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REPORT

Heterozygous Mutations in *OAS1* Cause Infantile-Onset Pulmonary Alveolar Proteinosis with Hypogammaglobulinemia

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Pulmonary alveolar proteinosis (PAP) is characterized by accumulation of a surfactant-like substance in alveolar spaces and hypoxemic respiratory failure. Genetic PAP (GPAP) is caused by mutations in genes encoding surfactant proteins or genes encoding a surfactant phospholipid transporter in alveolar type II epithelial cells. GPAP is also caused by mutations in genes whose products are implicated in surfactant catabolism in alveolar macrophages (AMs). We performed whole-exome sequence analysis in a family affected by infantile-onset PAP with hypogammaglobulinemia without causative mutations in genes associated with PAP: *SFTPB*, *SFTPC*, *ABCA3*, *CSF2RA*, *CSF2RB*, and *GATA2*. We identified a heterozygous missense variation in *OAS1*, encoding 2′5′-oligoadenylate synthetase 1 (OAS1) in three affected siblings, but not in unaffected family members. Deep sequence analysis with next-generation sequencing indicated 3.81% mosaicism of this variant in DNA from their mother's peripheral blood leukocytes, suggesting that PAP observed in this family could be inherited as an autosomal-dominant trait from the mother. We identified two additional *de novo* heterozygous missense variations of *OAS1* in two unrelated simplex individuals also manifesting infantile-onset PAP with hypogammaglobulinemia. PAP in the two simplex individuals resolved after hematopoietic stem cell transplantation, indicating that OAS1 dysfunction is associated with impaired surfactant catabolism due to the defects in AMs.

Lung surfactant is synthesized and stored in alveolar type II epithelial cells.[1] It is secreted into the alveolar spaces and reduces surface tension at air-liquid interfaces. Surfactant proteins (SP)-B and SP-C are highly hydrophobic and essential for the surface activity of lung surfactant.[2] ATP-binding cassette A3 (ABCA3) transports surfactant phospholipids into lamellar bodies where they bind SP-B and SP-C to form surfactant.[3] Secreted lung surfactant is partially recycled by type II epithelial cells, and the remainder is taken up and catabolized by alveolar macrophages (AMs). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is implicated as essential for catabolism of lung surfactant, as well as the proliferation and maturation of human AMs.[4]

Pulmonary alveolar proteinosis (PAP) is caused by lung surfactant system homeostasis dysfunction[5] and can be categorized into four types: autoimmune PAP (APAP) (MIM: 610910), secondary PAP (SPAP), genetic PAP (GPAP), and unclassified PAP. APAP is caused by excess production of autoantibodies against GM-CSF.[6] AMs of APAP-affected individuals show a typical foamy appearance.[7] SPAP is associated with underlying malignancies or blood diseases, such as myelodysplastic syndrome (MIM: 614286).[8] GPAP is caused by mutations in several genes.[9,10] SP-B, encoded by one of the genes associated with GPAP, *SFTPB* (MIM: 178640), is required for the maturation of SP-C. SP-B deficiency (MIM: 265120) is an autosomal-recessive disease characterized by respiratory distress syndrome (RDS) (MIM: 267450) at birth, which then develops into infantile type PAP.[11] Abnormalities in SP-C (encoded by *SFTPC* [MIM: 178620]) (MIM: 610913) and ABCA3 (encoded by *ABCA3* [MIM: 601615]) (MIM: 610921) are likely to yield manifestations of GPAP and interstitial pneumonitis.[12–14] Mutations in genes encoding GM-CSF receptor (*CSF2RA* [MIM: 306250] and *CSF2RB* [MIM: 138981]) are also associated with GPAP (MIM: 300770, 614370) in infants and adults.[15–17] Mutations in *GATA2* (MIM: 137295) are associated with monocytopenia and mycobacterial infection (MonoMAC) syndrome (MIM: 614172), which shows a broad spectrum of clinical manifestations, including PAP.[18]

In this study, we performed whole-exome sequence (WES) analysis in a family affected by infantile-onset PAP with hypogammaglobulinemia that had no causative mutations in genes associated with PAP: *SFTPB*, *SFTPC*, *ABCA3*, *CSF2RA*, *CSF2RB*, and *GATA2*. In addition, we

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Table 1. Demographic Characteristics of Five Affected Individuals in This Study

	A-II-1	A-II-3	A-II-4	B-II-1	C-II-1
Consanguineous parents	no	no	no	no	no
Gestation (weeks)	38	36	39	39	39
Birth weight (g)	2,896	2,852	2,290	2,140	3,410
Gender	male	male	female	female	female
Age at HSCT	not done	151 d	not done	8 m	11 m
Diagnosis of PAP	AU	BAL+AU	BAL+AU	BAL	BAL
Recurrent infection with hyperreactivity	unknown	unknown	yes	yes	yes
Hypogammaglobulinemia	unknown	unknown	yes	yes	yes
Leukocytosis with normal distribution	yes	yes	yes	yes	yes
Splenomegaly before treatment	yes	yes	yes	unclear	yes
Small and non-foamy AMs	yes	yes	yes	unclear	yes
Age at onset of respiratory dysfunction	39 d	39 d	49 d	2 m	5 m
Respiration status at birth	well	well	well	well	well
Respiration status after BAL	not done	unchanged	unchanged	improved	unchanged
Respiration status after IVIG	unknown	unknown	improved	improved	improved
Outcome	dead/91 d	dead/163 d	dead/11 y	dead/3 y	alive/19 m
Cause of death	respiratory failure	respiratory failure	respiratory failure	renal failure	alive

Abbreviations: AU, autopsy; BAL, bronchoalveolar lavage; HSCT, hematopoietic stem cell transplantation; d, day; m, month; y, year.

further studied two unrelated simplex individuals that also presented with infantile-onset PAP with hypogammaglobulinemia. A summary and details of the affected individuals are shown in Table 1 and Supplemental Note.

This study was conducted in accordance with the Declaration of Helsinki and the national ethical guidelines, and was approved by the Ethics Committees of Hokkaido University Faculty of Medicine and Graduate School of Medicine (16-001), Yokohama City University Graduate School of Medicine, and Tokyo Medical and Dental University. Parents of all individuals included in the study provided written informed consent for genetic analyses and publication. The genomic DNA of two affected siblings (A-II-1 and A-II-3) from family A was extracted by phenol/chloroform methods and stored at –80°C (Figure S1A). The genomic DNA samples of other family members were extracted from heparinized peripheral blood samples using SepaGene (Sankojunyaku). All DNA samples were amplified with an Illustra GenomiPhi DNA Amplification Kit (GE Healthcare). In addition, we established Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) from the two siblings, one affected (A-II-4) and one unaffected (A-II-2), and extracted their DNA. WES analysis was performed as described previously.[19] Briefly, 3 μg of whole-genome amplified DNA from peripheral blood or DNA samples from EBV-LCL were used in sample preparation. Genome partitioning was performed with SureSelect Human All Human Exon v4 (Agilent Technology) according to the manufacturer's protocol. The samples were run on a HiSeq2000 (Illumina) with 101-bp paired-end reads and 7-bp index reads. Reads were mapped to the human reference genome (GRCh37.1/hg19) by Novoalign 2.08.02. Variants were called with Genome Analysis Toolkit v1.6-5 and annotated using ANNOVAR (2012feb).

To identify the causative variants, we selected variants based on the following criteria. For the autosomal-dominant (*de novo*) model, we first removed synonymous variants and then selected (1) variants not found in our in-house exome data (n = 153 controls), (2) variants not registered in dbSNP 135 (see Web Resources) or NHLBI Exome Sequencing Project (ESP5400) (see Web Resources), (3) variants outside segmental duplication, (4) variants not observed in either parents or an unaffected sibling, and (5) variants shared among all three affected children. For the homozygous model, we first removed synonymous variants and then selected (1) rare variants ($n \leq 1/153$) in our in-house exome data, (2) variants with minor allele frequency (MAF) ≤ 0.01 in ESP5400, (3) variants outside segmental duplication, and (4) variants shared in three affected children. For autosomal compound heterozygous variants, we first removed synonymous variants and then selected (1) rare variants ($n \leq 1/153$) in our in-house exome data, (2) variants with MAF ≤ 0.01 in ESP5400, (3) variants outside segmental duplication, and (4) variants shared among three affected children. To estimate the mosaic variation in their parents, we counted the reads with variation using the BAM file with Integrative Genomic Viewer (see Web Resources). To confirm the presence of the variant allele in peripheral blood, we sequenced

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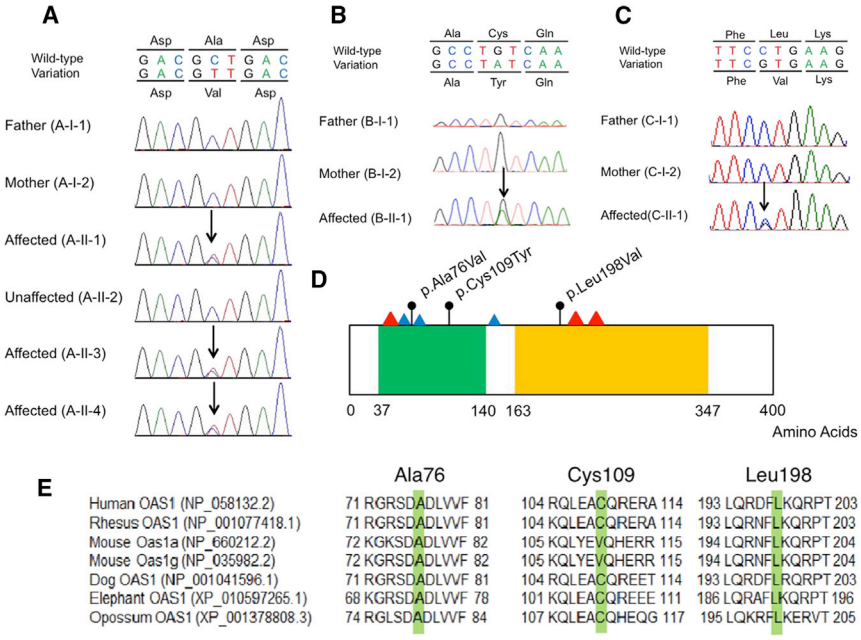

Figure 1. *OAS1* Variants in Three Families with PAP and Evolutionary Conservation and Variations in *OAS1*

(A–C) Genetic analysis for *OAS1* variation. Sanger sequencing demonstrated variations of (A) c.227C>T (p.Ala76Val) in family A, (B) c.326G>A (p.Cys109Tyr) in family B, and (C) c.592C>G (p.Leu198Val) in family C. Black arrows show the positions of the variations.

(D) Schematic representation of *OAS1* mutations. Black circles indicate the variants identified in this study. Blue and red triangles indicate metal binding sites (Asp75, Asp77, and Asp148) and ATP binding sites (Ser63, Lys213, and Gln229), respectively. Green and yellow boxes show the nucleotidyltransferase (NTP_transf_2) domain and 2′,5′-oligoadenylate synthetase 1, domain 2, and C terminus (OAS1_C) domain, respectively, predicted using the SMART program (see Web Resources).

(E) Evolutionary conservation of Ala76, Cys109, and Leu198 amino acids in human OAS1. These amino acids are highlighted in green. Six orthologous sequences were aligned using the multiple sequence alignment program, Clustal Omega (see Web Resources).

the 151-bp amplicon covering the candidate *OAS1* (MIM: 164350) variant by deep sequence analysis using MiSeq with Miseq Reagent Kit v1 (300 cycles) (Illumina). Genomic DNA samples from two unrelated simplex individuals from families B and C (B-II-1 and C-II-1) (Figures S1B and S1C) were subjected to PCR amplification of *OAS1* with primers to cover three transcriptional variants sharing the same exons 1 to 4 (RefSeq: NM_016816.3, NM_002534.3, and NM_001032409.2) as listed in Table S1. Direct sequence analysis of *OAS1* was performed as described previously.[20] Nucleotide sequences were compared with the reported reference sequence of *OAS1* (RefSeq: NM_016816.3).

The quality of WES performance is summarized in Table S2. The exonic regions were not well covered using the whole-genome amplified samples (64.6%–89.6% of coding sequences by ≥20× reads). Although we first considered that the condition in family A was inherited in an autosomal-recessive manner, there were no causative recessive variants (Tables S3 and S4). Unexpectedly, one *de novo* missense variant, c.227C>T (p.Ala76Val) in *OAS1* (RefSeq: NM_016816.3), was shared by all three affected individuals but not by unaffected family members (Figure 1A and Table S5). This variant was not registered in the ESP5400, Exome Aggregation Consortium, or Human Genetic Variation Database and was predicted to be "tolerated" by SIFT and "polymorphism" by MutationTaster, but "probably damaging" by PolyPhen-2 (see Web Resources) (Table 2). This amino acid is located between the metal binding sites (Asp75 and Asp77) in the NTP_transf_2 domain (Figure 1D) and is evolutionarily conserved from opossum to human (Figure 1E). Biological parentage was confirmed based on nine microsatellite markers. As either of the parents could have this variant as a mosaic, we performed deep sequencing of the parents' amplicons covering this variant and detected the mutant T allele at a frequency of 3.81% in the mother (Table S6).

Furthermore, we found two additional heterozygous *OAS1* variants occurring *de novo* in two unrelated simplex individuals with infantile-onset PAP with hypogammaglobulinemia (B-II-1 and C-II-1) (Figures S1B and S1C). A variation of c.326G>A (p.Cys109Tyr) in *OAS1* was observed in B-II-1 but not in her parents (Figure 1B). This missense variation was "tolerated" by SIFT and "polymorphism" by MutationTaster, but "possibly damaging" by PolyPhen-2 (Table 2). Another variation of c.592C>G (p.Leu198Val) in *OAS1* (observed in C-II-1 but not in her parents) (Figure 1C) was predicted as "tolerated" by SIFT, "benign" by PolyPhen-2, and "polymorphism" by MutationTaster (Table 2). Neither p.Cys109Tyr nor p.Leu198Val are included in ESP5400, Exome Aggregation Consortium, or Human Genetic Variation Database.

To assess the impact of p.Ala76Val, p.Cys109Tyr, and p.Leu198Val on protein structure, we mapped these variations onto the crystal structures of a double-stranded RNA (dsRNA)-bound form of human OAS1 (hOAS1) and an apo form of porcine oligoadenylate synthetase 1 (pOAS1) (PDB: 4ig8 and 1px5, respectively) (Figure 2A).[21,22] There are structural differences between the RNA-bound and apo forms. The conformation of OAS1 is stabilized in a catalytically active form, which is capable of binding to ATP and Mg^{2+} ions upon binding to dsRNA. Ala76 in hOAS1 (Ala75 in pOAS1) adjoins the two active site aspartic acid residues, Asp75 and Asp77 (Asp74 and Asp76

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Table 2. *OAS1* Mutations Identified in Three Unrelated Families with GPAP

Variants	Prediction Software			Grantham Score
	SIFT	PolyPhen-2	MutationTaster	
c.227C>T (p.Ala76Val)	0.08 (tolerated)	0.936 (probably damaging)	polymorphism	64
c.326G>A (p.Cys109Tyr)	0.18 (tolerated)	0.715 (possibly damaging)	polymorphism	194
c.592C>G (p.Leu198Val)	1.00 (tolerated)	0.349 (benign)	polymorphism	32

in pOAS1), which bind to Mg^{2+} ions. The side chain of Ala76 is involved in a hydrophobic core that would define the active site structure in the RNA-bound form, indicating that the p.Ala76Val variant would affect enzymatic activity. There are structural differences around Cys109 between the RNA-bound and apo forms. The side chain of Cys109 in hOAS1 (Cys108 in pOAS1) is involved in a hydrophobic core of the apo form of OAS1, which is rearranged upon dsRNA binding. Leu198 in hOAS1 (Leu197 in pOAS1) is involved in a hydrophobic core, which is located close to the ATP binding pocket. Thus, the p.Leu198Val variant in hOAS1 (corresponding to p.Leu197Val in pOAS1) might impair ATP binding and enzymatic activity.

The free energy changes for each variant in a dsRNA-bound form of hOAS1 and an apo form of pOAS1 were calculated using the FoldX software through the YASARA interface.[23,24] After energy minimization with the "repair object" command, free energy change upon each variation was calculated with the "mutate residue" command through the YASARA interface. The FoldX calculation showed a marked increase in free energy associated with the p.Cys109Tyr variant in hOAS1 (p.Cys108Tyr in pOAS1) in the apo form of OAS1 (Figure 2B). This result suggested that p.Cys109Tyr likely disrupts the hydrophobic core of the apo form and the importance of this residue for regulation of enzymatic activity by dsRNA. FoldX predicted only a small impact of p.Ala76Val and p.Leu198Val on protein folding (Figure 2B).

The mechanisms by which the heterozygous mutations of *OAS1* described above cause PAP are unknown, and functional studies are currently underway. The OAS1 protein, encoded by *OAS1*, is a member of the 2-5A synthetase family essential for innate immune response against viral infection.[25–27] OASs are induced by interferons and use adenosine triphosphate in 2′-specific nucleotidyl transfer reactions to synthesize 2′,5′-oligoadenylates. These molecules activate latent RNase L, which results in degradation of both viral and endogenous RNA. In addition to antiviral activity, the OASs were reported to be involved in fundamental cellular functions, such as cell growth and differentiation, gene regulation, and apoptosis.[28] A single-nucleotide polymorphism (SNP), rs10774671, causing *OAS1* splicing variant p46 but not p48 and p52, is associated with increased susceptibility to plasma leakage and shock in individuals infected with dengue virus-2, indicating that immune overreaction could be triggered by the specific *OAS1* genotype, at least in dengue virus infection.[29] Although the functions of OAS1 on AMs have not been fully elucidated, expression of *OAS1* in AMs could be induced by oxidative stress.[30] Two SNPs are associated with severe acute respiratory syndrome (SARS) susceptibility: the G-allele of non-synonymous A/G SNP rs1131454 in exon 3 (p.Ser162Gly) located near the dsRNA binding domain and SNP rs2660 in the 3′ untranslated region of *OAS1*, possibly associated with expression of each transcriptional variant.[26,27] This association suggests the immune-modulating function of OAS1 protein especially in the respiratory system. Therefore, we speculated that the heterozygous mutations of *OAS1* observed in the three families, presumably gain-of-function mutations, might be associated with exaggerated immune reaction especially in AMs in response to viral infections, leading to dysfunction of AMs and impaired catabolism of lung surfactant.

There are a number of possible characteristic features of this disease, including infantile onset. All affected individuals were term infants and had no respiratory symptoms at birth. The onset of respiratory symptoms was distributed from 39 days to 5 months. The first symptoms appeared like a viral infection in most of the individuals, including C-II-1, who was positive for respiratory syncytial virus (RSV), cytomegalovirus, and subsequently coronavirus NL63 in sputum specimens during onset. Viral infections may trigger the onset of PAP via the mechanisms proposed above.

Hypogammaglobulinemia was observed in three affected individuals (A-II-4, B-II-1, and C-II-1) and was unclear in two individuals that died at 91 days (A-II-1) and 163 days (A-II-3). Three individuals that survived beyond the first year of life (A-II-4, B-II-1, and C-II-1) showed low levels of serum IgG, IgM, and IgA, although they had no B cell deficiency. Although the level of endogenous IgG was unknown due to repeated intravenous immunoglobulin (IVIG) administration, serum IgM and IgA levels increased gradually during the course of the disease. Hypogammaglobulinemia observed in affected individuals may be due to impaired B cell function by an as yet unknown mechanism caused by heterozygous *OAS1* mutations.

Small and non-foamy AMs were observed in four affected individuals (A-II-1, A-II-3, A-II-4, and C-II-1) and were undetermined in one individual (B-II-1). In contrast with the large and foamy AMs observed in APAP,[31] small and non-foamy AMs may indicate dysfunction of maturation and/or phagocytosis of AMs rather than impaired catabolism

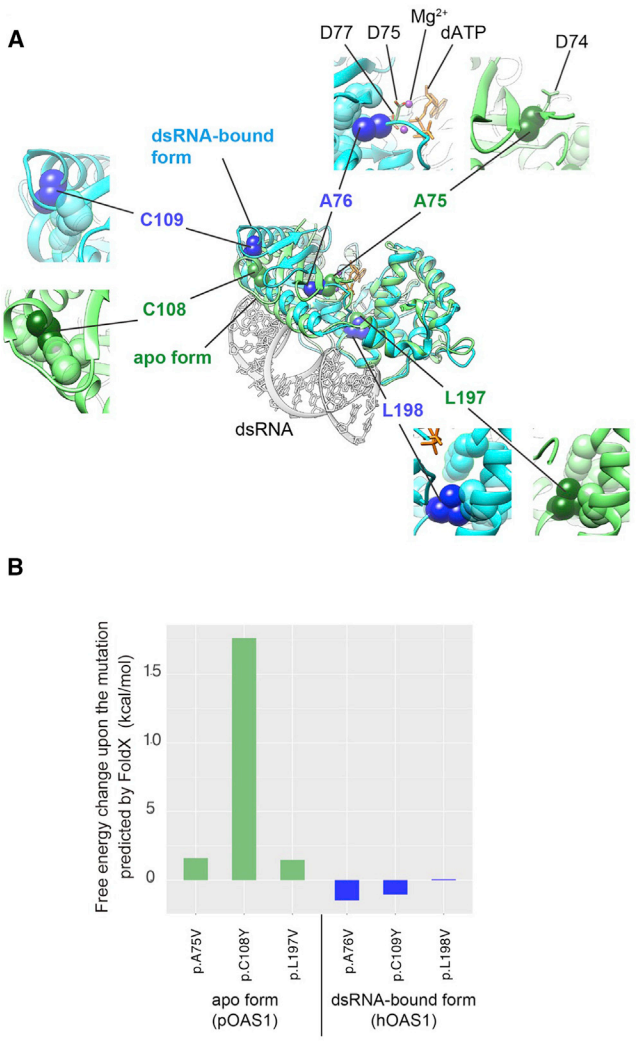

Figure 2. Impact of the Identified Variants on Protein Structure
(A) Mapping of the mutations on the crystal structures of a double-stranded RNA (dsRNA)-bound form of human oligoadenylate synthetase 1 (hOAS1) and an apo form of porcine OAS1 (pOAS1) (PDB: 4ig8 and 1px5, respectively). The superimposed structures of the dsRNA-bound (cyan) and apo (green) forms of OAS1 are presented as ribbon diagrams. The stick model is shown in gray. The altered residues are shown as van der Waals spheres in blue and dark green in the dsRNA-bound and apo forms, respectively. The substrate analog, 2′-deoxy ATP (dATP), and magnesium ions are shown as orange sticks and purple balls, respectively. Close-up views around the mutation sites are shown separately for the dsRNA-bound and apo forms. In the close-up views, some side chains of the hydrophobic residues around the mutation sites are shown as translucent spheres.
(B) The free energy changes associated with each variant in a dsRNA-bound form of hOAS1 and apo form of pOAS1 by the FoldX software are shown.

of phagocytosed lung surfactant. Although we performed BAL four times in A-II-4 and there were many CD14-positive small and non-foamy AMs every time, this finding was based on a single bronchoalveolar cell cytopathology specimen showing fewer than 20 cells in most of the individuals. Further studies in larger numbers of affected individuals with this disease are necessary.

Three individuals (A-II-4, B-II-1, and C-II-1) had overwhelming inflammation, which was not clear but was confirmed at autopsy in two individuals (A-II-1 and A-II-3). Splenomegaly, possibly related to hyperinflammation, was observed in four individuals (A-II-1, A-II-3, A-II-4, and C-II-1) before the initiation of replacement therapy with IVIG. Exogenous IgG improved respiratory symptoms and decreased systemic inflammatory responses in A-II-4, B-II-1, and C-II-1. However, AMs in bronchoalveolar lavage (BAL) fluid from A-II-4 under sequential IVIG showed no improvement in viability or phagocytosis of AMs (data not shown). Furthermore, A-II-4 showed gradual progression of PAP. Thus, immunoglobulin administration may be able to suppress uncontrolled inflammatory responses, without being able to improve dysfunction of AMs in terms of surfactant phagocytosis or catabolism. As inflammation plays an important role in the pathophysiology of PAP,[32] immunomodulation by IVIG may have some effect on the clinical course of PAP.

B-II-1 died from renal failure due to glomerulosclerosis following hematopoietic stem cell transplantation (HSCT). Glomerular diseases, including glomerulosclerosis, are increasingly observed especially in individuals with graft versus host disease after HSCT.[33] As renal biopsy was performed at the end stage of renal failure, it was difficult to determine whether glomerulosclerosis observed in B-II-1 was associated with HSCT. As another affected individual, A-II-4, showed proteinuria before initiation of monthly IVIG, OAS1 dysfunction itself could cause glomerular diseases. Long-term observation and accumulation of more individuals with this disease are necessary to evaluate this association.

Lung transplantation is a curative treatment for GPAP caused by mutations in genes encoding surfactant proteins or genes encoding a surfactant phospholipid transporter in alveolar type II epithelial cells.[34] On the other hand, HSCT is effective for GPAP caused by mutations in genes that could be responsible for surfactant catabolism in AMs.[35] In this study, two affected individuals (B-II-1 and C-II-1) received successful HSCT and recovered completely from PAP. C-II-1 has had cord blood engraftment for 2 years without any respiratory symptoms. It has been reported that AMs are derived from precursor cells in bone marrow.[36] Pulmonary transplantation of differentiated wild-type AMs derived from bone marrow in GM-CSF receptor-β-deficient mice showed long-term effectiveness due to proliferation of transplanted AMs.[37] Although IVIG could transiently improve the clinical condition, HSCT is the most effective therapy for this disease at present.

In summary, heterozygous mutations in *OAS1* cause infantile-onset PAP with hypogammaglobulinemia possibly caused by dysfunction of AMs. Infantile-onset leukocytosis without abnormal distribution, splenomegaly, and hyperreactivity were the most prominent findings. HSCT should be considered a curative treatment for this disease.

Supplemental Data

Supplemental Data include Supplemental Note, five figures, six tables, and Acknowledgments and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.01.019.

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Web Resources

dbSNP, https://www.ncbi.nlm.nih.gov/projects/SNP/
ExAC Browser, http://exac.broadinstitute.org/
Human Genetic Variation Database, http://www.hgvd.genome.med.kyoto-u.ac.jp/
IGV, http://www.broadinstitute.org/igv/
Multiple Sequence Alignment, https://www.ebi.ac.uk/Tools/msa/clustalo/
MutationTaster, http://www.mutationtaster.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
RefSeq, https://www.ncbi.nlm.nih.gov/RefSeq
SIFT, http://sift.bii.a-star.edu.sg/
SMART, http://www.smart.embl-heidelberg.de/

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