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CHOICE

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

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## 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-R $\beta$ c 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-R $\beta$ c 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.<sup>1</sup> PAP is clinically divided into three distinct forms: autoimmune, secondary and hereditary PAP.<sup>1</sup> 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.<sup>2–3</sup> Very rarely, there are unclassified cases in which neither GM-CSF autoantibodies nor underlying diseases are confirmed.<sup>4</sup> Of significance is the fact that mutant mice deficient in GM-CSF or its receptor spontaneously develop PAP<sup>5–6</sup> and some hereditary cases exhibit defects in GM-CSF receptor (GM-CSF-R) expression.<sup>7–8</sup>

GM-CSF-R is composed of the binding  $\alpha$  chain and the common  $\beta$  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-R $\alpha$  gene (*CSF2RA*) in hereditary PAP cases, which is located in the pseudoautosomal region of PAR1 on the X and Y chromosomes.<sup>7–8</sup>

Dirksen *et al* reported GM-CSF-R  $\beta$ c 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.<sup>9</sup> However, this mutation is now known as a single nucleotide polymorphism in normal subjects<sup>10</sup> (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.<sup>4</sup>

### 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  $1 \times 10^5$  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 U/ml penicillin in a humidified atmosphere of 5% CO<sub>2</sub> 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  $1 \times 10^6$  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% CO<sub>2</sub> 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.<sup>7</sup>

### Immunoblotting

Blood mononuclear cells were cultured on 24-well culture plates at  $5 \times 10^5$  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.<sup>7</sup> Primary antibodies used for detection included anti-human GM-CSF-R $\beta$ c (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-human GM-CSF-R $\alpha$ , anti-human STAT5 (Santa Cruz Biotechnology), and anti-human phosphor-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.<sup>7</sup> 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-R $\alpha$ ) (eBioscience, San Diego, California, USA) and anti-human CD131 (common  $\beta$ ) (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).

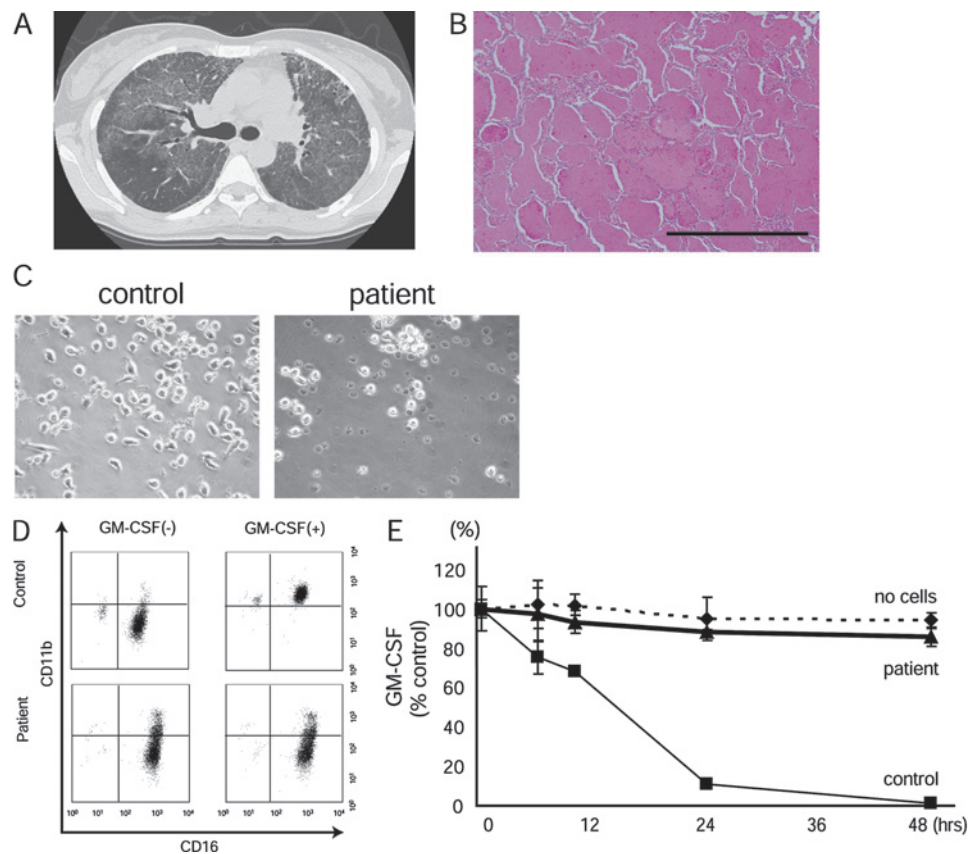
### RESULTS

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 PaO<sub>2</sub> 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).<sup>7</sup> 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-R $\beta$ c (figure 2B). Using flow cytometry, the expression of GM-CSF-R $\alpha$  on patient monocytes was comparable to the control; however, expression of GM-CSF-R $\beta$ c 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: AB573019) (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-glass 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 $\times$ ). 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  $\pm$  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.



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.<sup>11</sup>

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.<sup>7, 12</sup> 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  $\beta c$  ( $-/-$ ) mice, proliferating capacity of bone marrow cells under GM-CSF stimulation was intact in heterozygous ( $+/-$ ) mice compared with that in wild-type ( $+/+$ ) mice.<sup>6</sup> 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

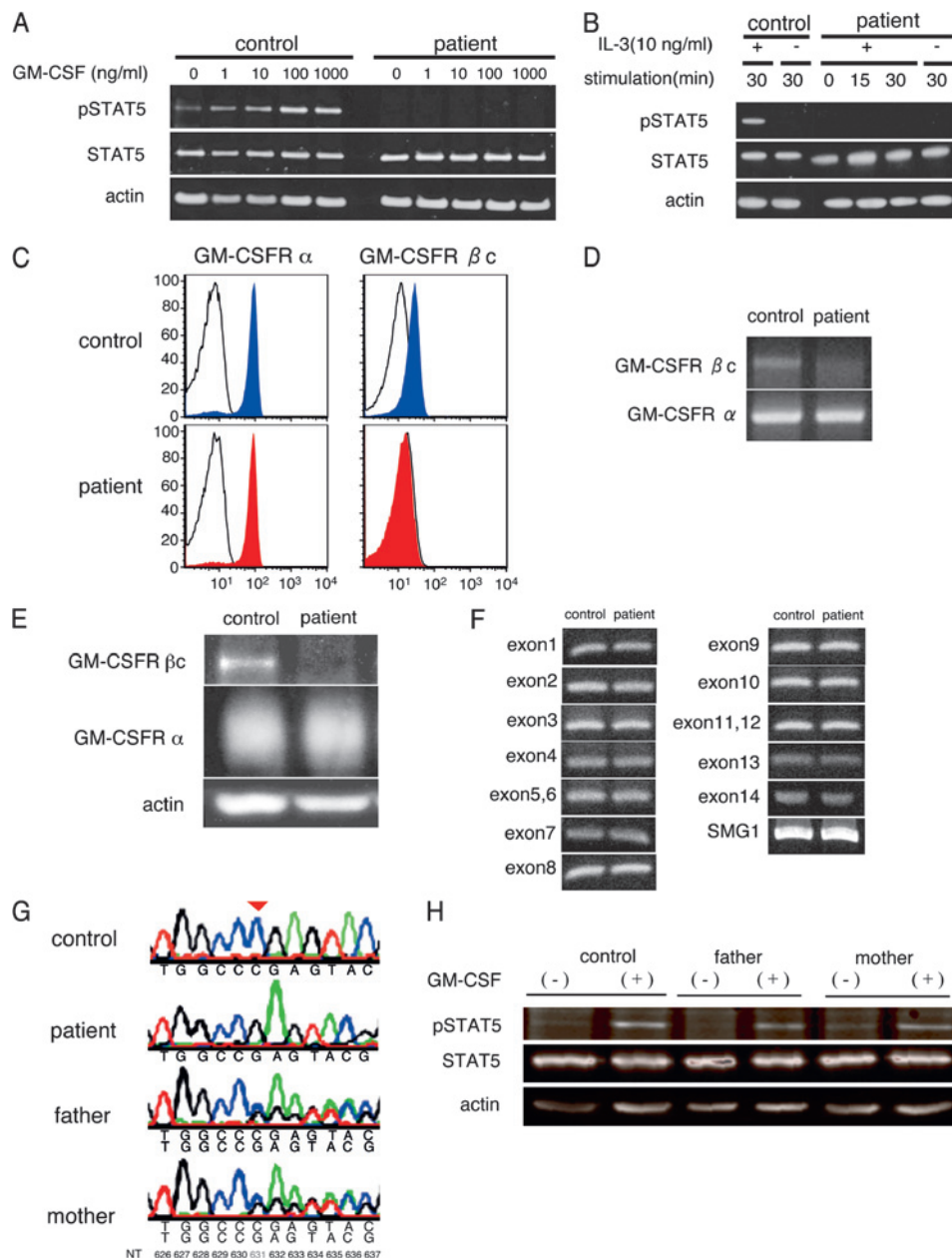
This is the first report identifying a gene defect in *CSF2RB* causing GM-CSF-R $\beta c$  deficiency and a signalling defect which caused adult-onset hereditary PAP. In a previous report, flow cytometry evaluation showed that four out of eight cases of hereditary PAP had complete GM-CSF-R $\beta c$  deficiency, whereas the remaining four cases showed reduced expression.<sup>9</sup> 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-R $\beta c$  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-R $\beta c$  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.<sup>13</sup> However, the defect in surfactant clearance was significantly milder in *CSF2RB* targeted mice than in GM-CSF deficient mice.<sup>14</sup> In this regard, Ding *et al* hypothesised that multiple intracellular signalling pathways are being activated by GM-CSF binding to the GM-CSF-R $\alpha$  alone.<sup>15</sup>

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-R $\alpha$ . 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

**Figure 2** Effect of granulocyte/macrophage colony-stimulating factor (GM-CSF) on functions of leucocytes, and expression and genetic analyses of GM-CSF-R $\beta$ c. (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-R $\alpha$  and  $\beta$ c 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-R $\beta$ c (upper),  $\alpha$  (middle) and actin (lower) on PBMCs by western blotting from a control (left) and the patient (right) using anti-GM-CSF-R $\beta$ c,  $\alpha$  and actin antibodies, respectively. No band was observed in the patient (right lane) at the position of band for GM-CSF-R $\beta$ c which was detected in the control (left lane), whereas bands for GM-CSF-R $\alpha$  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.



the absence of infectious disease. In this regard, the late onset of PAP in the present case was distinctive and unique among  $\beta$ c deficiency.

Taking the previous report into consideration,<sup>7</sup> 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-R $\alpha$  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.<sup>12</sup> 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.<sup>16</sup>

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.<sup>17</sup> Fukuno *et al* described a case of PAP secondary to myelodysplastic syndrome that resolved after successful cord blood transplantation.<sup>18</sup> 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.<sup>19</sup>

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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.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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