Hypomorphic mutations in *PRF1*, *MUNC13-4*, and *STXBP2* are associated with adult-onset familial HLH

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Familial hemophagocytic lymphohistiocytosis (HLH) is a rare primary immunodeficiency disorder characterized by defects in cell-mediated cytotoxicity that results in fever, hepatosplenomegaly, and cytopenias. Familial HLH is well recognized in children but rarely diagnosed in adults. We conducted a retrospective review of genetic and immunologic test results in patients who developed HLH in adulthood. Included in our study were 1531 patients with a clinical diagnosis of HLH; 175 patients were 18 years or older. Missense and splice-site sequence variants in *PRF1*, *MUNC13-4*, and *STXBP2* were found in 25 (14%) of the adult patients. The A91V-PRF1 genotype was found in 12 of these patients (48%). The preponderance of hypomorphic mutations in familial HLH–causing genes correlates with the later-onset clinical symptoms and the more indolent course in adult patients. We conclude that late-onset familial HLH occurs more commonly than was suspected previously. (*Blood*. 2011;118(22): 5794-5798)

Introduction

Familial hemophagocytic lymphohistiocytosis (familial HLH; MIM 267700)[1] is an inherited immune deficiency characterized by the overactivation and excessive proliferation of macrophages and T lymphocytes. This leads to infiltration and damage of organs, including the liver and the CNS. Classic clinical features include prolonged fevers, cytopenias, hepatosplenomegaly, and signs of immune activation. Hemophagocytosis is the hallmark of HLH in general, but it may not present at early stages of the disease. The functions of natural killer (NK) cells and cytotoxic T lymphocytes are reduced or absent. familial HLH in children is rapidly fatal without the appropriate immunosuppressive treatment followed by hematopoietic stem cell transplantation.

To date, defects in at least 7 genes (ie, *PRF1*, *MUNC13-4*, *STX11*, *RAB27A*, *STXBP2*, *SH2D1A*, and *BIRC4*) are known to be associated with familial HLH.[1-7] The protein products of the 5 autosomal-recessive (AR) genes *PRF1*, *MUNC13-4*, *STX11*, *RAB27A*, and *STXBP2* are all involved in the PRF1-dependent cytotoxic pathway, so affected patients often demonstrate abnormal function of cytotoxic lymphocytes. Perforin is constitutively contained within the secretory granules of all cytotoxic lymphocytes, facilitating the entry of granule contents into target cells that are infected, spent, or dangerous to the immunologic well-being of the organism, which results in the cytotoxic response. Mutations in the other AR genes interfere with the delivery of the cytotoxic granules to the contact surface with target cells and their extrusion of contents into the contact zone. Most studies on the AR forms of familial HLH focus on patients who present with symptoms of the disease within the first years of life.[8-11] Only a few cases with later onset have been reported.[12-15]

In this study, we describe the genetic and immunologic findings of adult patients in North America who have been referred to the Diagnostic Center for Heritable Immunodeficiency at Cincinnati Children's Hospital Medical Center (CCHMC) for testing.

Methods

This study was approved by the CCHMC institutional review board. During the 5-year study period, 1531 patients were referred for genetic testing due to a suspected diagnosis of HLH and/or associated conditions, as determined by their referring physicians. Clinical and demographic features were collected based on the information provided by ordering physicians on the test requisition form.

Genomic DNA was obtained from blood, BM, B-lymphoblastoid cell lines, and autopsy tissues using a standard procedure. All coding exons and at least 50 base pairs of the adjacent intronic region of the PRF1, MUNC13-4, and STXBP2 genes were amplified by PCR, followed by bidirectional sequencing. Detailed methodologies have either been published previously[2-5] or available upon request. Mutation nomenclature is based on the recommendations of the American College of Medical Genetics and the Human Genome Variation Society.

Several in silico analysis methods for the prediction of functional consequences of sequence variants were used to provide initial classification. In particular, we used SIFT (http://blocks.fhcrc.org/sift/SIFT.html), which uses evolutionary information from homologous proteins,[16] and PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/), which incorporates structural information into classification rules.[17] The Grantham Scale[18] was also used to evaluate the significance of amino acid substitutions.

Minor allele frequency was found in the National Center for Biotechnology Information single-nucleotide polymorphism database and also identified through testing 50 unrelated individuals from a southern Ohio control

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Table 1. Adult patients with sequence variants in *PRF1*, *MUNC13-4*, and *STXBP2*

Patient ID	Age at onset, y	Sex	Ethnicity	Symptoms/clinical diagnosis †	PRF1 results	MUNC13-4 results	STXBP2 results	NK-cell function	% perforin in NK cells
P1	18	M	A	HLH	10 C > T (R4C)/98 G > A (R33H)‡	NL/NL	ND	14.8	ND
P2	18	M	W	HLH	1066 C > T (R356W)/1349C > T (T450M)	NL/NL	ND	ND	ND
P3	18	F	W	ALL without remission; suspected HLH	272 C > T (A91V)/NL	NL/NL	ND	Absent	90
P4	18	F	W	HLH	272 C > T (A91V)/NL	NL/NL	ND	1.6 (low)	ND
P5	18	M	W	Mycosis fungoides, lymphadenopathy; suspected HLH	272 C > T (A91V)/NL	NL/NL	ND	ND	ND
P6	18	F	B	HLH	NL/NL	1579 C > T (R527W)/NL	ND	ND	ND
P7	18	M	W	Neutropenia; suspected HLH	272 C > T (A91V)/1042 G > A (R348H)‡	ND	ND	ND	ND
P8	19	M	W	MAS in infancy, severe vasculitis with CNS involvement	272 C > T (A91V)/NL	NL/NL	ND	3.3	81
P9	19	F	H	Suspected HLH	1229 G > A (R410Q)†/NL	NL/NL	ND	ND	75 (low)
P10	19	F	W	Suspected HLH	272 C > T (A91V)/563 C > T (P188L)‡	NL/NL	ND	2.2 (low)	39 (low)
P11	19	F	W	HLH	NL/NL	753 + 3G > A/NL	ND	Absent	98
P12	19	M	A	EBV encephalitis and seizures; suspected HLH	ND	2240 G > A (S747N)‡/2553 + 5C > G‡	ND	ND	ND
P13	19	F	W	HLH	NL/NL	753 + 3G > A/NL	ND	ND	ND
P14	21	F	W	Suspected HLH	1310 C > T (A437V)‡/NL	NL/NL	ND	Absent	61 (low)
P15	23	F	W	HLH	1066 C > T (R356W)/1066 C > T (R356W)	NL/NL	ND	ND	7 (low)
P16	24	M	W	Suspected HLH	NL/NL	2174 A > G (E725G)‡/NL	ND	Absent	ND
P17	24	M	W (Arabic)	HLH	NL/NL	NL/NL	1782(*12) g > a/1782(*12) g > a	0.3 (low)	69 (low)
P18	25	F	W	Suspected HLH	272 C > T (A91V)/NL	NL/NL	795-4C > T/ NL	3.7	ND
P19	25	F	H	HLH	445 G > A (G149S)/695 G > A (R232H) + 272 C > T (A91V)	NL/NL	ND	2.2 (low)	Absent
P20	28	F	A	Suspected HLH	10 C > T (R4C)/NL	NL/NL	ND	ND	ND
P21	28	M	W	HLH; male sibling died with HLH	272 C > T (A91V)/NL	182 A > G (Y61C)‡/NL	NL	ND	ND
P22	30	M	A	Suspected HLH	ND	1369 C > A (L457M)‡/2447 + 61_2447 + 150del 90‡	ND	38.4	97
P23	66	F	W	HLH	272 C > T (A91V)/NL	NL/NL	ND	ND	ND
P24	74	M	W	HLH	272 C > T (A91V)/NL	ND	ND	3.8	61 (low)
P25	75	M	W	HLH	272 C > T (A91V)/272 C > T (A91V)	NL/NL	NL	low§	24 (low)

NL indicates normal, wild type; ND, not done; W, nonHispanic white; B, black; A, Asian; H, Hispanic; and LU, lytic units.
*Mutation occurred at the 3′LITR region.
†Information collected was based on physician's notes on the requisition form.
‡Previously unreported variants not found in our control population.
§Test was done in another institution so the number is not available.

Table 2. Summary of genetic test results in 1531 patients with suspected HLH

Patients			Sequence variants found*							
			PRF1		*MUNC13-4*		*STXBP2*		Total no. of variants	Variants found in patients, %
Age, y	Count	% of total	2 variants	1 variant	2 variants	1 variant	2 variants	1 variant		
0-1	415	27	70	32	27	47	1	5	182	44
1-5	483	32	16	31	13	37	9	5	111	23
5-12	241	16	0	16	2	15	6	5	44	18
12-18	217	14	3	21	2	10	2	5	43	20
18+	175	11	7	11	2	5	1	1	27	15
Total	1531	100	96	111	46	114	19	21	407	27

*In some patients, mutations and sequence variants were found in > 1 gene.

population (race: 83.4% white, 11.8% black, 1.6% Asian, 1.6% Hispanic, and 0.2% Native American). NK-cell cytotoxicity and perforin expression were analyzed as described previously at the Diagnostic Immunology Laboratory at Cincinnati Children's Hospital.[19-21] The results were compared with the normal ranges for age-matched controls that have been obtained in our laboratory.

Results

In total, 1531 patients were referred for genetic testing due to a suspected diagnosis of HLH and/or associated conditions as determined by their referring physicians. Of these patients, 175 were 18 years of age or older. Twenty-five of the adult patients (14%) possessed mutations or sequence variants in *PRF1*, *MUNC13-4*, or *STXBP2* (Table 1). All of the mutations were either missense base substitutions or splicing site variations. In comparison, in patients with age of onset below 18 years, we found mutation(s) in *PRF1*, *MUNC13-4*, and *STXBP2* in ~ 30% of the patients, which included nonsense, missense, splice site, and other type of deleterious mutations. Table 2 shows the number of sequence variants found in different age groups in patients with clinical suspected HLH. In some of these younger patients, mutations and sequence variants were found in > 1 gene.

Twelve missense mutations and sequence variants were identified in *PRF1* in 18 patients. The A91V-*PRF1* genotype was found in 12 patients in both heterozygous and homozygous states. Eight sequence variants in *MUNC13-4* were found in 7 patients; 2 were splice-site changes and 5 were missense mutations. Two splice-site sequence variants in *STXBP2* were identified in 2 patients. Two patients (P18 and P21) were double heterozygous, with the A91V-*PRF1* genotype in the *PRF1* locus and a second mutation in either *STXBP2* or *MUNC13-4*. More than 1/2 of the patients (13 of 25) carried only one mutation in one of these genes, which are inherited in an autosomal recessive fashion. It is possible that other types of mutations (eg, gross deletions, insertions, or complex rearrangements) were not able to be detected by the methodology used in this study or that these patients possess mutations in additional genes that have not yet been discovered to be associated with HLH.

Eleven novel sequence variants were identified in this study. In silico analyses of the predicted structural effects of the missense variants are summarized in Table 3.[13,22-24] A91V, P188L, R356W, and T450M in *PRF1* and Y61C in *MUNC13-4* are consistently predicted to be pathogenic, with no presence in general healthy populations except A91V (4%-7% by multiple studies).[20,24] The sequence variant A91V-*PRF1* and its relationship with familial HLH have been studied extensively in recent years.[25,26] Numerous clinical studies[13-15,24] have documented variability in cytotoxic function in individuals who are heterozygous or homozygous for the A91V substitution. Most of the evidence suggests that although

Table 3. Missense variants and their significances predicted by in silico analyses

Gene	Base change	AA change	SIFT	PolyPhen	Grantham scale*	Conservation (in mammals)	MAF, %	Reference(s)
PRF1	10 C > T	R4C	Not tolerated	Inconclusive	180	Not conserved	2-4	NCBI
PRF1	98 G > A	R33H	Not tolerated	Benign	29	Not conserved	0	This study
PRF1	272 C > T	A91V	Not tolerated	Possibly damaging	64	Conserved	4-9	14,15,20,24-26
PRF1	445 G > A	G149S	Not tolerated	Benign	56	Conserved	0	22
PRF1	563 C > T	P188L	Not tolerated	Probably damaging	98	Conserved	0	This study
PRF1	695 G > A	R232H	Tolerated	Benign	29	Conserved	0	3,15,17
PRF1	1042 G > A	V348M	Tolerated	Benign	21	Not conserved	0	This study
PRF1	1066 C > T	R356W	Not tolerated	Possibly damaging	101	Conserved	0	22
PRF1	1229 G > A	R410Q	Tolerated	Benign	43	Conserved	0	This study
PRF1	1310 C > T	A437V	Not tolerated	Benign	64	Conserved	0	This study
PRF1	1349 C > T	T450M	Not tolerated	Probably damaging	81	Conserved	0	13
MUNC13-4	182 A > G	Y61C	Not tolerated	Probably damaging	194	Conserved	0	This study
MUNC13-4	1369 C > A	L457M	Not tolerated	Benign	15	Conserved	0	This study
MUNC13-4	1579 C > T	R527W	Not tolerated	Probably damaging	101	Not conserved	2	28, NCBI
MUNC13-4	2174 A > G	E725G	Tolerated	Benign	98	Conserved	0	This study
MUNC13-4	2240 G > A	S747N	Tolerated	Benign	46	Not conserved	0	This study

NCBI indicates National Center for Biotechnology Information; and MAF, minor allele frequency.
*Grantham scale: < 50, conservative; 51-100, moderately conservative; 101-150, moderately radical; and > 151, radical.

it is a "milder" or hypomorphic mutation, A91V is not clinically neutral.[27,28] The predictions for the rest of the sequence variants are largely inconclusive.

Perforin expression was measured by flow cytometry in 7 of the patients with *PRF1* sequence variants. The level of perforin correlated well with the genetic findings in adult patients. Perforin was absent or severely low in patients with biallelic mutations in *PRF1*, whereas slightly decreased or at the lower normal level in patients with 1 mutation (Table 1). Results of NK-cell functional testing were available for 14 patients. NK-cell function was typically observed to be low in these adult patients with HLH, but was not closely correlated with the number of mutations identified in *PRF1*, *STXBP2*, or *MUNC13-4*.

Discussion

In young children, the presenting symptomatology of familial HLH has been well described[9,29] and forms the basis for the diagnostic criteria. In older children and adults, a broader spectrum of clinical phenotypes (eg, encephalitis, autoimmune lymphoproliferative disease, acute lymphoblastic leukemia, aplastic anemia, and systemic onset juvenile idiopathic arthritis)[11,14,15,30-35] have been reported. In some of these reported cases, patients were cleared from infections before the overt diseases, and therefore a negative clinical history of HLH does not rule out this syndrome. In patients with familial HLH who carry hypomorphic genetic defects, residual NK- and T-cell function, although decreased, may be sufficient to prevent the development of clinical HLH for many years. In the patient cohort of the present study, 12% of patients were 18 years and older at the time of diagnosis. Two patients apparently developed the first symptoms of HLH in their mid-seventies. The observations of double heterozygosity in 2 adult patients (P18 and P21), with the A91V-*PRF1* genotype in the PRF1 locus and a second mutation in either *STXBP2* or *MUNC13-4*, are very interesting. Although there is no known direct interaction among these 3 proteins, additive effects are possible given that they all play critical roles in the perforin-dependent cytotoxic pathway. Additional research is required to further define the functional effects of these genetic alterations and to determine the optimal treatment for adult patients with HLH. Anecdotal information from physicians who submitted samples for testing indicated that a range of treatments have been used, including steroid therapy alone, HLH-94–type therapy, and, in a young adult with recurrent disease, allogeneic hematopoietic stem cell transplantation.

With increasing awareness about HLH, more adult patients and/or patients with milder and/or relapsing clinical courses are being identified. In this study, we found that almost all of the sequence variations in *PRF1*, *MUNC13-4*, and *STXBP2* carried by adult patients were either missense or splice-site changes representing hypomorphic mutations that played a contributing role in the development of late-onset HLH when patients were challenged by viral infection and other types of environmental stresses. Genetic and immunologic diagnostic testing and timely HLH-directed treatment should be considered for adult familial HLH patients.

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Authorship

Contribution: K.Z. and A.H.F. designed the research, collected, analyzed, and interpreted the data, and wrote the manuscript; K.A.R. analyzed the results and wrote the manuscript; R.A.M., P.S.K., and M.B.J. collected the data and reviewed the manuscript; D.K., J.A.J., and J.V. performed the research and collected the data; J.M. analyzed the data and wrote the manuscript; and Q.W. collected the data and created the tables.

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