Familial hemophagocytic lymphohistiocytosis in an adult patient homozygous for A91V in the perforin gene, with tuberculosis infection

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Perforin gene (*PRF1*) mutations have been reported in 20-30% of patients with familial hemophagocytic lymphohistiocytosis (FHL), an autosomal recessive disorder of infancy and early childhood that impairs or abolishes lymphocyte cytotoxicity. We report the first case of FHL in an adult patient homozygous for A91V in *PRF1* with tuberculosis. The monozygotic twin of the patient is healthy. A91V confers genetic susceptibility for the development of FHL, but is not enough to trigger the disease on its own. We discuss the role of the A91V change together with *M. tuberculosis* infection as synergistic factors in the late onset of FHL.

Key words: perforin, familial hemophagocytic lymphohistiocytosis, A91V mutation, tuberculosis.

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Hemophagocytic lymphohistiocytosis (HLH) is an immune disorder characterized by uncontrolled activation of T cells and macrophages and overproduction of inflammatory cytokines.[1,2] The primary form of HLH or FHL is genetically determined, whereas the secondary form occurs in association with infections (mostly viral, but also bacterial, fungal, mycobacterial and parasitic), malignancies and autoimmune disorders. These two forms may be difficult to distinguish from each other. Mutations of *PRF1* (FHL type 2) and *MUNC 13-4* (FHL type 3) genes are the most frequent genetic defects in FHL patients. *PRF1* gene codes for perforin, a protein expressed by natural killer (NK) cells and cytotoxic T lymphocytes (CTL). Perforin plays a critical role in the cytolytic process. Impaired cytotoxicity by NK cells and CTL is crucial in the pathophysiology of FHL type 2.[3] The onset of this disease may be triggered by infections, especially those caused by Epstein-Barr virus (EBV). FHL usually affects infants, but adult onset has been exceptionally reported.[4-6]

We report here our investigations of a family with eight siblings. Two monozygotic twin males in this family together with another brother all carry the homozygous A91V *PRF1* change. However, their clinical phenotypes differ: one of the twins has FHL and pulmonary tuberculosis, the identical twin is healthy, and the third brother suffers from chronic lymphocytic leukemia (CLL).

Case report

A 49-year old male patient (II-2), born of non-consanguineous parents from Formentera Island (Spain), was admitted to the hospital because of fever and pancytopenia. A diagnosis of tuberculous spondylitis had been made at the age of 29. The father of the patient (I-1) died of cirrhosis, the mother (I-2) died of B-cell non-Hodgkin's lymphoma (NHL), one brother (II-1) died of gastrointestinal bleeding, another brother (II-8) has CLL, the monozygotic twin (II-3) remains asymptomatic with a healthy son (III-1), and three sisters (including two monozygotic twins) (II-4, II-6, II-7) and one brother (II-5) are healthy. The last one had a son (III-2) who had a sudden death (Figure 1A).

Five days before admission, the patient developed periorbital swelling and was treated with corticosteroids. Laboratory tests performed on admission yielded the following results: hemoglobin 10.4 g/dL (normal: 13-16.5), leukocyte count 1300/μL (4100-11000), neutrophils 800/μL (2500-7500), platelets 14000/μL (130000-390000), lactate dehydrogenase 1498 U/L (240-480), ferritin 7000 ng/mL (30-400), direct bilirubin 3.27 mg/dL (0.1-0.3), aspartate aminotransferase 321 U/L (2-38), alanine aminotransferase 102 U/L (2-41), alkaline phosphatase 383 U/L (40-129), triglycerides 277 mg/dL (50-200) and soluble interleukin-2 receptor (sCD25) 63190 pg/mL (0-1900). Serologic tests for

EBV and cytomegalovirus indicated past infection. A test for the human immunodeficiency virus (HIV) was negative. A chest radiography showed no abnormalities. On admission, examination of a stained bone marrow aspirate revealed isolated histiocytes with hemophagocytosis. Immunophenotyping by flow cytometry of bone marrow revealed no abnormal cellularity, and the karyotype was normal.

Imipenem and antituberculosis therapy was started. A new bone marrow aspirate, 6 days later, showed histiocytes with evident hemophagocytosis (Figure 1B). Therapy with the HLH-94 protocol[7] was started, but the patient died of pulmonary hemorrhage 10 days after starting treatment. A sputum sample smear obtained on the second day in hospital was negative for acid-fast organisms, but a culture became positive for *M. tuberculosis* two months later. Blood and bone marrow cultures were negative for bacteria and mycobacteria.

Autopsy revealed numerous histiocytes with hemophagocytosis in the bone marrow and spleen, and an alveolar hemorrhage in the lung. Cultures of these tissues were negative for mycobacteria. Immunological features of the family members are detailed in Table 1.

Design and Methods

Lymphocyte populations and subpopulations in whole blood were analyzed by flow cytometry. Perforin expression was evaluated in all living family members by flow cytometry using anti-perforin antibody (δG9 clone) (BD Biosciencies, San Jose, CA, USA).[8] NK activity (in percent specific lysis) was tested in family members II-3, II-8 and III-1 and in two healthy controls by standard procedures.[9] Serum interleukin-6, interferon-γ, tumor necrosis factor-α (Bender MedSystems, Vienna, Austria) and sCD25 (R&D, Abingdon, UK) were measured in duplicate by enzyme linked immunosorbent assays (ELISA). DNA samples were obtained from the family as well as from 32 healthy blood donor who were natives of Formentera Island. Exons 2 and 3 of the *PRF1* gene coding region were amplified using polymerase chain reaction (PCR). PCR products were purified and sequenced as previously reported.[10]

Results and Discussion

To our knowledge, we report the first case of FHL in an adult patient homozygous for A91V in the *PRF1* gene. Up to now, A91V homozygosity has been described in only three subjects, an adult who was healthy until reported,[11] a child with acute lymphoblastic leukemia,[12] and a 5-year old patient with FHL.[13] Our patient developed this disease in adulthood in the setting of *M. tuberculosis* infection. Tuberculosis-associated HLH has rarely been reported; it can take place in patients with other medical conditions such as hemodialysis, HIV infection or aplastic anemia, and carries a high mortality.

Some researchers believe that the A91V substitution represents a neutral polymorphism because the fre-

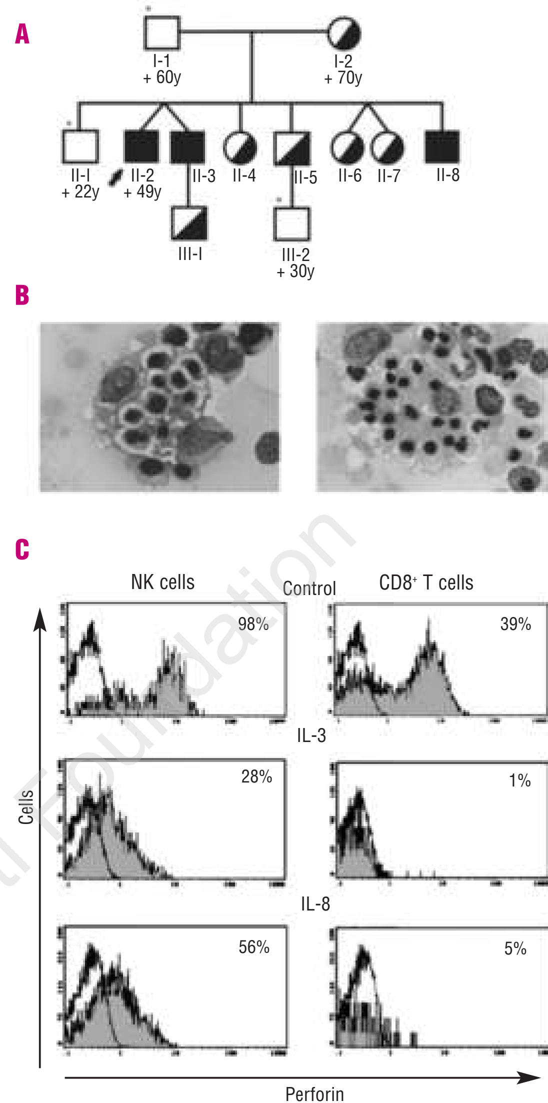

Figure 1. A. Family pedigree. Pedigree of the family members indicating the subjects homozygous for the change (black squares) or heterozygous for A91V (half black squares or circles) in *PRF1*. Asterisks indicate the family members in whom mutational analysis was not conducted. The males are represented by squares and the females by circles. The patient with FHL is indicated by an arrow. Deceased subjects are indicated with a cross together with the age of death. **B.** Morphologic findings in the bone marrow. Histiocytes containing phagocytosed erythroid precursors in the bone marrow aspirate smear (magnification ×1000). **C.** Perforin expression pattern in NK and CD8+ T cells from a healthy control, II-3 and II-8. Subject II-3 (monozygotic twin of the patient) and II-8 (patient's brother with CLL), both homozygous for the A91V change in the *PRF1* gene, have a partial deficiency of perforin; the perforin expressed in NK cells was moderately decreased, while the perforin expressed in CD8+ T cells was extremely reduced. (Open histograms represent staining with an isotype control antibody).

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Table 1. Immunological laboratory features and viral serologic tests of the family members.

	II-2 (Patient)	II-3	II-4	II-5	II-6	II-7	II-8	III-1	Normal values
A91V change	V/V	V/V	A/V	A/V	A/V	A/V	V/V	A/V	
SCD25 (pg/mL)	63130	882	527	1020	774	735	1192	1043	(0-1900)
IL-6 (pg/mL)	210	0	12	22	0	24	0	0	(0-25)
IFN-γ (pg/mL)	270	0	0	0	0	0	0	2	(0-5)
TNF-α (pg/mL)	479	0	0	0	0	0	0	0	(0-5)
NK cells (%)	44	12	22	10	19	18	4	11	(3-18)
NK cells (μL)	180	230	638	180	401	493	296	549	(80-700)
CD8+ T cells (%)	9	11	21	20	17	15	2	23	(10-37)
CD8+ T cells (μL)	30	222	609	360	359	411	101	1155	(250-1300)
Perforin in NK cells	Not tested	Decreased	Normal	Normal	Normal	Normal	Decreased	Normal	
Perforin in CD8+ T cells	Not tested	Extremely reduced	Normal	Normal	Normal	Normal	Extremely reduced	Extremely reduced	
NK activity	Not tested	Normal	Not tested	Not tested	Not tested	Not tested	Moderate decreased	Normal	
Positive viral serologies	EBV, CMV, HSV-1, VZ, Measles	EBV, HSV-1, VZ, Measles	EBV, CMV, HSV-1, VZ Measles	EBV, CMV, HSV-1,VZ, Measles	CMV, HSV-1, VZ, Measles	CMV, HSV-1 VZ, Measles	HSV-1, Measles	VZ, Measles	

EBV denotes Epstein-Barr virus, CMV cytomegalovirus, VZ varicella-zoster virus, HSV-1 herpes simplex virus type 1.

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quency of this change is too high for the rarity of FHL (heterozygosity between 3% to 17. 5%).[8,14,15] We have confirmed the high frequency of A91V (21%) in 32 blood donor natives from Formentera Island (7 heterozygous out of 32). The mild role of the double A91V mutation as a susceptibility factor in FHL is further blurred by the fact that the genetically identical twin is healthy, despite having encountered several viruses (see Table 1 and pedigree in Figure 1A) capable of triggering this disease.[3,11] Several FHL patients with late presentation have been shown to carry the A91V change, although it is not exclusive for this type of discrepant clinical features between FHL siblings.[5,6]

It was not possible to investigate perforin expression and NK cell cytotoxicity in the patient, but a reduced expression of perforin was shown in the monozygotic twin (II-3), II-8 and III-1. The defective perforin expression moderately affected NK (normal in III-1), but was severe in CD8+ T lymphocytes (Figure 1C). These different expressions of perforin in two cell types can be related to different mechanisms of intracellular processing or externalization of the protein. The functional assay of NK lytic activity yielded normal results, similar to that obtained in healthy controls (Table 1). Perforin-dependent cytotoxic activity in CD8+ lymphocytes was not tested. Amino acid 91 in perforin is located in a domain without structural analogos,[16] suggesting that the role of this part of the molecule is not of major importance. *In vitro* models for analysis of the A91V mutation show a greater impairment of perforin expression and function. Voskoboinik *et al*. reported that A91V perforin was expressed at decreased levels and had a lytic capacity below 50% that of wild-type perforin in a rat basophil leukemia (RBL) cell line transfected with the A91V construct.[16] Trambas *et al*. demonstrated that A91V perforin is not recognized using an antibody raised against native perforin (δG9 clone), suggesting that A91V causes a conformational change and reduces the production of the active form of perforin, resulting in defective cytotoxic function.[17] Risma *et al*. studied the expression of mutant perforins in transfected RBL-1 cells; the mutations chosen were all reported as either compound heterozygous or homozygous and were seen in patients with FHL type 2. This study concluded that A91V is a milder class I perforin mutation with partial maturation and reduced but detectable perforin and NK function.[13] The different degrees of severity observed among our natural and *in vitro* models for the same genetic defect could be attributed to the redundant contribution of other protein pathways or genetic systems that modulate the perforin genetic mutation *in vivo*.

A possible link between *PRF1* mutations and an increased risk of developing lymphoma or leukemia has been suggested.[5,8,12,18] In this family, the homozygous

A91V brother (II-8) has CLL and the heterozygous mother of the patient (I-2) died of NHL. These observations suggest that significant epigenetic influences could play a role in the development of distinct clinical phenotypes in A91V homozygous subjects. It has recently been shown that DNA methylation patterns are indistinguishable during the early years of life in monozygotic twins, but change remarkably in older twins, affecting the gene expression portrait.[19] Although the mechanisms that regulate perforin expression are incompletely understood, it is known that methylation of a promoter specific region of the gene regulates protein expression.[20]

On the basis of these results, A91V can be considered a mutation that contributes to the pathophysiology of FHL. This genetic defect causes moderate and severe reduction of perforin expression in NK and $CD8^+$ cells, respectively, but NK lytic function is normal. The fact that tuberculosis had been diagnosed 20 years before suggests that the infection alone was not a factor strong enough to trigger the disease. It seems that a concomitant stimulus (e. g. corticosteroid therapy in our patient), epigenetic factors or co-inherited genetic abnormalities were needed for the development of FHL in a patient with homozygous A91V *PRF1* mutation.

All authors meet the criteria for being contributing authors.
JB, MUA, RC, MAd and FJV were responsible for the clinical care of the patient. EM, LMA, MG, EPA, JC, AG and AA performed the flow cytometry analysis and the analysis of the perforin gene mutation status. JG, EF-C and RU-M performed the study of the NK cell activity.
All authors were involved in the discussion and revision of the manuscript and gave their permission for the final version submitted for publication.
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