11 Gene (*STX11*) Mutation in Three Argentinean Patients with Hemophagocytic Lymphohistiocytosis

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Abstract

Introduction—Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disease with major diagnostic and therapeutic difficulties, basically comprising two different conditions: primary and secondary forms. Recent advances regarding molecular diagnosis may be useful to distinguish from one another, especially in sporadic cases starting in early infancy.

Materials and Methods—In this report, we evaluated three Argentinean patients with clinical suspicion of HLH, but without family history. We excluded mutations in the perforin gene but identified in the three patients a novel homozygous deletion (c. 581_584delTGCC; p.Leu194-ProfX2) in the gene-encoding syntaxin 11 (*STX11*), causing a premature termination codon.

Results and Conclusion—Each parent from the three unrelated families resulted heterozygous for this deletion confirming the diagnosis of familial hemophagocytic lymphohistiocytosis type 4. Patients shared the same single-nucleotide polymorphism profile in *STX11* gene, and genotyping at ten microsatellites surrounding this gene support the presence of a single-haplotype block carrying the novel mutation.

Keywords

Familial hemophagocytic lymphohistiocytosis; primary immunodeficiency; syntaxin 11 gene; founder mutation

Introduction

The diagnosis of familial hemophagocytic lymphohistiocytosis (FHL) [1, 2], a genetically heterogeneous immunologic disorder affecting young children, can be established by the presence of clinical criteria and can be confirmed by molecular genetic testing. Clinical characteristics include: prolonged fever, cytopenias affecting two or three of the peripheral blood lineages, splenomegaly, hypofibrinogenemia, hemophagocytosis, defective natural killer (NK) cell function, hyperferritinemia, and high plasma concentrations of soluble IL2 α receptor [3]. With regard to the genetic defects in FHL, three causative gene abnormalities are known today: *PRF1* [4, 5], *UNC13D* [6], and *STX11* [7]. This third gene, whose defect was recently associated with FHL type-4, encodes a protein, syntaxin 11, postulated to play a role in intracellular trafficking. Although its precise role in the pathogenesis of FHL is not known, its prominent expression in phagocytes and antigen presenting cells [8] and its involvement in vesicle trafficking suggest, like other primary forms of FHL, a defective granule secretory pathway [9–12].

The *STX11* gene consists of two exons and covers a genomic interval of 37 kb. The coding region comprises only exon 2, and mutations seem to be greatly restricted to patients of Turkish origin [13–15]. Early identification of the underlying genetic defect is crucial for patients without family history of hemophagocytic lymphohistiocytosis (HLH), so as to rapidly treat them by hematopoietic stem cell transplantation (HSCT), the only curative approach so far. In the present study, we identified an identical novel mutation of *STX11*

gene in three unrelated patients lacking family history of HLH that can be attributed to a single haplotype.

Materials and Methods

Sequencing Analysis of Genomic DNA

After informed consent, blood was obtained for genetic analysis from the probands and their parents. Blood from healthy control subjects was also obtained after informed consent.

Total DNA was isolated from EDTA-anticoagulated whole blood samples, and the complete open-reading frames for *PRF1* and *STX11* were analyzed by direct sequencing using ABI 310 and ABI 3130 (Applied Biosystems). The primers for the amplification of the two coding exons of *PRF1* (exons 2 and 3) and for the coding exon of *STX11* (exon 2) as well as the polymerase chain reaction (PCR) conditions have been previously described [5, 7].

Regions containing the single-nucleotide polymorphisms (SNPs) found in intron 1 of *STX11* gene (see Table II and Genome Variation Server; <http://gvs.gs.washington.edu>) were amplified by PCR, these products directly sequenced using ABI Prism BigDye (v1.1) terminators and analyzed on an ABI 3130 Sequencer (PE Applied Biosystems). Primer sequences and annealing temperatures are available upon request. The frequency of minor alleles was estimated from a sample of 30 unrelated subjects of Argentinean origin.

STX11 Expression Analysis

Expression of *STX11* RNA was analyzed by reverse transcriptase PCR with templates made by oligo(dT) primed reverse transcription of poly(A)⁺ RNA obtained from peripheral blood mononuclear cells (Trizol, Invitrogen) and with specific primers previously designed [10].

Peripheral blood lymphocytes were lysed in 1% Triton X-100 in Tris-buffered saline on ice; nuclei and other debris were removed by centrifugation; proteins were separated by 10% SDS-PAGE and analyzed by immunoblotting using a syntaxin 11 specific antiserum as described previously [16].

Microsatellite Region Analysis

Ten microsatellite markers adjacent to *STX11* (D6S1684, D6S308, D6S310, D6S409, D6S1704, D6S1003, D6S1703, D6S1649, GATA184A08, and D6S311) were amplified using fluorescent (6-FAM) labeled primers. PCR products were electrophoresed on an ABI3130 DNA sequencer (PE Applied Biosystems). Analyses and assignment of marker alleles were carried out using Genotyper software (PE Applied Biosystems).

Mutation Leu194ProX2 Age Estimation

In order to estimate the age of the most recent common ancestor (MRCA) of the haplotype carrying the Leu194ProfsX2 mutation, a previously described likelihood method was modified and applied [17, 18]. Briefly, the number of generations to MRCA is predicted from the first recombination signal on a set of markers located at variant distances on both sides of Leu194ProfsX2. Recombination rates for the intervals among the selected

extragenic markers were estimated by means of a robust lineal regression on published data from 102 polymorphic sites located along a region of 20 Mb to each side of Leu194ProfsX2 and incorporated into the Marshfield linkage map (<http://research.marshfieldclinic.org/genetics>). Linkage (Kosambi centiMorgans, cM) was inferred for the ten microsatellites analyzed based on sequence position on chromosome 6. The correspondence between genetic and physical distances over the whole region was estimated to be 1.12 cM per Mb. False recombination signal due to mutation was corrected, for a constant mutation rate of 0.00056 per generation and locus [19]. Correction for unseen recombination events was deprecated. The analysis was performed assuming that the five haplotypes carrying the mutation (both from patient 1 parents, both from patient 2 parents, and only one from patient 3 parents, with the latter being an inbred family) diverged independently from a common ancestral haplotype (star genealogy).

Results

Case Reports

Patient 1—A 20-month-old previously healthy female child presented with fever, hepatosplenomegaly, and enlarged lymph nodes. Two months later, she developed cytopenias and was referred to our hospital. The family history was unremarkable for immunodeficiency, except for a deceased young boy in the maternal branch of the kindred. The workup was consistent with the diagnosis of HLH, according to diagnostic guidelines. The initial laboratory findings, before chemotherapy was started, included anemia, neutropenia, thrombocytopenia, hypertriglyceridemia, coagulopathy with hypofibrinogenemia, hepatic enzyme abnormalities with mild cholestasis, and hyperferritinemia (Table I). The bone marrow aspirate showed hemophagocytic features, and in the liver biopsy, an accumulation of lymphocytes and mature macrophages was found. She was started on the HLH-2004 treatment protocol with achievement of a short period of remission. A few weeks after the chemotherapy was stopped, she was hospitalized because of a low respiratory tract infection and neutropenia; hepatosplenomegaly, elevated aminotransferases, and hypertriglyceridemia were observed. She improved with supportive care and broad-spectrum antibiotics. There was no evidence of the disease during the following 8 months, when she was readmitted to the hospital with pneumonia, fever, hepatosplenomegaly, enlarged abdominal lymph nodes, and cytopenias (neutropenia and thrombocytopenia). Hemophagocytosis was proven in a bone marrow biopsy. Surprisingly, she responded well to antibiotics with only residual neutropenia. She was released from the hospital without specific treatment and remained in good health for 2 months. By that time, FHL4 was verified, and because of the frequent reactivations of the disease following infections, continuation therapy was started until the accomplishment of HSCT, which was performed with a matched unrelated donor. Unfortunately, she died a few months later because of transplant-related complications.

Patient 2—She is a 2-year-old girl at the time of writing, born to non-consanguineous parents. At 13 months of age, she was admitted to the hospital with fever, haematological cytopenias, hepatosplenomegaly with cholestatic hepatitis, erythematous cutaneous rash, and neurological involvement (peripheral facial palsy with spinal fluid mononuclear pleocytosis

and elevated protein levels). Initial bone marrow specimens were not conclusive. Since clinical and laboratory diagnostic criteria for HLH were fulfilled (see Table I), she was started on HLH-2004 protocol. Central nervous system disease resolved with systemic therapy, so intrathecal methotrexate was not used. Remission was gradually achieved with no signs of active disease. The familial form of the disease, FHL4, was proven. She is now under chemotherapy and supportive treatment waiting for the accomplishment of HSCT.

Patient 3—A girl of a consanguineous Argentinean family with unremarkable personal history for immunodeficiency was referred at 3 years of age to our hospital for fever, cervical lymphadenopathy, hepatosplenomegaly, cutaneous rash, and pancytopenia without microbiological findings in the initial workup. Since HLH was suspected, laboratory and histopathological evaluation were performed, confirming the diagnosis. The bone marrow specimen showed hemophagocytic features. HLH-2004 treatment was initiated with good clinical response. She is 3 years and 3 months of age at the time of writing, with confirmed diagnosis of FHL4 and under continuation chemotherapy treatment waiting for the accomplishment of HSCT.

Detection of Homozygous Mutation in STX11 Gene in the Three Patients

After exclusion of *PRF1* gene mutation by genomic sequencing of the entire coding region and normal perforin protein level by flow cytometric analysis (data not shown), we performed *STX11* gene evaluation in the three patients. Direct gene sequencing showed in exon 2, an identical homozygous 4-bp deletion in all affected patients (Fig. 1a). Exon 2 contains all of the gene-coding sequence, and the 4-bp deletion leads to a frame shift with generation of a premature termination codon after two altered residues (Fig. 1b). The p.Leu194ProfsX2 mutation was also identified heterozygously in the six parents from the three families (Fig. 1a and data not shown) but not in 100 control chromosomes nor in previous reports containing *STX11* mutations [7, 10, 13, 14].

To determine if the mutated *STX11* is expressed in the patient's mononuclear cells, RNA from patients 2 and 3 was amplified by RT-PCR resulting in apparent normal-sized transcripts in which direct sequencing revealed the 4-bp deletion seen in genomic DNA (Fig. 1c and data not shown). The premature stop codon generated by this novel mutation is predicted to lead to a truncated protein lacking the SNARE domain (Fig. 1b), thought to act as a protein–protein interaction module in the assembly of a SNARE protein complex, probably mediating most if not all vesicular membrane fusion events in eukaryotic cells. To determine if a truncated form of Stx11 protein is expressed in peripheral blood lymphocytes (PBL) from homozygous patients, a Western blot analysis was performed with a well-characterized anti-Stx11 polyclonal serum raised against the first 15 amino acids [20] (Fig. 1d). In lysates of PBL from healthy donors, a 35-kDa protein corresponding to the predicted molecular mass of Stx11 was detected, while complete absence of protein recognized by this antibody has been shown from patients' PBL.

Identical SNP Profile of STX11 in Patients with Leu194ProfsX2

The 4-bp deletion was not previously described in international reports and constitutes the first *STX11* mutation identified in Argentinean patients, strongly suggesting a founder

effect. SNP profile of *STX11* would be helpful for investigating whether the Leu194ProfsX2 mutation was inherited among patients as a single shared allele. We compared five intragenic SNPs, with minor allele frequencies higher to 12% and listed in the Genome Variation Server (<http://gvs.gs.washington.edu>), between the patients and a control group of 30 Argentinean subjects (Table II). All patients with p.Leu194ProfsX2 mutation shared the same SNP profile, primarily wild type at each locus.

A Same Homozygous Haplotype Block Surrounding the Novel Mutation in the Three Patients

To further discern whether Leu194ProfsX2 represents a single haplotype, ten microsatellites surrounding the *STX11* gene (according to the genetic and physical maps available at <http://genome.ucsc.edu> and www.ncbi.nlm.nih.gov) were genotyped in patients and in their parents (Fig. 2). The analysis of marker loci around *STX11*, namely D6S1684, D6S308, D6S310, D6S409, D6S1704, D6S1003, D6S1703, D6S1649, GATA184A08, and D6S311, revealed homozygosity in a 4.8-Mb physical interval in patients from families 1 and 2. Patient 3, being from a consanguineous family, also shared the same haplotype although she was homozygous in a larger interval (Fig. 2). Heterozygous parents presented the common haplotype carrying mutated *STX11*, while the remaining allele was different in all of them (Fig. 2).

In order to estimate the age of the MRCA of the haplotype carrying the novel mutation, a previously described likelihood method was modified and applied on our set of markers located at variant distances on both sides of the disease mutation [17, 18]. For patient 3, only one of the two haplotypes carrying the mutation was considered since the family was inbred. The age of the MRCA of the Leu194ProfsX2 mutation was estimated to be nine generations before patients' parents (95% confidence interval, 2 to 21). Assuming that one generation is 25 years, this corresponds to ≈ 225 years (95% confidence interval, 50 to 525 years; excluding a shared grandparent with a confidence of 99%).

Discussion

In this study, we identified a previously undescribed *STX11* mutation on three Argentinean, *PRF1*-negative, unrelated HLH patients. So far, mutation registry for FHL4 (<http://bioinf.uta.fi/STX11base/>) only contains data from families of Turkish/Kurdish origin. Regarding the scarcity of the FHL syndrome, the occurrence of the same mutation in three unrelated families would suggest an FHL founder mutation in Argentina. In evaluating this by *STX11* gene SNP profiles and locus microsatellite genotyping, we observed that this novel mutation can be actually attributed to a single haplotype, shared by all carrier parents of the three homozygous patients. To estimate the age of the most recent common ancestor carrying the disease causing mutation, we choose a slightly modified likelihood method [18], originally described by Génin et al., due to its validated efficiency in a very small number of affected individuals and suitability to estimate the age of rare mutations [17]. We found that the most recent common ancestor of these parents should have lived 225 years ago (95% confidence interval, 50–525).

Founder mutations have been described for several primary immunodeficiencies, including the most frequently observed *PRFI* mutation in patients of African ancestry [21]. Population in Argentina has a genetically heterogeneous ethnic background mainly conferred by the integration of European immigrants to the native population. In these patients, although lacking consistent data regarding a particular ethnic background, we cannot exclude, from their family names, a Basque ancestry in all of them. Noteworthy, Basque settlement in Argentina took place approximately by the same time of the estimated occurrence of *STX11* mutation.

Different reports suggested that the Stx11 protein takes part of the vesicle–plasma membrane fusion during exocytosis of the granules from cytotoxic T cells and NK cells [10, 11, 22]. The homozygous mutation p.Leu194ProfsX2 found in all three investigated patients was predicted to result in a premature termination codon. In spite of the presence of normal-sized transcripts of *STX11*, Western blot analysis with an anti-Stx11 polyclonal serum revealed no protein in patients' mononuclear cells. Thus, these patients are comparable to previously described patients with other *STX11* mutations also leading to absence of detectable protein in NK or in T cells or to a truncated protein lacking the functionally relevant C-terminal segment [10].

Recent attempts to evaluate genotype–phenotype correlation in a large cohort of genetic heterogeneous FHL patients suggest that patients with *STX11* mutations may have a less severe disease due to a low frequency of early onset (less than 6 months old) and pathological cerebrospinal fluid as measurement of neurological disease [15]. The three patients in the present study could be considered as a homogeneous genotype group sharing the same mutation in *STX11* gene. None of them was, as previously reported, less than 6 months at diagnosis; in fact, they were between 13 to 35 months of age at onset, but patient 2 presented neurological involvement at this time, raising the question of the role of the mutation itself in the neurological expression of FHL. Similarly, Rudd et al. [14], reporting two FHL4 patients with severe developmental delay, who carried the same molecular defect as three other patients without neurological symptoms, questioned the specificity of the *STX11* mutation in the neurological disease. Further studies may be necessary to clarify this point.

Abnormalities in the function of NK cells have been observed in the great majority of patients with all forms of FLH. Cytolytic activity of freshly derived PBLs against K562 target cells (referred to as NK activity) showed in our patients a variable degree of reduction at onset, from almost absent to half the activity of the reference value (Table I). Consistent with this, the expression of CD107a on NK cells, a marker of granule exocytosis [23], was not completely abrogated in the two patients available for this study (Table I), although it was greatly impaired (16% and 23% relative to the mean value from healthy donors). In previous results obtained by disrupting Stx11 expression in normal NK cells, the partial, rather than complete, inhibition of killing was suggested to reflect the activity of the residual Stx11 or possible partial redundancies between Stx11 and other regulators of cell-mediated cytotoxicity [11]. In the present study, the absence of protein in Western blot assay would suggest that a mechanism independent of Stx11 could be responsible for the residual cytotoxic activity observed in our patients. This is in line with a report showing that IL-2

stimulation partially restores degranulation and cytotoxicity in NK cells from FHL4 patients even in those with no detectable Stx11 protein [10].

The diagnosis of familial hemophagocytic lymphohistiocytosis may be difficult, particularly in patients without affected relatives. Actually, despite its name, family history is often negative in this recessive disease, being an early identification of the underlying genetic defect, crucial to rapidly treat patients by HSCT, the only curative approach so far. Indeed, the genetic study of patients with HLH remains the accurate method for discrimination between the secondary and the genetic form, as well as to discriminate between genetic subtypes of the disease due to lack of other reproducible laboratory tools.

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Fig. 1. Identification of a novel *STX11* mutation associated with familial hemophagocytic lymphohistiocytosis (FHL). **a** DNA sequence of *STX11* in patient 1 and in the mother of patient 1 depicted by DNA electropherograms. **b** Schematic representation of the genomic organization of the *STX11* gene and the corresponding Stx11 protein. The coding region (*shaded*) comprises only exon 2, and the mutation site is shown by an *arrow*. Protein representation contains an amino-terminal helical domain called H_{abc}, a carboxy-terminal helical region called H3 (SNARE core motif) and a transmembrane anchor domain (TMD). The predicted translational arrest is indicated. **c** Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *STX11* in patients 2 (*P2*) and 3 (*P3*). Patients (*lanes 1* and *2*) showed bands which are indistinguishable from those derived from normally spliced mRNA (*lane 3*). Size markers are shown on the *right*, *C* cDNA used for RT-PCR was derived from a healthy control, *NC* negative control (no cDNA in the reaction mixture). **d** Western blot analysis of Stx11 protein expression showed absence of an approximately 35-kDa band in patient 2 (*P2*) and 3 (*P3*; *lanes 2* and *4*, respectively); *C* lysates from peripheral blood lymphocytes derived from healthy controls (*lanes 1* and *3*). Beta-actin was used as positive control

Fig. 2. Family pedigrees and haplotype analysis of polymorphic markers spanning the *STX11* locus on chromosome 6q24. The markers were ordered according to the Genome Browser of UCSC Genome Bioinformatics (<http://genome.ucsc.edu/index.html>). Black circles represent affected individuals. Diagonal bar indicates deceased patient. Shaded box or circle identify the *STX11* mutation carriers. The most plausible ancestral haplotype carrying the *STX11* mutation is shaded. The shared haplotype block is denoted in bold being the three patients homozygous in this region. NA not analysed

Table 1

Familial, Clinical, and Laboratory Findings at Onset

	Patient 1	Patient 2	Patient 3	HLH diagnostic criteria
Country or origin	Argentina	Argentina	Argentina	
Consanguinity	No	No	Yes	
Sex	Female	Female	Female	
Age at onset (months)	20	13	35	
Fever	Yes	Yes	Yes	
Splenomegaly	Yes	Yes	Yes	
Hemoglobin	88 g/L	98 g/L	75 g/L	<90 g/L
Platelets	12×10 ⁹ /L	52×10 ⁹ /L	10×10 ⁹ /L	<100×10 ⁹ /L
Neutrophils	0.98×10 ⁹ /L	0.51×10 ⁹ /L	0.62×10 ⁹ /L	<1.0×10 ⁹ /L
Triglycerides	394 mg/dl	243 mg/dl	548 mg/dl	265 mg/L
Fibrinogen	1.06 g/L	1.82 g/L	1.08 g/L	1.5 g/L
Ferritin	807.9 ug/L	1,556 ug/L	905.6 ug/L	500 ug/L
SolubleIL-2 receptor	ND	26,000 U/ml	27,000 U/ml	2,400 U/ml
NK-cell activity ^a	(25:1) 22%	(25:1) 3%	(25:1) 15%	Low or absent
CD107 on NK cells ^b	ND	(1:1) 5%	(1:1) 7%	
Hemophagocytosis	Yes ^c	Not conclusive ^c	Yes ^c	
Neurological disease	No	Yes	No	
Treatment protocol	HLH-2004	HLH-2004	HLH-2004	
Remission	Yes	Yes	Yes	
Relapse	Yes ^d	No ^e	No ^e	
HSCT	Yes	No	No	
Outcome	Deceased	Alive with no active disease	Alive with no active disease	

ND not determined

^aNormal range 30–50% (our laboratory reference data) when effector to target ratio is 25:1^bNormal percentage of CD107a⁺ NK cells when stimulated with the K562 cell line (ratio 1:1), 30% (mean of nine healthy individuals, range 24–35%)^cBone marrow specimen

Under continuation therapy₂
Discontinued treatment_p
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STX11 SNPs in the Patients and in Their Parents**Table II**

<i>STX11</i> gene	Family 1			Family 2			Family 3			
dsSNP ID ^a	Alleles ^b	Patient 1	Mother	Father	Patient 2	Mother	Father	Patient 3	Mother	Father
rs11155347	A/T	T/T	T/T	T/A	T/T	T/T	T/T	T/T	T/A	T/T
rs1322452	G/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/G	A/A
rs7756004	G/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/G	A/A
rs7764327	T/A	A/A	A/A	A/T	A/A	A/A	A/A	A/A	A/T	A/A
rs17073478	A/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T

^aIncluded in NCBI build 36 as 144517716, 144517984, 144521223, 144535034, and 144537619, respectively (with minor-allele frequencies (%) of 13, 48, 14, 13, and 44, respectively)

^bMinor alleles, with frequencies (%) in Argentina of 32, 37, 30, 30 and 0, respectively, are shown in *italics*