

**Missense mutations in the perforin (*PRF1*) gene as a cause of hereditary
cancer predisposition**

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Abstract

Perforin, a pore-forming toxin released from secretory granules of NK cells and CTLs, is essential for their cytotoxic activity against infected or cancerous target cells. Bi-allelic loss-of-function mutations in the perforin gene are invariably associated with a fatal immunoregulatory disorder, familial haemophagocytic lymphohistiocytosis type 2 (FHL2), in infants. More recently, it has also been recognized that partial loss of perforin function can cause disease in later life, including delayed onset FHL2 and haematological malignancies. Herein we report a family in which a wide range of systemic inflammatory and neoplastic manifestations have occurred across three generations. We found that disease was linked to two missense perforin gene mutations (encoding A91V, R410W) that cause protein misfolding and partial loss of activity. These cases link the partial loss of perforin function with some solid tumours that are known to be controlled by the immune system, as well as haematological cancers. Our findings also demonstrate that perforin gene mutations can contribute to hereditary cancer predisposition.

Introduction

Perforin (PRF, encoded by the *PRF1* gene) is a pore-forming toxin¹ stored in the secretory granules of cytotoxic lymphocytes. During an immune response, these 'killer' cells form an immune synapse with a virus-infected or cancer target cell, and release PRF and granzyme serine proteases into the synaptic cleft.² PRF forms membrane pores that are essential for cytotoxic lymphocyte pro-apoptotic serine proteases, granzymes, to enter the target cell cytoplasm, where they trigger apoptosis.³

Bi-allelic mutations in *PRF1* that completely abrogate function are classically associated with the paediatric immunoregulatory disorder familial haemophagocytic lymphohistiocytosis type 2 (FHL2),⁴ which accounts for 30-60% of all FHL cases.⁵ However, it is possible that FHL2 represents one extreme of a spectrum of diseases caused by PRF deficiency.⁶ Thus, missense mutations causing partial loss of expression or function of PRF are more often associated with later atypical onset FHL2⁷ and/or haematological malignancy.⁸⁻¹² It is unknown whether PRF deficiency can predispose to solid tumours, nor has a possible contribution of *PRF1* mutations to familial cancer ever been explored.

Herein, we describe a UK family of Italian origin, in which adult onset FHL2, haematological and various solid tumours have affected three generations. We show that these diseases are consistently associated with *PRF1* missense mutations, with defective protein folding causing partial loss of PRF expression. These findings highlight a novel hereditary cancer predisposition syndrome associated with *PRF1* mutations.

Methods

DNA sequencing

Oligonucleotide primers used for *PRF1* sequencing are available on request.

PRF expression

PBMNCs were surface stained using anti-CD56-PE, CD4-PerCP, CD8-APC, then fixed, permeabilized, and stained with cytofix/cytoperm and anti-human PRF-FITC or isotype control (BD Biosciences). 100,000 lymphocytes were acquired on FACs Calibur and analysed by sequential gating using CellQuest Pro (BD Biosciences).

Assessment of R410W function

Primary murine *Prf1*^{-/-} CD8⁺ T cells transgenic for the OT1 TCR were transiently transfected with either WT or mutant R410W PRF cDNA, as described previously.^{11,13} Cells were then used in four-hour ⁵¹Cr release assays against OVA257 (SIINFEKL peptide) labelled EL-4 thymoma target cells.^{11,13} % specific ⁵¹Cr release was estimated as described.¹¹

Study approval

All clinical investigation was conducted according to Declaration of Helsinki principles. All participants gave full informed consent for analysis of PRF expression and *PRF1* mutation screening, and for the results to be reported in this study.

Results and discussion

A family pedigree showing members in whom *PRF1* was analysed is shown in Figure 1. Further details, including *PRF1* mutational analysis and NK cell PRF expression are summarised in Table 1.

The index case, a 43 year-old woman (II-1), presented with lethargy, pyrexia and moderate splenomegaly. She was found to have pancytopenia, hyperferritinaemia, hypertriglyceridaemia and extensive haemophagocytosis in her bone marrow, leading to a diagnosis of HLH, based on the HLH-2004 diagnostic criteria. Despite extensive investigation, no acquired cause for HLH could be found; rather, the diagnosis of late onset FHL2 was supported by mutations in *PRF1* and reduced intracellular PRF expression (Figure 2). Treatment was according to the HLH-1994 protocol, but the patient developed progressive multi-organ failure and died.

Subsequently, two family members also carrying *PRF1* mutations and with reduced intracellular PRF presented with *de novo* leukaemia. The index case's niece (III-1) was diagnosed with acute lymphoblastic leukaemia (ALL), and her uncle (I-3) with chronic myelomonocytic leukaemia (CMML), which subsequently transformed to acute myeloid leukaemia (AML) (Table 1). A link between *PRF1* mutations and lymphoid malignancies has been reported,^{8,10,11} but to our knowledge this is the first case of CMML in association with PRF deficiency.

Further investigation of the family's medical history revealed that the father of the index case (I-1), who carried the same mutations as his daughter (II-

1), had developed multiple primary malignancies as an adult, including renal cell carcinoma at 54 years of age, but had never been affected by HLH (Table 1). No other predisposing environmental or genetic cancer risk had been identified. A brother of the index case (II-2) developed intracranial glioma, which was rapidly fatal despite radiation therapy. Genotyping was not performed, but it could be deduced from his parents' genotype that he would have carried at least one *PRF1* mutation, with a 50% probability of two mutations. To our knowledge, this is the first report of an association between human *PRF1* mutations and solid tumours, some of which have previously been described to be under the control of the immune system.^{14,15}

The A91V allele identified in this family is by far the commonest hypomorphic *PRF1* variant, being found in ~8% of Caucasians.⁵ A91V adversely affects PRF folding and this is typically thought to markedly reduce PRF levels and the cytotoxicity of CTL/NK cells.^{16,17} Although misfolded A91V PRF is detectable by standard methodologies in healthy A91V heterozygotes,¹⁷ earlier reports suggested that A91V homozygote patients (or those who co-inherited A91V together with a null mutation) have severely reduced or absent PRF levels.^{16,18} In analysing patient II-3, who had bi-allelic A91V mutations but has remained healthy, we demonstrated, for the first time, that A91V homozygosity is indeed compatible with reduced, but still detectable PRF levels (Figure 2). This discrepancy may be related to PRF levels being assessed at times when a patient is extremely ill.

In contrast, the R410W mutation, that also affects this family, has not been previously investigated. We found that R410W causes near-total loss of function, as the activity of *Prf1*^{-/-} mouse CTLs transfected with the mutant was

<5% of the CTLs reconstituted with wild-type PRF (Figure 3A). As with other cancer-associated *PRF1* mutations,¹¹ when R410W-transfected T cells were cultured at reduced temperature (30°C), their activity was restored to wild-type level (Figure 3B). The fact that the loss of activity of R410W is temperature-dependent strongly suggests that protein misfolding is responsible for its functional impairment.¹¹

A91V and R410W both lead to PRF misfolding, which reduces but does not abolish PRF activity. Such *in vitro* studies on PRF mutants have previously been shown to be strong predictors of their behaviour *in vivo* in carriers of these mutations.^{11,17} Whereas the complete loss of PRF function typically presents in early childhood as FHL2, the subtotal loss of PRF activity causes systemic inflammatory disease that is delayed beyond infancy (as in the index case II-1) or may present as a different immunopathology.⁶ In these instances, consistent with Burnet's hypothesis of tumour immune surveillance,¹⁹ reduced cytotoxic lymphocyte activity caused by partial loss of PRF may be expected to predispose to neoplasia, and this is indeed supported by previous studies.⁹⁻¹² It is not clear, however, why these malignancies thus far reported in humans in association with PRF deficiency are predominantly haematological. It is possible that PRF defects lead to a reactive proliferation of the lymphoid and histiocytic pools, as typically observed in FHL, causing genomic instability and a predisposition to neoplasia in one of these lineages. Alternatively, haematological malignancies may be more susceptible to immune control than solid tumours and may thus arise more frequently in the absence of a fully functional immune system. This report supports the strong link between partial PRF deficiency and haematological malignancy. Critically, it also shows for the first time that

multiple solid tumours, some known to be controlled by immune system,^{14,15} can occur in humans in this context, thus validating earlier investigations in murine models, where many types of solid tumour, including prostate, mammary and lung carcinoma, were controlled by PRF-dependent cytotoxicity.^{20,21} Overall, in the current study, the penetrance of PRF1 mutations was over 50% with 5 out of 9 carriers affected by disease (Table 1). Excluding patient II-2 (where genetic analysis was not performed), a similar penetrance was observed in both carriers of mono-allelic *PRF1* mutations (2 out of 4 carriers affected) and family members with bi-allelic mutations (2 out of 4 carriers affected).

Of particular note, the cases discussed in this report involve many family members and several generations. This is highly suggestive of a hereditary cancer predisposition syndrome,²² and we believe this is the first association of *PRF1* mutations with such a disorder. Such findings align closely with a recent report highlighting increased incidence of cancer in relatives of patients with FHL.²³

Although hereditary cancer syndromes are typically autosomal dominant with incomplete penetrance, the pattern of inheritance is less clear in this family, with both mono-allelic and bi-allelic carriers of *PRF1* mutations developing disease. One explanation for this variable pattern in inheritance is that PRF mutants such as A91V have been previously shown to exhibit dominant negative activity,^{17,24} and thus may cause pathology when only present on one allele. Furthermore, although *PRF1* mutations may be acting alone, it is possible that another undetected mutation may be present in this family. For example, it has recently been shown that patients with FHL may co-inherit mutations in two different genes regulating lymphocyte cytotoxicity, although this is a rare event

leading to FHL mostly at a younger age.²⁵ Besides, in the context of the family described here, co-inheritance of three pathological mutations would be required, with one leading to defective perforin secretion (*UNC13D* or *STX11* or *STXBP2*). While this is theoretically possible, the probability of such an event is exceedingly small. Alternatively, PRF deficiency may be acting in concert with classical cancer predisposition genes unrelated to cytotoxic lymphocyte function. The latter possibility potentially opens up a new paradigm, which we believe warrants further investigation: that deficiency of an extrinsic tumour suppressor such as PRF may co-operate with an intrinsic genetic defect to predispose to familial cancer syndromes.

Authorship Contributions

M.S.C. researched the study, collected and analysed the data, and wrote the manuscript; K.G. performed experiments and contributed to writing of the manuscript; I.H. performed experiments; M.L. supervised research and contributed to writing of the manuscript; N.P. collected clinical data and contributed to writing of the manuscript; M.S. collected clinical data; J.T. supervised research and contributed to writing of the manuscript; I.V. supervised research and contributed to writing of the manuscript.

J.T. and I.V. contributed equally to this study

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Figure and Table Legends

Figure 1. Characterization of PRF1 defects within the family. Family pedigree showing the relationship between family members in which PRF1 analysis was performed. An arrow indicates the index case. Each family member's cancer history is also indicated (H=haematological malignancy; S=solid cancer; DF=disease free; U= unknown cancer history).

Figure 2. Intracellular PRF expression in selected family members. Intracellular PRF expression in CD56+CD8- NK cells in patients I-1, II-3, II-5 and II-6 (serving as WT perforin control); in parenthesis is the median fluorescence intensity. Of note, patient II-3, who is homozygous for the A91V mutation but has remained healthy, demonstrates reduced, but appreciable PRF levels.

Figure 3. Determining the nature of the R410W mutation. (A) At 37°C, reconstitution of *Prf1*^{-/-} CTLs with R410W mutant PRF leads to cytolytic activity (assessed by ⁵¹Cr release assays) <5% of *Prf1*^{-/-} CTLs reconstituted with wild-type perforin. (B) At 30°C (a permissive temperature for protein folding)¹¹, *Prf1*^{-/-} CTLs transfected with R410W mutant PRF have cytolytic activity that is indistinguishable from *Prf1*^{-/-} CTLs transfected with wild-type PRF. A complete recovery of R410W activity indicates that the only reason for its marginal activity at 37°C is a severe misfolding, and the mutation itself does not affect PRF function.

Table 1: Summary of PRF defects and associated disease in family members. Analysis of PRF included *PRF1* genotype (assessed by Sanger sequencing) and PRF expression in NK cells (assessed by intracellular FACS). The penetrance of PRF mutations was found to be over 50% with 5 out of 9 carriers affected by disease, including solid tumours.

Figures and Tables

Figure 1

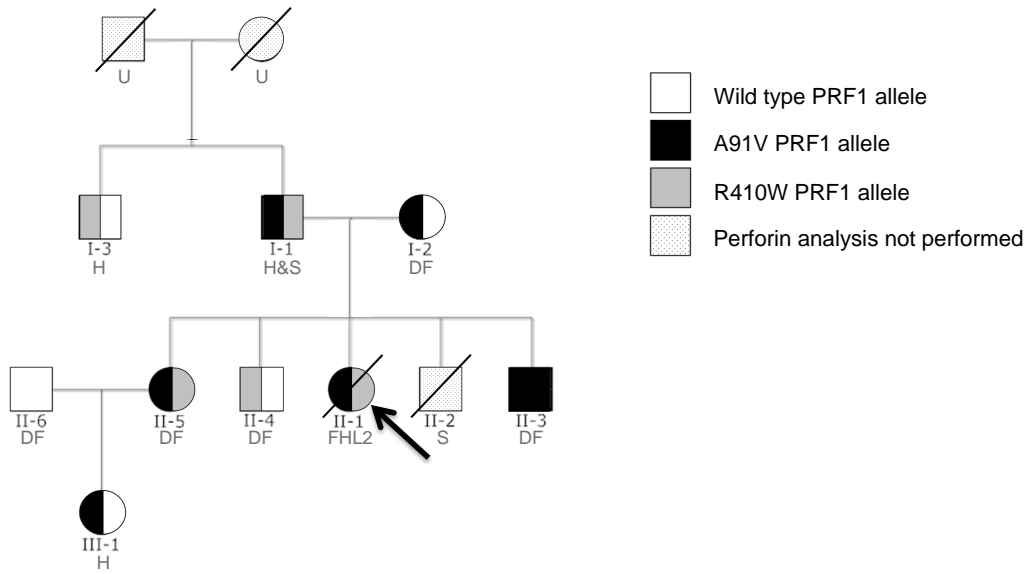


Figure 2

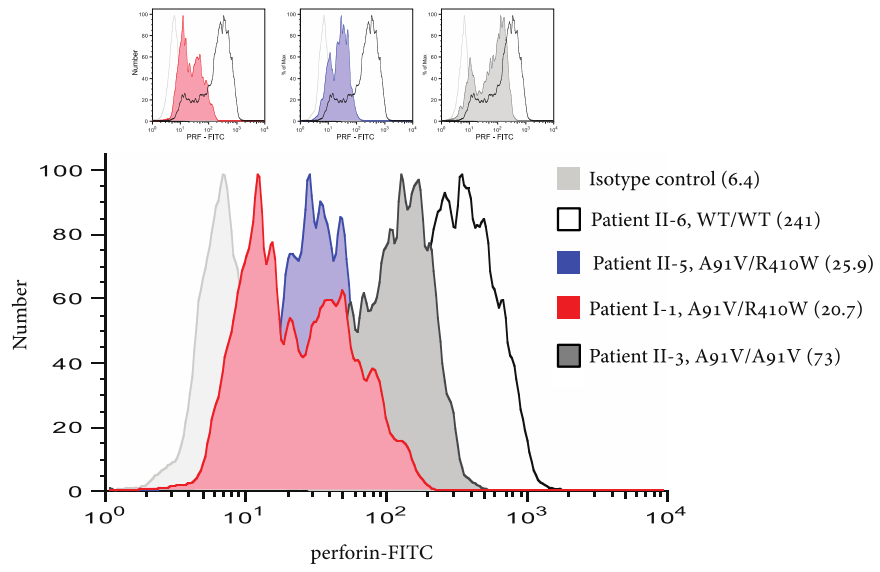


Figure 3

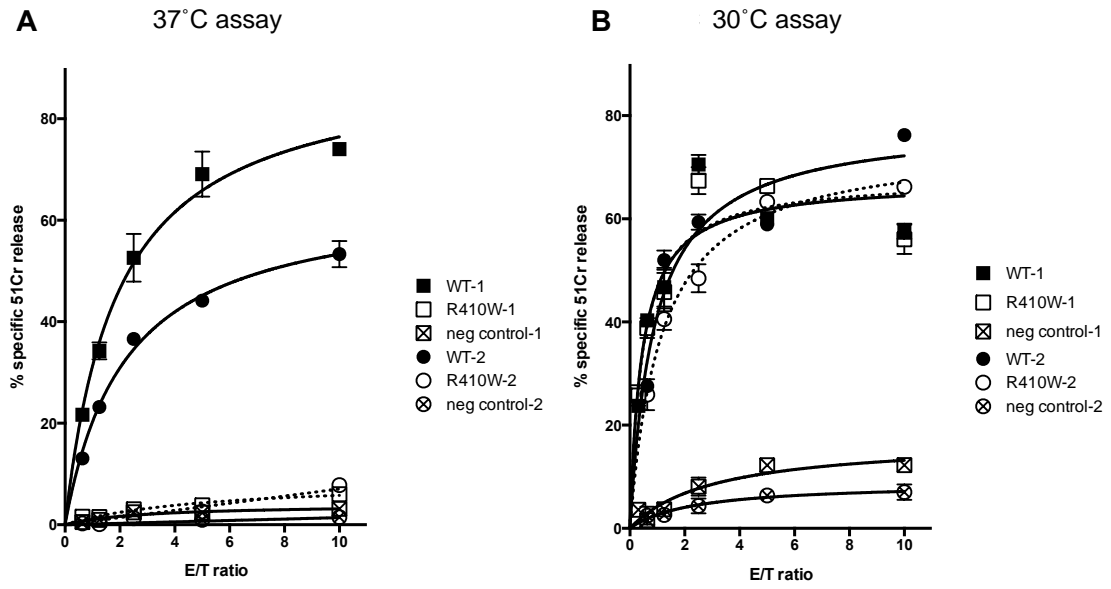


Table 1

Patient	Relationship to index case	Age at testing	PRF1 genotype	PRF expression in NK cells	Disease (age at presentation)
I-1	Father	76y	A91V/R410W	58%	Renal carcinoma (54y) Prostate carcinoma (71y) Gastric carcinoma (77y) AML (81y)
I-2	Mother	76y	A91V/WT	86%	Disease free
I-3	Uncle	70y	R410W/WT	70%	CMML (70y)
II-1	Index case	43y	A91V/R410W	65%	FHLH (43y)
II-2	Brother	Not tested	Not tested	Not tested	Glioma (40y)
II-3	Brother	49y	A91V/A91V	72%	Disease free
II-4	Bother	40y	R410W/WT	88%	Disease free
II-5	Sister	35y	A91V/R410W	47%	Disease free
II-6	Brother-in-law	49y	WT/WT	89%	Disease free
III-1	Niece	8y	A91V/WT	65%	ALL (8y)