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

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## Design and Methods

*(Online exclusively as a supplementary appendix)*

### Patients selection

All patients had FHL diagnosed on the basis of the current diagnostic criteria,[1,2] including mutation analysis.

### UNC13D gene analysis

Genomic and mRNA sequences of the UNC13D gene were retrieved from the National Center for Biotechnology Information (LOC201294; mRNA XM_113950). Genomic DNA was prepared from the peripheral blood samples obtained from the patients and their family members. UNC13D gene sequence obtained by the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) was analyzed and compared with the reported gene structure using the dedicated software SeqScape using an ABI Prism 3130 Sequence Detection System (Applied Biosystems). Sequence primers used for amplification are available on request. For UNC13D cDNA analysis total RNA was extracted from peripheral blood lymphocytes by the 6100 Nucleic Acid PrepStation protocol (Applied Biosystems).

cDNA was obtained by reverse transcriptase-PCR performed on the first-strand cDNA synthesized with the High Capacity cDNA Archive Kit (Applied Biosystems). UNC13D cDNA was amplified obtaining 10 fragments directly sequenced, in both directions, with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence primers used for amplification are available on request. Ten healthy controls were studied to assess the correct *UNC13D* RNA splicing.

When splicing errors were observed, we analyzed the sequence of the related intronic regions.

### Construction of UNC13D clones for splicing analysis

When unusual-sized products suggestive of splicing errors were identified, these products were cloned and sequenced to characterize RNA structure.

The PCR product was cloned into pCRTOPO-Blunt II-TOPO vectors. Cloning reactions and transformation of E.Coli TOPO10 competent cells were performed according to the TOPO TA Cloning kit instructions (Invitrogen). Ten positive transformants (white colonies) were screened and sequenced to investigate how the mutations affect Munc13–4 mRNA splicing.

### Immunoblotting

Western Blot analysis of Munc13-4 protein was performed as previously described.[3] Briefly, NK cells lysates were resolved by SDS gel electrophoresis (SDS-PAGE) on NuPAGE 4-12% Bis-Tris gels (Invitrogen, Paisley, United Kingdom) under reducing conditions. Proteins were transferred to nitrocellulose membranes (Invitrogen, Paisley, United Kingdom) and then incubated for 16 hrs at 4°C with rabbit anti-Munc13-4 antibody [a gift from Hisanori Horiuchi]. Membranes were incubated for one hour with HRP-labeled anti-rabbit Ig secondary antibody (Jackson ImmunoResearch Laboratories). The membranes were normalized using a rabbit anti-actin antibody (Sigma-Aldrich, Irvine, United Kingdom).

### In silico analysis

All the exons of UNC13D gene with the adjacent intronic sequences were analyzed, using computer programs available on the web, to assess mRNA processing. To find auxiliary splicing regulation motifs (ESEs, ISEs) we used a web query tool (RegRNA: *http://regrna.mbc.nctu.edu.tw*), and ISEs were defined according to its coding. To predict the presence and to analyze the strengths of cryptic splice sites we used the tool NNSPLICE (*http://www.fruitfly.org/seq_tools/splice.html*), with a minimum score cut-off for splice site predictions of 0.4, that allowed the identification of more than 83% of sites with less than 5% false positive.

## References

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