[haematologica]
2004;89:325-329

Mutation analysis of *HPS1*, the gene mutated in Hermansky-Pudlak syndrome, in patients with isolated platelet dense-granule deficiency

JAVIER CORRAL
ROCIO GONZÁLEZ-CONEJERO
NURIA PUJOL-MOIX
PERE DOMENECH
VICENTE VICENTE

A B S T R A C T

Background and Objectives. Isolated platelet dense granule (PDG) deficiency is a heterogeneous disorder frequently found among patients with mild to moderate bleeding diatheses. However, the molecular basis of this disorder is unknown. Genes involved in other rare bleeding disorders with associated reduction in the numbers of platelet dense-granules may play a role in isolated PDG deficiency. Among such genes, *HPS1* is known to play a key role in the genesis of PDG and as many as 18 different *HPS1* mutations have been identified in patients with Hermansky-Pudlak syndrome. Recently, we have identified subjects with one *HPS1* heterozygous mutation displaying significant reductions in PDG without the clinical phenotype of Hermansky-Pudlak syndrome. This suggested that *HPS1* mutations could be involved in isolated PDG deficiency.

Design and Methods. We sequenced all coding exons, and flanking intron regions of *HPS1* in 16 patients with mild to severe PDG deficiency, most of whom had mild bleeding episodes. Nine patients reported a familial history of bleeding diathesis with PDG deficiency. We also evaluated the prevalence of HPS1 variations in 215 controls. Transmission electron microscopy was used to evaluate the number and morphology of PDG from patients and selected controls.

Results. No patient with PDG deficiency carried severe mutations of the *HPS1* gene. We identified 6 previously described and 5 new polymorphisms in the HPS1 gene. Platelet electron microscopy in controls carrying these polymorphisms revealed that they did not significantly modify the number or morphology of PDG.

Interpretation and Conclusions. Mutations affecting the *HPS1* gene play a minor role in isolated PDG deficiency. These results support a molecular heterogeneity responsible for the number and morphology of PDG.

Key words: *HPS1* gene, platelet-dense granules, bleeding, genetics.

From the Universidad de Murcia, Centro Regional de Hemodonación, Spain (JC, RG-C, VV); Departament d'Hematologia, Hospital Santa Creu i Sant Pau, Barcelona. Universitat Autònoma de Barcelona, Spain (NP-M); Servei d'Hematologia i Hemoteràpia, Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Barcelona, Spain (PD).

*Correspondence: Dr. Javier Corral, Centro Regional de Hemodonación, Ronda de Garay s/n. 30003 Murcia (Spain)
E-mail: javier.corral@carm.es*

The key role of platelets in normal and pathological hemostasis and vascular remodelling is mediated by effector molecules that platelets secrete at sites of vascular injury. These molecules are contained in dense granules and α granules within the platelets. Platelet dense granules (PDG) contain small molecules (nucleotides, serotonin, catecholamines, ions and pyrophosphate) and have bound relevant adhesive platelet glycoproteins (Ib-IX, IIb/IIIa, P-selectin, and granulophysin).[1] Accordingly, these organelles play a key role in the amplification of platelet activation. Thus, a reduction in the number or contents of PDG will impair platelet function, increasing the risk of bleeding.

Disorders of platelet secretion are a consequence of abnormalities affecting PDG. These disorders are a relatively frequent cause of mild to moderate bleeding diatheses, characterized mainly by mucocutaneous bleedings (epistaxis, menorrhagia, and easy bruising).[1] Patients with these disorders show a mild to moderate prolonged bleeding time, abnormal platelet secretion induced by several platelet agonists, and impaired platelet aggregation. The definitive diagnosis is achieved by measuring significant quantitative deficiencies of PDG and/or of their electron-dense contents by means of transmission electron microscopy.[2] It is known that 10-18% of patients with congenital abnormalities of platelet function have PDG deficiency,[3] but despite being a relatively frequent disorder, the molecular basis of isolated PDG deficiency is not known.

Several rare and heterogeneous congenital bleeding disorders such as Chediak-Higashi syndrome, Wiskott-Aldrich syndrome, and thrombocytopenia with absent

Table 1. Clinical, laboratory, transmission electron microscopy and genetic features of patients with single platelet dense granule deficiency.

	Age/ sex	Plt[1]	Bleeding[2]	BT[3] (min)	Aggregation[4]	PDG (%)[5]	PDG morphology[6]	FH[7]	HPS1 gene[8]
1	8/F	400	EB, MB	9	ADP, Epi, Col	55	RC, FC	Yes	ISV5 G–35A+/- C636T (L212)+/-
2	20/M	200	MB, STO	11.5	Normal	50	FC	Yes	C636T (L212)+/- A1808G (Q603R)+/- C1472T (P491R)+/-
3	26/M	170	MB	17	Epi	65	LED	Yes	C636T (L212) +/+ ISV16 A-15G+/-
4	64/F	300	MB	17	Normal	52	RC	Yes	No polymorphism
5	8/F	95	–	7	Normal	45	Normal	Yes	No polymorphism
6	32/F	350	EB, MB, STO	7.5	Normal	33	LED	Yes	C11T (V4A) +/- ISV16 A-15G+/- C636T (L212) +/-
7	60/F	90	EB, MB	>20	Epi, Col, AA	6	Normal	No	C636T (L212) +/- ISV16 A-15G+/- ISV14 GG+7+8CT +/-3'UTR-C2115T +/-
8	26/F	140	EB, MB	15	Epi	70	RC, FC	No	C636T (L212) +/- A1808G (Q603R) +/- C1472T (P491R) +/-
9	19/F	100	EB, STO	13,5	Epi	55	Normal	No	C11T (V4A) +/ - ISV16 A-15G+/- C636T (L212) +/+ 10
10	42/F	275	EB, STO	8	Normal	55	Normal	Yes	ISV14 GG+7+8CT +/+ ISV16 A-15G+/+
11	10/F	450	EB	6	Normal	37	RC, FC	No	C636T (L212) +/ -ISV16 A-15G+/- ISV14 GG+7+8CT +/-
12	31/M	110	–	11	Normal	50	FC	No	C636T (L212) +/+
13	20/F	320	EB, STO	9	Col	50	Normal	Yes	No polymorphism
14	44/F	165	EB, MB	6.5	Risto	20	Normal	Yes	ISV5 G–35A +/+ G847T (G283W) +/-
15	26/F	110	–	9	ND	60	Normal	Yes	C297T (T99) +/- C1472T (P491R) +/- ISV14 GG+7+8CT +/-ISV16 A-15G+/- A1808G (Q603R) +/-
16	6/F	450	EB, MB, STO	7	Normal	66	FC, EG	Yes	C636T (L212) +/-

[1]Plt: platelets ×10⁹/L (microscopy counts); [2]bleeding: EB: easy bruising; MB: mucous bleeding (epistaxis, menorrhagia, gingivorrhagia); STO: surgical, traumatic or obstetric bleeding. 3BT: bleeding time (Ivy method). [4]Impaired aggregation response to: Epi: epinephrine; AA: arachidonic acid; Col: collagen; Risto: ristocetin; ND: not determined. [5]Number of platelet dense granules per mm² of platelet area, expressed as % of the mean normal numbers. [6]Predominant dysmorphic traits of platelet dense granules: RC: reduced solid core; FSC: fragmented solid core; EG: empty granule with no visible core; LED: low electron-density. [7]FH: familial story of PDG deficiency. [8]Genetic modifications identified in the HPS1 gene, indicating the nucleotide change and the affected codon (in brackets). The presence of the polymorphism in heterozygous or homozygous state is also indicated (+/- and +/+, respectively).

radii, exhibit PDG deficiency, but Hermansky-Pudlak syndrome is the most typically associated disease.[4] Hermansky-Pudlak syndrome is a rare autosomal recessive disorder manifested by occulocutaneous albinism, a bleeding tendency, and in some cases ceroid-lipofuscin-lysosomal storage disease. The storage pool defect arises from defects in formation or trafficking of lysosomes and related organelles, including melanosomes and PDG.[4] The molecular basis for Hermansky-Pudlak Syndrome is complex and heterogeneous, involving different genetic loci.[5] However, the majority of patients have mutations in the *HPS1* gene. As far as we know, 18 allelic mutations have been reported in *HPS1*, including the North-West Puerto-Rican founder effect (a 16-bp duplication within exon 15 identified in homozygosis in about 400 patients with Hermansky-Pudlak syndrome from this country).[4] Half of these mutations affect exon 5 and 11, a fact which suggests two mutational hot spots.[6,7]

The *HPS1* gene has 20 exons, and it encodes a protein with 700 amino acids and a molecular mass of 79.3 KDa.[8] Recent experiments have demonstrated that this protein plays a key role in the genesis of multiple cytoplasmic organelles in different tissues and in particular PDG.[9] Finally, we have identified two relatives of a Spanish patient with Hermansky-Pudlak syndrome who carry only one *HPS1* mutation (insC974) in a heterozygous state; these individuals have a low number of PDG and impaired platelet function without clinical symptoms.[10]

All these data suggest that the *HPS1* gene might be relevant in isolated storage pool deficiency.

Design and Methods

Patients

This study included 16 unrelated patients with mild to severe deficit of PDG and mild bleeding episodes, most of whom had been previously characterized.[11] The clinical features and laboratory results of these patients are summarized in Table 1. Deficiency of PDG was determined and characterized by transmission electron microscopy. Table 1 also shows the number and morphologic features of the PDG in these patients. Remarkably, nine patients had a familial history of PDG deficiency. There was no clinical or biological evidence of any condition or drug known to affect platelets. We

Table 2. Prevalence of HPS1 polymorphisms in 16 patients with single platelet dense granule deficiency, controls from the same country, and other healthy populations. The amount of platelet dense granules of selected healthy individuals is also indicated.

Polymorphism	*Method detection*	*Controls*		*Other populations*[1]		*Patients PDG deficiency*
		% (allele)	*PDG by TEM*[2]	*Caucasian %*	*Asian %*	*% (allele)*
T11C (V4A)	ASRA Bgl I	3.9% (0.038)	Normal (N=2)	ND	ND	12.5% (0.063)
C297T (T99)	Sequence	ND	ND	7.1%	40%	6.3% (0.031)
ISV5 G-35A	ASRA Nde I	13.9% (0.081)	Normal (N=2)	ND	ND	12.5% (0.094)
C636T (L212)	ASRA Xho I	54.1% (0.311)	Normal (N=1)	ND	ND	62.5% (0.406)
G847T (G283W)	ASRA BseN I	9.5% (0.047)	Normal (N=2)	7.1%	0	6.3% (0.031)
ISV11 T+13C	SSCP	1% (0.010)	Normal (N=1)	0	6.7%	0
insC974	SSCP	0	50% (relatives of HPS patient N=2)[3]	ND	ND	0
ISV14 GG+7+8CT	Sequence	ND	ND	42.9%	63.3%	25.0% (0.156)
C1472T (P491R)	Sequence	ND		21.4%	40%	18.8% (0.094)
A1808G (Q603R)	ASRA Msp I	14.1% (0.070)	Normal (N=2)	21.4%	40%	18.8% (0.094)
ISV16 A-15G	Sequence	ND	ND	42.6%	63.3%	43.8% (0.250)
C2115T	ASRA Pst I	1% (0.005)	Normal (N=2)	ND	ND	6.3% (0.031)

[1]*Data from Bailin et al.*[11] [2]*Number of platelet dense granules determined by transmission electron microscopy. N: number of healthy subjects investigated.* [3]*Data from González-Conejero et al.*[8] *ASRA: allelic specific restriction assay. ND: not determined. HPS: Hermansky-Pudlak syndrome.*

also evaluated the prevalence of *HPS1* variations in 215 controls. Patients and controls gave their informed consent to enter the study, which was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Transmission electron microscopy

Platelet electron microscopy was used, as previously described,[11] to examine the number of dense granules and their ultrastructural morphology in all patients with PDG deficiency and in selected controls with genetic modifications affecting the *HPS1* gene.

Venous blood samples were taken without anticoagulant, immediately fixed in glutaraldehyde at 37°C, and postfixed in osmium tetroxide in White's buffer. The number of PDG per μm^2 of platelet area was counted. The results were compared with those previously obtained in a group of 15 healthy individuals.[11] The mean number of PDG per μm^2 of these healthy individuals was taken to be equivalent to 100%, and subject with values under 80% were considered to have PDG deficiency. We also assessed PDG morphology.

DNA analysis

All coding exons of the HPS1 gene (exons 3-20), and their flanking intron regions, were amplified by polymerase chain reaction (PCR) using previously described primers and conditions.[10] PCR products were purified from 1.5% agarose gels using Ultraclean Gel Spin (Mobio, Solana Beach, CA, USA). The sequencing reaction was performed with an ABI Prism Big Dye Terminator Cycle sequencing kit on an automated sequencer type 377 (Perkin-Elmer Applied Biosystems, Washington Cheshire, UK), with forward and reverse primers used for amplification. Genetic changes identified in the *HPS1* gene were evaluated in controls by allele specific restriction assay or single stranded conformational polymorphism (SSCP) (Table 2).

Results

HPS1 gene analysis in patients with isolated PDG deficiency

Neither the insC974 mutation (frequently described in patients with Hermansky-Pudlak syndrome of non-Puerto Rican origin) nor other mutations affecting exon 11 (the main mutational hot spot of this gene), nor mutations in exon 5 (a second mutational hot spot)[6,7] were identified in patients with isolated PDG deficiency. In brief, no patient carried severe mutations in the *HPS1* gene. However, our search did reveal 10 nucleotide changes according to the published sequence of the HPS1 gene.[12] Six of these changes were previously described as common single nucleotide polymorphisms,[7,12] but we also identified 4 unreported modifications: exon 3: C11T (Val4Ala); ISV5: G–35A; exon 7: C636T (Leu 212); and exon 20: C2115T, affecting the 3'-untranslated region of the gene, 12 positions after the stop codon (according to the sequence of Bailin *et al.*, nucleotide 1 begins at the first nucleotide).[12] Table 1 shows the distribution of these polymorphisms. Finally, the published cytosine ISV8 +29 is probably a mistake or a rare genetic change identified by Bailin *et al.*,[12]

because all sequenced patients and two controls displayed adenine in a homozygous state at this position. We did not identify any of the other polymorphisms described in the *HPS1* gene in our patients.

Prevalence of HPS1 genetic modifications in controls

In order to determine whether genetic changes identified in the *HPS1* gene are rare mutations or common polymorphisms, we investigated their prevalence in the control group. We only studied those modifications that were identified by restriction assay or SSCP. Thus, C297T (Thr99), ISV14 GG+7+8CT, and ISV16 A-15G were not evaluated in controls. The C1472T (Pro491Arg) was not directly studied, but this polymorphism seems to be strongly linked to the A1808G (Gln603Arg), as these polymorphisms displayed identical frequency in different populations (Table 2), and we detected complete linkage disequilibrium (Table 1). Mutations affecting exon 11, the main *HPS1* mutational hot-spot, were also evaluated in controls by SSCP.[10] Table 2 summarizes these results. We did not observe the insC974 mutation in controls, but we identified one additional polymorphism (ISV11 T+13C) that was previously reported to be present in 3% of the Asian population, but not in the Caucasian population.[12] The frequency of *HPS1* polymorphisms was similar among patients with isolated PDG deficiency and among controls (Table 2).

Transmission electron microscopy of platelets from controls carrying HPS1 polymorphisms

In order to test the role of *HPS1* polymorphisms in PDG deficiency, we studied the dense granules in platelets from healthy subjects carrying the studied polymorphisms. No *HPS1* polymorphism was significantly associated with severe reduction in the number of PDG or changes in PDG morphology (Table 2).

Discussion

Platelet dense granule deficiency is frequently observed among patients with congenital abnormalities of platelet function.[3] The notable family clustering of PDG deficiency supports the role of genetic factors in this disorder.

Mutations affecting different genes have been identified in patients or animal models with rare syndromes associated with PDG deficiency such as Chediak-Higashi syndrome, Wiskott-Aldrich syndrome, thrombocytopenia with absent radii, or Hermansky-Pudlak syndrome,[4] although, as far as we know, no genetic defect has been implicated in isolated PDG deficiency. All genes involved in organogenesis of PDG and those involved in disorders with PDG deficiency are candidates to play a role in isolated PDG deficiency. We studied the *HPS1* gene because of the key role of this gene in the organogenesis of PDG, its relevance in Hermansky-Pudlak syndrome, the number of mutations identified, and the identification of carriers of *HPS1* mutations without clinical signs but with a significant reduction in the number of PDG.[4-10]

In fact, we speculated that mutational hot-spots of the *HPS1* gene, specifically the insC974 mutation, common among non-Puerto Rican patients with Hermansky-Pudlak syndrome,[6,7,9,13] could be present in the general population and might be responsible for PDG deficiency. However, we did not detect this mutation or other *HPS1* mutations in either the general population or patients with isolated PDG deficiency. In accordance with this, a study sequenced the *HPS1* gene in one patient with isolated PDG deficiency did not find any significant mutations.[13] The *HPS1* polymorphisms identified in our study played no significant role in the number or morphologic features of the PDG. These data strongly suggest that the *HPS1* gene plays only a minor role in isolated PDG deficiency.

The synthesis and/or processing of melanosomes, lysosomes and PDG are under a common and complex genetic control. In the mouse, at least 15 genes are involved in a phenotype resembling the human Hermansky-Pudlak syndrome.[14] The disease is likewise genetically heterogeneous in humans. Thus far, mutations in 7 orthologous genes have been found in patients with Hermansky-Pudlak syndrome.[15] All these genes are candidates to be involved in isolated PDG deficiency. Mutations affecting *HPS1* and 4 have been identified in patients with severe forms of Hermansky-Pudlak syndrome, always including hypopigmentation. Patients with HPS2 (carrying mutations in the β-3A subunit of adaptor complex-3 (*AP-3*) always have neutropenia. However, patients with defects in HPS 3, 5 and 6 (and probably also HPS7) have relatively mild clinical phenotypes, often with no apparent hypopigmentation, but only bleeding problems.[16] According to these data and our results, *HPS 3*, *5* and *6* genes are perfect candidates to be screened in cases with isolated PDG deficiency.

JC was responsible for the conception of the study, performed the genetic analyses together with R G-C, and wrote the paper. N P-M and PD were responsible for the clinical data and analyzed and interpreted the electron microscopy data. VG was responsible for the design of the study and for the final approval of the version to be submitted. The authors reported no potential conflicts of interest.

We thank ML Lozano, J Rivera, and E Pérez for their helpful discussion and Dr. JM Duran (Hospital La Línea de la Concepción, Cadiz, Spain) and Dr. A Blanco (Hospital de Palamós, Girona, Spain) for samples from patients with deficiency of platelet dense-bodies.

Manuscript received September 9, 2003. Accepted December 18, 2003.

References

1. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. Platelets 2001; 12:261-73.
2. White JG. Use of the electron microscope for diagnosis of platelet disorders. Semin Thromb Hemost 1998;24:163-8.
3. Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred six patients. Blood 1987;70:620-3.
4. Huizing M, Anikster Y, Gahl WA. Hermansky-Pudlak syndrome and Chediak-Higashi syndrome: disorders of vesicle formation and trafficking. Thromb Haemost 2001;86:233-45.
5. Gahl WA, Brantly M, Kaiser-Kupfer MI, Iwata F, Hazelwood S, Shotelersuk V, et al. Genetic defects and clinical characteristics of patients with a form of oculocutaneous albinism (Hermansky-Pudlak syndrome). N Engl J Med 1998;338:1258-64.
6. Hermos CR, Huizing M, Kaiser-Kupfer MI, Gahl WA. Hermansky-Pudlak syndrome type 1: gene organization, novel mutations, and clinical-molecular review of non-Puerto Rican cases. Hum Mutat 2002;20:482-91.
7. Albinism Database. International Albinism Center. University of Minnesota. In: Albinism Database. International Albinism Center. University of Minnesota. Mutations of the Hermansky-Pudlak syndrome-1 gene (HPS1) associated with Hermansky-Pudlak syndrome. Available at URL: http://www.cbc.umn.edu/tad/hps1mut.htm
8. Oh J, Bailin T, Fukai K, Feng GH, Ho L, Mao JI, et al. Positional cloning of a gene for Hermansky-Pudlak syndrome, a disorder of cytoplasmic organelles. Nat Genet 1996;14:300-6.
9. Feng L, Novak EK, Hartnell LM, Bonifacino JS, Collinson LM, Swank RT. The Hermansky-Pudlak Syndrome 1 (HPS1) and HPS2 genes independently contribute to the production and function of platelet dense granules, melanosomes, and lysosomes. Blood 2002;99:1651-8.
10. Gonzalez-Conejero R, Rivera J, Escolar G, Zuazu-Jausoro I, Vicente V, Corral J. Molecular, ultrastructural and functional characterization of a Spanish family with Hermansky-Pudlak syndrome: role of insC974 in platelet function and clinical relevance. Br J Haematol 2003;123:132-8.
11. Pujol-Moix N, Hernandez A, Escolar G, Espanol I, Martinez-Brotons F, Mateo J. Platelet ultraestructural morphometry for diagnosis of partial δ-storage pool disease in patients with mild platelet dysfunction and/or thrombocytopenia of unknown origin. A study of 24 cases. Haematologica 2000;85:619-26.
12. Bailin T, Oh J, Feng GH, Fukai K, Spritz RA. Organization and nucleotide sequence of the human Hermansky-Pudlak syndrome (HPS) gene. J Invest Dermatol 1997;108: 923-7.
13. Oh J, Ho L, Ala-Mello S, Amato D, Armstrong L, Bellucci S, et al. Mutation analysis of patients with Hermansky-Pudlak syndrome: a frameshift hot spot in the HPS gene and apparent locus heterogeneity. Am J Hum Genet 1998;62:593-8.
14. Ciciotte SL, Gwynn B, Moriyama K, Huizing M, Gahl WA, Bonifacino JS, et al. Cappuccino, a mouse model of Hermansky-Pudlak syndrome, encodes a novel protein that is part of the pallidin-muted complex (BLOC-1). Blood 2003;101:4402-7.
15. Li W, Zhang Q, Oiso N, Novak EK, Gautam R, O'Brien EP, et al. Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). Nat Genet 2003; 35:84-9.
16. Huizing M, Helip-Wooley A, Dorward H, Claassen D, Hess R, Gahl WA. Hermansky-Pudlak syndrome: a model for abnormal vesicle formation and trafficking. Pigment Cell Res 2003;16:584.