Human Immunology 74 (2013) 14–17

Contents lists available at SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Rapid Communication

Functional impact of A91V mutation of the *PRF1* perforin gene

Natalia Martínez-Pomar [a,*], Nallibe Lanio [a], Neus Romo [b], Miguel Lopez-Botet [b,c], Núria Matamoros [a]

[a] Department of Immunology, University Hospital of Son Espases, Palma de Mallorca, Spain
[b] Immunology Unit, University Pompeu Fabra, Barcelona, Spain
[c] IMIM (Hospital del Mar Research Institute), Spain

ARTICLE INFO

Article history:
Received 3 May 2012
Accepted 3 October 2012
Available online 13 October 2012

ABSTRACT

Perforin (*PRF1*) gene mutations have been associated with Familial Hemophagocytic Lymphohistiocytosis type 2 (FHL2). Substitution p.A91V (c.272C>T) in exon 2 was first described as a neutral polymorphism. Nonetheless, recent clinical evidence and functional assays, suggest a potential pathogenic role for p.A91V, especially in compound heterozygous individuals. Moreover, p.A91V homozygosity has been linked to various pathological states including FHL and lymphocytic leukaemias.

In the present report we evaluated the impact of this mutation in a compound heterozygous A91V/G149S 31 year-old asymptomatic female. Functional assays revealed low perforin expression levels, as well as an impaired NK cell-mediated cytotoxicity, partially reconstituted after incubation with IL-2. These results support that p.A91V mutation, associated to another mutated *PRF1* allele, may potentially predispose seemingly healthy carriers to suffer a milder FHL2 clinical phenotype, including later onset of the disease. Thus, clinical monitoring of p.A91V carrier individuals bearing another mutation in *PRF1* is warranted.

1. Introduction

FHL is a heterogeneous disorder characterized by a hyperinflammatory syndrome with fever, hepatosplenomegaly, cytopenia and, less commonly, central nervous system involvement. FHL is a lethal autosomal recessive defect [1] that typically occurs in infancy or early childhood [2]. To date, described genetic defects associated with this pathology disrupt mechanisms involved in triggered or activation-induced cell death. Perforin-encoding *PRF1* was the first gene linked to FHL2 [3] being responsible for 15–30% of cases [4]. Perforin is a pore-forming cytolytic protein synthesized and stored in secretory granules of cytotoxic T lymphocytes (CTLs) and natural killer cells (NK). Its function is essential for killing virus-infected or transformed cells through the granule exocytosis pathway [5]. The exact contribution of the various *PRF1* mutations reported in the literature is not yet clear. The pathophysiological potential of the common variant c.C271T (p.A91V), found in up to 20% of healthy individuals [6,7] remains to be fully elucidated [8–10]. Initial studies considered A91V a benign polymorphism [11,12] but recent reports have proposed it as a disease-modifying gene [13–15]. Functional studies associated A91V to decreased cytotoxicity activity [16–18] but most clinical observations implying a pathogenic role for A91V have been limited by the small sample size. In the current study we describe a healthy 31 year-old female compound heterozygous for A91V/G149S who displayed diminished perforin expression levels and impaired NK and CD8+ T cell-mediated cytotoxicity.

2. Material and methods

2.1. Subjects

We studied a woman native from Morocco whose daughter developed FHL. The study protocol was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by our hospital research ethics committee. Informed consent was obtained from all subjects.

2.2. PRF1 mutation analysis

Genomic DNA was extracted from peripheral blood (QIAamp DNA Blood mini Kit) in accordance with the manufacturer's Instructions. Mutation analysis of PRF1 gene was performed by direct DNA sequencing using specific primers. In all samples the PRF1 gene encoding exon 2 and 3 was amplified using primers as follows: for exon 2, forward (F2) 5′-CCTTCCATGTGCCCTGATAATC-

* Corresponding author. Address: Department of Immunology, University Hospital of Son Espases, Ctra. Valldemosa, 79, E-07010 Palma de Mallorca, Spain. Fax: +34 871 909 951.
E-mail address: natalia.martinez@ssib.es (N. Martínez-Pomar).

0198-8859/$36.00 - see front matter © 2012 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.humimm.2012.10.011

3′ and reverse (R2) 5′-GAAGCAGCCTCCAAGTTTGATTG-3′; and for exon 3, forward (F3) 5′-CAGTCCTAGTTCTGCCCACTTA-3′ and reverse (R3) 5′-CTAATGGGAATACGAAGACAGCC-3′. PCR analyses were carried out using standard reaction mixes, and PCR products were purified (QIAquick PCR purification Kit) and sequenced directly on an automated sequencer (ABI-3100; Applied Biosystems) adding the following pair of internal primers to improve sequence analysis: reverse (R2int) 5′-GCCCTCCTGTAGGGCATTTTC-3′ and forward (F2int) 5′-AGTGGACACACAAAGGTTCCTG-3′. Sequence data were analyzed with Chromas software and compared with the reported gene structure (gene number 190339, NCBI).

2.3. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). An intracellular staining was performed using the Cytofix/Cytoperm kit (Becton Dickinson, CA) as described by the manufacturer. Briefly, cells were incubated with anti-CD3-PerCP and anti-CD56-PE (BD Pharmingen, CA). After washing, cells were permeabilized and incubated with the anti-Perforin-FITC mAb (BD Pharmingen, CA). Samples were analyzed by flow cytometry in a FACScan (Becton Dickinson, CA); data were processed with Cell Quest Pro (Becton Dickinson, CA) and FlowJo (Tree Star, Inc., OR) softwares. Appropriate isotype-matched control mAbs were used to assess nonspecific binding. The surface expression of a panel of activating NK cell-associated receptors (i.e. NKG2A, NKG2D, NKG2C, NKp46, NKp30 and CD16) was analysed by multicolour flow cytometry as previously described [19].

2.4. Cytotoxicity assays

The cytolytic activity of PBMCs, freshly isolated or incubated 48 h with hrIL-2 (1000 U/ml), was analyzed against the human K562 erythroblastoid leukaemia cell line in a 4-h ^{51}Cr-release assay (effector/target ratios ranged from 100/1 to 12.5/1). All assays were set up in triplicate and specific lysis was calculated as previously described [19].

3. Results and discussion

FHL2 frequently occurs in children under 2 years of age but atypical forms with delayed onset have been reported [13]. In the present report we studied a woman whose daughter (G149S homozygous) presented severe FHL in early infancy (2 month old). Both parents were carriers and furthermore, the mother presented the A91V variant in trans (Fig. 1). Perforin expression is mainly confined to NK cells, as well as CD8+, CD56+, TcRγδ+ and some activated CD4+ T cells [20–22]. An analysis of NK and T cells from the asymptomatic A91V/G149S compound heterozygous revealed decreased perforin expression levels as compared to a healthy control (Fig. 2); as expected, perforin was undetectable in cells from the (G149S/G149S) patient. The proportions of CD56^{dim} and CD56^{bright} NK cell subsets and the expression of NK cell-associated receptors (i.e. NKG2A, NKG2D, NKG2C, Nkp46, NKp30, NKp44 and CD16) were comparable in both individuals (data not shown).

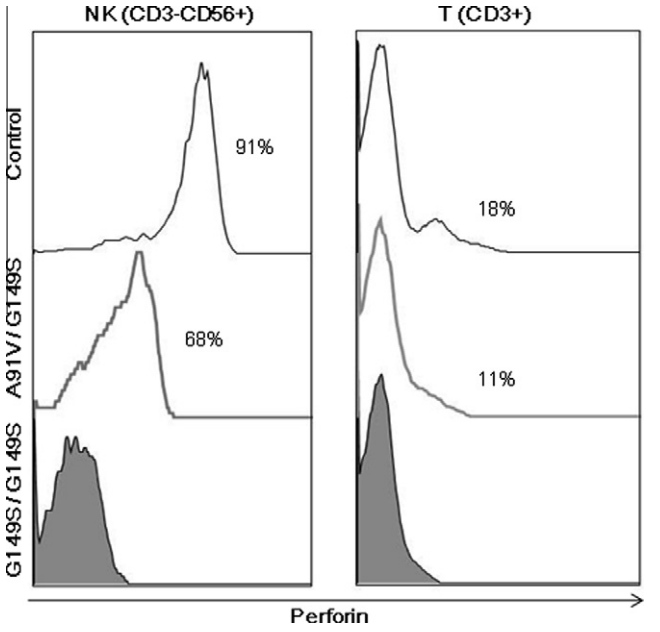

Fig. 2. Perforin expression analysis in T and NK cells. Perforin levels in T and NK cells were lower in the compound heterozygous individual as compared with a healthy control sample. Cells from the G149S homozygous patient did not express perforin.

Cytotoxicity mediated by PBMC derived from the compound heterozygous (A91V/G149S) individual was tested against the NK-sensitive K562 cell line, in parallel to samples from the homozygous (G149S/G149S) FHL patient and a healthy control. As shown in Fig. 3A, compound heterozygous cells displayed an impaired cytotoxic function against K562 cells, comparable to perforin-deficient G149S/G149S cells. When the samples were tested after in vitro IL-2 stimulation, cytotoxicity was enhanced in A91V/G149S cells and, to a lesser extent, in G149S/G149S cells, remaining in every case lower than that of control cells (Fig. 3B).

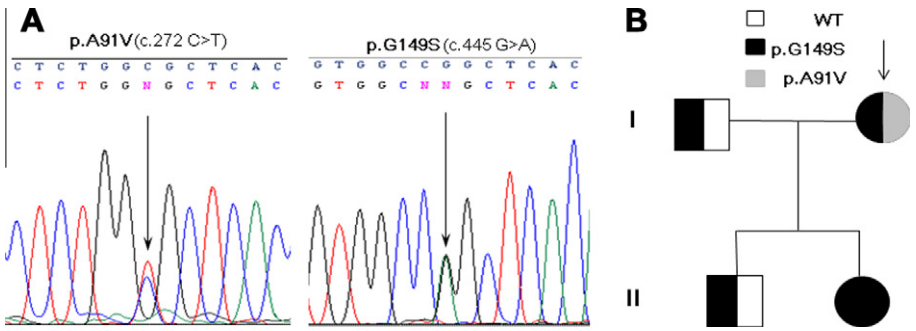

Fig. 1. Molecular analysis of the *PRF1* gene and family pedigree. (A) The DNA sequence of *PRF1* exon 2 (ENST00000373209) for the proband revealed a compound heterozygous gene profile with two mutations in trans: c.272 C>T and c.445 G>A. These mutations resulted in aminoacid substitutions p.A91V and p.G149S, respectively. (B) Family pedigree analysis for both mutations. Index case is indicated by an arrow. Circles represent females; squares, males; half-filled symbols *PRF1* mutations.

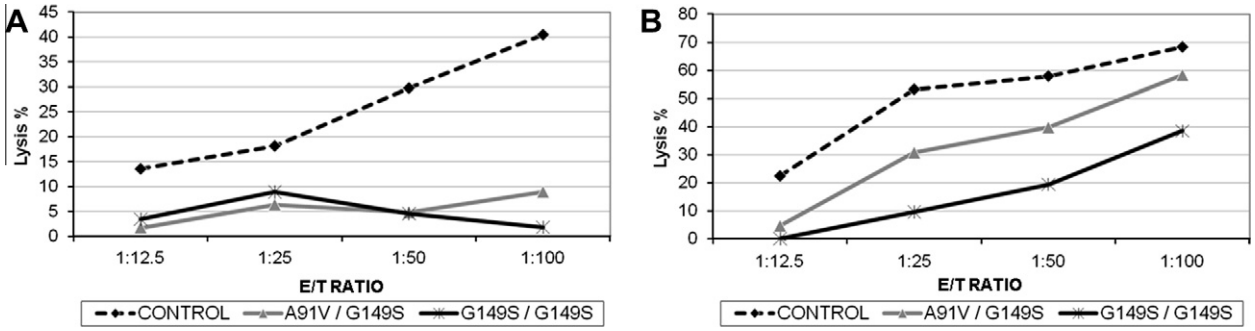

Fig. 3. NK cell-mediated cytotoxicity assays. (A) PBMC samples from the homozygous G149S/G149S patient, the compound heterozygous (A91V/G149S) individual and a healthy donor were tested in parallel against the NK-sensitive K562 cell line at different effector/target (E/T) ratios. (B) Cytotoxicity assays were carried out employing as effector cells the same PBMCs samples tested in (A), previously incubated for 48h with IL-2 (1000 U/ml).

Analysis of predicted structural and functional effects of the A91V variant was tested using Polyphen (Polymorhism Phenotyping) (http://genetics.bwh.harvard.edu/pph/) and the Sorting Intolerant From Tolerant (http://blocks.fhcrc.org/sift/SIFT.html) programs, predicting a possibly damaging (PSIC score difference: 1.791) and affect protein function (score 0.01), respectively.

The high frequency of the A91V mutation in healthy populations and the low numbers of FHL patients harboring this mutation has rendered its putative pathogenic role a controversial issue. Yet, its association to an impaired in vitro perforin function has been reported [23,24]. Moreover, a recent retrospective review [25] of genetic and immunological observations in patients developing late FHL revealed that around 7% presented the A91V genotype. Moreover, Chia et al. identified a subgroup of individuals from non-consanguineous families with two mutated *PRF1* alleles, in which onset of FHL was markedly delayed or presented an hematological malignancy. Some of these patients were found to be A91V homozygous or compound heterozygous with a second *PRF1* mutated allele. Our data are consistent with the observations of Chia et al. [23], who demonstrated that cytotoxic function of mutant A91V cells was completely rescued by culturing at 30 °C.

The ability of IL-2 stimulation to partially rescue the NK cell function has been previously reported in patients with type 2 FHL [26], pointing out the involvement of perforin-independent cytolytic mechanism(s) (e.g. TRAIL).

In summary, our results further support that this frequent polymorphism associated to another *PRF1* allele mutation may predispose seemingly healthy carriers to suffer a milder clinical phenotype, including later onset of the disease. Thus, a close clinical follow-up of p.A91V carrier individuals bearing another *PRF1* mutation is recommended. Moreover, the high incidence of p.A91V in the general population warrants an extended molecular analysis of this mutation in carrier relatives of FHL2 patients.

Acknowledgments

This work was supported by Grant Plan Nacional I+D (SAF2010-22153-C03-01) from University Pompeu Fabra.

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(2):417–33.
[2] Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2007;48(2):124–31.
[3] Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science 1999;286(5446):1957–9.
[4] Göransdotter EK, Fadeel B, Nilsson-Ardnor S, Söderhäll C, Samuelsson A, Janka G, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. Am J Hum Genet 2001;68(3):590–7.
[5] Voskoboinik I, Dunstone MA, Baran K, Whisstock JC, Trapani JA. Perforin: structure, function, and role in human immunopathology. Immunol Rev 2010;235(1):35–54.
[6] Busiello R, Fimiani G, Miano MG, Aricò M, Santoro A, Ursini MV, et al. A91V perforin variation in healthy subjects and FHLH patients. Int J Immunogenet 2006;33(2):123–5.
[7] Zhang K, Johnson JA, Biroschak J, Villanueva J, Lee SM, Bleesing JJ, et al. Familial haemophagocytic lymphohistiocytosis in patients who are heterozygous for the A91V perforin variation is often associated with other genetic defects. Int J Immunogenet 2007;34(4):231–3.
[8] Molleran Lee S, Villanueva J, Sumegi J, Zhang K, Kogawa K, Davis J, et al. Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. J Med Genet 2004;41(2):137–44.
[9] Mehta PA, Davies SM, Kumar A, Devidas M, Lee S, Zamzow T, et al. Perforin polymorphism A91V and susceptibility to B-precursor childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Leukemia 2006;20(9):1539–41.
[10] Mancebo E, Allende LM, Guzmán M, Paz-Artal E, Gil J, Urrea-Moreno R, et al. Familial hemophagocytic lymphohistiocytosis in an adult patient homozygous for A91V in the perforin gene, with tuberculosis infection. Haematologica 2006;91(9):1257–60.
[11] Zur Stadt U, Beutel K, Weber B, Kabisch H, Schneppenheim R, Janka G. A91V is a polymorphism in the perforin gene not causative of an FHLH phenotype. Blood 2004;104(6):1909.
[12] Feldmann J, Le Deist F, Ouachée-Chardin M, Certain S, Alexander S, Quartier P, et al. Functional consequences of perforin gene mutations in 22 patients with familial heamophagocytic lymphohistiocytosis. Br J Haematol 2002;117(4):965–72.
[13] Clementi R, Emmi L, Maccario R, Liotta F, Moretta L, Danesino C, et al. Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. Blood 2002;100(6):2266–7.
[14] Trizzino A, Zur Stadt U, Ueda I, Risma K, Janka G, Ishii E, et al. Genotype–phenotype study of familial haemophagocytic lymphohistiocytosis due to perforin mutations. J Med Genet 2008;45:15–21.
[15] Sánchez IP, Leal-Esteban LC, Alvarez-Álvarez JA, Pérez-Romero CA, Orrego JC, Serna ML, et al. Analyses of the PRF1 gene in individuals with hemophagocytic lymphohystiocytosis reveal the common haplotype R54C/A91V in Colombian unrelated families associated with late onset disease. J Clin Immunol 2012;32(4):670–80.
[16] Voskoboinik I, Thia MC, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. Blood 2005;105(12):4700–6.
[17] Trambas C, Gallo F, Pende D, Marcenaro S, Moretta L, De Fusco C, et al. A single amino acid change, A91V, leads to conformational changes that can impair processing to the active form of perforin. Blood 2005;106(3):932–7.
[18] Voskoboinik I, Sutton VR, Ciccone A, House CM, Chia J, Darcy PK, et al. Perforin activity and immune homeostasis: the common A91V polymorphism in perforin results in both presynaptic and postsynaptic defects in function. Blood 2007;110(4):1184–90.
[19] Sáez-Borderías A, Romo N, Magri G, Gumá M, Angulo A, López-Botet M. IL-12-dependent inducible expression of the CD94/NKG2A inhibitory receptor regulates CD94/NKG2C + NK cell function. J Immunol 2009;182(2):829–36.
[20] Rukavina D, Laskarin G, Rubesa G, Strbo N, Bedenicki I, Manestar D, et al. Age-related decline of perforin expression in human cytotoxic T lymphocytes and natural killer cells. Blood 1998;92(7):2410–20.
[21] Rutella S, Rumi C, Lucia MB, Etuk B, Cauda R, Leone G. Flow cytometric detection of perforin in normal human lymphocyte subpopulations defined by expression of activation/differentiation antigens. Immunol Lett 1998;60(1):51–5.

[22] Kawamura T, Kawachi Y, Moroda T, Weerasinghe A, Iiai T, Seki S, et al. Cytotoxic activity against tumour cells mediated by intermediate TCR cells in the liver and spleen. Immunology 1996;89(1):68–75.
[23] Baran K, Dunstone M, Chia J, Ciccone A, Browne KA, Clarke CJ, et al. The molecular basis for perforin oligomerization and transmembrane pore assembly. Immunity 2009;30(5):684–95.
[24] Chia J, Yeo KP, Whisstock JC, Dunstone MA, Trapani JA, Voskoboinik I. Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. Proc Natl Acad Sci U S A 2009;106(24):9809–14.
[25] Zhang K, Jordan MB, Marsh RA, Johnson JA, Kissell D, Meller J, et al. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial hemophagocytic lymphohistiocytosis. Blood 2011;118(22):5794–8.
[26] Schneider EM, Lorenz I, Müller-Rosenberger M, Steinbach G, Kron M, Janka-Schaub GE. Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis. Blood 2002;100:2891–8.