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

Takeshi Tanaka,1 Natsuki Mitoi,2 Yoshiko Tsukihashi,1 Ryuishi Tazawa,2 Chinatsu Kaneko,2 Takahito Nei,2 Toshiyuki Yamamoto,3 Tomayoshi Hayashi,4 Tsutomu Tagawa,5 Takeshi Nagayasu,5 Futoshi Kuribayashi,5 Koya Ariyoshi,1 Koh Nakata,2 Konosuke Morimoto1

ABSTRACT
Background Disruption of granulocyte/macrophage colony-stimulating factor (GM-CSF) signalling causes pulmonary alveolar proteinosis (PAP). Rarely, genetic defects in neonatal or infant-onset PAP have been identified in CSF2RA. However, no report has clearly identified any function-associated genetic defect in CSF2RB.

Methods and results The patient was diagnosed with PAP at the age of 36 and developed respiratory failure. She was negative for GM-CSF autoantibody and had no underlying disease. Signalling and genetic defects in GM-CSF receptor were screened. GM-CSF-stimulated STAT5 phosphorylation was not observed and GM-CSF-Rβc expression was defective in the patient’s blood cells. Genetic screening revealed a homozygous, single-base deletion at nt 631 in exon 6 of CSF2RB on chromosome 22, which caused reductions in GM-CSF dependent signalling and function. Both parents, who were second cousins, showed no pulmonary symptoms, and had normal GM-CSF-signalling, but had a CSF2RB allele with the identical deletion, indicating that the mutant allele may give rise to PAP in an autosomal recessive manner.

Conclusions This is the first report identifying a genetic defect in CSF2RB that causes deficiency of GM-CSF-Rβc expression and impaired signalling downstream. These results suggested that GM-CSF signalling was compensated by other signalling pathways, leading to adult-onset PAP.

INTRODUCTION
Pulmonary alveolar proteinosis (PAP) is a rare lung disease in which lipoproteinaceous material is accumulated 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 unclassified cases in which neither GM-CSF autoantibodies 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. Recently, two groups reported mutations in the coding region of the GM-CSF-Rα gene (CSF2RA) in hereditary PAP cases, which is located in the pseudoautosomal region of PAR1 on the X and Y chromosomes.7 8

Dirksen et al reported GM-CSF-Rβc deficiency in four patients with PAP diagnosed during the neonatal to infant period. In one patient, a point mutation in CSF2RB resulted in an amino acid change at codon 602 from proline to threonine.9 However, this mutation is now known as a single nucleotide polymorphism in normal subjects10 (NCBI SNP Database ID rs1801122; http://www.genecards.org/), and thus, no report has clearly identified gene defects in CSF2RB disrupting GM-CSF signalling. Here, we describe the first adult-onset case with a single-base deletion in exon 6 of CSF2RB, resulting in severe reductions in GM-CSF dependent signalling and function in the blood cells.

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 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, Washignton, 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 U/ml 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 U/ml 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.

**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 IL-3 (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. Primary antibodies used for detection included anti-human GM-CSF-Rα (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-human GM-CSF-Rα, anti-human STAT5 (Santa Cruz Biotechnology), 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 second 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. Briefly, heparinised whole blood was collected and incubated in the absence or presence of 10 mg/ml human GM-CSF. Blood was incubated with a phycoerythrin (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 CD151 (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 PAH 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). 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). Clearance 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’s PBMCs (figure 2A). Similarly, phosphorylated STAT5 was not observed in the IL-3-stimulated PBMCs, suggesting defective signalling mediated by GM-CSF-Rβc (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 amplions 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 amplion 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
Figure 1 Pulmonary alveolar proteinosis (PAP) diagnosis and granulocyte/macrophage colony-stimulating factor (GM-CSF) functional analysis on leucocytes. (A) Chest high-resolution CT demonstrating diffuse homogenous ground-grass opacity and crazy-paving appearance. (B) Section of lung biopsy specimen stained with H&E, demonstrating accumulation of amorphous eosinophilic materials in alveolar space (total magnification 40×). Bar: 1.0 mm. (C) Phase contrast microscopic images of monocytes from the patient (right) or a control (left) incubated with 10 ng/ml of GM-CSF for 5 days. Results shown are representative of independent experiments. (D) Flow cytometry analyses for the expression of CD11b on neutrophils from a control (upper) and the patient (lower) incubated with (right) or without (left) 10 mg/ml GM-CSF. Results shown are representative of independent experiments. (E) Time course for absorption of exogenous GM-CSF by peripheral blood mononuclear cells (PBMCs) from the patient or a control subject. Cells were incubated as described in methods section. Results are expressed as a percentage of initial GM-CSF concentration. Data are expressed as mean±SE. GM-CSF in the culture supernatant was unchanged up to 48 h in the patient PBMCs, whereas it was rapidly decreased in the control, indicating that GM-CSF was not absorbed by the patient’s cells.

**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. 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. However, the defect in surfactant clearance was significantly milder in CSF2RB targeted mice than in GM-CSF deficient mice. In this regard, Ding et al hypothesised that multiple intracellular signalling pathways are being activated by GM-CSF binding to the GM-CSF-Rβ alone.

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-Rβc. 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 was evaluated by western blotting. PBMCs from the patient or a control subject were incubated with IL-3 (0–10 ng/ml). Results shown are representative of independent experiments.

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.

Figure 2 Effect of granulocyte/macrophage colony-stimulating factor (GM-CSF) on functions of leucocytes, and expression and genetic analyses of GM-CSF-Rβc. (A) GM-CSF stimulated phosphorylation of STAT5 in peripheral blood mononuclear cells (PBMCs). PBMCs from the patient or a control subject were incubated with GM-CSF (0–1000 ng/ml). Phosphorylated STAT5 (pSTAT5) was detected by western blotting. As positive controls, total STAT5 (STAT5) and actin were detected. Results shown are representative of independent experiments. (B) IL-3 stimulated phosphorylation of STAT5 in PBMCs was evaluated by western blotting. PBMCs from the patient or a control subject were incubated with IL-3 (0–10 ng/ml). Results shown are representative of independent experiments. (C) Flow cytometry analysis for the expression of GM-CSF-Rα and βc on monocytes from a control (upper) and the patient (lower). (D) RT-PCR analysis of mRNA in PBMCs from a control (left) or the patient (right) using primers specific for cDNA of CSF2RA (lower) or CSF2RB (upper). (E) Detection of GM-CSF-Rβc (upper), α (middle) and actin (lower) on PBMCs by western blotting from a control (left) and the patient (right) using anti-GM-CSF-Rβc, α and actin antibodies, respectively. No band was observed in the patient (right lane) at the position of band for GM-CSF-Rα which was detected in the control (left lane), whereas bands for GM-CSF-Rα and actin were clearly detected in both the patient and the control. Enhanced chemiluminescence was used for the detection of the signal. Results shown are representative of independent experiments. (F) Comparison of PCR amplicons for the 14 exons (exon1–14) from genomic DNA of CSF2RB between a control and the patient.

(G) Nucleotide sequence of CSF2RB from a control, the patient and patient’s parents, encoding nt 626–637 (numbered relative to the initiation codon; GenBank accession NM_000395). (H) GM-CSF stimulated phosphorylation of STAT5 in PBMCs. PBMCs from a control, the patient’s father and mother were incubated with or without GM-CSF (10 ng/ml). pSTAT5 was detected by western blotting. As positive controls, total STAT5 and actin were detected. Results shown are representative of independent experiments.
Acknowledgements The authors thank Drs Bruce Trapnell, Takuji Suzuki and Jun Tohyama for helpful advice and discussions.

Funding This work was supported in part by grants from the Japanese Ministry of Education and Science, the Ministry of Health, Labor and Welfare of Japan (H15-trans-014), and the Japanese Society for the Promotion of Science (200390230, 20059130).

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.

Provenance and peer review Not commissioned; externally peer reviewed.

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