Adult-onset hereditary pulmonary alveolar proteinosis caused by a single-base deletion in CSF2RB

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INTRODUCTION
Pulmonary alveolar proteinosis (PAP) is a rare lung disease in which lipoproteins accumulate in the alveoli and terminal bronchioles, resulting in the development of respiratory failure.1 PAP is clinically divided into three distinct forms: autoimmune, secondary and hereditary PAP.1 Autoimmune PAP is associated with the disruption of granulocyte/macrophage colony-stimulating factor (GM-CSF) signalling caused by high levels of GM-CSF autoantibodies in the lung.2 3 Very rarely, there are uncatalogued cases in which neither GM-CSF autoantibody nor underlying diseases are confirmed.4 Of significance is the fact that mutant mice deficient in GM-CSF or its receptor spontaneously develop PAP5 6 and some hereditary cases exhibit defects in GM-CSF receptor (GM-CSF-R) expression.7 8 GM-CSF-R is composed of the binding α chain and the common β chain, which is also utilised by interleukin-3 (IL-3) and IL-5. Binding of GM-CSF initiates the Jak2, STAT5 and PI3K/Akt pathways.9

METHODS
Subjects
This study was approved by the institutional review boards of Nagasaki University and Niigata University. Written informed consent was obtained from the patient, her parents and all control volunteers. Blood mononuclear cells were collected from the subjects. Genetic counselling was performed for the patient and her parents before and after genetic analyses.

GM-CSF autoantibody measurement
GM-CSF autoantibody concentrations in the serum and bronchoalveolar lavage fluid (BALF) were measured by ELISA as described previously.4

Isolation of blood mononuclear cells and monocytes
Heparinised blood was obtained and blood mononuclear cells were prepared by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). To isolate CD14 positive monocytes from blood mononuclear cells, human CD14 microbeads (MACS; Miltenyi Biotec, Auburn, California, USA) were applied for magnetic sorting.

Differentiation of monocytes to macrophages in vitro
Peripheral blood monocytes from a control subject and the patient were cultured in a 24-well culture
plate (BD Falcon, Franklin Lakes, New Jersey, USA) at 1×10^5 cells per well with 10 ng/ml of recombinant human GM-CSF (Leukine, Berlex, Seattle, Washington, USA) in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/ml streptomycin and 100 μl/penicillin in a humidified atmosphere of 5% CO₂ at 37°C. Morphological changes were evaluated after 5–14 days under phase contrast microscopy.

**GM-CSF clearance assay**

To assess receptor-mediated binding and uptake of exogenous GM-CSF, blood mononuclear cells from the patient and control volunteer were cultured in 24-well culture plates at 1×10^6 cells per well in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 mg/ml streptomycin and 100 μl/penicillin in a humidified atmosphere of 5% CO₂ at 37°C. Recombinant human GM-CSF was added at a concentration of 100 pg/ml. Subsequently, GM-CSF concentration in the supernatant of each well was measured by ELISA (R&D Systems, Minneapolis, Minnesota, USA), as previously described.7

**Immunoblotting**

Blood mononuclear cells were cultured on 24-well culture plates at 5×10^5 cells per well. Cells were incubated with GM-CSF (0–1000 ng/ml) or IL3 (0–10 ng/ml) for 15 min. Lysed protein extracts were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane, and assessed by standard western blotting procedures, as previously described.7 Primary antibodies used for detection included anti-human GM-CSF-Rβc (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-human GM-CSF-Rα, anti-human STAT5 (Santa Cruz Biotechnology, Santa Cruz, California, USA), and anti-human phospho-STAT5 (Millipore, Billerica, Massachusetts, USA) antibodies. Peroxidase labelled anti-rabbit IgG antibody (Sigma-Aldrich, St Louis, Missouri, USA) was used as secondary antibody and visualised with ECL plus (GE Healthcare, Waukesha, Wisconsin, USA). To enhance the signal, immunoreactions enhancer solution (Can Get Signal, TOYOBO, Osaka, Japan) was used according to the manufacturer’s instructions. Actin was measured as a loading control for each sample using anti-actin antibody (Santa Cruz Biotechnology).

**Flow cytometry**

CD11b expression of neutrophils was analysed by flow cytometry (FACSCalibur, Beckton Dickinson, Franklin Lakes, New Jersey, USA) as previously described.7 Briefly, heparinised whole blood was collected and incubated in the absence or presence of 10 ng/ml human GM-CSF. Blood was incubated with phycocerythrin (PE) conjugated mouse anti-human CD11b (BD Pharmingen, Franklin Lakes, New Jersey, USA) and a fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD16 (BD Pharmingen). Each triplicate sample was analysed. Expression of GM-CSF receptors was analysed using anti-human CD116 (GM-CSF-Rα) (eBioscience, San Diego, California, USA) and anti-human CD131 (common β) (eBioscience).

**Reverse transcription PCR**

Total RNA was extracted from blood mononuclear cells using RNA Easy Plus Mini Kit (QIAGEN, Hilden, Germany) and was reverse transcribed with random hexamer primers using the SuperScript III First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, California, USA). The cDNAs were subjected to semi-quantitative RT-PCR analysis using PrimeSTAR GXL DNA polymerase (TaKaRa Bio, Otsu, Japan) with CSF2RA-specific primer (supplementary table 2).

**Nucleotide sequencing**

PCR products were purified using QIAquick DNA extraction kit (QIAGEN) and subjected to nucleotide sequencing using BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) and CSF2RB-specific primer (supplementary table 2).

**Array-CGH**

Array-comparative genomic hybridisation (aCGH) analysis was performed using the Agilent 105A Whole Human Genome Oligo Microarray kit according to the manufacturer’s protocol (Agilent Technologies, Palo Alto, California, USA). Genomic DNA was extracted from peripheral blood using the QIAquick DNA extraction kit (QIAGEN).

**RESULTS**

In 2000, a 36-year-old woman, previously healthy, with normal chest radiograph at a health check-up 1 year earlier, gradually developed dyspnoea on exertion. She was diagnosed as having PAP by typical findings on chest radiography (figure 1A), bronchoalveolar lavage (BAL), and lung histopathological examination (figure 1B). She had undergone whole lung lavage (WLL) five times within 4 years and had subsequently started home oxygen therapy. In 2005, the arterial blood gas analysis on room air showed a PaO₂ of 44.8 mm Hg. Her clinical parameters and course are summarised in supplementary figure 1 and supplementary table 1, respectively. Bone marrow cells showed mild hypocellularity with normal cell differentials and a normal karyotype of bone marrow cells. GM-CSF autoantibodies were not detected in either the serum or BALF (supplementary table 1).

We decided to screen for GM-CSF signalling abnormalities because GM-CSF concentration was extremely high in her serum (124.8 pg/ml).7 The patient’s monocytes incubated with GM-CSF failed to differentiate into macrophages with a viability of less than 50% within 5 days (figure 1C). The baseline expression of CD11b, a complement receptor of neutrophils, was up-regulated in the patient compared with the control, which was augmented by incubation with GM-CSF in the control but not in the patient (figure 1D). Cleavage of GM-CSF by the patient’s peripheral blood mononuclear cells (PBMCs) was impaired (figure 1E). We then analysed signal activated by GM-CSF in PBMCs. When PBMCs were stimulated with increasing concentrations of GM-CSF (0-1000 ng/ml), phosphorylated STAT5 was observed in the healthy control but not in the patient (figure 2B). Using flow cytometry, the expression of GM-CSF-Rα on patient monocytes was comparable to the control; however, expression of GM-CSF-Rβc was not detected (figure 2C). These results were confirmed at mRNA level by RT-PCR (figure 2D) and immunoblotting (figure 2E).

cDNA of CSF2RA reverse transcribed from the mRNA of the patient PBMCs had no mutations or deletions (data not shown). PCR amplicons for 14 exons of CSF2RB from patient PBMCs appeared similar to the control in size and expression (figure 2F). Screening of nucleotide sequences from the PCR amplicon for each exon revealed a homozygous single-base deletion at nt 631 in exon 6 (accession number: AB575019) (figure 2G), causing a frame shift that gave rise to a stop codon at nt 789 (supplementary figure 2A). This deletion has not been reported in the
SNPs database (UCSC Genome Browser Bioinformatics (http://genome.ucsc.edu/) and the JSNP Japanese Single Nucleotide Polymorphism Database (http://snp.ims.u-tokyo.ac.jp/index.html)). The same deletion was recognised at the same codon in the patient’s buccal mucosa, indicating that this change occurred in the germ line (data not shown). Further evaluation using array-comparative genomic hybridisation (aCGH) demonstrated no interstitial deletion encompassing the region of CSF2RB (supplementary figure 2B). Taken together with the expression defect on PBMCs, mRNA harbouring a homozygous premature termination codon in the patient’s CSF2RB gene may be degraded by nonsense-mediated mRNA decay system.11

The nucleotide sequence of exon 6 in CSF2RB from the PBMCs of both parents demonstrated heterozygous deletion at the same codon as the patient (figure 2G); however, GM-CSF stimulated STAT5 phosphorylation was normal (figure 2H). In this regard, Suzuki et al reported a case with CSF2RA mutation whose parents showed heterozygous mutations but normal STAT5 phosphorylation under GM-CSF stimulation.7 12 Although CSF2RB and CSF2RA are different genes, it may support our finding that a heterozygous mutation in GM-CSF receptor genes does not cause the signalling defect. Furthermore, according to the functional analysis in Be (-/-) mice, proliferating capacity of bone marrow cells under GM-CSF stimulation was intact in heterozygous (+/-) mice compared with that in wild-type (+/+ ) mice.5 Although both parents were free from any pulmonary disease, they had been second cousins with a history of three miscarriages between them. Therefore, the homozygous single-base deletion in CSF2RB was probably inherited from both parents in an autosomal recessive manner.

**DISCUSSION**

This is the first report identifying a gene defect in CSF2RB causing GM-CSF-Rβc deficiency and a signalling defect which caused adult-onset hereditary PAP. In a previous report, flow cytometry evaluation showed that four out of eight cases of hereditary PAP had complete GM-CSF-Rβc deficiency, whereas the remaining four cases showed reduced expression.9 All of these diagnoses were made within the first 17 months of infancy when all patients became symptomatic. Four patients with a complete defect in GM-CSF-Rβc expression had severe respiratory failure and required oxygen therapy. A point mutation within CSF2RB was found in one patient, but was later recognised to be present in 6% of 184 multinational normal subjects as mentioned above.

Targeting deletion of GM-CSF-Rβc in mice has been shown to give rise to the development of PAP between 6 and 14 weeks after birth, which can be reversed by bone marrow transplantation and haematopoietic reconstitution by 8–12 weeks after the transplant.13 However, the defect in surfactant clearance was significantly milder in CSF2RB targeted mice than in GM-CSF deficient mice.14 In this regard, Ding et al hypothesised that multiple intracellular signalling pathways are being activated by GM-CSF binding to the GM-CSF-Rα alone.15

Thus, we speculate that the onset of PAP in the present case might have been delayed by the signals from extremely elevated GM-CSF in the lung, which was conveyed through GM-CSF-Rα. Alternatively, some factors other than GM-CSF in the lung may have compensated for the function of alveolar macrophages. This was supported by the presence of mild leukocytosis on admission and remarkably increased M-CSF levels in BALF, in
the absence of infectious disease. In this regard, the late onset of PAP in the present case was distinctive and unique among deficiency.

Taking the previous report into consideration, the detection of phosphorylated STAT5 in blood mononuclear cells stimulated with GM-CSF and/or IL-3 is likely to be a useful and convincing method of screening for defects in GM-CSF-Rα signalling. This case had some similarities of phenotype of PAP and molecular biomarkers (serum GM-CSF, serum SP-D, GM-CSF in BAL, M-CSF in BAL, CD11b SI, STAT5 phosphorylation, and GM-CSF clearance) with CSF2RA mutations cases. Increased GM-CSF levels in BALF and serum are important markers since autoimmune PAP is typically characterised by neutralisation of GM-CSF in the lung. Thus, an elevated serum free GM-CSF level is useful in distinguishing between autoimmune and GM-CSF receptor dysfunction PAP.

Bone marrow transplantation would represent the most effective treatment for our case. In this regard, Dirksen et al reported that allogeneic bone marrow transplantation for a patient with acute myelogenous leukaemia successfully led to remission of secondary PAP. Fukuno et al described a case of PAP secondary to myelodysplastic syndrome that resolved after successful cord blood transplantation. Replacement of impaired alveolar macrophages with stem cell derived normal alveolar macrophages might correct the defect seen in PAP. However, the safety and efficacy of bone marrow or cord blood transplantation for this patient should be further evaluated.
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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the institutional review boards of Nagasaki University and Niigata University.

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