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Case Report Open Access

"Identification of a Deletion in *Stxbp2* Causative of Familial Hemophagocytic Lymphohistiocytosis Type 5"

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Abstract

Hemophagocytic lymphohistiocytosis (HLH) is a rare, life-threatening immune deficiency, characterized by a hyper-inflammatory syndrome. The familial form of HLH (FHL) is caused by mutations in genes associated with lymphocyte granule-mediated cytotoxicity. Mutations in *Stxbp2* (Sintaxin binding protein 2) gene result in defect of Munc18-2 protein, the causative defect of the subtype defined as FHL5. Functional tests as intracytoplasmic expression of perforin and surface expression of CD107a, help to direct genetic analysis. Different mutations have been described in the FHL-related genes known so far (*PRF1, UNC13-D, STX11*, *Stxbp2*): missense, nonsense, splicing, regulatory, small deletions/insertions. Recently a pathogenic inversion of 253 KB upstream of the 3' *UNC13D* gene has been reported. Here we describe a new deletion causative of FHL5. We confirmed the deletion by Real-Time PCR and by CGH-array. We finally documented by western blot the absence of expression of Munc18-2 protein in our patient. These data shows the need to introduce new diagnostic strategies in order to screen mutations not detected by the methods classically used.

Keywords: FHL5; *Stxbp2*; CGH

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a rare disease characterized by a hyper inflammatory syndrome. Fever, splenomegaly, thrombocytopenia, high levels of ferritin, soluble CD25 levels, and triglyceride, and low levels of fibrinogen are the main presenting features. Bone marrow aspiration may show hemophagocytosis by activated macrophages [1]. The familial form of the disease (FHL) is a genetically heterogeneous disorder caused by mutations in genes involved in the secretory lysosome dependent exocytosis pathway [2]. Five subtypes have been described so far and the gene is known for four of these: FHL type 2 (OMIM 603553) associated with mutations in PRF1 gene coding for the pore forming protein perforin; FHL type 3 (OMIM 608898) caused by mutations in the *UNC13D* gene coding for the secretory regulator Munc13-4; FHL4 (OMIM 603552) caused by mutations in *STX11* gene; more recently a new subtype was defined as FHL5 (OMIM 613101) caused by mutations in *StxBP2* gene encoding Munc18-2 protein [3,4]. Most FHL patients present within the first few years of life, and the course of the disease is usually rapidly fatal unless promptly treated and then cured with hematopoietic stem cell transplant [5-7]. The identification of biallelic mutations defining a genetic subtype is therefore essential to confirm the diagnosis, indicate transplantation, and offer a correct counseling.

Munc18-2 belongs to the Sec1/Munc18-like (SM) protein family, whose members is all long about 600 amino acid residues and is involved in regulation of SNARE-mediated membrane fusion events. Munc18-2 is specifically involved in the regulation of vesicle transport to the plasma membrane by the interaction with Syntaxin-11. This interaction is eliminated by the mutations of *StxBP2* which may lead to a decreased stability of both proteins [3].

Insufficient function of Munc18-2 in neutrophils is associated with defective mobilization of the granules.

Some clinical presentations of FHL5 seem to be different from the typical phenotype of FHL2 and FHL3. Indeed gastrointestinal manifestations were found in many patients such as diarrhea, gastroesophageal reflux, and abdominal pain; renal tubular alteration was also found in a patient. Probably this could be due to the presence of altered Munc18-2 protein in cells other than CTL, including intestinal and renal epithelium [8,9].

These defects contribute to the ineffective elimination of bacterial triggers (usually *E. coli*) and down-regulation of immune responses that result in a sustained hyper inflammatory state of gastrointestinal system [8-11].

FHL5 has been reported to account for up to 20% of cases of FHL in the German series [10]. Recent reports suggest that it is not restricted to a specific geographic region [2]. Since 2010, 47 different mutations of *StxBP2* have been described: 21 missense/nonsense, 10 splicing/regulatory and 16 small deletion/insertion mutations [web resource: HGMD].

Here we report the presenting features of a patient with FHL5, and the results of the genetic study in her family, in which a deletion of the *StxBP2* gene was documented.

Method

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Cytofluorimetric screening

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. Perforin expression on NK cells (CD3-CD56+ cells of PBL) was detected by intracellular staining (after fixation and permeabilization) with BD Pharmingen™ reagent set and cytofluorimetric analysis, as previously reported [12].

Resting and activated NK cells were tested in degranulation assay quantifying cell surface CD107a expression, as previously described [13]. Briefly, anti-CD107a-PE mAb was added during the cell culture for 2 hours at 37°C in 5% CO_2. Thereafter, the cells were stained with anti-CD56-APC and anti-CD3-PerCP mAb and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). All reagents were from BD Biosciences (Oxford, UK). Surface expression of CD107a was assessed in the CD3-CD56+ cells. Results were evaluated as ΔCD107a (i.e., % CD107a+ cells of stimulated - % CD107a+ cells of unstimulated sample) and defined defective when lower than the 10th percentile of healthy controls [13,14].

Mutation analysis

Genomic DNA was isolated from peripheral blood samples using BioRobot EZ1 Workstation (Qiagen, Milan, Italy). *StxBP2* gene was amplified by standard methods using specific primers, including the adjacent intronic sequences for identification of splice-site variants. PCR product was directly sequenced, in both directions, with the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Foster City, California, USA). Sequences obtained using an ABI Prism 3130XL Sequence Detection System (Applied Biosystems) were analyzed and compared with the reported gene structure using the dedicated software SeqScape (Applied Biosystems).

Real time PCR

Real-time PCR amplification was performed with the 7500 Real-Time PCR System 7 (Applied Biosystems®). Briefly 20 ng of DNA were amplified using the SYBR® Green PCR master mix with the following cycle conditions: initial denaturation at 95°C for 3 minutes, followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing for 60 seconds, and extension at 72°C for 60 seconds (Fast SYBR® Green Master Mix Protocol).

Fluorescence measurements were obtained during the elongation step. Each template was tested in at least five replicates to estimate the mean Ct. Exon 2 was used as internal reference and DNA from a normal control as calibrator.

CGH-Array

Array-CGH analysis was performed using Agilent SurePrint G3 Human CGH Microarray Kit, 1x1M (Agilent Technologies, Santa Clara, CA, USA) with 2.1 KB overall median probe spacing (1.8 KB in Refseq genes). Labelling and hybridisation were performed following the protocols provided directly by Agilent: 500 ng of purified DNA of the patient and of a control of the same sex (Agilent) were double digested with RsaI and AluI enzymes (Agilent) for 2 h at 37°C, obtaining products between 200 bp and 500 bp in length. Each digested sample was labelled for 2 h, minimizing light exposure, using the Agilent Genomic DNA Labelling Kit, using Cy5-dUTP for the patient DNA and Cy3-dUTP for the reference DNA. Labelled products were column purified (Agilent) and prepared combining test and control sample. After probe denaturation and pre-annealing with 50 µg of Human Cot-1 DNA (Invitrogen), hybridization was performed at 65°C for 40 h in a rotating oven at 20 rpm. After two washing steps the array slide was scanned with the Agilent C Scanner. The spot intensities were measured and the image files quantified using the Agilent Feature Extraction 1.5.1.0 software. Text outputs from the quantitative analyses were imported into Agilent Cyto Genomics Edition 2.0.6.0 software (Agilent Technologies). Breakpoint positions were reported according to GRCh37/hg19.

Western blot analysis

Western blot analysis of Munc18-2 protein was performed as previously described [15].

Case Study

A female baby (UPN 904), aged 47 days, second child from related parents of Egyptian origin, developed fever, splenomegaly, cytopenia (anemia, neutropenia and thrombocytopenia), with low levels of fibrinogen and high levels of ferritin. Therefore she was referred to our HLH Registry [1] for confirmation of the diagnosis, functional and genetic studies. The diagnosis was confirmed, according to the Histiocyte Society criteria [5].

Flowcytometry analysis showed normal perforin expression but almost undetectable degranulation capacity. Based on that, we started mutation analysis of the degranulation-related genes. Direct sequencing showed no mutations in *UNC13D* and *STX11*, while the analysis of *StxBP2* showed no amplification of exons 17-19 (Figure 1). The analysis of genomic DNA of both parents showed normal amplification profile and normal sequences; yet polymorphisms were present in homozygosis in the last three exons of the gene on both parents. This supported the hypothesis of a deletion of this region in the family.

To address this issue, we first performed Syber Green RT-PCR that revealed the loss of the genomic region spanning exons 18-19, while exon 2 was used as a reference (Figure 2). To confirm this result and to map the deleted region, we performed CGH-Array analysis that showed a deletion of the terminal portion of the gene (Figure 3). To document the effect of the deletion on the protein we used western blot analysis that showed protein absence (Figure 4).

Conclusion

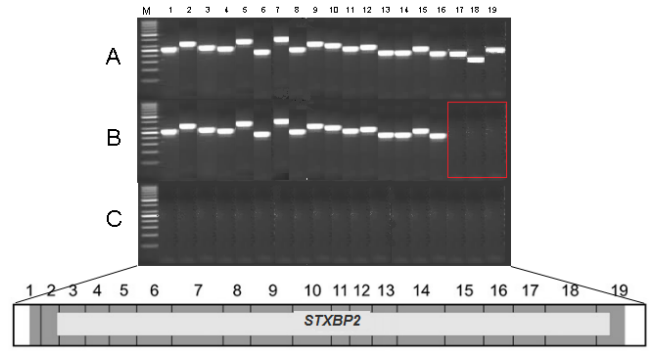


Figure 1: Gel electrophoresis of *StxBP2* gene.
A) Positive Control Lane: the figure shows the amplification of exons 1 to 19 of *StxBP2*. First line is DNA Molecular Weight Marker XIV 100 base pair (Roche). B) UPN 904 Lane: The figure shows the amplification of exons 1 to 16 and the absence of PCR product for exons 17, 18 and 19 (highlighted in red box). In the first line there is DNA Molecular Weight Marker XIV 100 base pair (Roche). C) No Template Control (NTC) Lane. The figure shows absence of amplification in NTC.

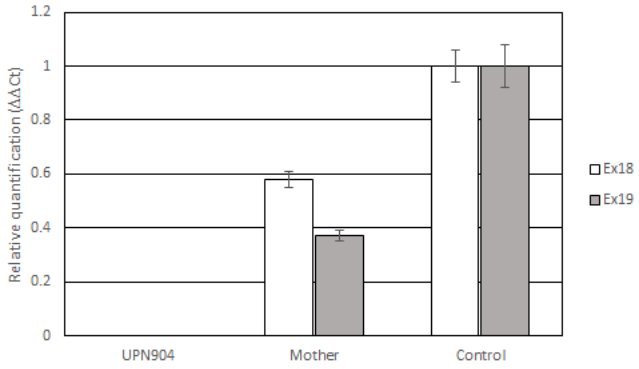


Figure 2: **Syber Green RT-PCR**. Real time PCR amplification of exon 18 and exon 19 in UPN 904, in her mother and in a normal control revealed biallelic loss of the genomic region spanning exons 18-19 in the patient while her mother showed a relative quantification suggestive for heterozygous loss of this region (ΔΔCt=0.58 for exon 18, ΔΔCt=0.37 for exon 19).

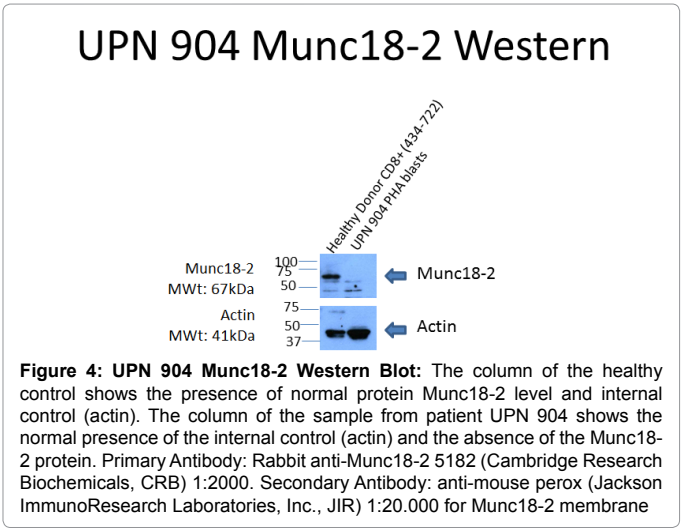
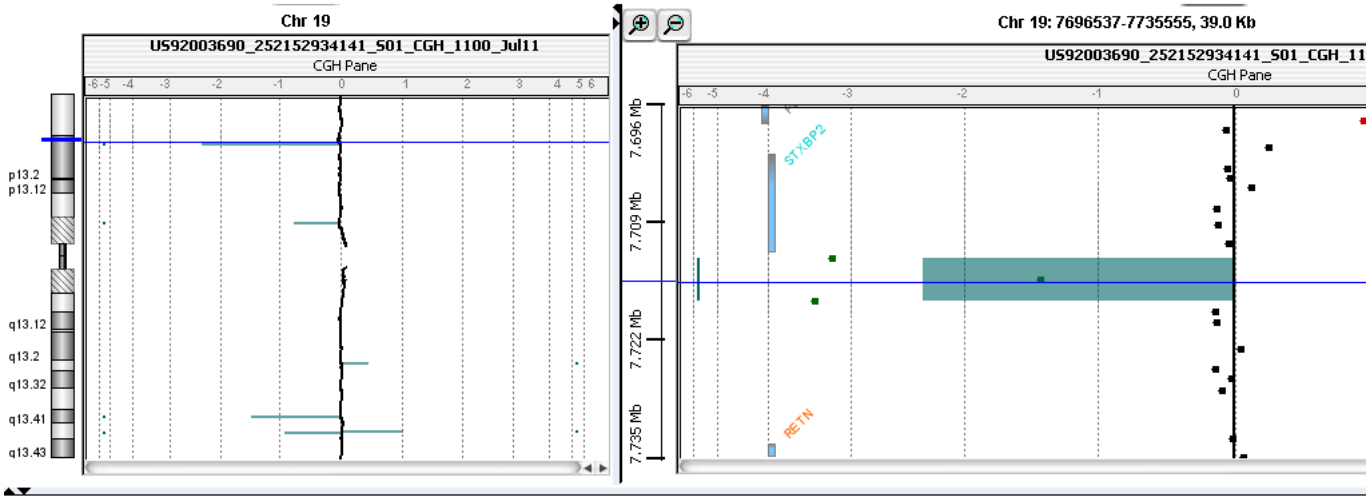
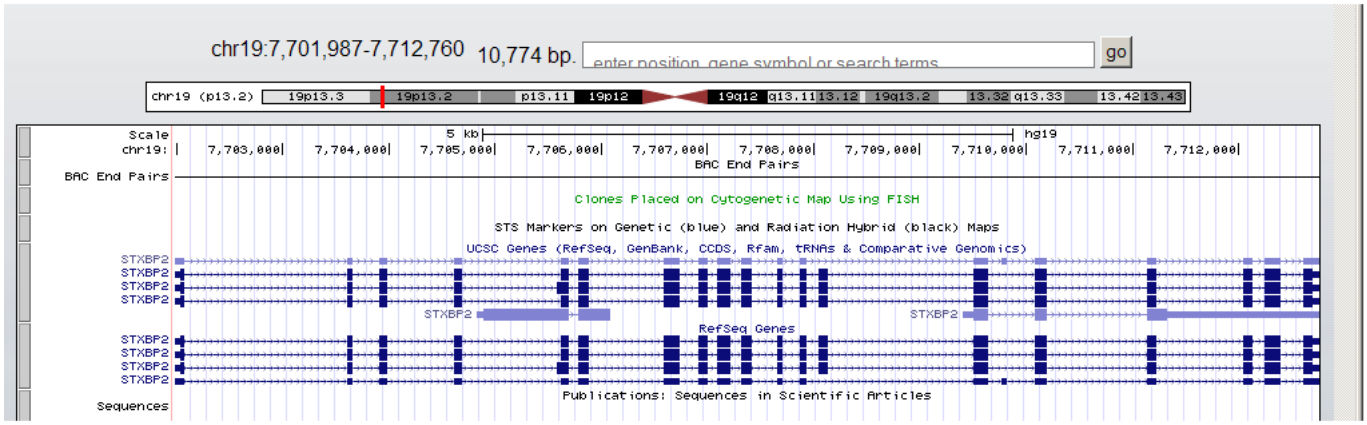


Figure 4: UPN 904 Munc18-2 Western Blot: The column of the healthy control shows the presence of normal protein Munc18-2 level and internal control (actin). The column of the sample from patient UPN 904 shows the normal presence of the internal control (actin) and the absence of the Munc18-2 protein. Primary Antibody: Rabbit anti-Munc18-2 5182 (Cambridge Research Biochemicals, CRB) 1:2000. Secondary Antibody: anti-mouse perox (Jackson ImmunoResearch Laboratories, Inc., JIR) 1:20.000 for Munc18-2 membrane



(a)



(b)

Figure 3: UPN 904 CGH-Array.
Schematic of the chromosome 19 region containing the 10,774 bp (7,701,987-7,712,760) microdeletion.
Panel (a) shows an ideogram of chromosome 19, together with the region encompassing the microdeletion.
Panel (b) shows the location and extent of the deletion detected in the pro-band reported here, involving the terminal portion of *StxBP2* gene. These graphics were taken from the UCSC genome browser (http://genome.ucsc.edu/).

Identification of genetic mutations in children with a clinical diagnosis of HLH is of paramount clinical relevance. Indeed, it has immediate therapeutic implications since it defines the indication to carry on an initial chemo-immunotherapy and bring the patient to early hematopoietic stem cell transplantation. Several genetic subtypes of FHL and related conditions have been identified over the last 15 years [2]. The initial flow-cytometry screening performed at the reference laboratories allows rapid identification of perforin as well SAP proteins, thus indicating specific mutation analysis [13,16,17]. Patients with defective degranulation at the CD107 expression assay are addressed to mutation analysis of the related genes (*UNC13D*, *STX11*, *StxBP2*) [13,14]. Mutations in *StxBP2* are associated with FHL5; the most recently identified genetic subset of this disease. In the small number of patients reported so far, different mutation have been observed [2,18]. Yet, deletions have never been reported in this gene. We report a child with homozygous deletion of the last three exons of *StxBP2*. To document this finding, we used a strategy of analysis including Sanger sequencing and CGH-array. With this approach, the mutation was characterized and confirmed on both parents, who are first cousins. Protein expression confirmed that this mutation was indeed pathogenic and accounted for the clinical picture of full-blown HLH with a very early onset.

This finding extends the spectrum of possible causative mutations in FHL5, like deletions/duplication, and draws attention to adapt new analysis strategies for selected patients.

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Disclosure of Conflict of Interest

None

Web Resources

HGMD, https://portal.biobase-international.com/hgmd/pro/gene.php?gene=*Stxbp2*

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