Journal of Clinical Immunology (2019) 39:138–141
https://doi.org/10.1007/s10875-019-00599-3

Auto-inflammation in a Patient with a Novel Homozygous *OTULIN* Mutation

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Received: 18 November 2018 / Accepted: 6 February 2019 / Published online: 22 February 2019

To the editor:

Homozygous mutations in OTULIN (Ovarian TUmor domain deubiquitinases with LINear linkage specificity) result in an auto-inflammation named OTULIN-related auto inflammatory syndrome (ORAS), characterized by prolonged recurrent fevers, joint swelling, gastrointestinal inflammation/diarrhea, failure to thrive, lipodystrophy, and painful erythematous rash with skin nodules [1, 2]. OTULIN deficiency manifests early in life and is potentially lethal.

OTULIN is a deubiquitinase that hydrolyzes linear ubiquitins [3]. Linear ubiquitination regulates various signaling pathways, including the NF-κB pathway. Ubiquitination of NEMO is mediated by Linear UBiquitin chain Assembly Complex (LUBAC). Patients with defects in a LUBAC component develop immunodeficiency and auto-inflammation and die in early childhood [4, 5]. OTULIN counter-regulates LUBAC activity by hydrolyzing linear ubiquitins, thereby avoiding unrestricted NF-κB activation. In the absence of OTULIN, the level of linear ubiquitin is not controlled and the immune system is activated, even in the absence of infection [4, 5].

Here, we report an Iranian newborn child (gestational age 35 weeks) from consanguineous parents (first cousins) (Fig. 1 panel I). Soon after birth, she developed abscesses (erythematous nodules) without fever or sepsis and consequently was hospitalized. The lesions were scattered over the chest and both extremities (Fig. 1 panel II). There was leukocytosis, especially neutrophilia with elevated ESR, CRP, and total IgG (Supplemental data, Table 1). Other immunological tests, including opsonization, chemotaxis, phagocytosis, and oxidative burst (nitroblue tetrazolium and dihydrorhodamine), were normal. Cultures of blood for *Mycobacterium tuberculosis* and of the lesions for gram negative and positive bacteria were negative at 2, 4, and 6 months (during hospitalizations). The patient was treated with broad-spectrum antibiotic therapy and

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10875-019-00599-3) contains supplementary material, which is available to authorized users.

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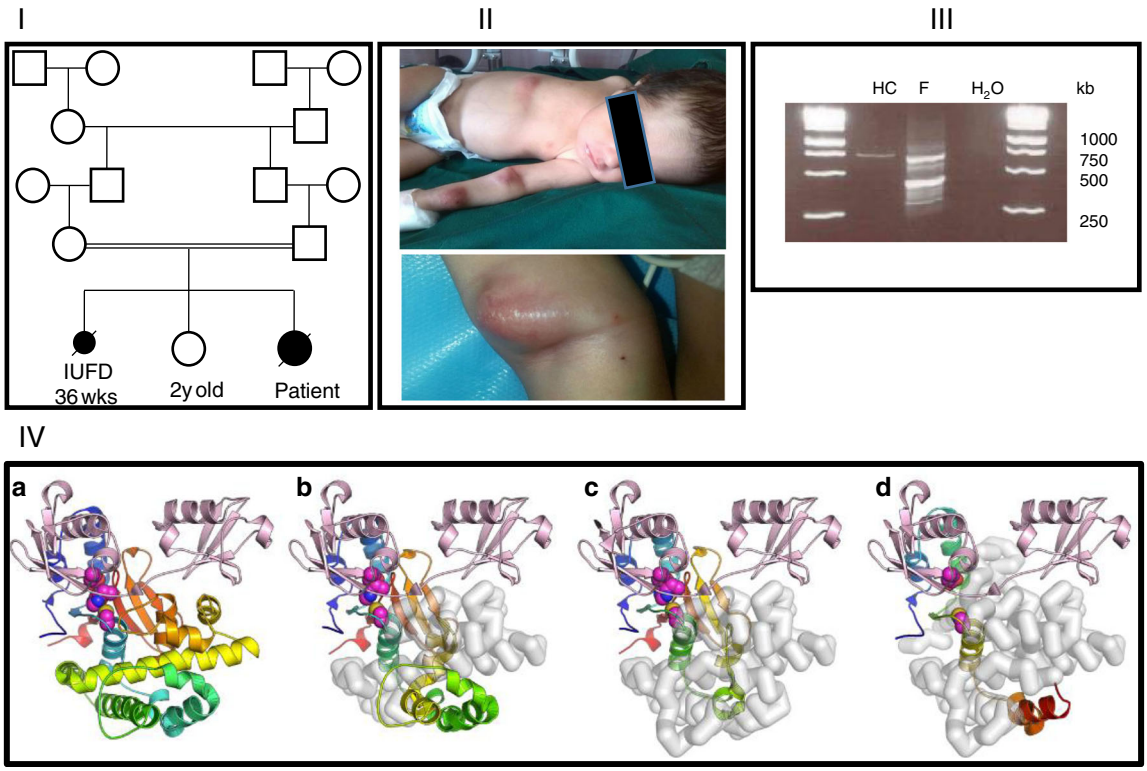

Fig. 1 Characterization of patient and OTULIN splice variants. Panel I: Pedigree from consanguineous family (IUFD, intrauterine fetal death). Panel II: Erythematous nodules in multiple areas. Panel III: Agarose gel electrophoresis of products obtained from RT-PCR using primers anchored in exon 4 and exon 7 of OTULIN and template cDNA from a healthy control (HC), the father (F; heterozygous carrier) and a negative control (H_2O). kb, kilo base; HC, healthy control. Panel IV: Modeling of OTULIN splice variants. Wild type and mutant OTULIN are depicted as a cartoon colored blue to red, from N to C termini, with the two ubiquitins depicted in light pink. The deleted stretches of amino acids are depicted as a transparent white tube in the mutant OTULIN. The catalytic residues of the OTULIN Ubiquitin complex are depicted as pink spheres. (A) Wild-type OTULIN. (B) Predicted OTULIN resulting from exon 6 skipping. (C) Predicted OTULIN resulting from skipping of exon 5 and exon 6. (D) Predicted OTULIN resulting from retention of 17 nucleotides from intron between exon 4 and 5 followed by skipping of exon 6

with interferon-γ, because the lesions pathologically resembled those found in phagocytic disorders. She remained well for 2 months but experienced an exacerbation of the previous lesions (at the same site, but more severe and outspread) at 2, 4, and 6 months of age (for which hospitalization was needed). These exacerbations coincided with the administration of routine vaccine injections (BCG, DTP, hepatitis B, MMR, and IPV). At the age of 8.5 months, the patient developed a high-grade fever with respiratory distress and mild hepatosplenomegaly. Chest X-ray showed pulmonary edema that led to her demise. The parents are healthy.

Whole-exome sequencing revealed a homozygous c.864 + 2 T > C variant in *OTULIN* (NM_138348). There was no other relevant homozygous mutation (Supplemental Data Table 2). Sanger sequencing (Supplemental Data Fig. 1) confirmed the homozygous *OTULIN* mutation in the patient and revealed a heterozygous mutation in the parents. This variant was not present in the gnomAD and in 1785 Iranian controls. In silico analysis of this variant predicted an alteration of the wild-type donor site which will probably affect splicing. Thus, we investigated the effect of the variant on splicing of OTULIN mRNA by reverse transcriptase (RT)-PCR analysis of cDNA derived from fibroblasts of a heterozygous carrier (father) and healthy control. As the patient died, no cells from the patient were available for analysis. For detailed analysis of splicing of intron 5–6, we (i) performed a PCR with primers anchored in exon 4 and 7, (ii) separated the reaction products by gel electrophoresis, and (iii) sequenced individual clones after pJet1.2 cloning (Thermo Fisher Scientific). In addition to the fully spliced mRNA PCR fragment (also found in the control), different smaller bands were uniquely found in PBMCs from the heterozygous carrier (Fig. 1 panel III). Sanger sequencing of individual clones revealed PCR fragments skipping exon 6, skipping exon 5 and exon 6, or retention of 17 nt between exon 4 and exon 5 followed by exon 6 skipping. Expasy translation software predicted that the observed in vivo splicing will result in a deletion of 90 amino acids, a deletion of 132 amino acids or a frameshift creating a stop codon (L157GfsX12), respectively. Surprisingly, expression of OTULIN protein in primary fibroblasts of the father was comparable to expression in control fibroblasts (data not shown). We hypothesize that the truncated protein is not stable and is degraded. IL-6 production of heterozygous carrier primary fibroblasts after 48 h stimulation with LPS (10 μg/mL), IL-1β (50 ng/mL), Poly I:C (12.5 μg/mL) and TNF-α (100 ng/

mL) was comparable to cytokine response of control primary fibroblasts (data not shown).

Modeling of the different splice variants starting from the crystal structure of OTULIN [using the homology module in the Molecular Operating Environment (The Chemical Computing Group, Montreal, Canada)] suggests instable/inactive OTULIN protein (Fig. 1 panel IV), which is in line with the observed clinical phenotype. Skipping of exon 6 results in the deletion of 90 amino acids (Fig. 1 panel IV B). Such modification leaves the active site area intact, but it leaves a large portion of the hydrophobic protein core exposed to the hydrophilic solvent resulting in an unstable protein. Furthermore, a loop which is required for coordinating the peptide linker between the consecutive ubiquitin domains in the active site, and thereby allowing catalysis to occur [3], is absent. Both alterations would result in an inactive OTULIN protein. A similar effect is observed when exons 5 and 6 are skipped, leading to a loss of 132 amino acids from the core of the protein (Fig. 1 panel IV C). Finally, OTULIN formed by retention of 17 nucleotides of the intron between exon 4 and 5 followed by skipping of exon 6 lacks the majority of the protein structure domain required for interacting with linear ubiquitins, resulting in an inactive protein (Fig. 1 panel IV D).

Previously identified disease-causing variants in OTULIN include L272P [1, 2]. Y244C and G174Dfs*2 [2]. All these disease-causing variants affect the deubiquitinase function of OTULIN. Leu 272 forms a part of the binding pocket for linear di-ubiquitin. The homozygous variant described in this report affects splicing and computer modeling predicts impaired deubiquitinase activity.

OTULIN deficiency is associated with enhanced NF-κB activity and TNFR1 signaling. Anti-TNF-α is considered the preferred therapy for OTULIN deficiency. The patient reported by both Damgaard et al. [1] and Zhou et al. [2] (L272P) did not respond to corticosteroids or an IL-1R antagonist (anakinra), but responded to anti-TNF treatment (infliximab). For the other two patients described by Zhou et al. [2], one patient (Y244C) responded to a high dose of Anakinra and the other patient (G174Dfs*2) partially responded to TNF inhibition (etanercept; dose not specified).

In conclusion, we report a novel disease-causing variant in OTULIN that affected splicing. The patient suffered from recurrent erythematous rash and skin nodules and systemic inflammation (resulting in fatal pulmonary edema). The diagnosis was made based on genetic evidence and RT-PCR analysis that confirmed the presence of multiple alternatively spliced transcripts in the father's RNA sample. Functional analysis could not be performed, but the truncated proteins are predicted to be inactive.

OTULIN deficiency is very rare and only 4 patients have been described in the literature. The phenotype is severe, potentially lethal and treatment with anti-TNF therapy is efficacious in controlling disease activity. Thus, it is critical to identify these patients early in life and treat them appropriately. This case highlights the burning need for early genetic diagnosis such that adequate therapy can be initiated timely.

Acknowledgements We kindly thank Saba Arshi, Mohammad Hassan Bemanian, Morteza Fallahpour, Alireza Nateghian, Farhad Abolhasan Chobdar, Davood Mansouri, Mehrnaz Mesdaghi, and Mojgan Kiani Amin for their cooperation and for their help in the work-up of the patient and taking care of the patient. We thank the patient and her family.

Research Funding This work was supported by a Geconcerteerde Onderzoeksactie (GOA) grant from the Research Council of the Catholic University of Leuven, Belgium.

Authors' Contribution Whole-exome sequencing, data analysis, and clinical diagnosis were done by Mohammad Shahrooei, Hassan Rokni-Zadeh, and Majid Changi-Ashtiani. Kian Darabi collected the data, Mostafa Manian gathered samples for analyses, and Farhad Seif made substantial contributions to design of the study and edited the draft. Mohammad Nabavi took care of the patient. Isabelle Meyts edited the draft. Jeroen Vrancken and Arnout Voet performed and described the modeling studies. Leen Moens and Xavier Bossuyt designed the experimental studies. Leen Moens performed the experimental analyses. Mohammad Nabavi and Xavier Bossuyt drafted the manuscript. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interests.

Ethical Approval Informed consent was obtained.

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