Two patients with Hermansky Pudlak syndrome type 2 and novel mutations in *AP3B1*

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ABSTRACT

Hermansky Pudlak syndrome type 2 (HPS2) is a rare disorder associated with mutations in the Adaptor Protein 3 (AP-3) complex, which is involved in sorting transmembrane proteins to lysosomes and related organelles. We now report 2 unrelated subjects with HPS2 who show a characteristic clinical phenotype of oculocutaneous albinism, platelet and T-lymphocyte dysfunction and neutropenia. The subjects were homozygous for different deletions within *AP3B1* (g.del180242-180866, c.del153-156), which encodes the AP-3β3A subunit, resulting in frame shifts and introduction of nonsense substitutions (p.E693fsX13, p.E52fsX11). In the subject with p.E693fsX13, this resulted in expression of a truncated variant β3A protein. Cytotoxic T-lymphocyte (CTL) clones from both study subjects showed increased cell-surface expression of CD63 and reduced cytotoxicity. Platelets showed impaired aggregation and reduced uptake of ^{3}H-serotonin. These findings are consistent with CTL granule and platelet dense granule defects, respectively. This report extends the clinical and laboratory description of HPS2.

Key words: Hermansky Pudlak syndrome, mutations, AP3B1.

Citation: Wenham M, Grieve S, Cummins M, Jones ML, Booth S, Kilner R, Ancliff PJ, Griffiths GM, and Mumford AD. Two patients with Hermansky Pudlak syndrome type 2 and novel mutations in AP3B1. Haematologica. 2010;95:333-337. doi: 10.3324/haematol.2009.012286

Introduction

Hermansky Pudlak syndrome type 2 (HPS2) (OMIM: #608233) is an autosomal recessive disorder comprising oculocutaneous albinism, platelet dysfunction, and immunodeficiency arising from neutropenia and T-lymphocyte dysfunction.[1] Although HPS2 shares the features of albinism and platelet dysfunction with other Hermansky Pudlak syndrome disorders, immunodeficiency is a unique feature of this HPS variant. The disease phenotype arises from mutations in the *AP3B1* gene, which encodes the β3A subunit of the Adaptor Protein 3 (AP-3) complex.[2] AP-3 mediates sorting of transmembrane proteins to lysosomes in all cells, but cells with secretory lysosomes, including melanocytes, platelets and leukocytes, are functionally impaired.[3-7]

Previous studies of cytotoxic T-lymphocyte (CTL) clones from a patient with HPS2 (P1) showed decreased cytotoxicity and enlarged lytic granules that failed to polarize correctly towards target cells.[5] We now provide a description of 2 further unrelated individuals (P2 and P3) with HPS2 associated with novel mutations in *AP3B1*.

Design and Methods

Generation of CTL clones

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Plaque PLUS (GE Healthcare), cloned by limiting dilution and stimulated with 1 μg/mL phytohemagglutinin-M (Roche Diagnostics), 100U/mL IL-2 (Roche) and 10^{6}/mL irradiated allogeneic PBMC. Clones were selected by FACS and cultured as described.[5]

Western blotting of AP3B1 subunits

CTL lysates were separated on 10% SDS-PAGE Tris-HCl gels, transferred to nitrocellulose membranes and probed with rabbit antibodies against the β3A, δ3, μ3A or σ3 subunits of AP-3 (gifts from M.S. Robinson, Cambridge, UK).[8,9]

Acknowledgments: the authors would like to thank Debbie Smyth for assistance with sequencing.
Funding: this work was supported by the Wellcome Trust to GMG. MW was supported by a Rhodes Scholarship.
Manuscript received on June 4, 2009. Revised version arrived on July 21, 2009. Manuscript accepted on July 22, 2009.
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AP3B1 mutation detection

Genomic DNA (gDNA) was isolated from CTL clones using the Wizard isolation kit (Promega). *AP3B1* exons and intronic flanking sequences were amplified by PCR and sequenced using the Big Dye reaction process (Applied Biosystems). Primers were as previously described[7] and PCR conditions are available on request. cDNA was synthesized using Superscript II reverse transcriptase kit (Invitrogen) from CTL mRNA. The AP3B1 coding sequence was then amplified by PCR in fragments smaller than 1200nt and sequenced as described above. Nucleotide variations were identified by comparison to NCBI reference sequence NM_003664 and corresponding gDNA sequence NC_00005.8.

Analysis of CTL clones

CTL were stained with mouse anti-CD63 and analyzed by FACS. Cytotoxicity assays against P815 targets were performed using the Cytotox96 kit (Promega) with 1 μg/mL anti-CD3 antibody (UCHT1; BD Biosciences).

Platelet function testing

Platelet aggregation was tested by measuring light transmission in platelet rich plasma to a panel of agonists as described previously.[10] The platelet dense granule pool was quantified by measuring ^{3}[H]-5HT uptake.[11]

Results and Discussion

Clinical phenotype

Subject P2 was the first child of healthy unrelated Maltese parents. She presented at ten weeks with pendular nystagmus and failure to fix and follow. She was noted to have albinoid fundi, hypopigmented skin and neutropenia. P2 had a stable neutrophil count of 0.2-0.7×10^{9}/L and has been managed with long-term oral antibiotic prophylaxis. There have been no major infections and P2 continues to thrive at three years and has no developmental

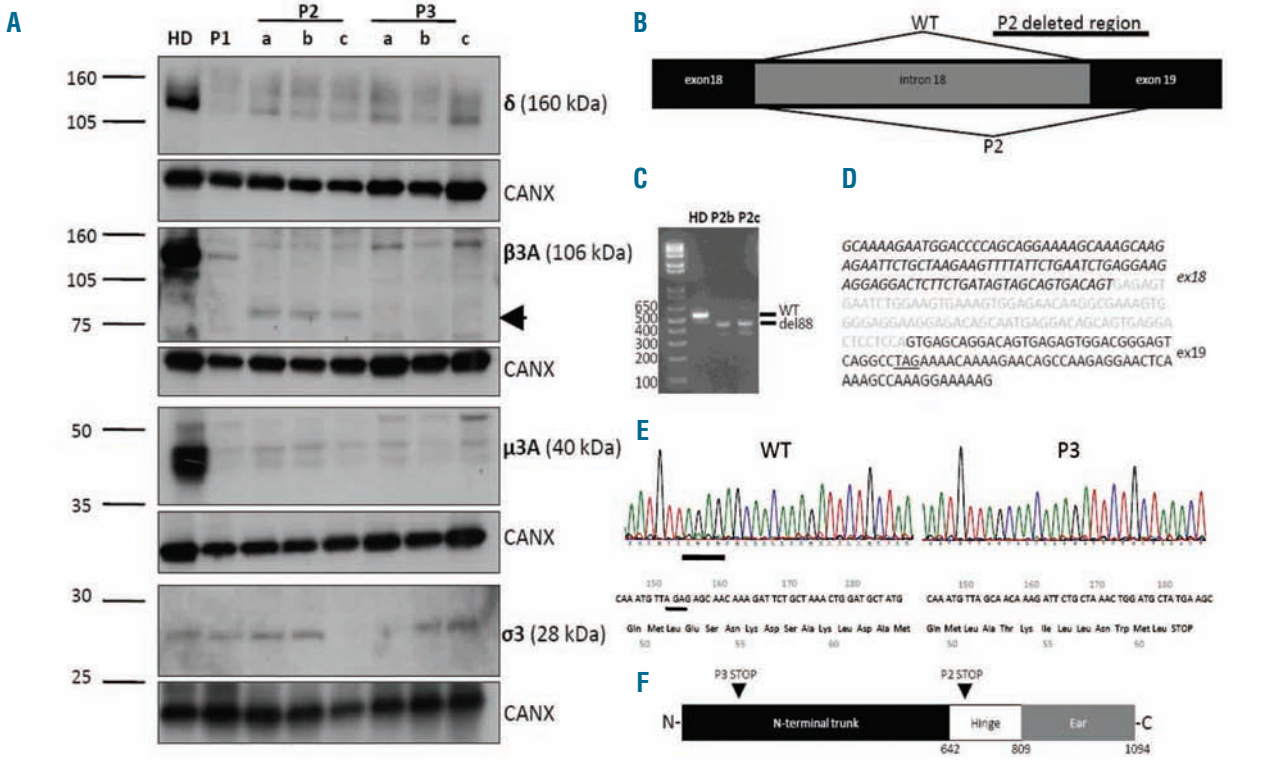

Figure 1. Identification of genetic and protein defects in HPS2 patients. (A) Western blots of CTL lysates, probed with antibodies against AP-3 subunits, then reprobed with an antibody against calnexin (CANX) as a loading control. We studied CTL clones from a healthy donor (HD), a previously reported subject with HPS2 (P1),[5] and subjects P2 (P2a-c) and P3 (P3a-c). Molecular weight markers (in kDa) are indicated at left, and the expected molecular weights of each subunit are given in brackets. The arrowhead indicates a smaller protein band observed using the antibody against β3A in CTL clones from P2. (B) The 624bp homozygous deletion in intron18-exon19 of AP3B1 in subject P2 includes the 3' end of intron 18 and the 5' end of exon 19, including the intron 18 splice acceptor site. In P2, this deletion is predicted to enable splicing of intron 18 using a cryptic acceptor site with exon 19, immediately adjacent to the deleted region. Wild-type intron 18 splicing (WT) and the predicted alternate splicing in P2 are shown. (C) *AP3B1* mRNA isolated from CTL clones from P2 (P2b and P2c) was studied by RT-PCR then subsequent PCR of fragments of the *AP3B1* coding sequence. The fragment containing coding sequence from exons 18 and 19 was smaller in clones from P2 than in a healthy donor, corresponding to a cDNA deletion of 88bp. Molecular weight markers (in bp) are shown at left. (D) Sequence analysis of the abnormal *AP3B1* transcript identified in CTL clones from P2 showed intact coding sequence from exon 18 (italicised) spliced to coding sequence from exon 19 (normal font), but with the loss of 88bp of exon19 coding sequence (shaded font). This deletion is predicted to cause a frame shift leading to a stop codon (underlined) 13 residues downstream of the abnormal splice site in exon 19. (E) Electropherogram of coding sequence of exon 2 of *AP3B1* from a healthy donor and P3, showing a homozygous 4bp deletion (underlined). This deletion is predicted to cause a frame shift leading to a stop codon. (F) Domain structure of β3A showing the positions of the predicted nonsense codons in P2 and P3. The substitution in P2 is predicted to cause expression of a truncated β3A protein of approximately 80kDa, which corresponds to the smaller protein band identified in Figure 1A.

delay. There has been no abnormal bleeding although surgery for congenital hip dysplasia was performed after preoperative platelet transfusion.

Subject P3 was a male infant of healthy unrelated English Caucasian parents who presented at 12 months with failure to thrive, developmental delay, oculocutaneous albinism and neutropenia (0.2×10^9/L). P3 was also dysmorphic with brachiocephaly, a long smooth philtrum and mildly anteverted nares. Since presentation, P3 experienced recurrent respiratory infections and bronchiectasis despite resolution of neutropenia with G-CSF and long-term oral antibiotic prophylaxis. Following varicella infection at 18 months and herpes stomatitis at 24 months, P3 developed transient hepatosplenomegaly, thrombocytopenia and hypertriglyceridemia suggestive of hemophagocytic syndrome. At 36 months, P3 developed pulmonary fibrosis. There has been no abnormal bleeding.

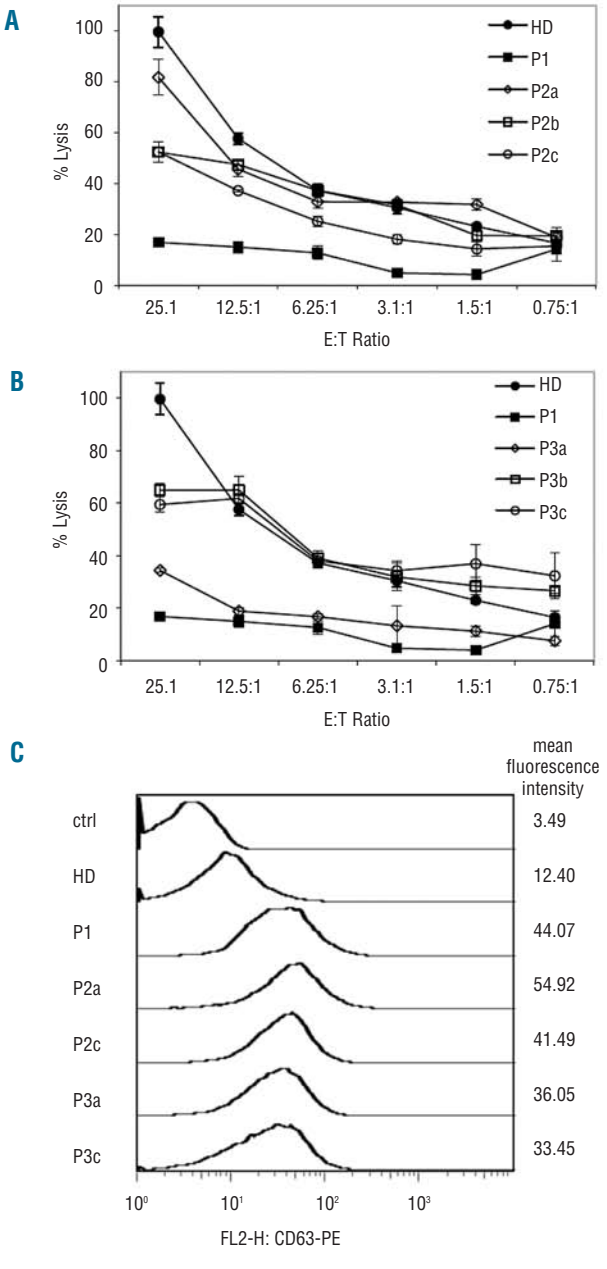

Figure 2. Phenotypic characterization of HPS2 CTL (A and B) CTL clones from a healthy donor (HD), a previously reported HPS2 patient (P1) and 2 new HPS2 patients (P2a-c and P3a-c) were studied in a cytotoxicity assay against P815 targets at different effector:target (E:T) ratios as indicated. The data presented indicate the percentage of target cell lysis after 4 h incubation and are representative of four independent experiments. Samples were assayed in triplicate (error bars represent standard deviation). (C) Plasma membrane expression of CD63 in CTL clones from a healthy donor (HD) and HPS2 patients P1, P2 (P2a, P2c) and P3 (P3a, P3c). HD cells stained with an isotype control antibody are also shown (ctrl). Mean fluorescence intensities are indicated.

Analysis of AP-3 complex subunit expression in CTL

Since the clinical phenotype of our study subjects resembled that of previous HPS2 patients, we studied expression of the AP-3 complex subunits in lysates from CTL clones. P2 and P3 showed markedly reduced expression of the β3A, δ, and μ3 subunits of AP-3 (Figure 1A). P2 showed an additional band using antibodies against β3A that was not present in healthy controls or other subjects with HPS2, suggesting expression of a truncated β3A protein of approximately 80kDa (Figure 1A).

Genetic analysis of AP3B1

Genomic DNA from P2 showed a homozygous 624bp deletion in *AP3B1* (g.del180242-180866) that comprised parts of intron 18 and exon 19 and included the wild-type intron 18 splice acceptor site (Figure 1B). The *AP3B1* cDNA fragment containing coding sequence from exons 18 and 19 was smaller than the expected size (Figure 1C). Sequencing showed this to be an abnormal transcript generated using a cryptic splice acceptor site in exon 19 causing an 88bp deletion in the *AP3B1* cDNA (c.del2078-2165). In addition to causing loss of coding sequence, this deletion caused a frame shift leading to a nonsense substitution (p.E693fsX13) 13 residues downstream of the abnormal splice acceptor site (Figure 1D). This is predicted to generate a truncated β3A protein of approximately 80kDa (Figure 1F) corresponding to the abnormal β3A band identified by Western blot (Figure 1A).

Genomic DNA from P3 showed a homozygous 4bp deletion in exon 2 of *AP3B1* (Figure 1E) that was confirmed by sequence analysis of *AP3B1* cDNA (c.del153-156). This deletion is predicted to cause a frame shift leading to a stop codon (p.E52fsX11) near the AP-3β amino terminus (Figure 1F). There was no discernible expression of β3A in P3 by Western blot.

Effect of AP-3 deficiency on CTL cytotoxicity and CD63 expression

In order to investigate CTL function in P2 and P3, we studied the ability of different CTL clones to lyse target cells in a cytotoxicity assay. For both subjects, there was variation in cytotoxic activity between CTL clones, with some clones killing up to 70% of targets, while others killed only a maximum 35% of targets (Figure 2A and B). Different CTL clones from P2 and P3 consistently showed increased CD63 expression at least two-fold greater than clones from healthy donors (Figure 2C), similar to P1.[5]

Platelet aggregation and storage pool

Since HPS2 has been associated with absent platelet dense granules, we studied platelet function in P3.

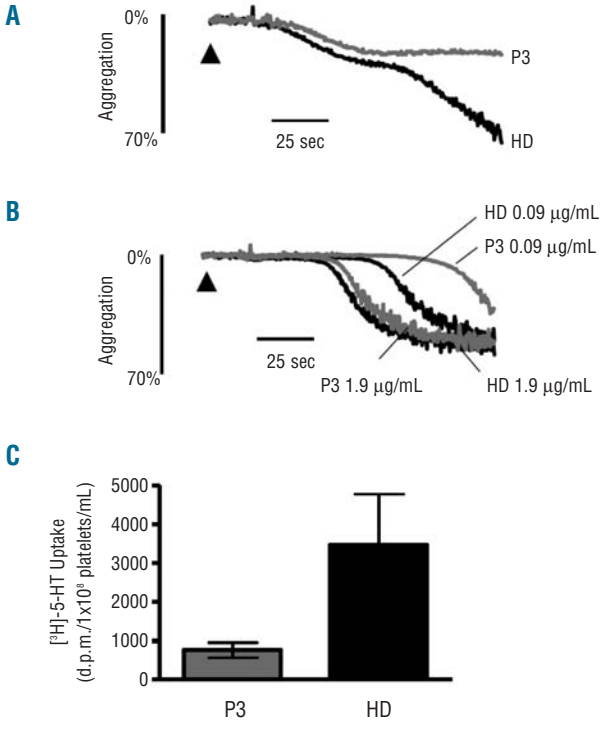

Figure 3. Platelet aggregation and [^{3}H] 5-hydroxytryptamine uptake in subject P3 (A-B) Platelets from P3 and a healthy donor control (HD) were studied by first isolating platelet-rich plasma (PRP) from citrated-blood by centrifugation. The platelet agonists adrenaline 30 µM (A) and collagen 0.09 µg/mL and 1.9 µg/mL (B) were then added to stirred PRP at the time point indicated by the arrow. Platelet aggregation was measured by determining the change in light transmission over 125s. (C) The uptake of ^{3}[H] 5-HT into platelet dense granules was measured by labeling platelets with 10 mCi of [^{3}H]-5-hydroxytryptamine for 1 h at 30°C before isolation from plasma by centrifugation in the presence of PGE1 and indomethacin. Platelets were re-suspended in Tyrodes-HEPES buffer and a 0.2 mL aliquot was then mixed with 4mL of scintillation fluid before analysis in a scintillation counter. Background signal, determined by scintillation counting of the supernatant of re-suspended labeled platelets, was subtracted from the value obtained from the platelet suspension.

Platelets showed reduced aggregation with 0.09 µg/mL collagen compared to a healthy donor control, although with 1.9 µg/mL collagen platelet aggregation was indistinguishable (Figure 3A). With 30 µM adrenaline, platelets from P3 showed absent secondary wave aggregation (Figure 3B). Platelet uptake of [^{3}H] 5-HT was markedly reduced compared to healthy donor control platelets (Figure 3C). These findings are consistent with absence of platelet dense granules.

We report the clinical and laboratory phenotype of 2 unrelated subjects with HPS2 in which homozygous deletion mutations in *AP3B1* caused frame shifts and premature stop codons, leading to absent (P3) or truncated (P2) expression of β3A. To date, HPS2 has been reported previously in only 9 individuals from 6 kindreds with different homozygous or compound heterozygous *AP3B1* mutations.[2,5,7,12-15] Previous studies have shown that absent β3A leads to instability and rapid degradation of the entire AP-3 complex.[2,16] The AP-3 mutations in this study also result in loss of the entire AP-3 complex, even when the truncated 80kDa form of β3A, encoding the N-terminal trunk and part of the hinge, is expressed.

Our study subjects and all previously reported HPS2 patients showed oculocutaneous albinism and immunodeficiency manifesting as recurrent bacterial and possibly viral infections. The immunodeficiency phenotype is consistent with neutropenia, although in P3 infections persisted despite improved neutrophil count with G-CSF treatment. This may indicate an additional immune defect in HPS2, possibly arising from CTL dysfunction. Subject P3 also showed dysmorphia and pulmonary fibrosis that have been previously reported in HPS2,[7,12-15] and had two episodes of hemophagocytic syndrome following common viral infections. Hemophagocytic syndrome has previously been reported in a single HPS2 patient,[15] although this patient also carried a mutation in one allele of *Rab27a*, a gene known to be involved in hemophagocytic syndrome. Subject P2 showed a less severe clinical phenotype than P3, possibly because of residual expression of a truncated β3A protein in P2, in contrast to absent expression in P3.

Laboratory analysis of P3 and P2 showed significant neutropenia which was resolved in P3 with G-CSF. The laboratory platelet phenotype in P3 was consistent with absent platelet dense granules, which is a consistent finding in the Hermansky Pudlak syndrome disorders but not specific for HPS2. CTL clones from both subjects showed markedly increased CD63 expression on the plasma membrane, as previously identified in all reported HPS2 patients. Whether this increased expression has any functional consequence in CTL is not yet known. Levels of CTL cytotoxicity in P2 and P3 varied compared to the previous HPS2 patient, P1.[5] Decreased CTL and NK cell cytotoxicity has been reported for other HPS2 patients,[7,15] and the reasons for the variable levels of killing seen in clones from P2 and P3 reported here are not yet understood. The physiological implications of reduced cell-mediated cytotoxicity are seen in the recurrent bacterial and viral infections observed in these and other HPS2 patients, although the relative contributions of CTL, NK cell and neutrophil defects to this phenotype are not yet fully determined.

Authorship and Disclosures

GMG, ADM, PA: study design, principal investigators, manuscript writing; MW: sequence analysis; CTL assays and manuscript writing; SG, SB: isolation of CTL clones; MLJ: platelet analysis; RK: sequence analysis; PA, MC, ADM: clinical data collection.

The authors reported no potential conflicts of interest.

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