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Clinical, molecular and cellular features of non-Puerto Rican Hermansky-Pudlak syndrome patients of Hispanic descent

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Abstract

Hermansky-Pudlak syndrome is an autosomal recessive condition characterized by a bleeding diathesis and hypopigmentation of the skin, hair and eyes. Some HPS patients develop other complications such as granulomatous colitis and/or a fatal pulmonary fibrosis. Eight genes have been associated with the condition, resulting in subtypes HPS-1 through HPS-8. The HPS gene products are involved in the biogenesis of specialized lysosome-related organelles such as melanosomes, platelet delta granules and others. HPS1 and HPS4 form a stable complex named BLOC-3, and patients with BLOC-3 or AP-3 deficiency develop pulmonary fibrosis. Therefore, it is important to subtype each HPS patient. HPS type 1 (HPS-1) occurs frequently on the island Puerto Rico due to a founder mutation. Here, we describe seven mutations, six of which are previously unreported, in the *HPS1*, *HPS4* and *HPS5* genes among patients of Mexican, Uruguayan, Honduran, Cuban, Venezuelan and Salvadoran ancestries. Our findings demonstrate that the diagnosis of HPS should be considered in Hispanic patients with oculocutaneous albinism and bleeding symptoms. Moreover, such patients should not be assumed to have the HPS-1 subtype typical of northwest Puerto Rican patients. We recommend molecular HPS subtyping in such cases, since it may have significant implications for prognosis and intervention.

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Conflict of interest

All authors declare no conflict of interest.

Keywords

albinism; bleeding diathesis; Hermansky-Pudlak syndrome; Hispanic descent; lysosome-related organelle; pulmonary fibrosis

Introduction

Hermansky-Pudlak syndrome (HPS; OMIM 203300) is a rare multisystemic, disorder characterized by oculocutaneous albinism, and a bleeding diathesis, sometimes accompanied by immunodeficiency, granulomatous colitis and/ or fatal pulmonary fibrosis (Hermansky and Pudlak, 1959; Gahl *et al*., 1998; Brantly *et al*., 2000; Schinella *et al*., 1980). These clinical manifestations are due to defects in the formation or function of lysosome-related organelles such as melanosomes in melanocytes, platelet delta granules, lung lamellar bodies and/or lytic granules of cytotoxic T-cells (Wei, 2006; Huizing *et al*., 2008). HPS is caused by defects in one of eight genes: *HPS1, AP3B1, HPS3* to *HPS8* (Oh *et al*., 1996; Dell'Angelica *et al*., 1999; Anikster *et al*., 2001; Suzuki *et al*., 2002; Li *et al*., 2003; Zhang *et al*., 2003; Morgan *et al*., 2006). With the exception of the gene encoding a subunit of AP-3 (HPS-2) (Dell'Angelica *et al*., 1999), the HPS genes encode novel proteins of unknown function that have no homology to any other protein and no recognizable motif. In addition, HPS gene products have been identified as subunits of at least three novel multi-protein complexes named **B**iogenesis of **L**ysosome-related **O**rganelles **C**omplex (BLOC)-1 through -3. BLOC-1 is a multimeric complex composed of HPS7 and HPS8, among other subunits (Starcevic *et al*., 2004; Li *et al*., 2003; Morgan *et al*., 2006). BLOC-2 is composed of HPS3, HPS5 and HPS6 (Di Pietro *et al*., 2004), while HPS1 and HPS4 are subunits of BLOC-3 (Martina *et al*., 2003; Nazarian *et al*., 2003). Deficiency of any of these complexes can affect intracellular trafficking of proteins (Dell'Angelica, 2004).

Patients who have mutations in the same BLOC exhibit similar phenotypes (Wei, 2006; Huizing *et al*., 2008). BLOC-3 deficient patients exhibit a relatively more severe phenotype of hypopigmentation, frequently develop granulomatous colitis, and suffer a fatal, adult-onset pulmonary fibrosis (Huizing *et al*., 2008; Anderson *et al*., 2003; Hermos *et al*., 2002). Patients with deficiency in BLOC-2 manifest a milder phenotype of variable hypopigmentation and sporadic granulomatous colitis, but pulmonary fibrosis has not been found in BLOC-2 patients (Huizing *et al*., 2001; Huizing *et al*., 2004; Huizing *et al*. 2009). So far, only two BLOC-1 patients have been reported, one HPS-7 patient (Li *et al*., 2003) and one HPS-8 family (Morgan *et al*., 2006), but no detailed clinical features were provided apart from hypopigmentation, silvery hair (in HPS-8) and a bleeding diathesis.

HPS is described in patients worldwide (Brantly *et al*., 2000; Anderson *et al*., 2003; Witkop *et al*., 1990; Huizing *et al*., 2001; Ito *et al*., 2005; Merideth *et al*, 2009; Vincent *et al*., 2009), but is common on the island of Puerto Rico. One of 1,800 Puerto-Ricans in the northwest part of the island suffers from HPS type 1 (HPS-1) (Witkop *et al*., 1990), due to a 16 bp duplication in exon 15 of the *HPS1* gene (Oh *et al*, 1996). In central Puerto Rico, one of 4,000 natives has HPS-3 due to another founder mutation, a 3,904-bp deletion in the *HPS3* gene (Anikster *et al*., 2001; Santiago-Borrero *et al*., 2006).

Here we report six non-Puerto Rican Hispanic HPS patients. All these individuals presented with pale to light skin color, nystagmus, and bleeding problems including epistaxis and easy bruising. Molecular analysis revealed that none of these patients carried the Puerto Rican 16 bp deletion in *HPS1* or the 3,904 bp deletion in *HPS3* founder mutations, but instead had other mutations in the *HPS1, HPS4* or *HPS5* genes. These cases emphasize the molecular variability of HPS among non-Puerto Rican, Hispanic HPS patients.

Results and Discussion

Clinical Findings

Patient HPS117-1 is a 29-year-old Mexican man who was seen for advanced pulmonary fibrosis and symptoms of inflammatory bowel disease. The diagnosis of HPS was suspected because he had albinism, nystagmus, and pulmonary fibrosis on CT scan and lung biopsy. The patient was referred to the NIH, where DNA analysis revealed a heterozygous mutation in exon 11 of *HPS1*, i.e., c.972delC; p. M325W*fs*X6 (Figure 1a and Figure S1a). On electron microscopic examination, the platelets had no dense bodies (Figure 2a). The patient reported relatively severe exertional dyspnea, fatigue, and a progressive cough present for more than a year. Pulmonary function tests revealed severe restriction and a severe reduction in gas exchange; the forced vital capacity (FVC) was 44% of predicted, total lung capacity (TLC) was 52% of predicted and diffusion capacity for carbon monoxide (DLCO) was 43% of predicted. Conventional and high-resolution chest computerized axial tomography (HRCT) scans showed diffuse bilateral interstitial infiltrates (Figure 2b). Biopsies from a colonoscopy revealed evidence of colitis in the sigmoid colon and rectum. The patient died from complications of end-stage pulmonary fibrosis at age 30.

Patient HPS118-1, the sister of HPS117-1, is a 39-year-old Mexican woman with mild pulmonary fibrosis, essential hypertension, and rheumatoid arthritis. She had 3 unaffected siblings, but her paternal great grandfather reportedly had albinism. Her initial NIH evaluation at age 32 revealed that she had a heterozygous mutation in *HPS1*: c.972delC; p. M325W*fs*X6 (exon 11) (Figure 1a); platelet electron microscopy confirmed the absence of dense bodies. She had no symptoms of lung disease, but a CT scan showed mild bilateral basilar peripheral interstitial lung opacities (Figure 2b). Pulmonary function testing showed mild restriction with a TLC of 70% of predicted and a mild reduction in diffusion capacity. At age 33, she was diagnosed with rheumatoid arthritis and essential hypertension, both of which are well controlled with medications.

Her recent evaluation at age 39 revealed some progression of her lung disease, but she remains asymptomatic. HRCT of the chest showed a mild increase in fibrosis in the lung bases, especially on the right, and continued involvement in the right middle lobe. Pulmonary function test results were stable, with an FVC 69% of predicted, TLC 70% of predicted, and an adjusted DLCO 64% of predicted.

Patient HPS125-5 is an 8-month-old boy of Mexican ancestry with brown hair (Figure 1a) and irides, decreased retinal pigmentation, and nystagmus. He had easy bruising (Figure S1c) and epistaxis, which led to the discovery of absent platelet dense bodies (Figure S1c).

He has no signs of colitis. DNA analysis showed a heterozygous mutation in *HPS5*: c. 1423delC; p.L475S*fs*X37 (exon 12) (Figure 1a).

Patient HPS150-4 is a 2-year-old boy of Uruguayan ancestry with HPS-4. He has blond hair, grey irides (Figure 1a), iris transillumination, nystagmus and pale fundi. He presented with easy bruising (Figure S1c). Bloody stools began at 18 months, along with epistaxis and prolonged gingival bleeding after trauma. His platelets showed no dense bodies. Genomic DNA revealed two compound heterozygous mutations in *HPS4*: c.45 G>A; p.W15X (exon 3) and c.47delA; p.N16I*fs*X11 (exon3) (Figure 1a).

Patient HPS163-1 is a 16-year-old male of Honduran-Salvadoran ancestry. He has pale skin and his blond hair darkened considerably since birth (Figure 1a); his irides are brown (Figure 1a), and nystagmus is present. Bruising was noted at age 13; his platelets have no dense bodies. Episodes of abdominal pain, bloody diarrhea, and anal fissures prompted treatment for Crohn's disease. Nausea caused a 20 pound weight loss; syncopal episodes and anemia let to a colectomy with end ileostomy. Repair of a perforated bowel required 18 units of platelets. There were no pulmonary symptoms. DNA analysis showed a homozygous mutation in *HPS1*, i.e., c.467_476del; p.Y156C*fs*X16 (exon 6) (Figure 1a).

Patient HPS353-5 is a 3 and 9/12 year-old boy of Cuban-Venezuelan ancestry. He has medium brown hair, brown irides (Figure 1a), and nystagmus with photosensitivity. Ocular albinism was considered but genetic testing for X-linked ocular albinism was negative. Although there was no excessive bleeding with previous surgeries (i.e. circumcision, strabismus or dental surgery), the patient presented with easy bruising (Figure S1c) of his lower extremities and two minor episodes of spontaneous epistaxis. On electron microscopic examination, his platelets had no dense bodies (Figure S1b). He had no manifestations of colitis. His visual acuity was OD 20/100 and OS 20/125 with pendular horizontal nystagmus, diffuse iris transillumination, optic disc pallor, and blond periphery of the retinae with no foveal reflex. DNA analysis revealed compound heterozygous mutations in *HPS5*, i.e., c.302_305delTTTG; p.V101G*fs*X3 (exon 5) and c.1,634+1G>A (intron 13) (Figure 1a).

Molecular and Cellular Analysis

DNA sequencing of all coding exons and intronic boundaries of the *HPS1, HPS3, HPS4, HPS5,* and *HPS6* genes (Wei, 2006; Huizing *et al*., 2008) revealed the mutations listed in Table 1.

In addition, the patients' fibroblast proteins were electrophoresed and immunoblotted using antibodies against HPS4 to evaluate the protein expression of BLOC-3, consisting of HPS1 and HPS4, and against HPS5 to evaluate BLOC-2, consisting of HPS3,HPS5, and HPS6 (Figure 3). Mutation in one member of a BLOC complex destabilizes the entire complex, causing degradation of the other proteins in the complex. Therefore, utilization of a specific antibody for one member of BLOC-3 (HPS4) or BLOC-2 (HPS5) assists in demonstrating deficiency of other members of the same complex (Nazarian *et al*., 2008).

These immunoblotting techniques also demonstrated the pathogenicity of certain mutations, in cases where we found only one mutation in an HPS gene. For example, HPS117-1 and

HPS118-1 carried a single copy of c.972delC in *HPS1*. This frame-shift mutation generates a premature termination codon, and causes nonsense-mediated RNA decay (Shotelersuk *et al*., 1998). It was previously found in Puerto Rican patients (Carmona-Rivera *et al*., 2010), but was also described in other Caucasians (Oh *et al*., 1998) and in an African-American (Merideth *et al*., 2009). In fact, the C-nucleotide at position 972 was recognized as an *HPS1* mutation 'hotspot' (Oh *et al*., 1998). However, no second mutation could be found in our patient's coding region of *HPS1*, so we used immunoblotting of HPS117-1 fibroblast extracts to illustrate a dramatic reduction in the levels of the HPS4 antibody signal, similar to that of HPS-1 control fibroblast extracts. This suggested lack of functional HPS1 (Figure 3a); the patients' second *HPS1* mutation probably involves a non-coding region of *HPS1*.

DNA analysis of patient HPS125-5 showed a previously unreported single copy mutation in *HPS5*, c.1423delC (Figure 1a), causing a frame-shift and generating a premature termination codon. No second mutation was found, but protein analysis showed complete absence of the HPS5 protein in this patient's fibroblasts (Figure 3a), supporting the diagnosis of HPS-5.

Sequence analysis of HPS150-4 showed two unreported compound heterozygous mutations in exon 3 of the *HPS4* gene, a nonsense mutation and a 1-bp deletion leading to a premature termination codon (Figure 1a). RNA transcripts from both alleles are likely to be degraded by nonsense mediated RNA decay, a prediction supported by complete absence of the HPS4 protein in the patient's fibroblasts (Figure 3a).

Similarly, an unreported homozygous 10-bp deletion in exon 6 of *HPS1*, found in HPS163-1 (Figure 1a), is predicted to result in nonsense-mediated RNA decay. We used qPCR (Griffin *et al*., 2005) to rule out hemizygosity in this patient; qPCR demonstrated the presence of two *HPS1* alleles, confirming homozygosity of the 10-bp deletion (data not shown). In addition, protein expression levels of HPS4 were reduced (Figure 3b), suggesting degradation of HPS4 in the absence of the HPS1 protein.

Patient HPS353-5 was compound heterozygous for two unreported mutations in *HPS5*, including a 4-bp deletion (TTTG) in exon 5 leading to a premature termination codon. The second mutation was a splice site variant in intron 13 (c.1,634+1G>A). To demonstrate aberration in splicing, we amplified *HPS5* cDNA using primers located in exon 11 and exon 16 (Figure 4a). In addition to the expected 694-bp PCR product, we found a band of approximately 570-bp (Figure 4b). Sequence analysis of the additional band demonstrated skipping of exon 13 (124-bp) (Figure 4c), altering the reading frame. Immunoblot analysis showed total absence of the HPS5 protein in fibroblasts of patient HPS353-5 compared to normal (Figure 3a), indicating that these two mutations have pathogenic implications for the *HPS5* gene product.

In summary, we report six non-Puerto-Rican Hispanic HPS patients with Mexican, Uruguayan, Honduran, Cuban, Venezuelan and Salvadoran ancestries, identifying mutations in the *HPS1*, *HPS4* and *HPS5* genes (Table 1). Importantly, we did not identify the two common Puerto-Rican HPS founder mutations, i.e., the 16-bp duplication in *HPS1* and the 3,904-bp deletion in *HPS3*, in our cohort. Hispanic patients with oculocutaneous albinism and bleeding symptoms should not be assumed to have the HPS-1 subtype typical of

northwest Puerto Rican patients. We recommend molecular HPS subtyping in such cases, since a diagnosis of the type of HPS allows anticipation of clinical complications, appropriate management and genetic counseling (Huizing *et al*., 2008; Gahl *et al*., 2002).

Materials and Methods

Patients and cells

All patients in this study were enrolled in the clinical protocol "Clinical and Basic Investigations into Hermansky-Pudlak Syndrome" (NCT00001456, www.clinicaltrials.gov), approved by the NHGRI Institutional Review Board, and adhered to Helsinki guidelines. All patients or their parents provided written, informed consent. Patients were diagnosed with HPS based on the presence of oculocutaneous albinism (i.e., decreased visual acuity, nystagmus, and some degree of hypopigmentation relative to family members) and the absence of platelet delta granules on whole-mount electron microscopy.

Primary cultures of skin fibroblasts, obtained from a 4-mm punch biopsy, were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum containing 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Electron microscopy of platelet delta granules

Presence of platelet delta granules in platelet-rich plasma was analyzed using whole mount and transmission electron microscopy as previously described (Witkop *et al*., 1987; Huizing *et al*., 2007).

Molecular analysis

Genomic DNA was isolated from patients' peripheral blood mononuclear cells using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA). All *HPS1*, *HPS3*, *HPS4*, *HPS5* and *HPS6* coding exons and flanking intronic boundaries were amplified by polymerase chain reaction (PCR) amplification, and subjected to bi-directional sequencing. RNA was extracted from fibroblast using the RNeasy Mini kit (Qiagen) and transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Primer sequences for amplifying exon 11–16 of *HPS5* (Figure 3) were: forward 5'-GACTGCAGATAAATTGGAGCATTT-3' and reverse 5'-GTAGCTTGGTCATTGCTTCTGCTG-3'.

Sequencing was performed using ABI BigDye Terminator chemistry (Applied Biosystems, Foster City, CA) with detection on an ABI 3130×l genetic analyzer (Applied Biosystems). Results were analyzed using Sequencher v4.9 software (Gene Codes Corporation, Ann Arbor, MI).

Pulmonary function testing and lung computed tomography scanning

Pulmonary function testing measurements were made using standard equipment according to American Thoracic Society recommendations as previously described (SensorMedics, Yorba Linda, CA) (Rouhani *et al*., 2009). Conventional and high-resolution computed tomography scans of the chest were performed as previously described, without intravenous

contrast during end-inspiration in the supine and prone positions, respectively (General Electric Medical Systems, Milwaukee, WI) (Gochuico *et al*., 2008).

Immunoblotting

Fibroblast pellets were lysed by incubation in ice-cold lysis buffer [50 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.5% w/v Triton X-100, 5mM EDTA, 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)] for 30 min at 4°C, followed by centrifugation at 16,000 ×g for 15 min. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). Total protein samples (20 μg) were separated on a 4–12% gradient NuPAGE Bis-Tris Gel (Invitrogen, Carlsbad, CA), and blotted to nitrocellulose membranes. Membranes were blocked with 10% bovine serum albumin (BSA) for 30 min at room temperature, followed by incubation with one of three primary antibodies, i.e., rabbit polyclonal antibodies against HPS4 (1:500) (H-150; Santa Cruz Biotechnology Inc, Santa Cruz, CA), rabbit polyclonal antibody against HPS5 (1:500) (Proteintech Group Inc, Chicago, IL), or mouse monoclonal antibody against α-tubulin (1:10,000) (Sigma, St. Louis, MO). Subsequently, the membranes were probed with either secondary donkey anti-rabbit or anti-mouse antibodies, Horseradish peroxidase linked (1:10,000; GE Healthcare, UK) followed by detection with enhanced chemiluminescence (ECL) Western Blotting substrate from Pierce (Rockford, IL), or with secondary IRDye® 800CW goat anti-rabbit or anti-mouse antibodies (1:10,000) for 1 hour at room temperature and detected using the Li-Cor Odyssey® Infrared Imaging System per the manufacturer's instructions (Li-Cor Biosciences, Lincoln, NE).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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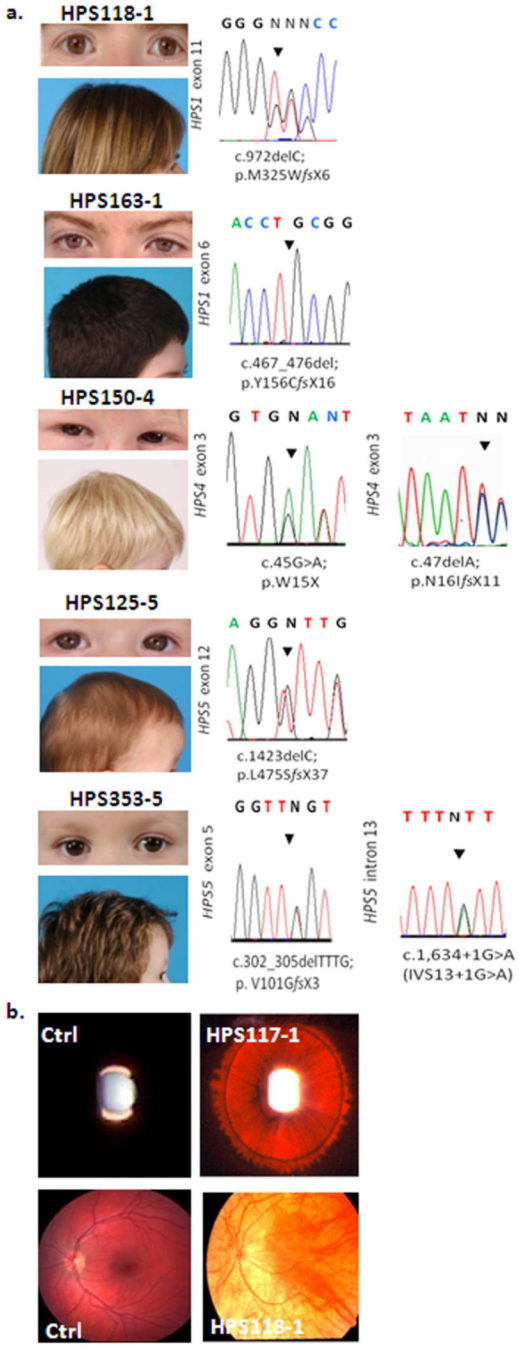

Figure 1. Clinical and molecular findings among non-Puerto Rican Hispanic HPS patients

(a) Pigmentation and sequencing chromatograms of all six patients reported in this study. All patients showed hypopigmented skin with hair colors ranging from blond to brown. (b) Representative images of iris transillumination (in HPS117-1) and pale fundus (in patient HPS118-1), compared to controls (Ctrl).

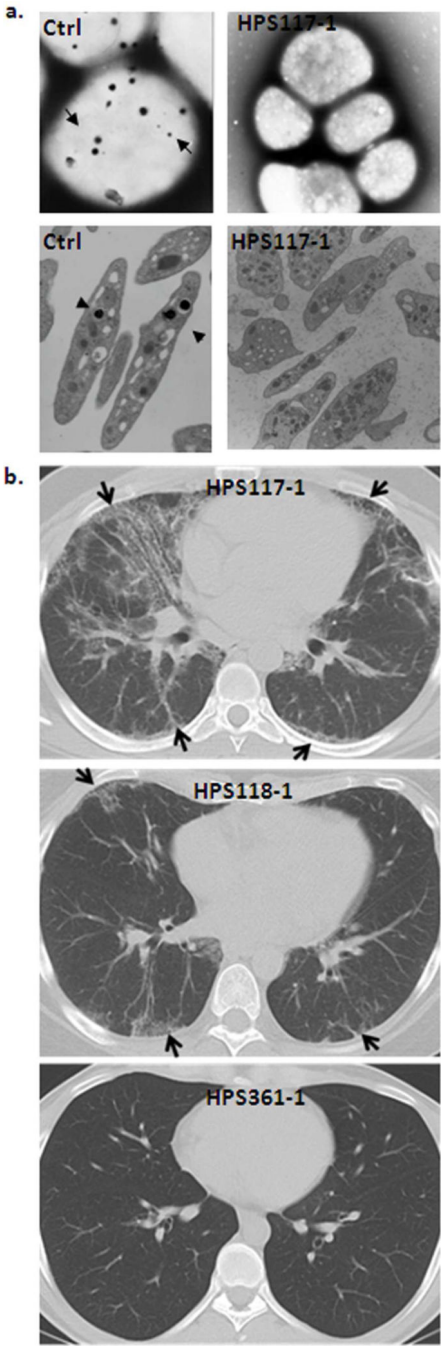

Figure 2. Hallmarks of HPS patients

(a) Representative whole mount (top panel) and transmission (bottom panel) EM images of selected patient compared to controls (Ctrl), showing absence of platelet delta granules (arrows in control images). (b) Conventional chest CT of HPS117-1 (top image) shows diffuse bilateral peripheral interstitial pulmonary infiltrates (arrows). A CT scan from HPS118-1 (middle image) shows mild bilateral interstitial opacities (arrows) predominantly in subpleural areas. In contrast, no pulmonary infiltrates were found in a CT scan from HPS361-1 (bottom image), who is a 22 year-old male with HPS-1 and no lung disease.

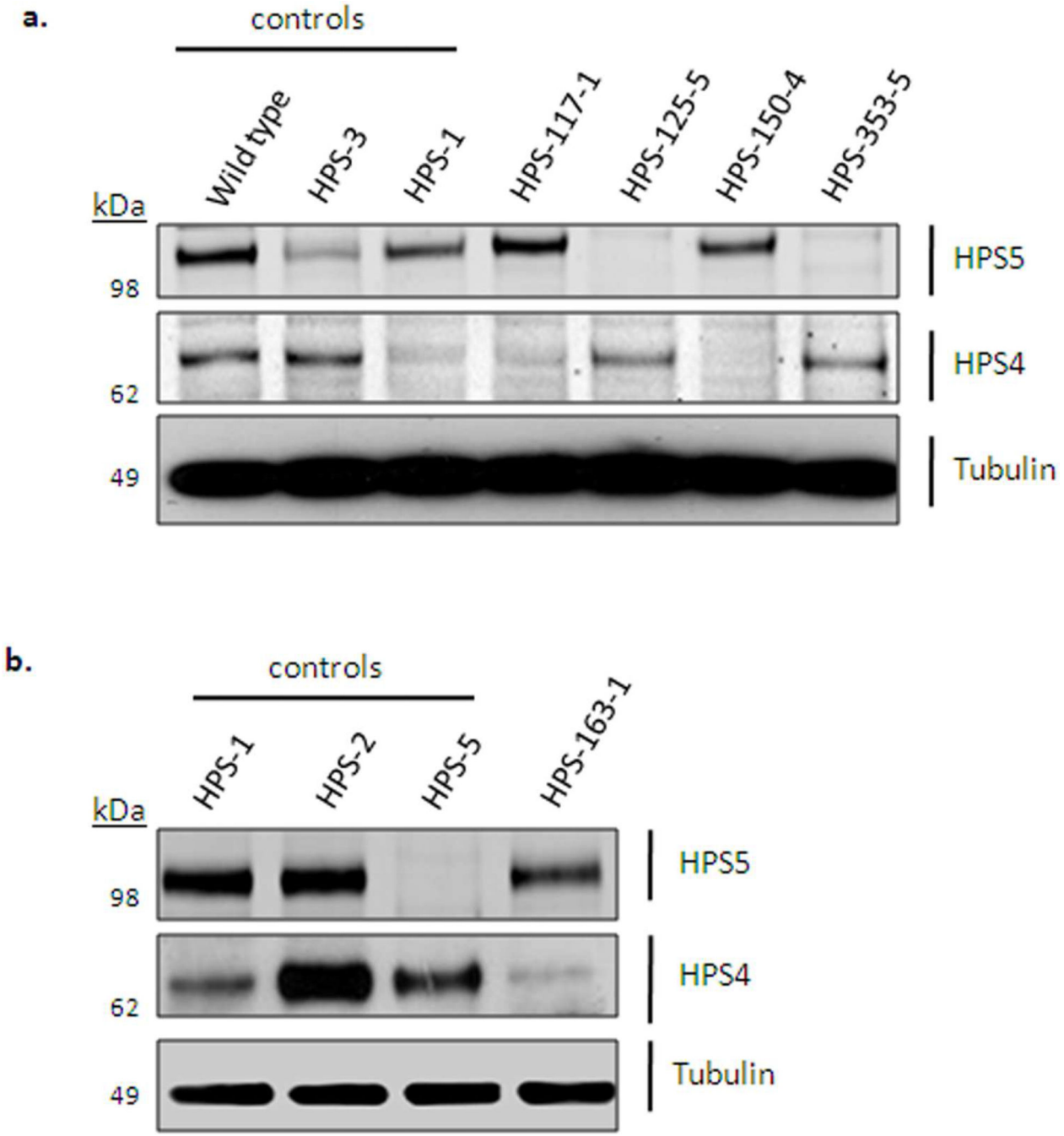

Figure 3. Immunoblot analysis of patients fibroblast extracts

Immunoblotting was performed with antibodies against HPS4 (to detect BLOC-3 defects), HPS5 (to detect BLOC-2 defects), and α-tubulin (loading control). (a) Normal, HPS-3, and HPS-1 fibroblast extracts were loaded as controls (lanes 1–3) and compared to the patients' protein expression. Patient HPS117-1 showed decreased HPS4 expression, similar to that of control HPS-1 cells (lane 3), suggesting an HPS1 defect. Patients HPS125-5 and HPS353-5 expressed no HPS5 protein, confirming an HPS5 defect. Patient HPS150-4 expressed no HPS4 protein, confirming an HPS4 defect. (b) Patient HPS163-1 showed reduced levels of

HPS4 protein, similar to the HPS-1 loading control (lane 1), suggesting HPS-1 disease in this patient.

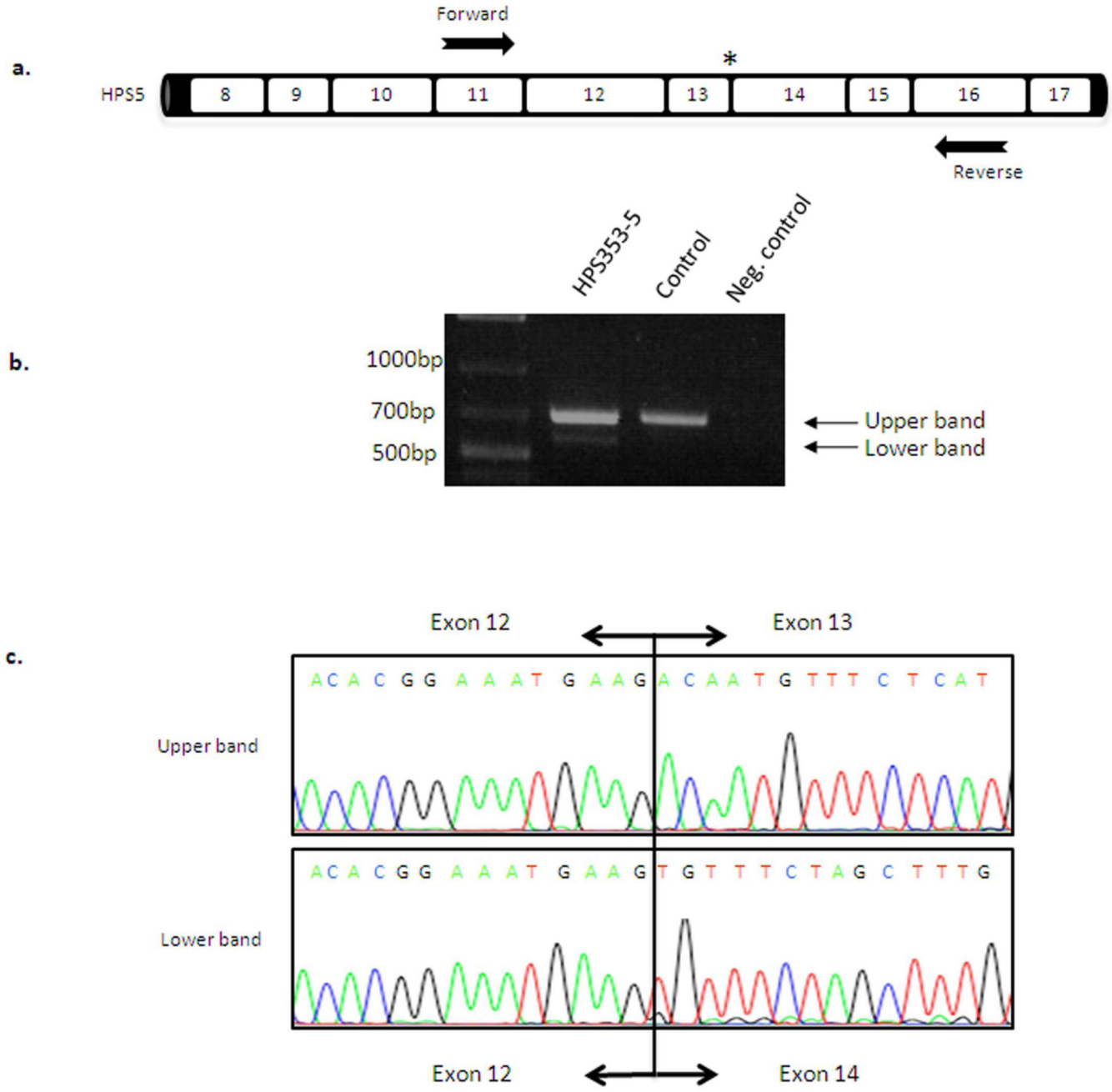

Figure 4. cDNA analysis of patient HPS353-5

(a) Schematic representation of the *HPS5* gene and location of the primers used for PCR analysis to detect splice-site alteration in patient HPS353-5. The patient's splice site variant c.1,634+1G>A (intron 13) is located one bp intronic from the exon12-exon13 boundary (asterisk). (b) cDNA amplification of exons 11–16 of the *HPS5* cDNA transcript showing the expected band of 694-bp and a lower molecular weight band around 570-bp. (c) Sequence analysis of the lower molecular weight band revealed skipping of exon 13 (124-

bp) in patient HPS353-5, confirming the pathogenicity of the novel *HPS5* splice site variant in this patient.

Table 1

Summary of clinical and molecular features in non-Puerto Rican Hispanic patients.*

Patient	Age (years)	Sex	Ancestry	Gene	Allele 1	Allele 2	Bleeding	Lung fibrosis	GI
HPS117-1	29	M	Mex	*HPS1*	c.972delC (ex 11) p.M325W*fs*X6	ND	Br	+ (FVC 44%)	−
HPS118-1	39	F	Mex	*HPS1*	c.972delC (ex 11) p.M325W*fs*X6	ND	Br	+ (FVC 69%)	−
HPS125-5	8 mo	M	Mex	*HPS5*	c.1423delC (ex 12) p.L475S*fs*X37	ND	E, Br	−	−
HPS150-4	2	M	Uru	*HPS4*	c.45G>A (ex 3) p.W15X	c.47delA p.N16I*fs*X11	E, Br	−	−
HPS163-1	16	M	Hon/Sal	*HPS1*	c.467_476del p.Y156C*fs*X16 (ex6)	c.467_476del p.Y156C*fs*X16 (ex6)	Br	−	+
HPS353-5	3	M	Cub/Ven	*HPS5*	c.302_305delTTTG p.V101G*fs*X3 (ex 5)	c.1,634+1G>A (in13)	E, Br	−	−

*Abbreviations: +, present; −, absent; *Br*, easy bruising; *Cub*, Cuban; *E*, epistaxis; *F*, female; FVC, Forced Vital Capacity; *GI*, gastrointestinal symptoms; *Hon*, Honduran; M, male; mo, months; *ND*, not determined; *Mex*, Mexican; *Sal*, Salvadoran; *Uru*, Uruguayan; *Ven*, Venezuelan