Pulmonary alveolar proteinosis caused by deletion of the GM-CSFRα gene in the X chromosome pseudoautosomal region 1

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Pulmonary alveolar proteinosis (PAP) is a rare lung disorder in which surfactant–derived lipoproteins accumulate excessively within pulmonary alveoli, causing severe respiratory distress. The importance of granulocyte/macrophage colony–stimulating factor (GM-CSF) in the pathogenesis of PAP has been confirmed in humans and mice, wherein GM-CSF signaling is required for pulmonary alveolar macrophage catabolism of surfactant. PAP is caused by disruption of GM-CSF signaling in these cells, and is usually caused by neutralizing autoantibodies to GM-CSF or is secondary to other underlying diseases. Rarely, genetic defects in surfactant proteins or the common β chain for the GM-CSF receptor (GM-CSFR) are causal. Using a combination of cellular, molecular, and genomic approaches, we provide the first evidence that PAP can result from a genetic deficiency of the GM-CSF α chain, encoded in the X-chromosome pseudoautosomal region 1.

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Pulmonary alveolar proteinosis (PAP) is a rare disorder of the lung caused by impaired surfactant homeostasis and is clinically characterized by the accumulation of lipoproteinaceous material within alveolar spaces, often leading to respiratory failure (1). Three forms of PAP have been described: congenital, secondary, and acquired. Congenital PAP can result from mutations in genes encoding the surfactant proteins B or C, or the common β chain (βc) of the receptor for GM-CSF (2–6). Secondary PAP develops in conditions in which there are reduced numbers or functional impairment of pulmonary alveolar macrophages, and has been associated with the inhalation of inorganic dust (silica) or toxic fumes, hematologic malignancies, pharmacologic immunosuppression, certain infections, and impaired βc expression (7–11). Acquired PAP is the most common form, usually occurring in adults, and is caused by neutralizing autoantibodies to GM-CSF (12–14).

The online version of this article contains supplemental material.

The importance of GM-CSF in the pathogenesis of PAP has been confirmed in humans and mice, wherein GM-CSF signaling is required for pulmonary alveolar macrophage catabolism of surfactant (12–17). In addition, mice with a targeted disruption of GM-CSF or βc genes developed PAP (15–17). Local expression of GM-CSF in the lungs of GM-CSF–deficient mice, or transplantation of bone marrow from normal mice into βc-deficient mice, corrected the defective metabolism of surfactant (18–19). Furthermore, administration of GM-CSF has been efficacious in some patients with acquired PAP (20).

Because the GM-CSFR is composed of a cytokine-binding GM-CSFR α chain (GM-CSFRα) subunit and the βc subunit (21,22), theoretically, deficiency of either subunit should

have the potential to result in PAP. We provide the first report that PAP can result from a genetic deficiency of the GM-CSFRα.

RESULTS AND DISCUSSION
Case history
A 4-yr-old female with Turner syndrome and respiratory insufficiency was diagnosed with PAP at age 3. Her past history was significant for respiratory failure caused by respiratory syncytial virus pneumonia in the first month of life, and a diagnosis of reactive airways disease. She presented with respiratory distress and hypoxemia, with a "crazy paving" pattern on chest imaging. Open lung biopsy revealed alveolar proteinaceous material without alveolar epithelial hyperplasia or chronic interstitial changes, and bronchoalveolar lavage revealed proteineous material and foamy macrophages, all of which are consistent with PAP. Analyses for mutations of the genes encoding surfactant proteins B and C and ABCA3 were negative (performed by the Johns Hopkins DNA Diagnostic Laboratory). Her serum GM-CSF concentration measured by ELISA (performed by the University of Michigan Cytokine Reference Laboratory) was 1,573.3 pg/ml (normal = 0–7.8 pg/ml), and anti–GM-CSF antibody concentration measured by ELISA (performed by the Cleveland Clinic Cytokine Biology Laboratory) was 0 ng/ml. Her complete

blood count and differential were normal. Worsening respiratory failure was managed intermittently by whole lung saline lavage under arteriovenous extracorporeal membrane oxygenator support, but pulmonary function progressively declined. Treatment with 20 µg/kg GM-CSF per day subcutaneously, which may be efficacious in some patients with acquired PAP (20), was not beneficial. Given the absence of surfactant gene mutations and anti–GM-CSF antibodies, the elevated baseline GM-CSF serum levels and the lack of clinical improvement with exogenous GM-CSF administration, and no evidence for other secondary causes of PAP, the potential for a defect in the GM-CSFR was investigated.

Lack of GM-CSFRα expression and function in the patient's monocytes
To test the hypothesis that the patient had a defect in GM-CSFR expression, flow cytometry was used to detect GM-CSFRα and βc on peripheral blood monocytes of the patient, as well as her father, mother, and sister. The mother expressed high levels of GM-CSFRα on virtually all of her monocytes, as demonstrated in Fig. 1 A by the single peak of staining on her CD14+ cells, as expected (23). In contrast, GM-CSFRα was undetectable on the patient's monocytes (Fig. 1 A). By comparison, the father and sister demonstrated bimodal patterns of GM-CSFRα expression on their monocytes (Fig. 1 A),
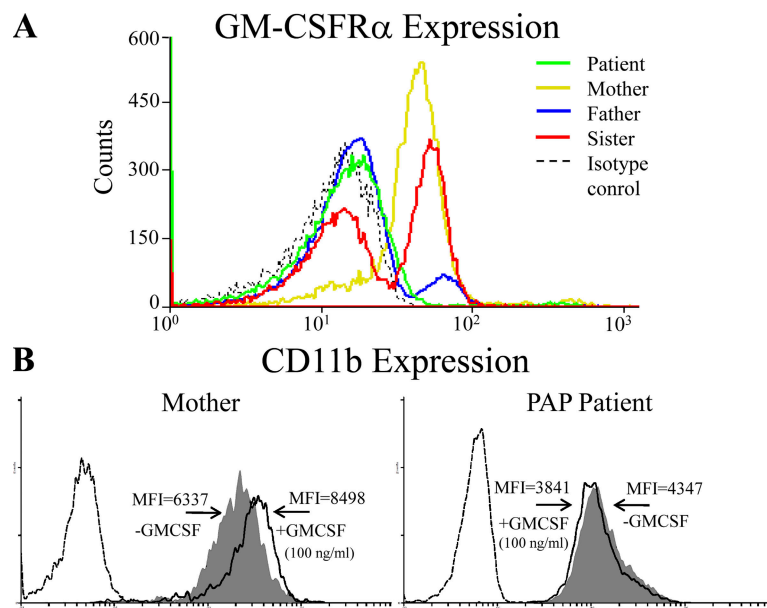

Figure 1. Absence of GM–CSFRα expression and responsiveness by the PAP patient's peripheral blood granulocytes. (A) CD14+ peripheral blood monocytes from the patient and family members were analyzed for the cell-surface expression of GM-CSFRα by flow cytometry. The dashed line is the isotype-negative control antibody. The patient does not express GM-CSFRα (green). The mother's monocytes give a single peak for GM-CSFRα expression (yellow). The father's and sister's monocytes give a bimodal pattern of staining for GM-CSFRα expression (blue and red). (B) Up-regulation of CD11b expression by stimulation of the patient's and mother's peripheral blood granulocytes with GM-CSF was measured by flow cytometry. The histograms demonstrate constitutive expression of CD11b by granulocytes from both the mother and the patient (shaded). Stimulation with 100 ng/ml GM-CSF resulted in up-regulation of CD11b on the mother's granulocytes, but not on the patient's granulocytes (solid line). The dashed lines are the isotype-matched controls. The MFI from the histograms from the mother's and patient's granulocytes before and after stimulation with 50 and 100 ng/ml GM-CSF were used to calculate the SI, as previously described (reference 25). The data were reproducible in duplicate samples and in two separate experiments.

indicating that only a subpopulation of the father's and the sister's monocytes expressed GM–CSFRα. All subjects expressed cell-surface βc (unpublished data), and βc function in the patient was indicated by the presence of normal numbers of peripheral blood eosinophils, which differentiate in response to IL-5 signal transduction through the IL-5Rα and βc (24).

To determine whether the absence of GM-CSFRα expression by the patient was accompanied by functional unresponsiveness to GM-CSF, GM-CSF–induced up-regulation of CD11b on granulocytes of the patient and her mother were measured by flow cytometry (Fig. 1 B), as previously described (25). Constitutive expression of CD11b was greater on the mother's granulocytes (mean fluorescence intensity [MFI] = 6,337) than the patient's granulocytes (MFI = 4,347). The mother's granulocytes had a 22.4 stimulation index (SI) with 50 ng/ml GM-CSF (MFI = 7,759) and a 34.1 SI with 100 ng/ml GM-CSF (MFI = 8,498). In contrast, the patient's granulocytes did not up-regulate CD11b in response to GM-

CSF, with a −1.2 SI at 50 ng/ml GMCSF (MFI = 4,294) and a −11.6 SI at 100 ng/ml GM-CSF (MFI = 3,841).

Absence of GM-CSFRα mRNA transcripts by the patient's leukocytes

The patient's karyotype indicated a 46Xi(Xq) genotype in which the Xi chromosome appeared to be of normal length and hybridized a probe for the X-chromosome pseudoautosomal region 1 (X-PAR1) region (Fig. 2 A). In contrast, the Xq chromosome had a truncated Xp arm and did not hybridize the X-PAR1 probe.

To determine whether the absence of GM-CSFRα protein expression was accompanied by impaired expression of GM-CSFRα mRNA transcripts, RT-PCR was used. For comparison, transcripts were also amplified for βc and the other βc-associated receptor molecules (IL-3Rα and IL-5Rα), as well as mRNA transcripts from genes flanking the GM-CSFRα gene within the X-PAR1 region (thymic stromal lymphopoietin
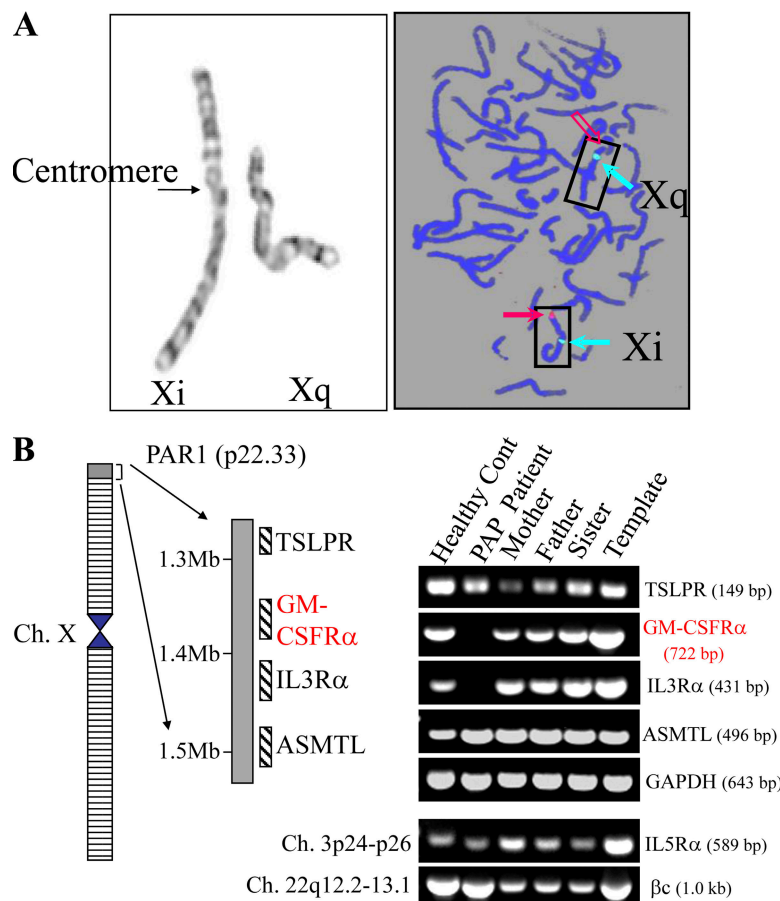

Figure 2. Molecular analysis of GM-CSFRα. (A, left) Karyotype analysis of the PAP patient demonstrating one grossly intact X chromosome (Xi) and one short X chromosome that is truncated in the P arm (Xq), characteristic of Turner syndrome. (A, right) FISH analysis of the PAP patient's chromosomes using a probe specific for the X centromere (teal) and a probe specific for the proximal PAR1 region of the X chromosome (red probe). The PAR1 probe hybridizes to the Xi chromosome but not the Xq chromosome. (B, left) Illustration of a subregion of the XPAR1, band p22.33. Highlighted in red is the GM-CSFRα gene, which is located between the genes for the TSLPR and the IL-3Rα, which is followed by the gene encoding *ASMTL* (B, right) RT-PCR analysis of the genes examined in PAR1 region, as well as genes encoding IL-5Rα (Ch. 3p24-p26) and βc (Ch. 22q12.2-13.1). The data were reproducible in three out of three experiments.

receptor [TSLPR] and acetylserotonin methyltransferase [ASMTL]). Results from the patient's leukocytes were compared with results from her family members and an unrelated healthy control (Fig. 2 B). The patient's family members and the control subject each expressed all of the transcripts. In contrast, transcripts for GM-CSFRα and IL-3Rα were not detected for the patient, whereas the other transcripts were detected. These data suggested a potential defect in the patient's X-PAR1 that affected both the GM-CSFRα and IL-3Rα genes.

Deletion of the patient's GM-CSFRα exons 5–13

To directly analyze the integrity of the GM-CSFRα gene structure, PCR amplification of each of the 11 exons encoding GM-CSFRα (exons 3–13) was performed (Fig. 3). In addition, a primer set for exon 8 of the *SMG1* gene on chromosome 16 was included as an internal PCR control (627 bp). Results with the patient's DNA were compared with those with DNA from three control cell lines. Although all 11 coding exons of GM-CSFRα were detected in each of the controls, only the first two coding exons (exons 3 and 4) of GM-CSFRα were detected in the patient's DNA; exons 5–13 were absent.

This is the first report of PAP resulting from a genetic deficiency of GM-CSFRα. Clinical expression of PAP caused by a genetic deficiency of GM-CSFRα would predictably re-

quire homozygous defects involving both of the GM-CSFRα alleles. However, because the GM-CSFRα gene is encoded in the PAR1 region of the X and Y chromosomes (26), Turner syndrome, which is caused by the absence or partial deletion of one of the X chromosomes (27), provides the biological circumstance of nature whereby a defect in one GM-CSFRα allele could result in the PAP phenotype.

The absence of mRNA transcripts for GM-CSFRα and IL-3Rα indicated that the PAR1 region of the Xi chromosome was abnormal, despite the positive hybridization signal from the fluorescence in situ hybridization (FISH) experiment. The presence of mRNA transcripts for TSLPR and ASMTL, whose genes flank the genes for GM-CSFRα and IL-3Rα, respectively, suggested a genomic deletion that affected ∼1 mb. Examination of the 11 coding exons for GM-CSFRα demonstrated deletion of exons 5–13, providing a genetic basis for the absence of mRNA transcripts and protein for GM-CSFRα. Because the IL-3Rα message was not detected in the patient, the genetic deletion affecting GM-CSFRα likely extends to the gene encoding IL-3Rα. However, impaired IL-3 responses do not result in a PAP phenotype in mice (17) and have not been associated with PAP in humans.

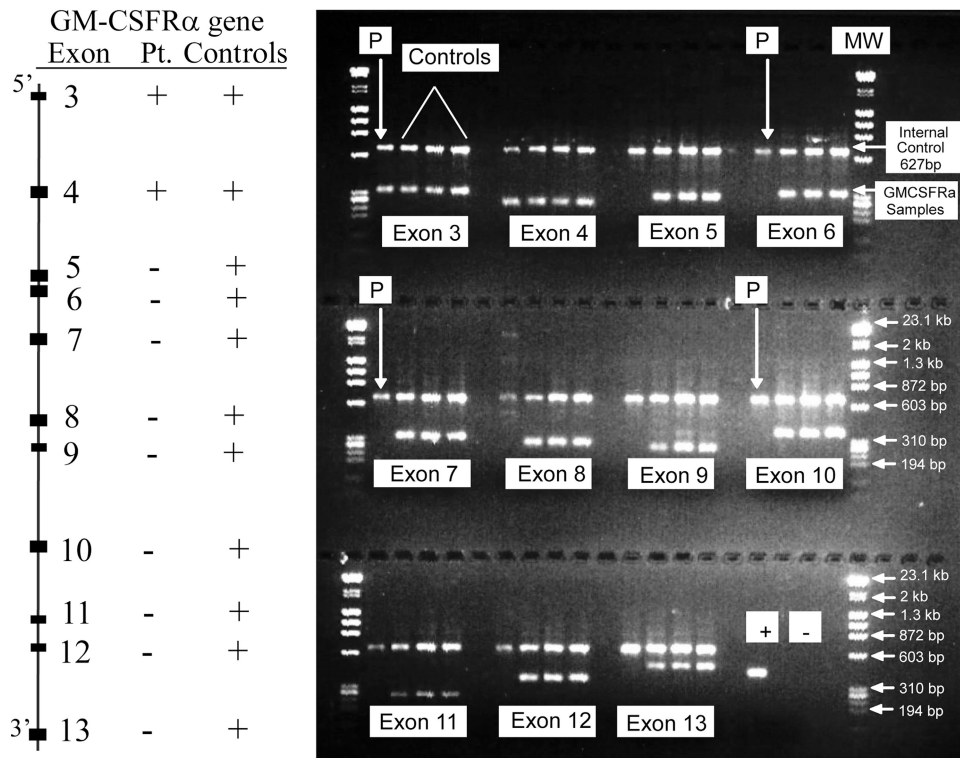Although the bimodal pattern of GM-CSFRα protein expression by the patient's father and sister was not accompanied

Figure 3. Genomic analysis of GM–CSFRα. (left) Genomic structure of the GM-CSFRα gene locus depicting the 11 coding exons (black boxes) and intronic regions (black line). (right) PCR amplification of the 11 exons (exons 3–13) encoding the GM-CSFRα gene using genomic DNA isolated from the PAP patient's leukocytes. Each GM-CSFRα primer set was designed to generate amplicons of 200–350 bp (bottom band), whereas the internal standard (*SMG1*, Ch. 16) was 627 bp (top band present in all lanes). The first lane in each group of amplicons is the PAP patient's DNA sample (P); the remaining three lanes are positive controls. Exons 3 and 4 are present, but exons 5–13 are missing from the patient's genomic DNA. The data were reproducible in three out of three experiments.

by a clinical phenotype, this raises the possibility that both the father and sister are carriers of a PAR1 partial deletion. If so, then such a deletion resides on the paternal X-PAR1, and not on the Y-PAR1, and would be consistent with the patient's Xi being of paternal origin. Alternatively, the patient's Xi-PAR1 deletion may have occurred de novo in the context of recombination and genomic rearrangement that resulted in the Turner genotype.

The PAR1 is essential for meiotic pairing and recombination of sex chromosomes (26, 28). Although all genes within the PAR1 escape X inactivation and are therefore candidates for haploinsufficiency disorders, the only previously known disease gene within the PAR1 is the short-stature homeobox (28). Although the PAR1 has a high frequency of recombination, the subregion encoding the GM-CSFRα gene apparently has a relatively low level of recombination (28). The frequency of mutations in GM-CSFRα that impair physiological responses to GM-CSF is unknown, but is likely to be rare even in large populations because the prevalence of PAP is low. However, the potential importance of GM-CSF responsiveness for granulocyte and pulmonary alveolar macrophage function leaves open the possibility that some degree of immune dysfunction could occur in the context of partial unresponsiveness to GM-CSF. Indeed, the recent demonstration of an impaired ability of granulocytes to respond to GM-CSF in autoimmune PAP (25) was confirmed in our patient by the inability of her granulocytes to up-regulate expression of CD11b after GM-CSF stimulation. Moreover, the immediate adjacency of the genes for TSLPR, GM-CSFRα, and IL-3Rα, all of which encode for receptors of relevance to allergic inflammation, also raises the intriguing possibility that mutations within this PAR1 region may have implications for the pathophysiology of allergic disorders.

The importance of GM-CSF signaling in PAP is underscored by the experimental observation that mice with a targeted disruption of GM-CSF or βc genes developed PAP (15–17). In addition, local expression of GM-CSF in the lungs of GM-CSF–deficient mice, or transplantation of bone marrow from normal mice into βc-deficient mice, corrected the defective metabolism of surfactant (18–19). GM-CSFRα–deficient mice have not been reported.

Diagnosing PAP caused by a genetic deficiency of GM-CSFRα has important therapeutic implications because, theoretically, bone marrow reconstitution from a healthy donor should result in leukocytes that express the GM-CSFRα and thereby a cure for the impaired surfactant homeostasis that underlies PAP, especially because such a strategy has been successfully demonstrated in mice with βc deficiency (19). Our patient underwent bone marrow reconstitution with an HLA-matched unrelated donor but died from a respiratory infection 4 wk after transplant, before recovery of immune competency. Nonetheless, the ability to screen for GM-CSFRα deficiency by flow cytometry provides a convenient test for evaluating infants with PAP not explained by other causes of GM-CSF unresponsiveness, and can be confirmed by molecular methods.

MATERIALS AND METHODS

Study subjects. The Institutional Review Board of Baylor College of Medicine approved this study. All subjects or their legal guardians gave written informed consent, and minors gave assent. In addition to the patient, study subjects included healthy unrelated controls and the patient's immediate family, which consisted of her mother and father, and a sister, all of whom were healthy. Blood was obtained in EDTA tubes from all subjects and was used immediately for analyses. In brief, the red blood cells were hypotonically lysed and the leukocytes were resuspended in PBS containing 2% FCS.

Flow cytometry. GM-CSFRα cell-surface expression on peripheral blood monocytes was analyzed on a flow cytometer (model XL; Beckman Coulter). Monocytes were identified using mouse mAb anti–human CD14–Alexa Fluor 488 (M5E2; BD). GM-CSFRα expression on monocytes was detected using mouse IgG1 mAb anti–human GM-CSFRα (4H1; Santa Cruz Biotechnology, Inc.) and rat mAb anti–mouse IgG1-PE. Background was determined using an isotype-matched negative control mAb (MOPC-21; BD). Data were analyzed using Summit software (Dako).

GM-CSF up-regulation of CD11b expression. Peripheral blood granulocytes were analyzed by flow cytometry for GM-CSF up-regulation of CD11b expression, as previously described (25), using mouse mAb anti–human CD11b (ICRF44; BD). In brief, freshly isolated white blood cells were either unstimulated or stimulated in vitro with 50–100 ng/ml human GM-CSF for 30 min. Cells were labeled with anti-CD11b–PE antibodies and gated on the granulocyte population, and CD11b expression was measured by flow cytometry. The GM-CSF–induced CD11b SI on granulocytes was calculated by taking the MFI of CD11b on GM-CSF–stimulated granulocytes minus the MFI of CD11b on unstimulated granulocytes, divided by the MFI of CD11b on unstimulated granulocytes, multiplied by 100, as previously described (25). Data were plotted using Excel software (Microsoft).

Karyotype and FISH analysis. High-resolution G-banded metaphase chromosome analysis was performed on the patient's peripheral blood lymphocytes. FISH was performed to detect the proximal region of the X-PAR1 using bacterial artificial chromosome (BAC) probe RP11-74L17, which hybridizes at 1.781–1.929 mb on Xp22.3. X chromosomes were identified using a BAC probe specific for the X centromere. Miniprep BAC DNA (100 ng) was labeled with Spectrum Orange-dUTP or Spectrum Green-dUTP (Vysis) and used as probes for FISH analysis, as previously described (29).

RT-PCR. Total RNA was extracted from peripheral blood leukocytes from the patient, family members, and an unrelated healthy control using the RNeasy Mini kit (QIAGEN). 3 µg of total RNA was reverse transcribed with Oligo dT primers using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). 1 µl of the cDNA reaction was used to amplify each cytokine receptor and control gene with specific primers, for 35 cycles. PCR amplification conditions were as follows: 5 min at 95°C for 1 cycle; 45 s at 94°C for 35 cycles; 1 min at 60°C for 1 cycle; 2 min at 72°C for 1 cycle; and 12 min at 70°C for a final cycle (GeneAmp 9700 PCR System; Applied Biosystems). PCR products were analyzed in a 1% (wt/vol) agarose gel containing ethidum bromide and were visualized by fluorescence. The PAP patient is missing mRNA transcripts for GM-CSFRα and IL-3Rα. Primer sequences are listed in Table S1 (available at http://www .jem.org/cgi/content/full/jem.20080759/DC1).

Amplification of the GM-CSFRα gene. Primer sets flanking each of the 11 coding exons (exons 3–13) of the GM-CSFRα (*CSF2RA*) gene were individually amplified. A primer set for exon 8 of the *SMG1* gene on chromosome 16 was used as an internal PCR standard. Each GM-CSFRα exon primer set was designed to generate amplicons of 200–489 bp, whereas the internal standard was 627 bp. Amplification of the patient's DNA and DNA from three normal control cell lines was performed with Multiplex mix (QIAGEN) using standard PCR conditions. A positive and a negative control for the PCR process were also performed using primers flanking exon 10 of the

ARAF gene on chromosome X and cell-line DNA (lanes +/−). Reactions were performed using 10 ng of each DNA, 3.2 pmol of each primer, and 40 cycles of amplification. Each sample was resolved by loading 4 μl of PCR product on a 2% agarose gel and was visualized by ethidium bromide fluorescence. The sizing ladder consists of both HaeIII-digested ΦX174DNA and HindIII-digested λDNA. Primer sequences are listed in Table S2 (available at http://www.jem.org/cgi/content/full/jem.20080759/DC1).

Online supplemental material. Primer sequences for RT-PCR of mRNA transcripts and for GM-CSFRα exons are provided in Tables S1 and S2, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080759/DC1.

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