

A hypomorphic recombination-activating gene 1 (*RAG1*) mutation resulting in a phenotype resembling common variable immunodeficiency

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Background: Recombination-activating gene 1 (*RAG1*) deficiency presents with a varied spectrum of combined immunodeficiency, ranging from a T⁺B⁺NK⁺ type of disease to a T⁺B⁺NK⁺ phenotype.

Objective: We sought to assess the genetic background of patients with common variable immunodeficiency (CVID).

Methods: A patient given a diagnosis of CVID, who was born to a consanguineous family and thus would be expected to show an autosomal recessive inheritance, was subjected to clinical evaluation, immunologic assays, homozygosity gene mapping, exome sequencing, Sanger sequencing, and functional analysis.

Results: The 14-year-old patient, who had liver granuloma, extranodal marginal zone B-cell lymphoma, and autoimmune neutropenia, presented with a clinical picture resembling CVID. Genetic analysis of this patient showed a homozygous hypomorphic *RAG1* mutation (c.1073 G>A, p.C358Y) with a residual functional capacity of 48% of wild-type protein.

Conclusion: Our finding broadens the range of disorders associated with *RAG1* mutations and might have important therapeutic implications. (*J Allergy Clin Immunol* 2014;134:1375-80.)

Key words: Common variable immunodeficiency, granulomatous lesion, recombination activation genes, differential diagnosis, mismanagement

The product of recombination-activating gene 1 (*RAG1*) is part of the complex responsible for initiating programmed DNA cleavage for rearrangement of the genes encoding the variable (V), diversity (D), and joining (J) segments of the immunoglobulin and T-cell antigen receptors in progenitor lymphocytes.^{1,2} *RAG1* might also have important functions subsequent to the generation of double-strand breaks by forming a transient cleaved signal complex with the recombining ends to facilitate DNA end processing and ligation by other nonhomologous end-joining proteins.³⁻⁵

The spectrum of primary immunodeficiency (PID) associated with *RAG1* mutations varies based on the nature of the defect and its quantitative and qualitative effects on V(D)J recombination. Mutations in *RAG1* account for approximately 10% of all cases of severe combined immunodeficiency (SCID), and nearly 60 cases have been reported to date.^{6,7} Complete *RAG1* deficiency (<1% activity of the wild-type protein) resulting from amorphic or null mutations of both alleles leads to an SCID phenotype with lack of T and B lymphocytes (T⁺B⁺NK⁺ SCID).^{8,9} Patients with this phenotype present with life-threatening infections and failure to thrive in early infancy. However, natural killer cell activity is not affected because of its independence from antigen receptor gene rearrangement.¹⁰⁻¹²

Patients with hypomorphic *RAG1* mutations that allow very low levels of recombination activity can present with different phenotypes characterized by residual development of T cells, B cells or both. These forms include Omenn syndrome (hepatosplenomegaly, lymphadenopathy, severe erythroderma, eosinophilia, increased serum IgE and autoantibody levels, colitis, and infiltration of oligoclonal populations of T cells),¹³⁻¹⁵ atypical Omenn syndrome (skin inflammation without T-cell expansion, low proportion of CD31 cells, and >3% B cells),¹⁶ and atypical SCID with $\gamma\delta$ T-cell expansion (autoimmunity, severe cytomegalovirus infection, and partially functioning B cells with a limited ability for antibody production).¹⁷⁻¹⁹ Finally, a delayed-onset form with generalized granulomatous lesions, severe varicella infection, normal T- and B-cell frequencies, progressive hypogammaglobulinemia, defective specific antibody production, and autoimmunity has recently been reported,^{6,20-22} which is caused by hypomorphic mutations that support significant residual levels of recombination activity. Some of the features reported in these patients overlap with those seen in patients with common variable immunodeficiency (CVID) and might lead to misdiagnosis and insufficient treatment based on immunoglobulin replacement

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Abbreviations used

CVID: Common variable immunodeficiency
 MALToma: Mucosa-associated lymphoid tissue lymphoma
 PID: Primary immunodeficiency
 RAG1: Recombination-activating gene 1
 SCID: Severe combined immunodeficiency
 SNP: Single nucleotide polymorphism

rather than curative hematopoietic stem cell transplantation. Here we report the case of a patient with RAG1 deficiency with granuloma who was initially given a diagnosis of CVID and treated as such.

METHODS**Clinical evaluation**

Informed consent for the studies performed was obtained from the patient's parents in accordance with the principles of the ethics committee of the Tehran University of Medical Sciences. An evaluation sheet was used to summarize patients' demographic information, including name, sex, date of birth, age of symptom onset, clinical symptoms, age at diagnosis, family history and consanguinity, previous history of medications and vaccinations, and laboratory data.

Immunologic assays

A complete blood count was evaluated by using a cell counter with anticoagulated whole blood. Serum IgG, IgA, and IgM levels were measured by means of turbidimetry (Behring Nephelometer; Behringwerke, Marburg, Germany), and lymphocyte subpopulations of CD3, CD4, CD8, and CD19 were counted by using flow cytometry (Partec PAS, Münster, Germany) at the time of the study. IgE levels were measured with an ELISA (Dr Fooko Laboratorien GmbH, Neuss, Germany).

Homozygosity mapping

Genomic DNA was extracted from whole blood by using standard methods. A genome-wide scan was conducted by means of homozygosity mapping with GENEHUNTER in which the patient was genotyped for a panel of 381 microsatellite markers. The Linkage Mapping Set MD-10 (Applied Biosystems, Foster City, Calif) formed the core marker set for the scan.

Exome sequencing

Library preparation and exome sequencing. Three micrograms of genomic DNA was randomly fragmented with the Covaris Acoustic System (Covaris, Woburn, Mass). Adapters were then ligated to both ends of the fragments. The adaptor-ligated DNA templates were purified by using Agencourt AMPure SPRI beads (Beckman Coulter, Fullerton, Calif), in which fragments with an insert size of approximately 200 bp were excised. Extracted DNA was amplified by using ligation-mediated PCR, purified, and hybridized to the Agilent Sure Select Human All Exon 50 Mb kit (Agilent Technologies, Santa Clara, Calif) for enrichment. The hybridized fragments were subsequently bound to streptavidin beads from which nonhybridized fragments were washed off after 24 hours. Captured ligation-mediated PCR products were subjected to quantitative PCR by using an Agilent 2100 Bioanalyzer to estimate the magnitude of enrichment. Each captured library was subsequently loaded on the HiSeq2000 Illumina sequencer (Illumina, San Diego, Calif), according to the manufacturer's protocol, and high-throughput sequencing was performed to reach the desired average sequencing depth.

Read mapping and variant analysis. After the raw image files were processed with Consensus Assessment of Sequence and Variation software (CASAVA, version 1.7) for base calling with default parameters, the sequences of each subject were generated as 90-bp paired-end reads. The

sequenced reads were aligned to the human genome reference (UCSC hg 19 version; build 37.1) with SOAP aligner (soap2.21) software.²³ Duplicated reads were filtered out, and only uniquely mapped reads were kept for subsequent analysis. SOAPsnp (version 1.03) software²⁴ was subsequently used with the default parameters to assemble the consensus sequence and call genotypes in target regions. For single nucleotide polymorphism (SNP) quality control, low-quality SNPs that meet one of the 4 following criteria were filtered out: (1) genotype quality of less than 20; (2) sequencing depth of the site of less than 4; (3) estimated copy number of greater than 2; and (4) distance from the adjacent SNPs of less than 5 bp. Small insertions/deletions were detected by using the Unified Genotype tool from GATK (version v1.0.4705)²⁵ after the high-quality reads were aligned to the human reference genome by using BWA (version 0.5.9-r16).²⁶

Analysis protocol for exome sequencing results.

Because the patient was born to a consanguineous family, he would be expected to show an autosomal recessive inheritance pattern. Thus we first filtered out the synonymous mutations and then eliminated the common variants (frequency >0.5% in dbSNP132, 1000 genomes, HapMap, and an in-house database). Nonsynonymous/splice-site changes and insertions/deletions were prioritized according to (1) the presence of homozygous or compound heterozygous variants based on a recessive inherited trait; (2) relation to PID disease-associated genes based on the RAPID Web site (<http://rapid.rcai.riken.jp/RAPID/index.html>); (3) predictive software scores for the likelihood for pathogenicity (eg, Polyphen-2 and SIFT); and (4) homozygosity mapping analysis²⁷ for detection of the homozygous region to further narrow down candidate genes.

Sanger sequencing

Twenty nanograms of genomic DNA was used in PCR by using exon-specific primers (forward primer, CTTTCAGCCAACTTGCAGCTC; reverse primer, CCTCTTTGCAATGGTGTCCAC) with the following conditions: 95°C for 3 minutes, followed by 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute for 38 cycles, and a final extension of 72°C for 7 minutes. The PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Stockholm, Sweden) and subsequently sequenced at the Macrogen Company (Seoul, South Korea) and Eurofins MWG Operon (Munich, Germany). The sequences were analyzed with the Lasergene software package (DNASTar, Madison, Wis).

Functional analysis

Recombination activity of the RAG1 mutant protein was analyzed by using a flow cytometry-based assay, as previously described.²⁸ Results were expressed as a percentage of recombination activity of the wild-type protein.

RESULTS

The patient was born to first-degree consanguineous parents in 1989 (Fig 1), with no history of PID in his family. He had a Persian ethnicity and presented at 4 years of age with recurrent pneumonia. He received the regular vaccines included in the Iranian children's program (eg, oral polio and measles, mumps, and rubella) without any complications. He subsequently had lymphoproliferative manifestations, including retroperitoneal para-aortic lymph node hyperplasia and hepatosplenomegaly (Coombs, Wright, and Widal test results were negative and the glucose-6-phosphate dehydrogenase level was normal), leading to pancytopenia. Serologic investigations did not show any direct anti-globulin or anti-platelet antibodies. *Leishmania donovani* bodies in his bone marrow biopsy specimen were documented, and meglumine antimoniate (Glucantime) was administered (20 mg/kg/d) for 10 days.

During the following 6 years, he was admitted to the hospital because of severe varicella infection and subsequent pneumonitis and pulmonary tuberculosis. Despite isolation of *Mycobacterium tuberculosis* bacillus from sputum samples,

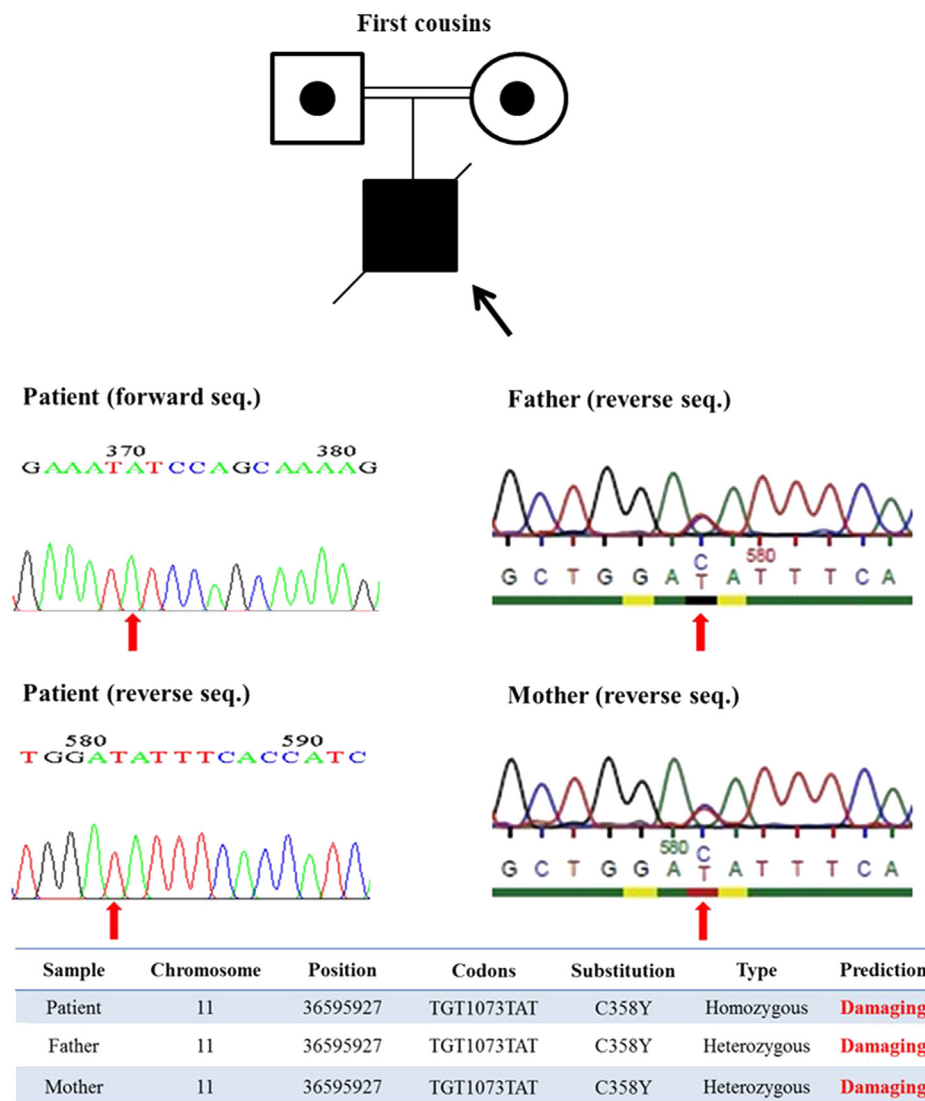


FIG 1. Pedigree and mutation analysis of a RAG1-deficient patient presenting as having CVID (arrows).

delayed hypersensitivity test to PPD was negative. Liver biopsy was performed because of long-term hepatomegaly refractory to treatment, which revealed granulomatous hepatitis. The immunologic profile of this patient was evaluated at 10 years of age and demonstrated hypogammaglobulinemia (IgG, 320 mg/dL; IgM, 30 mg/dL; IgA, 15 mg/dL; blood type O yet with an absence of anti-A and anti-B isohemagglutinins), a largely preserved distribution of T-cell and natural killer cell subsets but with a B-cell lymphopenia (CD3, 78.2%; CD4, 55.2%; CD8, 20.0%; CD19, 5.7%; CD20, 3.11%; CD20, 5.7%; CD16, 11.9%; and CD56, 6.9%) and normal neutrophil function and complement levels (Nitro Blue Tetrazolium test, 90%; 50% of total complement hemolytic activity, 96%; C3, 79 mg/L; C4, 25 mg/L; Table I). According to these data and the clinical and laboratory criteria of PID suggested by the International Union of Immunological Societies Expert Committee on PID, a tentative diagnosis of CVID was made and treatment with intravenous immunoglobulin was initiated.

Because of early finger clubbing, high-resolution computed tomography was performed and showed cylindrical bronchiectasis in the lower lobes of both lungs and atelectasis of the right

middle lobe. Pulmonary function tests revealed a mild mixed obstructive/restrictive pattern (FEV₁, 63.6%; forced vital capacity, 60%; maximum midexpiratory flow at 25% to 75% of forced vital capacity, 45%). CD19⁺CD27⁺IgD[−] memory B cells from the patient were 0.0017% of peripheral blood lymphocytes (0.03% of B cells), suggesting a reduced frequency of class-switch recombination.

Mutations in all known gene defects associated with CVID (including *CD19*; *CD20*; *CD21*; *CD81*; inducible costimulator [*ICOS*]; transmembrane activator, calcium-modulator, and cyclophilin ligand interactor [*TACI*]; *B-cell activation factor receptor* [*BAFFR*]; and LPS-responsive beige-like anchor [*LRBA*]) were ruled out by the exome sequencing data.

Homozygosity mapping suggested one linkage to a 71.68-Mb region on chromosome 11 (11p15.1-11q14.3, $P = 1.8 \times 10^{-42}$; Table II). Because this patient was born to a consanguineous family, he would be expected to show an autosomal recessive inheritance pattern. We first prioritized the exome sequencing results for nonsynonymous homozygous mutations, PID-associated genes, and mutations that were probably damaging, as justified by

TABLE I. Immunologic data of a CVID-like patient with RAG1 deficiency

Parameter	4 y	7 y	10 y	11 y	12 y	14 y	Reference range
Leukocytes (cells/ μ L)	8,000	2,400	4,000	3,400	2,100	1,200	4,300-11,400
Lymphocytes (% of leukocytes)	15	68	48	23	28	26	28-67
Neutrophils (% of leukocytes)	85	30	45	60	60	64	45-70
Hemoglobin (mg/mL)	8.4	8.1	9.6	9.9	10.0	10.3	12-15
Platelets (cells/ μ L)	75,000	66,000	59,000	79,000	120,000	110,000	150,000-500,000
Eosinophils (% of leukocytes)	2	2	1	1	8	8	0-3
Absolute T cells (cells/ μ L)	—	—	1,501	—	—	157	1,100-4,100
Absolute B cells (cells/ μ L)	—	—	109	—	—	50	200-1,400
Absolute natural killer cells (cells/ μ L)	—	—	228	—	—	105	70-120
IgG (mg/mL)	—	—	580*	260	320	450	600-1,500
IgM (mg/mL)	—	—	<40	20	<40	<20	50-370
IgA (mg/mL)	—	—	<20	10	<20	<10	80-380
IgE (mg/mL)	—	—	2	4	5	5	0-5
Isohemagglutinin titer	—	—	0	0	0	0	>1/8

*Time of start of treatment with intravenous immunoglobulin.

TABLE II. Homozygosity mapping of a patient with CVID with RAG1 deficiency

Chromosome	Length (Mb)	Start position	End position	P value
1	19.81	69650962	89465553	2.2×10^{-12}
1	16.85	95105523	111955492	6.1×10^{-11}
5	27.33	90383142	117710846	1.7×10^{-18}
8	16.81	107275147	124084081	6.5×10^{-13}
10	1.30	98646501	109945399	2.9×10^{-7}
10	10.65	121492422	132145048	1.4×10^{-7}
11*	71.68	20044296	91722855	1.8×10^{-42}
16	35.4	22048426	57446002	2.9×10^{-12}
18	5.92	555760	6478249	6.1×10^{-6}

Value in boldface is the highest statistically significant.

*Where we identified our novel homozygous RAG1 mutation.

predictive software. We subsequently found a novel homozygous mutation (c. G1073A, p. C358Y) in the *RAG1* gene, which is predicted to be probably damaging by using Polyphen 2 (<http://genetics.bwh.harvard.edu/pph2/>). This mutation was confirmed by means of Sanger sequencing in the patient (homozygous form) and his parents (heterozygous form), who were asymptomatic (Fig 1). Furthermore, we performed a functional test for RAG1, and the results were consistent with a mild phenotype, atypical SCID with granuloma with a residual recombination activity of $48.8\% \pm 0.6\%$ of wild-type protein, as measured by using an artificial green fluorescent protein substrate (Fig 2).

Despite regular follow-up and adequate immunoglobulin therapy, the patient progressed clinically with giardiasis and a mucosa-associated lymphoid tissue lymphoma (MALToma) at 11 years of age (refractory to eradication MALToma therapy), esophageal candidiasis, autoimmune neutropenia (absolute neutrophil count, $768/\mu$ L; perinuclear pattern anti-neutrophil cytoplasmic antibody, 92 IU/mL), atrophic tonsils and lymphopenia (absolute lymphocyte count, $312/\mu$ L), and moderate hydro-nephrosis at 13 years of age. The patient died at the age of 14 years of combined respiratory and renal failure.

DISCUSSION

To our knowledge, this case is the eighth patient with RAG1 deficiency in the world who presented with a phenotype of late-onset leaky SCID with granuloma and the one clinically most similar to CVID. Although the diagnosis of CVID is based on

exclusion of other known forms of PID, no case has been reported to have a presentation of a typical CVID as a consequence of RAG1 deficiency. Furthermore, it is not cost-effective to consider *RAG1* gene analysis in all patients with a diagnosis of CVID. Therefore, development of some hallmarks and clues might help physicians to select cases for mutation analysis.²⁹

Among the published patients, most were male (62.5%). The median age of onset was 2 years (range, 0.8-5 years), and the median age of granuloma formation was 6 years (range, 2.2-14 years). The late onset in these patients, which differs from onset in patients with other typical forms of SCID, is due to a gradual reduction in the number and function of B cells and the residual presence (and, to some extent, function) of T lymphocytes. This phenomenon leads to a delay in diagnosis of these cases to a median age of 7 years (range, 4-14 years) and clinical mismanagement.^{6,7,20-22,30}

The delayed onset of disease and milder clinical phenotype of these patients are consistent with residual levels of recombination activity of the mutant RAG1 protein (ranging from <5% to 50% of wild-type protein),^{6,7,20-22,30} which is sufficient to support partial preservation of a diversified T-cell repertoire. A similar leaky phenotype has also been demonstrated in mice homozygous for the S723C Rag1 mutation.³ Recombination activity of the C358Y RAG1 mutant protein was performed by using a recently described method in which flow cytometry-based measurement of green fluorescent protein expression serves as a rapid read-out of RAG1 function on an intrachromosomal substrate. With this assay, the C358Y RAG1 mutant had 48% recombination activity of wild-type RAG1. This result should not be confused with the condition in heterozygous healthy carriers of null *RAG1* mutations who express lower amounts of RAG1 protein that is functionally intact, enabling efficient V(D)J recombination and generation of a diversified T- and B-cell repertoire. By contrast, even 50% reduction of recombination activity at the protein level has been shown to affect generation of a diversified pool of V(D)J rearrangements at the endogenous *Igh* locus.²⁸

Although most reported patients carried compound heterozygous mutations (Fig 3),^{6,7,20-22,30} the current case is the fourth patient with a homozygous *RAG1* mutation. Indeed, efforts at finding a correlation between a given mutation and the clinical outcome have failed. However, there have been some reports in which the same mutation resulted in a different clinical picture

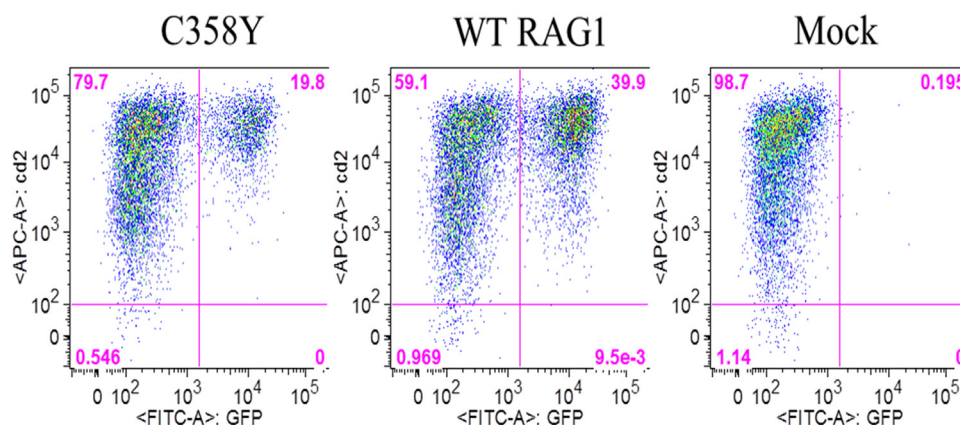


FIG 2. The A-Mul transformed $Rag1^{-/-}$ murine pro-B-cell line was first transduced with a retrovirus carrying a single copy of INV V(D)J substrate containing an inverted green fluorescent protein (GFP) flanked by recombination signal sequences. A clone with a single integrant was selected and transduced with a retrovirus coexpressing either human wild-type (WT) RAG1 and human CD2, mutant C358Y RAG1 and human CD2, or human CD2 alone. V(D)J recombination activity of the C358Y mutant was calculated by measuring GFP expression normalized to that observed in the presence of WT RAG1. One representative experiment of 3 is shown. APC, Allophycocyanin; FITC, fluorescein isothiocyanate.

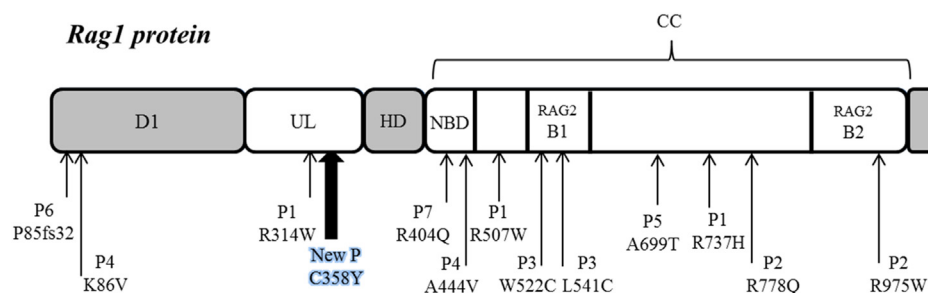


FIG 3. The location of RAG1 mutation in patients with leaky SCID and a granulomatous phenotype by compound heterozygous (P1-P4) and homozygous mutations (P5-P7 and New P). CC, Catalytic core; HD, homeodomain; NBD, nanomer binding domain; UL, ubiquitin ligase activity.^{6,7,20-22,30}

of SCID, which is suggestive of modifying roles of as yet unknown genetic or epigenetic mechanisms.^{7,16,31,32}

Although RAG1 defects are characterized by B-cell lymphopenia, 20% of patients, mostly those with consanguineous parents, present a B^+ phenotype with viral infections and BCG infection.⁷ In the patient reported here, circulating B cells were detectable but markedly reduced in number. Immunoglobulin serum levels in patients with atypical forms of RAG deficiency can be variable, ranging from normal³⁰ to selective IgA deficiency^{6,22} or agammaglobulinemia.²⁰ One patient with leaky SCID caused by compound heterozygosity for RAG2 mutations (p.T77N and C451A) has been reported and this patient was initially given a diagnosis of CVID.⁶ A variable number of peripheral B cells with clonotypic restrictions have also been observed in mice with homozygous hypomorphic *Rag1* mutations.^{3,33}

One of the most important shared features of leaky SCID and CVID in our case was immune dysregulation. Inflammatory and autoimmune manifestations, including autoimmune cytopenia,^{21,30} vitiligo,³⁰ and myasthenia gravis,²² have been reported in 57% of known leaky cases of RAG1 mutations associated with granuloma. Impaired receptor editing and defects of central and peripheral T-cell tolerance have been reported in patients and animal models with RAG deficiency and might account for the autoimmunity of the disease.^{34,35} Furthermore, inflammatory complications caused by increased IL-1 levels, a skewed

T_H2 cytokine profile, and oligoclonal expansion of T-cell clonotypes^{36,37} have been reported in 50% of these patients, causing organomegaly and lymphadenopathy.^{21,30}

Granulomatous lesions restricted to the skin or also involving other organs have been reported in the majority of patients with hypomorphic RAG1 mutations and a delayed-onset presentation.^{6,7,20-22,30,38} Although skin involvement was not documented in our case, this might still represent an important factor in the differential diagnosis between RAG1 deficiency and CVID because cutaneous granulomatous lesions are rare in the latter.³⁹ Moreover, granulomas in RAG1-deficient patients are mostly necrotizing,^{6,20,22,30} whereas patients with CVID more frequently show a noncaseating type.^{40,41} Nonetheless, the observation that not all patients with leaky RAG1 mutations manifest immune dysregulation indicates that epigenetic factors, environmental factors, or both play important roles in shaping the clinical phenotype.^{7,34}

Our patient had a MALToma, which has not been previously reported in patients with delayed-onset RAG1 deficiency. However, an EBV-induced lymphoma has been described in one case,⁶ and the overall clinical pattern of lymphoid malignancies in these patients is compatible with CVID.⁴² The pathophysiology of cancers in RAG1-deficient patients with leaky SCID can be explained by aberrant generation of broken DNA ends and premature release of the postcleavage complex, which might engage in inter-locus transrearrangements and activation of proto-oncogenes,

although this has not been clearly demonstrated in patients with CVID.^{3,6}

One of the most important points for differing CVID from RAG1 deficiency is the pattern of infection, in which severe systemic viral infections, especially varicella (in 83.3%), are more prevalent in RAG1-deficient patients with granuloma, suggesting a dysregulated response to viral antigens.⁴³

In summary, this case illustrates the need for careful assessment of patients with hypogammaglobulinemia and granulomatous lesions. Establishing a correct differential diagnosis between CVID and RAG deficiency (or other forms of leaky SCID) in such cases is essential to initiation of appropriate treatment and improvement of survival and quality of life.

Clinical implications: Patients with a diagnosis of hypogammaglobulinemia, granulomatous lesions, and a gradual decrease in B- and T-cell numbers should be investigated for RAG deficiency. In these patients it is essential to promptly initiate appropriate treatment with curative hematopoietic stem cell transplantation rather than immunoglobulin replacement.

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