High Frequency of Hermansky–Pudlak Syndrome Type 1 (HPS1) Among Japanese Albinism Patients and Functional Analysis of HPS1 Mutant Protein

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Hermansky–Pudlak syndrome (HPS) is an autosomal recessive disorder characterized by oculocutaneous albinism (OCA), bleeding tendency, and lysosomal accumulation of ceroid-like material. Seven genetically distinct subtypes of HPS are known in humans; most are rare outside of Puerto Rico. Here, we describe the analysis of the HPS1 gene in 24 Japanese OCA patients who lacked mutations in the four genes known to cause OCA (TYR/OCA1, P/OCA2, TYRP1/OCA3, and MATP/OCA4), and the identification of eight different HPS1 mutations in ten of these patients, four of which were novel (W583X, L668P, 532insC, 1691delA). An IVS5 + 5G → A splice consensus mutation was particularly frequent, the result of a founder effect for this allele in Japanese patients. Functional analysis by transfection of the L668P variant into Hps1-mutant melan-ep mouse melanocytes showed that this missense substitution is pathologic, resulting in an Hps-1 protein that is unable to assemble into the biogenesis of lysosome-related organelles complex-3.

Key words: albinism/biogenesis of lysosome-related organelles complex/founder effect/haplotype analysis/ Hermansky–Pudlak syndrome

Abbreviations: BLOC, biogenesis of lysosome-related organelles complex; HPS, Hermansky-Pudlak syndrome; HRP, horseradish peroxidase; OCA, oculocutaneous albinism; SNP, single nucleotide polymorphism; SSCP, single-strand conformation polymorphism

†The first two authors contributed equally to this work.

Hermansky–Pudlak syndrome (HPS; MIM# 203300) is an autosomal recessive disorder characterized by oculocutaneous albinism (OCA), bleeding tendency, and lysosomal accumulation of ceroid-like material. Seven genetically distinct subtypes of HPS are known in humans; most are rare outside of Puerto Rico. Here, we describe the analysis of the HPS1 gene in 24 Japanese OCA patients who lacked mutations in the four genes known to cause OCA (TYR/OCA1, P/OCA2, TYRP1/OCA3, and MATP/OCA4), and the identification of eight different HPS1 mutations in ten of these patients, four of which were novel (W583X, L668P, 532insC, 1691delA). An IVS5 + 5G → A splice consensus mutation was particularly frequent, the result of a founder effect for this allele in Japanese patients. Functional analysis by transfection of the L668P variant into Hps1-mutant melan-ep mouse melanocytes showed that this missense substitution is pathologic, resulting in an Hps-1 protein that is unable to assemble into the biogenesis of lysosome-related organelles complex-3.
sensory mutation was particularly frequent, a result of a founder effect for this allele in Japanese patients. Functional analysis of the L668P variant showed that this missense substitution is pathologic, lacking the ability to assemble into the BLOC-3.

Results

Mutations of the HPS1 gene and phenotypes of the Japanese patients To assess the occurrence of HPS1 in the 24 patients who lacked mutations in the TYR (OCA2), P (OCA2), TYRP1 (OCA3), or MATP (OCA4) gene among 93 unrelated Japanese OCA patients, we screened for mutations in the HPS1 gene. As shown in Table I, among ten of these patients we detected a total of eight different pathologic mutations (W583X, L668P, 288delT, 532insC, 1323insA, 1691delA, and IVS5+5G→A), four of which were novel (W583X, L668P, 532insC, and 1691delA). Analysis of DNA of 101 unrelated normally pigmented Japanese individuals detected only one of these variants, IVS5+5G→A, in a single heterozygous individual (Table II). This IVS5+5G→A mutation, which we previously showed as being pathologic with a sample of patient 5, causing aberrant pre-mRNA splicing with skipping of exon 5, and consequent frameshift in the resulting HPS1 mRNA (Suzuki et al, 2004), was observed in seven of the ten patients, three of whom were homozygous for this mutation (Table I). Thus, among these 93 Japanese patients thought to have OCA, mutations in HPS1 occurred in 11% of the patients.

Clinical phenotypes of these ten patients with HPS1 are summarized in Table I. All of the patients showed some pigmentation, which, although variable, was generally more than in patients with tyrosinase-negative OCA (OCA1A). Prolonged bleeding time was confirmed in six of ten patients. The other four patients all had clinical evidence of a bleeding diathesis, e.g., purpura on lower limbs or frequent epistaxis, but their bleeding times were not tested. Pulmonary fibrosis, confirmed by radiography and high-resolution computerized tomography of the chest, was found only in patient 4, who was 60 y old and had severe dyspnea. Patient 8, who was 39 y old, had no symptoms of pulmonary fibrosis or colitis. The other eight patients were not examined for these complications, as they appeared too young to exhibit symptoms.

Functional analysis of the L668P substitution by complementation of HPS1-null mouse melanocytes The L668P missense substitution involves an amino acid residue conserved among most known species, including human, mouse, rat, Xenopus, Fugu, and Ancylostoma, although divergent in Drosophila (Y) and Mosquito (F). Furthermore, the L668 substitution was not detected in any of the 101

Table I. Mutations and clinical phenotype of Japanese patients with HPS1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Maternal Mutation</th>
<th>Paternal Mutation</th>
<th>Hair color</th>
<th>Iris color</th>
<th>Nystagmus</th>
<th>Bleeding time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>3 mo</td>
<td>IVS5+5G→A</td>
<td>1691delA</td>
<td>Brown</td>
<td>Gray</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>10 mo</td>
<td>W583X</td>
<td>974insC</td>
<td>Light brown</td>
<td>Brown</td>
<td>+</td>
<td>20 min</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>6 mo</td>
<td>974insC</td>
<td>288delT</td>
<td>Blond</td>
<td>Gray</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>60 y</td>
<td>IVS5+5G→A</td>
<td>IVS5+5G→A</td>
<td>Blond</td>
<td>Blue</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>2 y</td>
<td>IVS5+5G→A</td>
<td>IVS5+5G→A</td>
<td>Blond</td>
<td>Blue</td>
<td>+</td>
<td>10 min</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>1 mo</td>
<td>532insC</td>
<td>1323insA</td>
<td>Blond</td>
<td>Reddish brown</td>
<td>+</td>
<td>13 min</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>1 y</td>
<td>L668P</td>
<td>IVS5+5G→A</td>
<td>Yellow</td>
<td>Blue</td>
<td>+</td>
<td>9.5 min</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>39 y</td>
<td>(IVS5+5G→A)</td>
<td>(L668P)</td>
<td>Light brown</td>
<td>Blue-brown</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>1.5 mo</td>
<td>974insC</td>
<td>IVS5+5G→A</td>
<td>Blond</td>
<td>Hazel</td>
<td>+</td>
<td>12 min</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>10 mo</td>
<td>IVS5+5G→A</td>
<td>IVS5+5G→A</td>
<td>Blond</td>
<td>Reddish brown</td>
<td>–</td>
<td>12 min</td>
</tr>
</tbody>
</table>

Novel mutations are in bold.

* A sample of patient 5 was used for the analysis for the IVS5+5G→A mutation causing aberrant pre-mRNA splicing (Suzuki et al, 2004).

** It was not determined which mutations were of maternal or paternal origin in this patient. The normal value for the bleeding time is <6 min. ND, not done; HPS, Hermansky–Pudlak syndrome.

Table II. Frequencies of mutations in Japanese patients with HPS1 and normally pigmented Japanese subjects

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Japanese patients with HPS1 (n=20)</th>
<th>Normally pigmented Japanese subjects (n=202)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS5+5G→A</td>
<td>10 (50%)</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>c.974insC</td>
<td>3 (15%)</td>
<td>0</td>
</tr>
<tr>
<td>c.288delT</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>c.1323insA</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>p.W583X</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>p.L668P</td>
<td>2 (10%)</td>
<td>0</td>
</tr>
<tr>
<td>c.532insC</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>c.1691delA</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
</tbody>
</table>

n, number of alleles examined; HPS1, Hermansky–Pudlak syndrome type 1.
Japanese control individuals tested. Together, these data indicated that residue L668 was functionally important and that L668P was not a common non-pathological polymorphism.

To assess the function of L668P-mutant HPS1 protein experimentally, we tested the ability of the wild-type versus mutant HPS1 polypeptides to constitute the BLOC-3 protein complex in mutant mouse melanocytes. We previously showed that cultured melanocytes derived from homozygous Hps1-null (melan-ep) and Hps4-null (melan-le) mutant mice both lacked the HPS1 protein, the apparent result of protein destabilization by the absence of either one of the complex components (Suzuki et al., 2002). To determine whether lack of the HPS1 protein likewise results in destabilization of the HPS4 protein, we raised antisera to the mouse HPS4 protein. As shown in Fig 1, western blot analysis showed that both melan-ep and melan-le melanocytes lack detectable ~ 80 kDa HPS4 protein, indicating that the HPS4 protein was destabilized in Hps1-mutant (melan-ep) melanocytes, just as the HPS1 protein was destabilized in Hps4-mutant (melan-le) cells (Suzuki et al., 2002). These data thus demonstrated that lack of either the HPS1 or HPS4 protein destabilized the other member of the BLOC-3. Unexpectedly, we also found that the steady-state quantity of HPS4 protein was reproducibly greater in Hps3-mutant melan-coa melanocytes in wild-type (melan-a) cells (Fig 1).

Chiang et al (2003) showed that overexpression of human HPS4 protein in melan-le melanocytes restores stability of endogenous HPS1 protein, most likely by allowing assembly of intact BLOC-3. We used an analogous approach to test whether the L668P mutant HPS1 protein could stabilize endogenous HPS4 protein in transfected melan-ep melanocytes. We transfected cells with cDNA expression plasmids encoding either wild-type or L668P mutant human HPS1 protein, and analyzed HPS1 and HPS4 protein expression by western blot assay. As shown in Fig 2, overexpression of normal human HPS1 protein in transfected melan-ep cells fully restored the stability of endogenous HPS4 protein (Fig 1). Overexpression of L668P-mutant HPS1 protein, however, failed to restore the stability of endogenous HPS4 (Fig 2). This result indicated that L668P-mutant HPS1 was functionally incapable of assembling with HPS4 in the BLOC-3 protein complex.

Haplotype analysis As discussed above, we found the HPS1 IVS5 + 5G → A mutation in seven of the ten Japanese HPS1 patients, three of whom were homozygous for this mutation. This allele thus appears to be common among Japanese HPS1 patients. To determine whether the multiple occurrences of this allele might derive from a common founder, we carried out haplotype analysis of patients and their parents for five single-nucleotide polymorphisms (SNP) located within the HPS1 gene (Fig 3). As shown in Table III, all alleles carrying the IVS5 + 5G → A mutation appear to share the SNP haplotype C–C–G–G–A. In contrast, among 101 unrelated normally pigmented Japanese controls, the C–C–G–G–A haplotype occurred with a frequency of 6.3%. Thus, the relatively high frequency of the HPS1 IVS5 + 5G → A mutation in Japanese patients appears to represent a founder effect for the mutation on this island nation.

Mutational analysis of the HPS4 gene We additionally screened for mutations in the HPS4 gene in the group of 14

Figure 1
Presence of Hermansky–Pudlak syndrome (HSP)4 protein in cultured mouse melanocyte lines. Cell extracts of wild-type melan-a (lane 1), Hps3-mutant (melan-coa; lane 2), Hps4-mutant (melan-le; lane 3), and Hps1—mutant (melan-ep; lane 4) melanocytes were immunoblotted using affinity-purified polyclonal antisera to mouse HPS4 protein. The mouse HPS4 protein reproducibly migrates at ~ 80 kDa on SDS-PAGE. Anti-β-actin antibody was used as a control for protein loading.

Figure 2
Expression of exogenous human Hermansky–Pudlak syndrome type 1 (HPS1) and endogenous HPS4 proteins in melan-ep cells. Melan-ep melanocytes were transfected using pIREShyg3-HPS1nor-mal (lane 1), pIREShyg3-HPS1mutantL668P (lane 2), and by pIREShyg3 as a mock (lane 3). Then the cell extracts were immunoblotted using the antibodies: Top: anti-HPS1 antibody; Middle: anti-HPS4 antibody; bottom: anti-β-actin antibody as a control for protein loading. Exogenous normal and L668P-mutant HPS1 protein were abundantly expressed in transfected cells. Endogenous HPS4 protein, however, was restored only in melan-ep cells transfected by the normal HPS1 expression plasmid.
OCA patients who remained undiagnosed. But we found no pathological mutations of the \textit{HPS4} gene, indicating that \textit{HPS4} may be rare among Japanese OCA patients.

\section*{Discussion}

This is a molecular analysis that documents a significant frequency of HPS among non-Puerto Rican patients with OCA. Remarkably, as many as ten of 93 Japanese patients with OCA were found to have HPS1, indicating that this disorder, previously considered rare, is actually one of the most common types of OCA among Japanese. Non-pigmentary features of HPS in these patients seem to be relatively mild, and thus attracted little clinical attention, resulting in these patients’ prior classification as having non-syndromic “OCA”. The diagnosis of HPS1 is very important, because it implies significant risk of major clinical complications that do not occur in non-syndromic OCA, including bleeding, pulmonary fibrosis, and colitis.

Only patient 4 developed pulmonary fibrosis. His dyspnea was severe. No other patients had symptoms or clinical evidence of either pulmonary fibrosis or colitis. Nevertheless, it is inappropriate to conclude that there is a low frequency of these complications among Japanese HPS1 patients, as eight of the ten patients studied here were younger than 2 y of age. The age of onset of pulmonary fibrosis in HPS is typically in the third or fourth decade (Davies and Tuddenham, 1976), and the age of onset of colitis in HPS is typically between 12 and 30 y (Schinella \textit{et al}, 1980). It will therefore be important to follow these Japanese pediatric HPS1 patients carefully over several decades to assess the occurrence of common HPS complications.

In Puerto Rico, HPS is relatively frequent, a specific \textit{HPS1} gene mutation having been amplified via the founder effect because of occult inbreeding and genetic drift (Fukai \textit{et al}, 1995). An analogous situation pertains to the IVS5 +5G → A mutation that we found in HPS patients from Japan, where a founder effect appears to account for a high frequency of this mutant allele among patients with OCA. We also identified the 974insC mutation in three of ten Japanese patients with HPS1. This mutation has been previously found in a number of European patients, with multiple independent mutational origins attributed to a frameshift hotspot associated with a polycytosine tract (Oh \textit{et al}, 1998). Our findings extend this mutation to an Asian population, most likely derived from at least one additional independent origin at this mutational hotspot.

The L668P missense substitution described here is only the second \textit{HPS1} missense substitution reported thus far, and subjected to functional analysis. The \textit{HPS1} protein interacts with the HPS4 protein in the BLOC-3 protein complex, which is required for the biogenesis of various lysosome-related organelles, including melanosomes. We have shown that L668P-mutant HPS1 protein, whereas stable, lacks the ability to stabilize the HPS4 protein, suggesting that L668P-HPS1 protein cannot assemble into BLOC-3.

\begin{table}[h]
\centering
\caption{Identified haplotype with the IVS5 +5G → A mutation}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Patient & Mutation & SNP1 & SNP2 & SNP3 & SNP4 & SNP5 \\
\hline
1 & IVS5 +5G → A & C & C & G & G & A \\
 & c.1691delA & C & A & C & C & G \\
4 (Homozygote) & IVS5 +5G → A & C & C & G & G & A \\
5 (Homozygote) & IVS5 +5G → A & C & C & G & G & A \\
7 & IVS5 +5G → A & C & C & G & G & A \\
 & p.L668P & C & C & G & C & A \\
8 & IVS5 +5G → A & C & C & G & C/G & A \\
 & p.L668P & C & C & G & C/G & A \\
9 & IVS5 +5G → A & C & C & G & G & A \\
 & c.974insC & C & A & C & C & G \\
10 (homozygote) & IVS5 +5G → A & C & C & G & G & A \\
\hline
Frequency (%) in normal Japanese\textsuperscript{a} & C: 91 & C: 49 & G: 57 & G: 13 & A: 57 \\
\hline
\end{tabular}
\textsuperscript{a}Two hundred and two normally pigmented Japanese alleles were analyzed. SNP, single-nucleotide polymorphism.
\end{table}
This may implicate L668 in stabilization of or even binding to HPS4. Interestingly, patients with the L668P mutation (patients 7 and 8 in Table I) manifested a relatively moderate HPS1 clinical phenotype that included congenital nystagmus, OCA with some pigmentation, and mild bleeding tendency (bleeding time: 9.5 min), suggesting that this variant may not be functionally null in vivo.

Finally, we unexpectedly observed that the amount of steady-state HPS4 protein is considerably greater in Hps3-null (melan-coa) melanocytes than in wild-type mouse melanocytes (see Fig 1). The HPS3 protein is a component of the BLOC-2 (Gautam et al, 2004), whereas HPS4, like HPS1, is a component of BLOC-3. These observations suggest that there may be some functional relationship between BLOC-2 and BLOC-3, although its nature remains to be elucidated.

Materials and Methods

Patients Twenty-four unrelated Japanese patients with OCA (six females and 18 males) who had no mutations in the TYR, P YTRP1, and MATP genes were included in this study. Patients’ clinical phenotypes are described in Table I. This study was approved by the ethics committee of the Nagoya University School of Medicine. Informed consent was obtained from each patient, or in the case of children from the patient’s parents. The study was conducted according to the Declaration of Helsinki Principles.

Mutation screening of the HPS1 gene DNA segments spanning each of the 20 HPS1 genomic exons were amplified using primers and PCR conditions described by Bailin et al (1997) and screened for mutations by the simultaneous single-strand conformation polymorphism (SSCP)/heteroduplex method (Lee et al, 1995). Two kinds of SSCP gels, with glycerol concentrations of 7% and 10%, were used to elevate the sensitivity of our mutation screening system. PCR products showing aberrant patterns were reamplified between BLOC-2 and BLOC-3, and sequenced directly using the genetic analysis system CEQ2000XL (Beckman Coulter, Fullerton, California).

DNA constructs Full-length normal human HPS1 cDNA was cloned in pc2r.1-TOPO (Invitrogen, Carlsbad, California), and subsequently thymine at nucleotide position 2003 was substituted by cytidine to create the L668P substitution by the site-directed mutagenesis method (Giebel and Spritz, 1990). The cDNA were then sequenced to confirm accuracy, and were inserted into mammalian expression plasmid pIREShyg3 (BD Biosciences, San Jose, California), creating pIREShyg3-HPS1null and pIREShyg3-HPS1mutantL668P, respectively.

Cell cultures and cell transfection Melan-a, melan-coa, melan-ep, and melan-le were cultured in RPMI-1640 medium with 10% fetal calf serum, 200 nM 12-O-tetradecanoyl phorbol 13-acetate, and 200 nM cholera toxin, in 10% CO2 as described (Suzuki et al, 2001). Melan-ep mouse melanocytes were cultured for 24 h and transfected with 1.6 μg pIREShyg3, pIREShyg3-HPS1-null, or pIREShyg3-HPS1mutantL668P per cm2 flask using Lipofectamine 2000 reagent (Invitrogen). Stable transfectants were selected in culture media containing 500 μg per mL hygromycin B (Invitrogen).

Antibody production and immunoblotting Polyclonal antiserum against mouse HPS4 protein was raised by immunizing rabbits with a synthetic peptide (486-DRTGFKPSPSGRH-498) as antigen, and were then affinity purified using a smaller peptide (490-FKPSPS-495) within the primary antigen peptide by Sigma Genos Japan (Hokkaido, Japan). Anti-HPS1 monoclonal antibody (mAb hHPS5) was previously described (Oh et al, 2000). Anti-β-actin monoclonal antibody was purchased from Sigma Genosysteme Japan. HRP-conjugated secondary antibodies were from DakoCytonomy (Glostrup, Denmark).

Cells were harvested by trypsin and centrifugation, washed twice with cold PBS, and were resuspended in CF-PBS lysis buffer (0.25 M sucrose, 25 mM KCl, 2.5 mM MgCl2, 0.01% SDS). Cell extracts from melanocytes were separated in 7.5% SuperSep polyacrylamide gels (Wako, Osaka, Japan) and transferred to Immobilon-P (Millipore, Billerica, Massachusetts). Membranes were blocked in 5% fat-free milk in PBS plus 0.05% Tween-20 for 1 h at room temperature, and were then incubated in blocking solution containing the appropriate primary antibody for 1 h at room temperature. Membranes were then washed extensively, incubated with appropriate HRP-conjugated secondary antibody for 1 h at room temperature, and washed again. Signals were detected using ECL plus western blotting detection reagents (Amersham Biosciences, Piscataway, New Jersey).

Haplotyping analysis Figure 3 shows the positions of five SNP derived from the genomic sequence of human chromosome 10 (Genbank accession no. NT_030059): SNP1 (IVS1-9C → T; SNP accession no. rs2296429) is located in intron 1 of HPS1 gene, SNP2 (nucleotide position 162C → A; accession no. rs1804689) in exon 2, SNP3 (IVS14 + 7G → C; accession no. rs2296433) in intron 14, SNP4 (1472C → G; accession no. rs2296434) in exon 15, and SNP5 (IVS16-15A → G; accession no. rs2296435) in intron 16. SNP1 and SNP2 were detected with a primer set for exon 2. SNP3 was detected with a primer set for exon 14. SNP4 was detected with a primer set for exon 15. SNP5 was detected with a primer set for exon 17. SSCP analysis and direct sequencing were used for genotyping. A haplotype of the allele with the mutation was constructed, based either on homozygosity or on the analyses of parental DNA. We also analyzed haplotypes of 101 unrelated normally pigmented Japanese as controls by the PCR-SSCP/heteroduplex method and direct sequencing, using the Estimating Haplotype-frequencies (EH) software program (Terwilliger and Ott, 1994; Curtis and Sham, 1995) to estimate the frequencies of five SNP in the controls.

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