

Characterisation of diverse *PRF1* mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis

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J Med Genet 2004;41:137–144. doi: 10.1136/jmg.2003.011528

Haemophagocytic lymphohistiocytosis (HLH, OMIM #267700, #603553) is a rare, lethal, cellular immunological disorder that draws attention to critical, genetically determined checkpoints in the regulatory pathways that normally maintain homeostasis within the immune system and ensure the natural termination of effector immune responses. HLH encompasses several entities, including the primary or familial form and the secondary forms associated with infections or malignancies.^{1,2} In all forms, HLH is believed to be a reactive process resulting from hyperactivation, proliferation, and migration of macrophages and type 1 T cells.³ Cytotoxic chemotherapy and/or immune suppressive therapy are usually effective in achieving symptomatic remission of HLH.^{4,5} The proven curative potential of haematopoietic stem cell transplantation underscores the premise that all forms of HLH result from genetic defects intrinsic to the immune system.^{5–7} Abnormalities in the function (but rarely the quantity) of cytotoxic immune cells, principally natural killer (NK) cells, but also cytotoxic T cells, have been observed in patients with HLH for nearly 20 years.^{8–11} Several genetic changes are thought to contribute to the development of HLH. Linkage analysis identified two loci at 10q21 and 9q21;^{12,13} additional studies provided evidence for the presence of at least one more locus.¹⁴ In 1999, the first molecular aetiology of HLH was described. Deficiency of perforin, a key lytic molecule of cytotoxic cells, was identified in a proportion of patients with HLH.¹⁵ Mutations of the coding sequence of *PRF1* have since been reported in approximately 20–30% of primary HLH cases.^{16,17} Most of the families involved in previous studies came from southern Europe, North Africa, Turkey, and Japan.^{16–20} Only four cases from North America have previously been published.²¹

In the present study, we investigated the immunophenotypes of HLH in relation to the presence or absence of disease causing mutations in *PRF1*. Where possible, clinical characteristics of patients with HLH were also compared. Using a comprehensive direct sequence analysis of the coding exons of the *PRF1* gene in combination with functional analysis of the affected cells, we have gained additional information about the distribution and nature of perforin mutations and their resulting phenotypes.

METHODS

Patients

A cohort of 50 North American families with children diagnosed with primary HLH were studied with approval by the internal review board of the Cincinnati Children's Hospital Medical Center. The majority of children were diagnosed and treated at institutions throughout the US and had blood samples sent to Cincinnati Children's Hospital for NK function testing, and perforin and granzyme B expression studies using flow cytometry. Most patients presented with hepatomegaly, splenomegaly, neutropenia,

Key points

- Thirteen novel mutations of the *PRF1* gene were observed in a large cohort of North American patients with primary haemophagocytic lymphohistiocytosis.
- The pathogenicity of two previously reported *PRF1* mutations, A91V and N252S, could not be conclusively confirmed in this population.
- With few exceptions, absent or decreased perforin protein levels correlated with the presence of *PRF1* mutations.
- Patients with *PRF1* mutations who demonstrated residual perforin expression had a later age of onset than patients with absent perforin expression.

and hypofibrinogenaemia. Lymphopenia, hypertriglyceridaemia, central nervous system involvement, fever, anaemia, and thrombocytopenia were also widely reported. Diagnosis was later confirmed through contact with the referring institution if the diagnosis at the time of testing was uncertain. Patients with viral associated HLH were not included in this study unless a positive familial history (multiple affected children or consanguinity) could be demonstrated. DNA samples were retained from 43 of the 50 families and later sequenced for mutations in *PRF1*.

PRF1 gene sequencing

Patients were screened for the presence of mutations in the coding exons and exon–intron boundaries of *PRF1* by PCR amplification of genomic DNA followed by direct sequencing of the PCR products. Genomic DNA was extracted from either fresh peripheral blood or from Epstein-Barr virus transformed B cell lines using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). PCR was used to amplify the coding exons 2 and 3 of the *PRF1* gene, including the exon–intron boundaries, using the following primers for exon 2: 5'-CCCTCCATGTGCCCTGATAATC-3' and 5'-AAGCAGCCTCCAAGTTTGATTG-3'; and exon 3: 5'-CCAGTCC TAGTTCTGCCCACTTAC-3' and 5'-GAACCCCTTCAGTCCAAG CATAAC-3'. Amplification of 500 ng of DNA was performed in a 50 µl assay of 1× PCR buffer, 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTP, 0.4 µmol/l of each primer, and 2.5 U *Taq* DNA polymerase (Life Technologies Inc., Rockville, MD, USA). Reaction conditions were 3 minutes at 95°C followed by 30

Abbreviations: HLH, haemophagocytic lymphohistiocytosis; NK, natural killer

cycles of 45 seconds at 95°C, 30 seconds at 60°C, 1 minute 45 seconds at 72°C; and then 10 minutes at 72°C. Primers used for sequencing were the same as those for amplification, with the addition of an internal exon 3 primer: 5'-CCATCACACCTCCATTAACGA-3'. Additionally, 5'-TTGGTCTAATGGGAATACGAAG-3' was used in place of the exon 3 reverse primer listed above for both PCR and sequencing to confirm the mutations near the 3' end in P23 and P28. Cycle sequencing using the BigDye terminator cycle sequencing reaction kit (version 2; Applied Biosystems, Foster City, CA, USA) was performed and the DNA fragments were separated on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Sequences were compared with the published *PRF1* sequence (GenBank accession no. M28393) using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA).

Perforin and granzyme B expression by flow cytometry

Whole blood was first surface stained with the following antibodies: TCR α / β -FITC, CD8-PerCP (BD Immunocytometry Systems, San Jose, CA, USA), and CD56-APC (Immunotech, Brea, CA, USA) for 20 minutes at room temperature. Red cells were then lysed for 10 minutes with FACSlyse (BD Immunocytometry Systems) and washed. The resultant white cell pellet was permeabilised using 500 μ l Cytotfix/Cytoperm (BD Pharmingen, San Diego, CA, USA) for 20 minutes, washed and stained with either PE conjugated anti-perforin (BD Pharmingen) or PE conjugated anti-granzyme B (Caltag Laboratories, Burlingame, CA, USA) or the respective isotype matched controls, for 30 minutes at room temperature. After washing, cells were resuspended in 1% paraformaldehyde and stored at 4°C prior to analysis by four color flow cytometry. Samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Natural killer cell function

K562 cells were loaded with radioactive chromium and then mixed with serial dilutions of a mononuclear cell preparation from a patient or control. The amount of chromium released into the supernatant after 4 hours of incubation was measured by gamma scintillation. The percentage lysis was calculated as:

$$\frac{(\text{cpm of sample} - \text{cpm of background})}{(\text{cpm of maximum} - \text{cpm of background})}$$

Lytic units were calculated from the curve of the percentage lysis *v* the dilutions and are defined as the number of effector cells needed to produce 10% lysis of 10³ target cells during the 4 hour incubation, as previously published.²²

Statistical analysis

Statistical comparisons were performed by the descriptive statistics program in Microsoft Excel using the two tailed *t* test, assuming equal variances.

RESULTS

Data representing 50 unrelated families (47 probands and 3 sets of parents of deceased affected children) with clinically confirmed HLH were analysed. For 25 families, ethnic background was specified, while the other 25 families self-reported as white with European ancestry. The patient group consisted of 26 males and 21 females ranging in age from 1 month to 16 years at diagnosis, with a median age of 7 months. Direct sequencing of PCR products spanning the two coding exons (exon 2 and 3) and exon-intron boundaries of the perforin gene revealed mutations in 25/43 families tested, including 13 previously unreported mutations (table 1). DNA was not available from seven patients, of whom five had normal or increased levels of perforin, and

two had minimally decreased perforin, either in intensity of staining or proportion of perforin expressing cells. The determination of pathogenicity of novel mutations was based on three factors: (a) association with disease (13/13); (b) absent or decreased perforin protein levels (10/10 tested); and (c) mutations resulting either in changes of conserved residues (10/10 missense) or in changes that likely affect the structure of the protein, either in the length of the protein (3/13) or the folding of the protein (1/13). Data from patients without *PRF1* mutations are presented in table 2. Fig 1 shows the distribution of previously reported mutations,^{15-21 23} together with the mutations described in this study, along the length of the gene. In addition to the presumed disease causing mutations, eight intragenic polymorphisms were also observed (table 3). Absent or decreased NK function was observed in 44/45 patients studied. Intracellular perforin protein levels were tested in 39 patients.

Frameshift and nonsense mutations

Three frameshift mutations, single nucleotide deletions, or insertions were detected. The 50delT in exon 2 was observed in 11 patients and is predicted to generate a truncated protein, whereas two novel frameshift mutations of exon 3 (1628insT and 1636delC) eliminate the naturally occurring translation stop codon of the *PRF1* gene, thereby extending the protein. One novel nonsense mutation, S150X, was also observed.

Missense mutations

Seventeen missense mutations, 10 of which are novel, were found in 17 probands. These missense mutations were not observed in >100 chromosomes from healthy individuals, and are scattered throughout the coding region of the gene. Fig 2 shows the location of the novel missense mutations in relation to the functional domains of the protein, along with an alignment of *PRF1* across several species.

Polymorphisms

In the present study, we identified eight intragenic polymorphisms (table 3), five of which have been previously described: R123H, T173T, A274A, H300H,¹⁶ and A91V.¹⁹ The R123H and T173T polymorphisms were the least common; both were observed only once, and in the same unaffected adult control. The H300H polymorphism was the most frequent, present in over half of the perforin alleles analysed.

Perforin expression by flow cytometry

To define the extent of perforin protein expression, NK and cytotoxic T lymphocyte cells from patients with HLH and presumed or known carriers were analysed by flow cytometry. In NK and cytotoxic T cells, markedly decreased or absent perforin expression measured by flow cytometry was observed in all patients with mutations of the *PRF1* gene, whereas those patients with primary HLH without *PRF1* coding region mutations demonstrated relatively normal perforin staining patterns (median values, 91% and 10%, respectively). Fig 3 shows perforin expression patterns of patients with various *PRF1* genotypes. Previous studies have shown that perforin expression measured by flow cytometry is decreased in obligate carriers of HLH and in some patients with other haemophagocytic syndromes.²¹

Granzyme B expression by flow cytometry

Granzyme B data are available for 16 patients with *PRF1* mutations and 15 patients without *PRF1* coding region mutations. Normal to increased expression of granzyme B was seen in 29/31 patients tested, in all their cytotoxic cells. P28 and P45 had slightly reduced proportions of granzyme B

Table 1 Profile of patients with primary HLH with *PRF1* mutations (n = 25)

Patient	<i>PRF1</i> mutation	<i>PRF1</i> polymorphism	Age/gender	Ethnicity	NK function (lytic units)	Perforin expressing NK cells	Perforin MCF of NK cells	Granzyme B expressing NK cells	Granzyme B MCF of NK cells
P22	665 A→G (H222R)*	None	1 month/M	Mixed: White/Asian	<0.01 ↓	<1% ↓	—	89%	642
P21	50delT (L17X)**	H300H**	2 months/M	African-American	<0.01 ↓	<1% ↓	—	98% ↑	908
P23	469 T→G (F157V)* 1628insT (frameshift)*	A274A* H300H*	2 months/F	White	<0.01 ↓	<1% ↓	—	ND	ND
P30	50delT (L17X)**	H300H**	2 months/M	African-American	<0.01 ↓	<1% ↓	—	98% ↑	4024 ↑
P33	50delT (L17X)* 1442 A→C (Q481P)*	H300H**	2 months/F	Hispanic	<0.01 ↓	<1% ↓	—	95%	2847 ↑
P9‡	50delT (L17X)**	H300H**	3 months/F	African-American	<0.01 ↓	<1% ↓	—	ND	ND
P59	50delT (L17X)**	H300H**	3 months/M	African-American	<0.01 ↓	<1% ↓	—	83%	1140 ↑
P28	666 C→A (H222Q)* 1636delC (frameshift)*	H300H*	3 months/M	White	<0.01 ↓	<1% ↓	—	57% ↓	964
P32	50delT (L17X)* 217 T→C (C73R)*	H300H**	4 months/M	African-American	<0.01 ↓	<1% ↓	—	99% ↑	3711 ↑
P26	50delT (L17X)**	H300H**	6 months/F	African-American	<0.01 ↓	<1% ↓	—	87%	3071 ↑
P31	50delT (L17X)**	H300H**	6 months/F	African-American	<0.01 ↓	<1% ↓	—	81%	817
P50‡	445 G→A (G149S)**	V145V** A154A** N252S** H300H**	6 months/M	Hispanic	<0.01 ↓	<1% ↓	—	87%	870
P11*	50delT (L17X)**	H300H**	8 months/M	African-American	<0.01 ↓	<1% ↓	—	ND	ND
P34	50delT (L17X)* 134 G→A (G45E)*	H300H**	8 months/F	African-American	<0.01 ↓	<1% ↓	—	79%	964
P57	673 C→T (R225W)**	None	2 years/M	Filipino	<0.01 ↓	91%	75 ↓	98% ↑	3514 ↑
P25*	445 G→A (G149S)* 895 C→T (R299C)*	V145V* A154A* N252S* H300H*	2 years/M	Portuguese	<0.01 ↓	ND	ND	ND	ND
P10‡**	133 G→A (G45R)* 160 C→T (R54C)*	A91V* H300H*	3 years/F	Hispanic	0.1 ↓	61% ↓	50 ↓	ND	ND
P24*	445 G→A (G149S)*	V145V* A154A* N252S* H300H**	4 years/F	Hispanic	<0.01 ↓	18% ↓	51 ↓	95%	1521 ↑
P29	673 C→T (R225W)**	None	5 years/F	White	0.1 ↓	53% ↓	29 ↓	95%	707
P12	449 C→A (S150X)* 673 C→T (R225W)*	A274A* H300H**	5 years/F	White	<0.01 ↓	ND	ND	ND	ND
P35	148 G→A (V50M)**	H300H**	7 years/M	Russian	9.2/0.01¶	9% ↓	44 ↓	96% ↑	803
P58**	3 G→A (M1I)* 208 G→T (D70Y)*	A91V* H300H*	8 years/M	White	0.01 ↓	<1% ↓	—	94%	2861 ↑
P1‡	116 C→A (P39H)* 445 G→A (G149S)*	H300H* H300H*	10 years/F	White	0.8 ↓	1% ↓	28 ↓	ND	ND
P27§**	3 G→A (M1I)* 938 A→T (D313V)*	A91V* H300H* A274A* H300H**	—	White	ND	ND	ND	ND	ND
P56§	50delT (L17X)**	H300H* H300H**	—	African-American	ND	ND	ND	ND	ND
Median values			6 months	—	0.01	<1%	—	95%	1052
Normal range			—	—	>3.2	<1 year: 94 (3) 1–15 years: 86 (5)	373 (129)	86 (9)	786 (286)

*Heterozygous; **homozygous.
‡Previously reported by Kogawa *et al.*
§Parents of a patient with HLH (patient unavailable).
¶A patient with variable NK function; the high and low values of six samplings are shown.
*Patients with N252S transition; **patients with A91V transition.
Novel mutations are in **bold** type.
MCF, mean channel fluorescence; ND, not done (test not performed).

expressing NK cells, but mean channel fluorescence measurements were normal.

Natural killer cell function

Of 45 patients tested, 35 had absent and 9 had decreased NK function. Patients with and without *PRF1* mutations had identical NK function as defined by median lytic units (tables 1 and 2). Patient P35, homozygous for V50M, showed normal NK function of all three cell types analysed (NK cells,

CD8+ T cells, and CD56+ T cells) on 3/6 occasions, despite markedly decreased perforin expression, in both the proportion of perforin expressing cells and intensity of staining. Cytotoxic T lymphocyte function was also decreased in this patient on repeated testing (data not shown). Three other patients with *PRF1* mutations (P1, P10, and P29) who demonstrated markedly decreased perforin protein expression by flow cytometry also showed minimal, but detectable, NK function at high effector:target ratios (table 1).

Table 2 Profile of patients with primary HLH without *PRF1* mutations (n = 18) or with normal proportion and/or intensity of perforin, but without available DNA for testing (n = 7)

Patient	<i>PRF1</i> mutation	<i>PRF1</i> polymorphism	Age/gender	Ethnicity	NK function (lytic units)	Perforin expressing NK cells	Perforin MCF of NK cells	Granzyme B expressing NK cells	Granzyme B MCF of NK cells
P36	None	H300H**	7 weeks/M	Nigerian	0.9 ↓	84% ↓	226 ↓	87%	674
P37	NA	NA	2 months/F	White	<0.01 ↓	93%	480	96% ↑	1198 ↑
P6‡	None	A274A* H300H*	2 months/F	White	<0.01 ↓	96%	296	99% ↑	1603 ↑
P53	NA	NA	2 months/F	White	<0.01 ↓	94%	974 ↑	92%	2095 ↑
P38	None	A274A* H300H*	3 months/M	White	0.2 ↓	93%	347	83%	1768 ↑
P13	None	A274A* H300H**	3 months/M	White	<0.01 ↓	ND	ND	ND	ND
P39	NA	NA	4 months/F	White	<0.01 ↓	90% ↓	1394 ↑	ND	ND
P7‡	None	A274A* H300H*	4 months/F	White	<0.01 ↓	83% ↓	492	ND	ND
P19¶	None	None	4 months/M	White	<0.01 ↓	ND	ND	ND	ND
P40¶	None	A274A* H300H*	5 months/M	Vietnamese	<0.01 ↓	85% ↓	335	94%	585
P41¶	None	H300H**	5 months/M	White	<0.01 ↓	ND	ND	ND	ND
P42	None	A274A* H300H*	7 months/M	White	<0.01 ↓	91%	780 ↑	ND	ND
P2‡	None	A274A* H300H*	7 months/F	White	<0.01 ↓	91%	368	91%	642
P51	None	H300H**	10 months/F	Hispanic	0.8 ↓	64% ↓	96 ↓	83%	1272 ↑
P47	None	H300H*	15 months/M	White	<0.01 ↓	83%	213 ↓	93%	1061
P43	None	A274A* H300H**	16 months/M	White	<0.01 ↓	94% ↑	306	97% ↑	831
P52	NA	NA	19 months/F	Australian	NA	84%	406	90%	1217 ↑
P49	NA	NA	21 months/F	Hispanic	NA	93% ↑	369	96% ↑	3238 ↑
P63	None	None	19 months/M	White	<0.01 ↓	ND	ND	ND	ND
P14‡	None	A91V* A274A* H300H*	3 years/F	Israeli	<0.01 ↓	ND	ND	ND	ND
P48	NA	NA	3 years/M	White	<0.01 ↓	99% ↑	662 ↑	99% ↑	3679 ↑
P54	NA	NA	12 years/M	Chinese	0.6 ↓	81%	228 ↓	92%	869
P55‡	None	A91V* H300H*	15 years/M	White	<0.01 ↓	ND	ND	ND	ND
P45	None	H300H*	16 years/M	White	0.04 ↓	14% ↓	292	32% ↓	736
P62§	None	A274A* H300H** H300H*	–	White	ND	ND	ND	ND	ND
Median values			7 months		0.01	91%	358	92%	1198
Normal range					>3.2	<1 year: 94 (3) 1–15 years: 86 (5)	373 (129)	86 (9)	786 (286)

*Patients with A91V transition.

*Heterozygous; **homozygous.

‡Previously reported by Kogawa *et al.*

§Parents of a patient with HLH (patient unavailable).

¶Known consanguinity.

MCF, mean channel fluorescence; NA, DNA not available; ND, not done (test not performed).

DISCUSSION

In the present study, the observed mutation frequency of *PRF1* among patients with primary HLH is at least 50%, which is higher than the reported frequency of European, Middle Eastern, and Japanese populations.^{16,17} The predominant mutation identified in this cohort was L17X, which was observed in all African-American patients studied. Intragenic polymorphism analysis of *PRF1* shows that all the 50delT mutations in African-American patients are associated with the homozygosity of H300H, and the absence of any other *PRF1* intragenic polymorphism, suggesting a common ancestral haplotype.

To examine whether missense mutations of *PRF1* occurred in particular sites of functional importance, an alignment of the amino acid sequence of *PRF1* was constructed across nine species (fig 2). Overall, the perforin protein was found to be highly conserved, with blocks of higher levels of conservation that correspond to residues that form the protein domains. Novel mutations reported here spanned all recognised functional domains of the perforin protein.

Three novel mutations resulting in defects of the N-terminal region of the protein were observed: G45E (P34), D70Y (P58), and C73R (P32). The human perforin protein begins with a signal peptide of 20 amino acids, which is removed in the endoplasmic reticulum. Significant lytic activity of the protein has been localised to the first 34 amino acid residues at the N-terminal region. Perforin expression was not detectable by flow cytometry and the NK function was severely decreased in all three patients, suggesting that the G45E, D70Y, and C73R mutations may change the stability of the perforin protein.

Seven novel mutations resulting in defects in and near the MAC PF region of the protein were identified. The C→A nucleotide change at position 449 in patient P12 produced a premature stop codon (S150X). The S150X mutation appeared as a heterozygous mutation in combination with a missense mutation, resulting in a presumably inactive protein of 150 amino acid residues missing both functional domains of the protein, the MAC and the C2. The MAC domain, an approximately 300 amino acid stretch in the

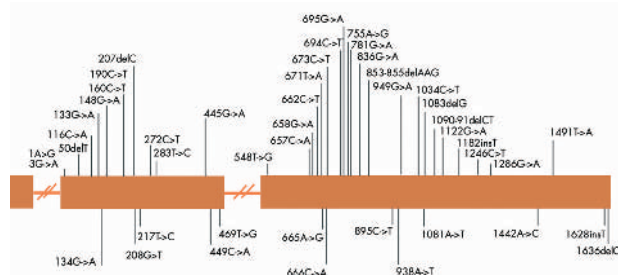


Figure 1 Schematic of the *PRF1* gene showing the distribution and location of all known *PRF1* mutations. The *PRF1* gene has three exons, two of which are coding. The mutations above the schematic gene structure designate previously reported mutations; new mutations observed in this study are shown beneath the schematic gene structure.

middle of the protein, is thought to be involved in pore formation, while the protein sequence between amino acid positions 409 and 521 shows homology to the C2 domain of protein kinase C. Similar homologies have been found in other proteins including synaptotagmin and phospholipase C delta,²⁴ in which this domain has been shown to bind phospholipids in a calcium dependent manner.

F157V (P23), H222R (P22), and H222Q (P28) are all mutations of well conserved residues; additionally, the histidine at position 222 is also conserved in this position in various MAC domains. The H222R (P22) and H222Q (P28) mutations occur in a region of the perforin protein that displays an amphipatic conformation in order to interact with and traverse the membrane lipid bilayer, and exhibits the highest degree of homology to the putative lipid binding domain of the complement components. Absent NK function in both patients suggests that mutations in this region interfere with the function of the protein.

Three novel mutations resulting in defects of the carboxy-terminal portion of the protein were observed. Normally, the 20 amino acids of the C-terminus are cleaved to yield a 60 kDa active form of the protein. This proteolytic cleavage occurs in an acidic compartment, removes a bulky glycan stretch, and allows the C2 domain to interact with phospholipid membranes and initiate pore formation. A→C at nucleotide 1442 (P33) changes glutamine at position 481 to proline. The Q481P change most probably affects folding of the C2 domain by breaking the beta sheet structure, thus altering the conformation of C2, and probably rendering the perforin inactive or labile.

In patient P23, a heterozygous single T insertion at nucleotide position 1628 eliminated the natural translation stop codon of perforin and added 30 more amino acids (GASRKPEWGRVVRTVSLERTSMLGLKGFQWEPGLSSYSX) to the carboxyterminal of the protein. Likewise, patient P28 had a single nucleotide deletion at position 1636 resulting in a frameshift that eliminated the stop codon and extended the protein by 56 amino acids (LQETGVGPC GENSELKGKDQYAWTEGVLTVGARAVFVPLDQACPTRDPHA AQDGFECGPTQIRKLSX). As activation of perforin occurs after the cleavage of 20 amino acid residues at the carboxyterminal end,²⁵ the addition of either 30 or 56 amino acid residues to the C-terminus may impair this cleavage and the subsequent activation of perforin, or may shorten the half life of the mutated protein.

In this series, the largest reported analysis of polymorphisms in *PRF1*, we find that non-disease causing polymorphisms occur in 69% of alleles. In earlier reports, the A91V transition has been described as both a polymorphism¹⁹ and more recently as a disease causing mutation with a milder phenotype.²³ We found this transition in the heterozygous

Table 3 Polymorphisms of the *PRF1* gene

Exon	Nucleotide change	Amino acid effect	Allele frequency
2	272 C→T	A91V	7/202 (3%)
	368 G→A	R123H	1/202 (0.5%)
	435 G→A	V145V	6/202 (3%)
	462 A→G	A154A	6/202 (3%)
	519 G→A	T173T	1/202 (0.5%)
3	755 A→G	N252S	5/202 (2%)
	822 C→T	A274A	30/202 (15%)
	900 C→T	H300H	136/202 (67%)

state in seven individuals. Three patients (P10, P27, and P58) had other biallelic disease causing *PRF1* mutations. Two patients (P14 and P55) with HLH had no other observed *PRF1* mutations. One asymptomatic sibling of a third patient with HLH (genotype unknown) demonstrated decreased levels of perforin protein and absent NK function (data not shown). The proband and parents were unavailable for study. One normal control demonstrated slightly decreased levels of perforin protein and normal NK function. The mothers of P27 and P58 both harboured M11 and A91V, which they transmitted to their offspring, presumably on the same allele. Likewise, the father of P10 carried both R54C and A91V. The observation of A91V in a normal control subject is consistent with this transition representing a polymorphism, particularly given the normal NK function in this individual. However, heterozygous carriers of *PRF1* mutations can also exhibit normal NK function despite decreased perforin expression.²¹ No patients have been observed thus far with only one other known disease causing *PRF1* mutation in combination with A91V, and no patients homozygous for A91V without other disease causing mutations have been seen. Although others have detected this genetic change in affected patients, we cannot conclude with certainty whether A91V is pathogenic. Future in vitro studies of the mutated perforin protein and a larger compilation of genotypes from both normal subjects and affected patients may resolve the putative pathogenicity of the A91V amino acid change.

V145V and A154A, two silent mutations, along with N252S were observed together in three patients (P50, P25, P24) with a G149S mutation and in one normal control. V145V and A154A were also observed with L17X, but without N252S, in the father of one patient who was a compound heterozygote for *PRF1* mutations (P33, table 1). Patient P50, homozygous for G149S, was also homozygous for V145V, A154A, and N252S. Both parents and an unaffected sibling were heterozygous at all four loci, suggesting a possible disease related haplotype. However, patient P1, a compound heterozygote carrying G149S, did not show V145V, A154A, or N252S, suggesting that multiple haplotypes that include the G149S mutation exist. Stepp *et al* and Feldman *et al* reported a patient that was heterozygous for N252S with no observed second mutation. We remain uncertain as to whether N252S contributes significantly to disease as we have observed this transition in one normal control subject who exhibited normal levels of perforin protein and normal NK function. Of the three patients with HLH who exhibited N252S, all demonstrated other biallelic mutations of *PRF1* that could account for their disease.

We report a 58% (25/43) frequency of perforin mutations among patients with primary HLH, leaving approximately half of familial HLH cases with no known aetiology. There was no significant difference between age at diagnosis when comparing patients with and without perforin mutations (median age at diagnosis was 6 months and 7 months, respectively, $p = 0.74$). However, when comparing patients

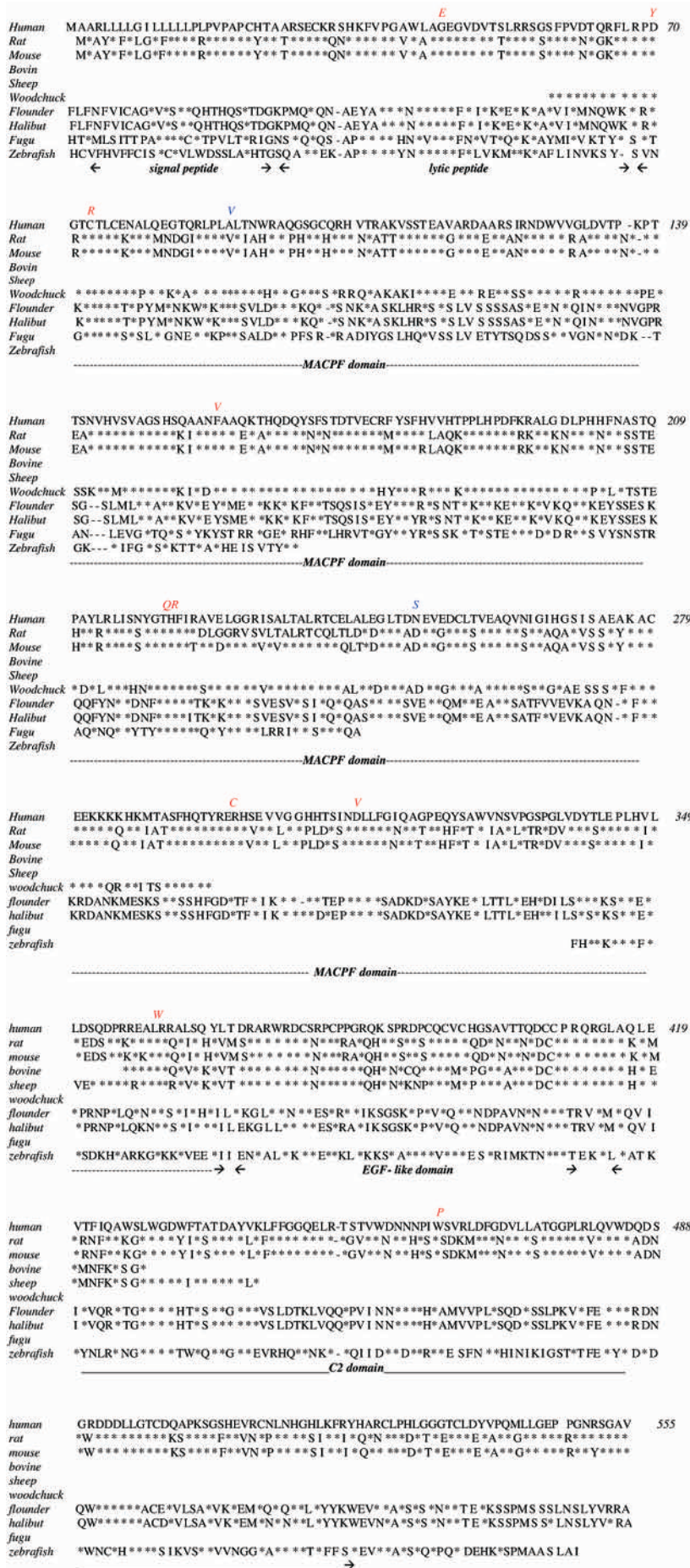


Figure 2 Alignment of human perforin protein with other species showing the functional domains of the protein. *Designates amino acid identity. Letters in red indicate novel missense mutations observed in this study. Letters in blue indicate the two transitions of undetermined pathogenicity, A91V and N252S.

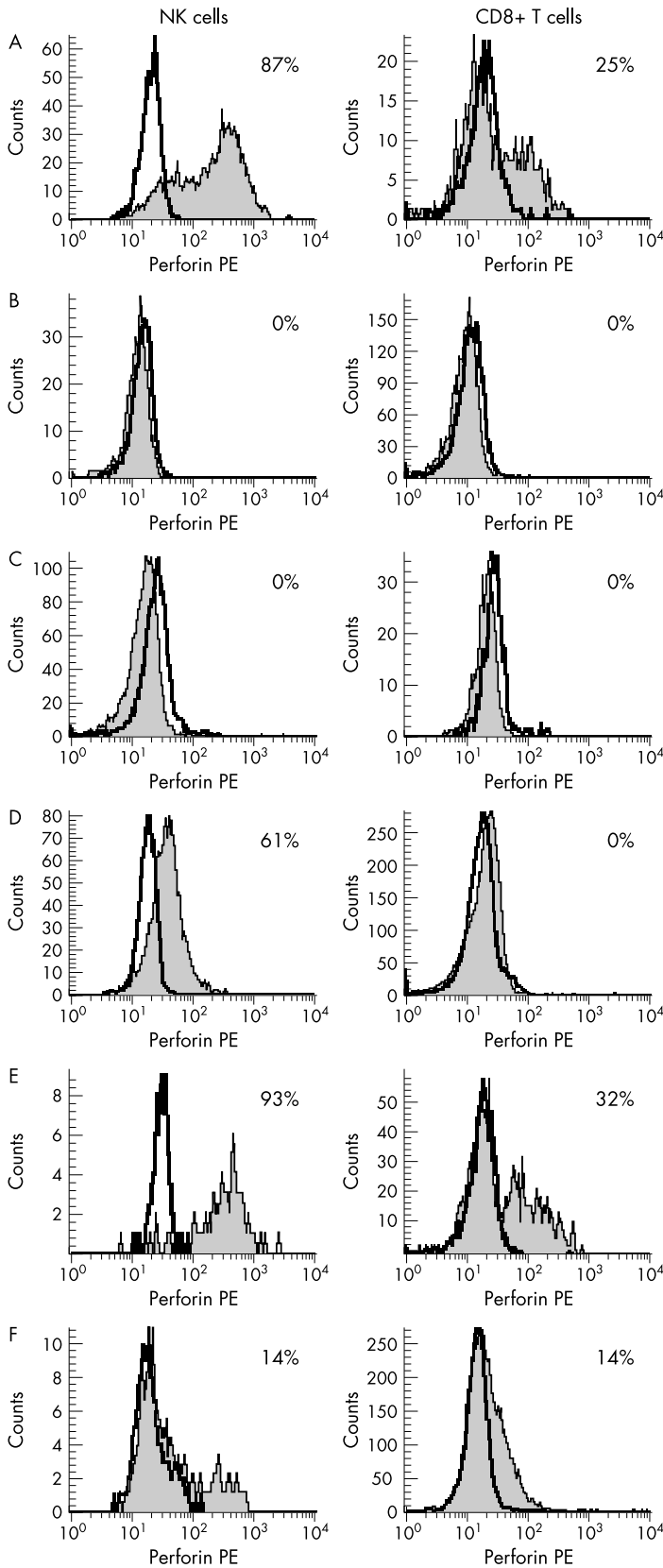


Figure 3 Flow cytometric histograms showing the perforin expression patterns in NK cells and CD8+ T cells of (A) a normal control; (B) patient P31, homozygous for 50delT; (C) patient P23, a compound heterozygote with no detectable perforin; (D) patient P10, a compound heterozygote with minimal detectable perforin; (E) patient P49, without *PRF1* mutation demonstrating a pattern similar to normal subjects; and (F) P45, a patient without *PRF1* mutation demonstrating decreased perforin expression in both NK and CD8+ T cells.

with *PRF1* mutation who expressed low levels of perforin *v* those with no detectable perforin, the median age of onset was 54 months *v* 3 months, respectively ($p \leq 0.001$). In one family with *PRF1* mutations, the proband (P12) presented at

5 years of age, while another sibling presented in infancy. As more *PRF1* gene status data become available, correlations between particular *PRF1* genotypes and clinical course may become evident.

All of the patients with *PRF1* mutations demonstrated absent or decreased perforin expression in NK cells by flow cytometry, in the proportion of perforin expressing cells and/or intensity of staining (table 1), and all but one (P35) demonstrated absent or markedly decreased NK cell function, suggesting that a single amino acid substitution can drastically affect the expression and/or function of the protein. The association of the V50M mutation with normal NK function is remarkable and warrants further investigation. The general conclusion drawn from the present study and from reports of others^{15–17} is that, with few exceptions, both missense and nonsense mutations produce phenotypes that are not distinguishable in severity.

Disease related mutations of human *PRF1* provide valuable insights into the role of individual domains in perforin function. The availability of a variety of mutations, nonsense, missense, and frameshift, throughout the perforin sequence provides a useful tool to dissect the structure–function relationships of the various domains. The distribution and location of the missense mutations identified in the present and previous studies illustrate that the mutations do not cluster within one domain. While the nonsense and frameshift mutations cause a truncated protein, the missense mutations affect amino acid residues that, in general, are conserved among different species. All the mutations, nonsense, missense, and frameshift, in the perforin gene had life threatening consequences for the patients with HLH.

ELECTRONIC DATABASE INFORMATION

Accession numbers and URLs for data presented in the study are as follows:

- Genbank: <http://www.ncbi.nih.gov/Genbank>.
- *PRF1* human: [NM005041] and [M28393]; *PRF1* rat: [P35763]; *PRF1* mouse: [P10820]; CO6 human: [NP000056]; CO8A human: [C8HUA]; CO8A rabbit: [P98136]; CO7 pig: [Q9TUQ3].
- Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim>.
- Familial haemophagocytic lymphohistiocytosis numbers: OMIM #267700 and OMIM #603553.

ACKNOWLEDGEMENTS

The authors thank Mr David Lee for assistance in establishing *PRF1* sequencing at CCHMC. This work was supported in part by the following grants: the Zachary Carter Memorial Fund, Ryan Marrocco Memorial Fund, and grants from the Histiocytosis Associations of America and Canada. J Sumegi was supported by grants from the Histiocytosis Association of America and the March of Dimes Foundation (grant number 6-FY2001-64).

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REFERENCES

- 1 Henter JI, Arico M, Elinder G, Imashuku S, Janka G. Familial hemophagocytic lymphohistiocytosis. Primary hemophagocytic lymphohistiocytosis. *Hematol Oncol Clin North Am* 1998;**12**:417–33.

- 2 Janka G, Imashuku S, Elinder G, Schneider M, Henter JI. Infection- and malignancy-associated hemophagocytic syndromes: secondary hemophagocytic lymphohistiocytosis. *Hematol Oncol Clin North Am* 1998;**12**:435–44.
- 3 Osugi Y, Hara J, Tagawa S, Takai K, Hosoi G, Matsuda Y, Ohta H, Fujisaki H, Kobayashi M, Sakata N, Kawa-Ha K, Okada S, Tawa A. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. *Blood* 1997;**89**:4100–3.
- 4 Henter JI, Samuelsson-Horne A, Arico M, Egeler RM, Elinder G, Filipovich AH, Gardner H, Imashuku S, Komp D, Ladisch S, Webb D, Janka G, the Histiocyte Society. HLH-94: a treatment protocol for hemophagocytic lymphohistiocytosis. *Med Pediatr Oncol* 1997;**28**:342–7.
- 5 Henter JI, Samuelsson-Horne A, Arico M, Egeler RM, Elinder G, Filipovich AH, Gardner H, Imashuku S, Komp D, Ladisch S, Webb D, Janka G, the Histiocyte Society. Treatment of hemophagocytic lymphohistiocytosis with HLH-94 immunochemotherapy and bone marrow transplantation. *Blood* 2002;**100**:2367–73.
- 6 Fischer A, Cerf-Bennussan N, Blanche S, Le Deist F, Bremond-Oury C, Leverger G, Schaison G, Durandy A, Griscelli C. Allogeneic bone marrow transplantation for erythrophagocytic lymphohistiocytosis. *J Pediatr* 1986;**108**:267–70.
- 7 Baker KS, Delaat CA, Steinbuch M, Gross TG, Shapiro RS, Loechel B, Harris R, Filipovich AH. Successful correction of hemophagocytic lymphohistiocytosis with related and unrelated bone marrow transplantation. *Blood* 1995;**86**(Suppl 1):387a.
- 8 Ladisch S, Poplack DG, Holiman B, Blaese RM. Immunodeficiency in familial erythrophagocytic lymphohistiocytosis. *Lancet* 1978;**i**:581–3.
- 9 Perez N, Virelizier JL, Arenzana-Seisdedos F, Fischer A, Griscelli C. Impaired natural killer activity in lymphohistiocytosis syndrome. *J Pediatr* 1984;**104**:569–73.
- 10 Arico M, Nespoli L, Maccario R, Montagna D, Bonetti F, Caselli D, Burgio GR. Natural cytotoxicity impairment in familial haemophagocytic lymphohistiocytosis. *Arch Dis Child* 1988;**63**:292–6.
- 11 Sullivan KE, Delaat CA, Douglas SD, Filipovich AH. Defective natural killer cell function in patients with hemophagocytic lymphohistiocytosis and in first degree relatives. *Pediatr Res* 1998;**44**:465–8.
- 12 Dufourcq-Lagelouse R, Jabado N, Le Deist F, Stephan JL, Souillet G, Bruin M, Vilmer E, Schneider M, Janka G, Fischer A, de Saint Basile G. Linkage of familial hemophagocytic lymphohistiocytosis to 10q21–22 and evidence for heterogeneity. *Am J Hum Genet* 1999;**64**:172–9.
- 13 Ohadi M, Lalloz MR, Sham P, Zhao J, Dearlove AM, Shiach C, Kinsey S, Rhodes M, Layton DM. Localization of a gene for familial hemophagocytic lymphohistiocytosis at chromosome 9q21.3–22 by homozygosity mapping. *Am J Hum Genet* 1999;**64**:165–71.
- 14 Graham GE, Graham LM, Bridge PJ, Maclaren LD, Wolff JEA, Coppes MJ, Egeler RM. Further evidence for genetic heterogeneity in familial hemophagocytic lymphohistiocytosis (FHLH). *Pediatr Res* 2000;**48**:227–32.
- 15 Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999;**286**:1957–9.
- 16 Goransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, Soderhall C, Samuelsson A, Janka G, Schneider M, Gurgey A, Yalman N, Revesz T, Egeler R, Jahnukainen K, Storm-Mathiesen I, Haraldsson A, Poole J, de Saint Basile G, Nordenskjold M, Henter J. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. *Am J Hum Genet* 2001;**68**:590–7.
- 17 Suga N, Takada H, Nomura A, Ohga S, Ishii E, Ihara K, Ohshima K, Hara T. Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. *Br J Haematol* 2002;**116**:346–9.
- 18 Clementi R, zur Stadt U, Savoldi G, Varoito S, Conter V, De Fusco C, Notarangelo LD, Schneider M, Klersy C, Janka G, Danesino C, Arico M. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. *J Med Genet* 2001;**38**:643–6.
- 19 Feldmann J, Le Deist F, Ouachee-Charadin M, Certain S, Alexander S, Quartier P, Haddad E, Wulffraat N, Casanova JL, Blanche S, Fischer A, de Saint Basile G. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. *Br J Haematol* 2002;**117**:956–72.
- 20 Ueda I, Morimoto A, Inaba T, Yagi T, Hibi S, Sugimoto T, Sako M, Yanai F, Fukushima T, Nakayama M, Ishii E, Imashuku S. Characteristic perforin gene mutations of haemophagocytic lymphohistiocytosis patients in Japan. *Br J Haematol* 2003;**121**:503–10.
- 21 Kogawa K, Lee SM, Villanueva J, Marmor D, Sumegi J, Filipovich AH. Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. *Blood* 2002;**99**:61–6.
- 22 Pross HF, Baines MG, Rubin P, Shragge P, Patterson MS. Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. *J Clin Immunol* 1981;**1**:51–63.
- 23 Clementi R, Emmi L, Maccario R, Liotta F, Moretta L, Danesino C, Arico M. Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. *Blood* 2002;**100**:2266–7.
- 24 Brose N, Hofmann K, Hata Y, Sudhof TC. Mammalian homologues of *Caenorhabditis elegans* unc-13 gene define novel family of C2-domain proteins. *J Biol Chem* 1995;**270**:25273–80.
- 25 Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO J* 1997;**16**:7287–96.