Mutations affecting mRNA splicing are the most common molecular defect in patients with familial hemophagocytic lymphohistiocytosis type 3

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ABSTRACT

Mutations of *UNC13D* have been described in patients affected by familial hemophagocytic lymphohistiocytosis (FHL3). The Munc13-4 protein contributes to the priming of the secretory granules. Mutation in this gene results in defective cellular cytotoxicity and the familial hemophagocytic lymphohistiocytosis clinical picture. Among reported mutations, few are predicted to impair splicing. Yet, functional impact of these mutations has not been addressed. We identified 18 out of 31 familial hemophagocytic lymphohistiocytosis families showing at least one mutation responsible for splicing error. We identified some known and three novel splicing mutations: one falls at the acceptor site of exon 11 and 2 are deep intronic mutations in IVS1 and in IVS30. We demonstrated that these deep intronic mutations affect regulatory sequences causing aberrant splicing. We report that *UNC13D* mutations leading to splicing errors represent the majority of mutations observed in familial hemophagocytic lymphohistiocytosis. This finding has implications for designing strategies for analysis of the families with suspected familial hemophagocytic lymphohistiocytosis.

Key words: lymphohistiocytosis, splicing.

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Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a genetically heterogeneous disorder characterized by a hyperinflammatory syndrome with fever, hepatosplenomegaly, cytopenia and sometimes central nervous system involvement.[1] The clinical picture may resemble that of leukemia, so bone marrow aspiration is usually performed early during the diagnostic work-up, allowing for hemophagocytosis by activated macrophages.

In most cases the natural course of HLH is rapidly fatal within a few weeks unless appropriate treatment, including corticosteroids, cyclosporine, etoposide, anti-thymocite globuline, can obtain transient disease control.[2-4] So far, only patients who underwent hematopoietic stem cell transplantation have been cured.[5-7]

Differential diagnosis of HLH may be difficult.[8] To this purpose, diagnostic guidelines for HLH have been established by the Histiocyte Society.[9,10] In particular, demonstration of frequent association with common pathogens, together with evidence of impaired natural killer cytotoxic activity, provided the rationale for considering HLH as a selective immune deficiency.[11-13] Starting from the original report by Farquhar *et al.* in 1952, autosomal recessive inheritance was proposed as the basis for the familial form of HLH (FHLH or FHL).

Since 2003, mutations of *UNC13D* (that encodes for the Munc13-4 protein) have been associated with FHL (FHL3, MIM 608898).[15] Munc13-4 is a critical effector of the exocytosis of cytotoxic granules priming cytotoxic granule fusion. Mutations in this gene impair the delivery of the effector proteins, perforin and granzymes, into the target cells resulting in defective cellular cytotoxicity and a clinical picture which appears very similar to that of FHL2 (MIM 603553),

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associated with PRF1 mutations.[16-19]

Recently, we screened the patient population for mutations of *UNC13D* and found that FHL3 accounts for a significant proportion of cases of FHL in Italy.[21] Among the observed mutations, scattered over the entire gene sequence, a few were reported to be responsible for impaired splicing of the gene products.[15,21-23] Six splice mutations are located in the exon/intron boundaries (322-1G>A, 753+1G>T, 754-1G>C, 1389+1G>A,1545-1G>T,1596+1G>C), 2 mutations (610A>G and 1847A>G) in the U1 exon recognition site, and only one (569+5G>A) in the intronic region. However, the functional implication of such mutations has not been widely addressed. Recently, an increasing number of disease-related mutations have been reported to be responsible for aberrant splice process,[22] increasing the focus of our research on mutations which might affect the splicing machinery. Notably, 15% of pathogenic mutations in mammalian genes affect mRNA splicing but, for some genes such as NF1 and ATM, nucleotide substitutions affecting the splicing process may account for up to 50% of all pathogenic mutations.

The splicing process involves many different proteins, including those of the *spliceosome*, which usually binds to the classical splice sequences located at the exon-intron borders (donor and acceptor sites). However, auxiliary sequences, scattered all over the gene, may serve as *exonic and intronic splicing enhancers* (ESE and ISE) that help in the recognition of exons.

In this report we show that *UNC13D* mutations leading to splicing errors are frequent in FHL3. Their detection and identification may be instrumental in the diagnosis of this disease.

Results and Discussion

We identified 31 families with FHL3 due to biallelic UNC13D mutations. Eighteen (58%) had at least one mutation predicted to be responsible for a splicing error (Table 1). A total of 7 mutations affecting splicing were identified. Only 2 of them, 753+1G>T and 1389+1G>A, had been already reported by other groups. The remaining 5 have only been observed in our patients: the 610A>G (M204V) and 1847A>G (E616G) (included in our recent report),[20] the previously unreported 952-1G>A, and in two families we found an allele containing deep (>10nt from the exon/intron boundary) intronic mutations (one had IVS1 +59C>T, +394C>T, +525G>T, and the other had IVS30+431delA).

Mutation 753+1G>T

This mutation, which falls at the donor site of exon 9, was found in ten families (UPN 6, 173, 180, 182, 196, 211, 225, 285, 363, 419). In five families it was at the homozygous state, while five were compound heterozygous for: 1847A>G (E616G) (n=2), 2346delGGAG (782fsX12), 1387C>T (Q463X), or 2570T>G (F857C).

A study of cDNA of 2 homozygous patients (UPN 285 and 419) showed that the only detected specific product was smaller than normal, indicating probable exon skipping. The amplification product was, therefore, sequenced and the whole exon 9 was found to be missing. In 3 patients, 2 homozygous (UPN 180 and 285), and one compound heterozygous (UPN 225), Western Blot analysis confrmed the absence of the Munc13-4 protein (*data not shown*).

Table 1. UNC13D mutations responsible for splicing errors observed in 18 patients with FHL3.

UPN publication	*Haplotype*	*Haplotype*	*Previous*
6	753+1 G>T	2346delGGAG (R782fsX12)	None
173	753+1 G>T	753+1 G>T	None
180	753+1 G>T	753+1 G>T	Ref 20
182	753+1 G>T	1387 C>T (Q463X)	None
196	753+1 G>T	2570 T>G (F857C)	Ref 20
198	1847 A>G (E616G)	1847 A>G (E616G)	Ref 20
211	753+1 G>T	1847 A>G (E616G)	None
225	753+1 G>T	1847 A>G (E616G)	Ref 20
237	1847 A>G (E616G)	2782 C>T (R928C)	Ref 20
249	1847 A>G (E616G)	1847 A>G (E616G)	Ref 20
285	753+1G>T	753+1 G>T	None
289	610 A>G (M204V), 2650 C>T (Q884X)	3226insG (1076fs)	Ref 20
350	1389+1 G>A	2346delGGAG (R782fsX12)	None
356	IVS30+431delA	2346delGGAG (R782fsX12)	None
363	753+1 G>T	753+1 G>T	None
383	IVS1+59 C>T,+394 C>T, +525 G>C	2346delGGAG (R782fsX12)	None
390	952-1 G>A	952-1 G>A	None
419	753+1 G>T	753+1 G>T	None

UPN: unique patient number.

Mutation 1847A>G

This mutation was found in five families (UPN 198, 211, 225, 237, and 249). Two were homozygous, and three compound heterozygous: two for 753+1G>T, and one for 2782C>T(R928C). It falls at two nucleotides from the end of exon 20, where it is predicted to disrupt U1 splice site recognition. To verify this, we cloned and sequenced cDNA from a homozygous patient, showing that 1847A>G disrupts splicing and results in several RNA products. Abnormal splicing results were found in 6 out of the 8 clones screened: 3 clones showed abnormal splicing of exon 20, 2 clones showed intronic *exonization* of 130 bp, and one clone showed an in-frame insertion of 54 intronic nucleotides between exons 1 and 2.

In 2 patients, one homozygous (UPN 249) and one compound heterozygous (UPN 237), Western Blot analysis confirmed the absence of the Munc13-4 protein (*data not shown*).

Mutations 952-1G>A and 1389+1G>A

These were found in one family each. The 952-1G>A, which was found in one homozygous patient (UPN 390), falls at the acceptor site of exon 11. The 1389+1G>A, which was found in combination with 2346delGGAG in one patient (UPN 350), falls at the

donor splice site of exon 15. Unfortunately, no material from these cases was available for protein study.

Mutation 610A>G (M204V)

Mutation 610A>G, (M204V), found in association with 2650C>T(Q884X) and 3226insG (1076fs) in one patient (UPN 289), is located at five nucleotides from the end of exon 7. The reported mutations make the sequence more closely complementary with U1snRNA, and might be expected to affect splicing, although there is no confirmed evidence of this.

Western Blot analysis documented the absence of the Munc13-4 protein (*data not shown*).

Deep IVS1 mutations

In one patient (UPN 383), the maternal allele carried 2346delGGAG, and the paternal three deep intronic mutations: IVS1+59C>T, +394C>T and +525G>T. All 3 involve putative intronic splicing enhancer (ISE) motifs: 2 of them created a new ISE (R0952 and R0987) and the other (+525G>T) disrupted a pre-existing ISE (R0952) (Figure 1). Due to the uncertain role of deep IVS1 mutations, we amplified the messenger RNA. Together with a normal-sized band we found a larger band suggesting an insertion; both bands were present in the father. Therefore, to characterize the splice error, the larger PCR product was cloned. By screening and sequencing ten colonies, we identified an intronic insertion of 130bp between exons 1 and 2 in five clones, and the retention of the intron between exons 3 and 4 in another clone (Figure 1). Western Blot analysis confirmed the absence of the Munc13-4 protein (Figure 1C).

Deep IVS30 mutations

We identified a patient (UPN 356) carrying a monoallelic mutation in the coding region 2346delGGAG; the patient showed the skipping of exon 31 at cDNA level (Figure2). To search for the genomic mutation responsible for exon 31 skipping in the mRNA, we sequenced the associated intron, and found a deep nucleotide deletion in intron 30: IVS30+431delA. Interestingly, this mutation occurs in a putative *intronic splicing silencer* (ISS, R1018), a cis-element that promotes exclusion of a specific exon from the mature mRNA.

Western Blot analysis confirmed the absence of the Munc13-4 protein (Figure 2).

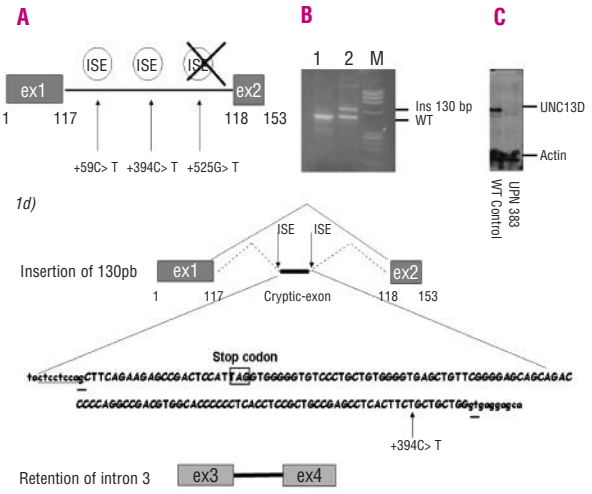

Figure 1. (A) Three deep IVS1 mutations involving ISE motifs in UPN 383 causing the insertion of a cryptic exon. (B) RT-PCR analysis of RNA from CTL showing a larger sized band due to an insertion of 130 bp (lane 2) that is absent in the control (lane 1), M= molecular marker (ºX174). (C) Western Blot showing the absence of UNC13D protein CTL from UPN 383. (D) Two clones identified in the patient carrying the IVS1 deep mutations (UPN 383), insertions and intron retention are represented by thick horizontal lines. The genomic localization (thick line) and sequence (capital letters) of the cryptic exon are shown. The splice site used by the pseudoexon are underlined. The location of the mutated base and of the ISE motifs are indicated (arrows). Inclusion of the cryptic exon introduces a premature stop codon (boxed).

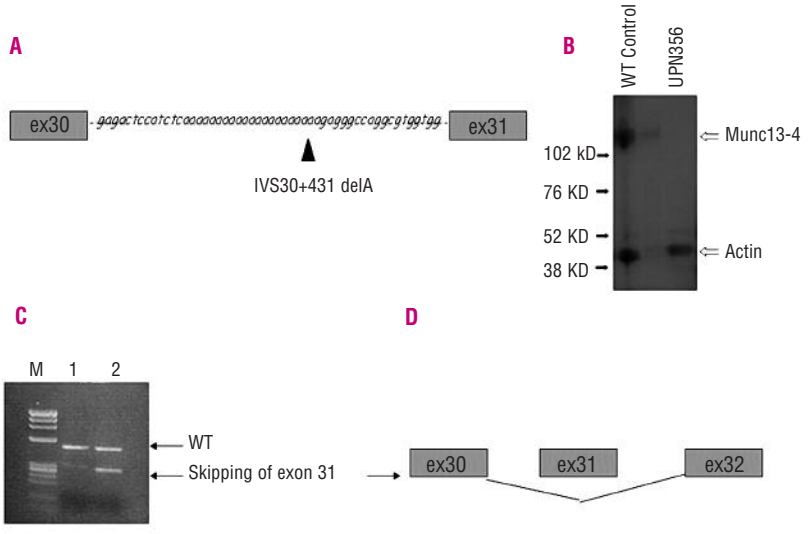

Figure 2. Mutation IVS30+431 delA. (A) Schematic representation of the mutated position in IVS30. (B) Western Blot showing the absence of UNC13D protein CTL from UPN 356. (C) RT-PCR analysis of RNA from CTL of UPN 356 showing a smaller sized band due to exon skipping. (D) Presentation of splicing consequence: exon 31 skipping is represented by thin diagonal lines connecting exon 30 and 32.

Cryptic-exon inclusion

We found the insertion of the same 130bp cryptic-exon in two unrelated families with FHL3 (UPN 249 and 383). By a BLAST search of GeneBank, the inserted sequence was identified as part of intron 1. This inserted sequence was identical in the 2 patients. The novel cryptic-exon is delimited at both 5' and 3' ends by ISE sequence; it includes the IVS1+394 nucleotide and a stop codon, located 7 codons after the inserted sequence. Cryptic splice sites used by the pseudoexon were predicted by NNSPLICE, assigning at both donor and acceptor sites a high score of 0.93 and 0.85 respectively.

UNC13D cDNA sequence in control subjects

To investigate whether the aberrant splicing found in our series represented normal alternative transcripts, we amplified cDNA from 10 healthy controls and assessed the correct UNC13D RNA splicing. No bands of unexpected size were found in any control cDNA. Furthermore, the entire correct cDNA sequence was obtained from 3 of these healthy subjects.

The diagnosis of HLH may be difficult. In particular, the clinical syndrome defined as HLH may be shared by patients with infection-associated, transient disease, usually cured by etiological anti-infectious therapy, sometimes associated to short-term immuno-suppressive therapy.[4,23] On the contrary, patients with FHL not only deserve a prompt, intensive treatment with chemo-immunotherapy, but can only be cured by hematopoietic stem cell transplantation (HSCT).[4,5-7] Therefore, the genetic study of patients with HLH, which at present remains the *gold standard* for discrimination between the *secondary* and the genetic form, is urgently needed for the families involved and the attending physicians.[8,16,24]

PRF1 mutations, associated with FHL2, account for only 40% of the population in our experience (*Aricò and Santoro, unpublished data*). Based on our preliminary studies, an additional 40% of our patients have FHL3 (*Aricò and Santoro, unpublished data*). The observation of some families with a single pathogenic mutation prompted us to investigate alternative defects of *Munc13-4*.

The splicing machinery may be affected by the size of the gene, with genes with more exons being more prone to splicing errors. The complexity of the *UNC13D* splicing process may suggest that, as already reported for *NF1, ATM, CHRNE* or *COL4A5*, it may be more vulnerable to pathogenic splicing errors.

The 753+1G>T is the most frequently observed mutation in our population, and this has also been reported by other groups.[14,21] It falls at the consensus site and causes the skipping of the adjacent exon. Yet, splice errors may also result from deep intronic mutations involving intronic splicing enhancer (ISE) motifs which promote exon recognition. By studying patient UPN 383, we documented that deep intronic mutations might either create a new ISE (IVS1+59C>T and +394C>T) or disrupt a pre-existing one (IVS1+525G>T). In particular, mutation IVS1+394C>T seems to promote the activation of the nearby splicing and the insertion of a cryptic exon. This finding is in keeping with observations in Alport syndrome and in the familial form of retinoblastoma in which the intronic exonization was caused by a deep intronic mutations in the *COL4A5* and *RB1* genes respectively.

Another unexpected finding was the identification of the same 130bp cryptic-exon activation in two unrelated families. This insertion is part of intron 1, is delimited by two ISE motifs at both 5' and 3' ends, it used strong cryptic splice sites (predicted by *in silico* analysis) and contains a stop codon leading to a truncated protein that will be degraded by nonsense-mediated decay activity.

In conclusion, we report that *UNC13D* mutations responsible for splicing errors are a very frequent cause of FHL3. In fact, over one half of the total pathogenic mutations arise from an *UNC13D* splice error. Although a few disease-causing splicing mutations at the canonical splice site sequences have been previously described, we describe the importance of deep intronic mutations which cause aberrant splicing in contributing to this disease. This is important when designing strategies for mutation analysis of *UNC13D* in patients with FHL. Although patients with a single pathogenic mutation identified by exon-by-exon approach are the most obvious targets for splicing studies, in these cases, the presence of two splicing, *hidden* mutations cannot be ruled out. Evidence of impaired degranulation as detected by the CD107a expression flowcytometry assay,[24] or defective protein at immunoblotting, may herald FHL3 and therefore strongly support the need for such an extended *UNC13D* analysis. Identification of *UNC13D* mutations allows genetic diagnosis of FHL3 in children and young adults, with immediate therapeutic implications, including indication for HSCT, selection of familial donor and prenatal diagnosis.

Authorship and Disclosures

AS: study design, supervisor of molecular study, analysis of the data, manuscript writing; SC: molecular study; AT: clinical data collection; GB: mutation analysis; CDF: clinical data collection; LDN: analysis of the results, manuscript writing; DP: cellular cytotoxicity study; GMG: protein and confocal study; MA: PI, study design, analysis of the clinical data, writing the manuscript, financial support. The authors reported no potential conflicts of interest.

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