

Rapid molecular diagnostics of severe primary immunodeficiency determined by using targeted next-generation sequencing

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Background: Primary immunodeficiency diseases (PIDDs) are inherited disorders of the immune system. The most severe form, severe combined immunodeficiency (SCID), presents with profound deficiencies of T cells, B cells, or both at birth. If not treated promptly, affected patients usually do not live beyond infancy because of infections. Genetic heterogeneity of SCID frequently delays the diagnosis; a specific diagnosis is crucial for life-saving treatment and optimal management.

Objective: We developed a next-generation sequencing (NGS)-based multigene-targeted panel for SCID and other severe PIDDs requiring rapid therapeutic actions in a clinical laboratory setting.

Methods: The target gene capture/NGS assay provides an average read depth of approximately 1000×. The deep coverage facilitates simultaneous detection of single nucleotide variants and exonic copy number variants in one comprehensive assessment. Exons with insufficient coverage (<20× read depth) or high sequence homology (pseudogenes) are complemented by amplicon-based sequencing with specific primers to ensure 100% coverage of all targeted regions.

Results: Analysis of 20 patient samples with low T-cell receptor excision circle numbers on newborn screening or a positive family history or clinical suspicion of SCID or other severe PIDD identified deleterious mutations in 14 of them. Identified pathogenic variants included both single nucleotide variants and exonic copy number variants, such as hemizygous nonsense, frameshift, and missense changes in *IL2RG*; compound heterozygous changes in *ATM*, *RAG1*, and *CMTA*; homozygous changes in *DCLRE1C* and *IL7R*; and a heterozygous nonsense mutation in *CHD7*.

Conclusion: High-throughput deep sequencing analysis with complete clinical validation greatly increases the diagnostic yield of severe primary immunodeficiency. Establishing a molecular diagnosis enables early immune reconstitution through prompt therapeutic intervention and guides management for improved long-term quality of life. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: Severe combined immunodeficiency, severe combined immunodeficiency newborn screening, next-generation sequencing, molecular diagnostics

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Abbreviations used

AT:	Ataxia telangiectasia
CGH:	Comparative genomic hybridization
CHARGE:	Coloboma, heart defects, choanal atresia, growth retardation, genital and/or urinary defects, ear anomalies and/or deafness
CNV:	Copy number variant
DGS:	DiGeorge syndrome
NK:	Natural killer
PIDD:	Primary immunodeficiency disease
SCID:	Severe combined immunodeficiency
SNV:	Single nucleotide variant
TREC:	T-cell receptor excision circle

Severe combined immunodeficiency (SCID) is one of the most severe primary immunodeficiencies and characterized by profound impairment of cellular and humoral immune responses.¹ Children with unrecognized and untreated SCID usually cannot live beyond infancy because of recurrent or life-threatening infections. However, if given a diagnosis within the first 3.5 months of life before infections develop, prompt treatment with hematopoietic stem cell transplantation or gene therapy can result in a greater than 90% survival rate. In addition to improved clinical outcomes, overall morbidity, mortality, and treatment costs are significantly reduced.²⁻⁵ Therefore the diagnosis of SCID is a medical emergency.

SCID is defined by the absence or significant deficiency of T lymphocytes. B cells can also be absent or nonfunctional in patients with SCID. During T-cell maturation in the thymus, small circular DNA episomes are generated as byproducts of T-cell receptor gene rearrangement. Therefore a low number of these T-cell receptor excision circles (TRECs) measured by using DNA isolated from dried blood spots in Guthrie card newborn screening specimens can serve as a surrogate biomarker for a potential diagnosis of SCID.^{6,7}

Since the Department of Health and Human Services recommended adding SCID to uniform newborn screening in May 2010, more than 30 states have begun screening for SCID, and additional states plan to offer the test shortly.⁸⁻¹¹ Pilot TREC screening programs have been assessed in European countries, such as France and the United Kingdom,^{12,13} and most recently, The Netherlands became the first nation in Europe to officially include SCID in its newborn screening program (<http://www.isns-neoscreening.org/news/123/2788/2015/04/25>).

The occurrence of SCID is estimated to be 1 in 58,000 births based on data from SCID newborn screening programs in 11 US states.¹⁴ This incidence rate approximately doubles the previous estimates of 1 in 100,000.¹⁵ More than 40 genes have been implicated to cause SCID and other severe primary immunodeficiency diseases (PIDDs; eg, familial hemophagocytic lymphohistiocytosis syndromes, zeta chain-associated protein of 70 kDa deficiency, MHC class II deficiency, and dedicator of cytokinesis 8 deficiency), and the list continues to expand.¹⁶⁻¹⁸ The most common type of SCID occurs because of deleterious mutations in the X-linked interleukin receptor common gamma chain gene (*IL2RG*) and accounts for 20% to 40% of cases.^{14,19-21} Defects in the IL-7 receptor α chain gene (*IL7R*) are the second most common cause of SCID.²¹ Molecular defects in the recombination-activating genes *RAG1* and *RAG2* affect the V(D)J recombination necessary for antigen receptor gene

assembly and represent the third most common form of SCID.²¹ Other causes of SCID include deficiency of the *ADA* gene, which encodes the enzyme adenosine deaminase in the purine catabolic pathway; the intracellular tyrosine kinase Janus kinase 3 gene (*JAK3*); and the Artemis gene *DCLRE1C* (DNA cross-link repair protein 1C) involved in V(D)J recombination/DNA repair.²¹ In addition, genetic disorders that can present with severe T lymphopenia at birth, such as coloboma, heart defects, choanal atresia, growth retardation, genital and/or urinary defects, ear anomalies and/or deafness (CHARGE) syndrome, DiGeorge syndrome (DGS), or trisomy 21, are also identified by using the TREC newborn screening assay.^{14,22-24}

The genetic heterogeneity of PIDDs makes a timely and accurate diagnosis challenging, hindering the prompt treatment of patients with SCID. The use of high-throughput next-generation sequencing (NGS) technologies to interrogate multiple genes simultaneously allows a rapid, cost-effective, genotype-based approach to molecular diagnosis.²⁵⁻²⁷ Here we report the high diagnostic yield of SCID and other severe PIDDs using targeted capture NGS-based comprehensive analyses.

METHODS

Specimens and sample preparation

Patient specimens were submitted to Baylor Genetics, previously Molecular Genetics Laboratories at Baylor College of Medicine, for NGS-based panel analyses. Samples with known mutations selected through the Center for Mendelian Genomics at Baylor College of Medicine were used for validation. The study was conducted under protocols approved for human subjects by the Baylor College of Medicine institutional review board. Additional DNA samples from cultured cells (Coriell Institute for Medical Research, Camden, NJ) and 1 normal saliva sample were also included for test development and validation.

Target capture and sequencing

Custom-designed NimbleGen SeqCap probes (Roche NimbleGen, Madison, Wis) were used for in-solution hybridization to enrich target sequences. Enriched DNA samples were indexed and sequenced on a HiSeq2000 sequencer (Illumina, San Diego, Calif) with 100 cycles of single end reads, according to the manufacturer's protocols. The insufficiently covered ($<20\times$) target exons were analyzed by using gene-specific amplicon-based Sanger sequencing.

Variant annotation and interpretation

Nucleotide changes observed in more than 5% of aligned reads were called and reviewed by using NextGENe software (SoftGenetics, State College, Pa). An RNA-oriented variant report setting and filter was used to examine variants in the noncoding RNA gene *RMRP*. Deleterious mutations and novel variants detected by using NGS were confirmed by means of Sanger sequencing.^{28,29} A coverage-based algorithm developed in-house, eCNVscan, was used to detect large exonic deletions and duplications.³⁰ The normalized coverage depth of each exon of a test sample is compared with the mean coverage of the same exon in the reference file, which is generated from 8 to 15 samples from the same or consecutive runs, to detect copy number variants (CNVs). Based on the validation results of array comparative genomic hybridization (CGH) experiments, ratios of less than 0.65 and greater than 1.35 are scored as deletions and duplications, respectively. All positive calls were further investigated and confirmed by using a second method.

RESULTS

Target enrichment and coverage

Coding regions and flanking intronic sequences of 191 genes associated with various primary immunodeficiency phenotypes

TABLE I. Genes in the severe PID panel and summary of capture and sequencing

NGS panel	Comprehensive SCID panel: 46 genes
Genes included	T-B+ SCID: <i>CD3D</i> , <i>CD3E</i> , <i>CORO1A</i> , <i>IL2RG</i> , <i>IL7R</i> , <i>JAK3</i> , <i>PTPRC</i> T-B- SCID: <i>ADA</i> , <i>AK2</i> , <i>DCLRE1C</i> , <i>LIG4</i> , <i>NHEJ1</i> , <i>PRKDC</i> , <i>RAG1</i> , <i>RAG2</i> Others: <i>AP3B1</i> , <i>CASP8</i> , <i>CD3G</i> , <i>CD8A</i> , <i>CHD7</i> , <i>CIITA</i> , <i>DOCK8</i> , <i>FOXN1</i> , <i>FOXP3</i> , <i>IKZF1</i> , <i>ITK</i> , <i>LCK</i> , <i>LYST</i> , <i>ORAI1</i> , <i>PNP</i> , <i>PRF1</i> , <i>RFK5</i> , <i>RFKANK</i> , <i>RFKAP</i> , <i>RMRP</i> , <i>SH2D1A</i> , <i>STAT5B</i> , <i>STIM1</i> , <i>STX11</i> , <i>STXBP2</i> , <i>TAP1</i> , <i>TBX1</i> , <i>TTC7A</i> , <i>UNC13D</i> , <i>XIAP</i> , <i>ZAP70</i>
No. of coding sequences	653
Target size	135,941 bp (coding exon sequences $\pm$ 20 bp)
Average coverage	1337 $\pm$ 374 per base
Enrichment	In-solution capture library (NimbleGen)
Sequencing information	Illumina HiSeq2000, 100 cycle, single end

and 5 genes located in the 22q11.2 deletion region, including the T-box gene *TBX1*, were enriched for sequencing (see Table E1 in this article's Online Repository at www.jacionline.org).³¹ Additional genomic sequences up to 320 bp upstream and 900 bp downstream of the mitochondrial RNA-processing endonuclease gene *RMRP* transcribed region, as well as more than 500 bp of the DNA sequences located after the stop codon of *IL2RG*, were also included in the capture library to allow detection of known pathogenic changes in these regulatory regions (see Fig E1, A, in this article's Online Repository at www.jacionline.org). From this library, 46 genes, including all known SCID-causing genes, with a total target size of 135,941 bases were clinically validated to create a severe PID panel (Table I). The average read depth was $1,337 \pm 374$ per base, and 97% of 653 coding sequences had a minimal read depth of greater than 100 $\times$. Only a total of 6 exons from 5 genes (*ADA*, the CD8 antigen alpha polypeptide gene *CD8A*, *JAK3*, the ORAI calcium release-activated calcium modulator 1 gene *ORAI1*, and *TBX1*) consistently showed insufficient coverage of less than 20 $\times$. In addition, a total of 15 exons of 5 genes (the adenylate kinase 2 gene *AK2*, the coronin 1A gene *CORO1A*, *DCLRE1C*, the lysosomal trafficking regulator gene *LYST*, and the signal transducer and activator of transcription 5B gene *STAT5B*) contain highly homologous sequences. These 21 exons are analyzed by means of gene-specific amplicon-based sequencing as a routing setting to ensure 100% coverage of all targeted regions.

Verification of known pathogenic single nucleotide variants and CNVs

We analyzed 13 DNA samples with known molecular defects and confirmed all deleterious variants (Table II). In addition to single nucleotide variants (SNVs), in patient S11, we identified a deletion of 10 nucleotides, 257_266delCAGCGCGGCT in *RMRP* (NR_003051), which was not analyzed previously. By using an NGS read depth-based CNV detection algorithm,³⁰ a heterozygous deletion encompassing *RAG1* and *RAG2* was detected in patient S12, who had known Potocki-Shaffer syndrome (OMIM#601224).

Although deleterious SNVs of *TBX1* have been reported,^{32,33} CNVs in Chr22q11.2 involving *TBX1* are more common causes of DGS.³⁴⁻³⁶ We have demonstrated the loss of one copy of *TBX1* in cell line NA17942 (patient S13), which is known to contain a 22q11.2 deletion (see Fig E1, B). Expanded copy number analysis with all genes included in the captured library revealed heterozygous deletion of 5 genes (the septin 5 gene

SEPT5, the glycoprotein Ib platelet beta subunit gene *GP1BB*, *TBX1*, the G protein subunit beta 1 like gene *GNB1L*, and *C22orf29*) mapped to the 22q11.2 DGS region.

Molecular analyses of infants with abnormal TREC newborn screening results or positive family history

Twenty infants ranging from 2 weeks to 14 months old were referred for severe PID panel analysis because of low TREC numbers in newborn screening tests ($n = 12$), family history ($n = 2$), or other signs of immunodeficiency ($n = 6$). Deleterious mutations or novel variants were identified and confirmed in 14 of the 20 patients (Table III).

Deleterious SNVs and small insertions/deletions detected. Hemizygous novel c.505C>T (p.Q169*) and frame-shift c.285_286dupTA (p.N96Ifs*52) variants were detected in the *IL2RG* gene of patients 1 and 2, respectively, which is consistent with the diagnosis of X-linked SCID and family histories of affected male subjects.

Patient 3, a male infant, was born with retinal coloboma, hypoplasia of heart chambers, coarctation of the aorta, choanal atresia, undescended testicles, and mild hydronephrosis, which is consistent with CHARGE syndrome. Newborn screening revealed undetectable TRECs and a complete absence of T cells. Severe PID panel analysis at 2 weeks of age identified a heterozygous nonsense mutation, c.7282C>T (p.R2428*), in the chromodomain helicase DNA binding protein 7 gene *CHD7* (NM_017780), confirming the clinical diagnosis. Patient 4 is a 3-week-old girl with a clinical presentation of T-B- natural killer (NK)+ SCID but no family history of immune deficiency. She was compound heterozygous for a pathogenic c.2924G>A (p.R975Q) variant (from the mother) and a novel c.1648G>A (p.D550N) missense variant (from the father) in *RAG1* (NM_000448). Patient 5 exhibited undetectable TRECS by means of newborn screening and a T-B+NK+ SCID phenotype. She was homozygous for a novel c.354C>A (p.C118*) stop codon mutation in *IL7R* (NM_002185).

Patient 6 is a 5½-month-old boy with low TREC numbers in newborn screening tests. The patient had T-cell lymphopenia and intermittent neutropenia. He had normal purine nucleoside phosphorylase enzyme activity and no evidence of maternal engraftment. NGS analysis of the 46 targeted PID genes detected 2 heterozygous changes, c.958C>T (p.R320W) and c.1556T>A (p.L519Q), in the forkhead box N1 gene *FOXN1* (NM_003593). The p.R320W mutation has been previously

TABLE II. Primary immunodeficiency–positive samples confirmed by using the targeted NGS-PIDD panel

Sample ID	Sex	Known diagnoses	NGS results*			
			Gene	Pathogenic variants	Zygosity	Inheritance
S1	M	CID, known <i>CORO1A</i>	<i>CORO1A</i>	c.248_249delCT (p.P83Rfs*11) c.1078delC (p.Q360Rfs*45)	Heterozygous	AR
S2	F	T–B+ SCID, known <i>JAK3</i>	<i>JAK3</i>	c.2297_2298delAGinsC (p.R767Efs*10) c.1204C>T (p.R402C)	Heterozygous	AR
S3	M	T–B+ SCID, Known <i>JAK3</i>	<i>JAK3</i>	c.452C>G (p.P151R) c.1924G>C (p.G642R)	Heterozygous	AR
S4	M	IPEX phenotype	<i>FOXP3</i>	c.737T>C (p.L246P)	Hemizygous	X-linked
S5†	M	IPEX phenotype	<i>FOXP3</i>	c.1147A>G (p.N383D)	Hemizygous	X-linked
S6†	M	IPEX phenotype	<i>FOXP3</i>	c.1147A>G (p.N383D)	Hemizygous	X-linked
S7	F	Mother of X-linked SCID proband	<i>IL2RG</i>	c.819delIT (p.I274Sfs*20)	Heterozygous	X-linked
S8	F	Mother of X-linked SCID proband	<i>IL2RG</i>	c.*308A>G	Heterozygous	X-linked
S9	F	Mother of X-linked SCID proband	<i>IL2RG</i>	c.184T>C (p.C62R)‡	Heterozygous	X-linked
S10	M	T–B–NK+ SCID, known <i>RAG1</i>	<i>RAG1</i>	c.2521C>T (p.R841W) c.2384A>G (p.D795G)	Heterozygous	AR
S11	F	CID with increased radiosensitivity	<i>ZAP70</i>	c.1505C>T (p.P502L) c.733G>A (p.G245R)	Heterozygous	AR
S12	M	Potocki Shaffer syndrome arr 11p13p11.2 (34056280-45112798)x1	<i>RMRP</i> <i>RAG1</i> and <i>RAG2</i>	257_266delCAGCGCGGCT One copy loss of <i>RAG1</i> and <i>RAG2</i>	Heterozygous	AR
S13 (NA17942)	M	DGS arr 22q11.21 (17030682-19792611)x1	<i>TBX1</i>	One copy loss of 5 genes, including <i>TBX1</i>	Heterozygous	AD

AD, Autosomal dominant; AR, autosomal recessive; CID, combined immunodeficiency; F, female; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; M, male.

*The previously reported deleterious changes are shown in boldface.

†Patients S5 and S6 are maternal half-brothers and thus both carry the same hemizygous c.1147A>G (p.N383D) change (NM_014009) in the DNA-binding forkhead domain of the *FOXP3* gene.

‡The T to G change at the same position, c.184T>G (p.C62G), is a reported pathogenic mutation (CM962691).

reported,³⁷ and p.L519Q is a rare variant with a frequency of 0.7% in the African population and less than 0.2% in the general population. Both changes affect highly conserved amino acids; arginine 320 is located in the forkhead DNA binding domain, and leucine 519 is in the C-terminal acidic activation domain.^{38,39} This patient showed no dysmorphic features and no skin, hair, or nail abnormalities, as expected from *FOXN1* deficiency. It seems possible that these 2 hypomorphic missense changes in *FOXN1* can only manifest T-cell lymphopenia.

Patient 7 harbored a heterozygous c.1275_1278delACTC (p.L426Tfs123*) novel frameshift change in the *FOXN1* gene and a heterozygous c.7538G>A (p.R2513Q) missense variant in the *CHD7* gene. The finding of the *CHD7* variant in the asymptomatic father argues against its role as the cause of the profound T-cell lymphopenia observed in patient 7. Lack of a second molecular lesion in *FOXN1* leaves the molecular explanation of disease as an open question for this patient. In addition, patient 7 did not have alopecia, as often seen in patients with *FOXN1* deficiency.

Patient with mosaic SCID. A pathogenic missense variant c.202G>A (p.E68K) was found in *IL2RG* in a 6-week-old boy (patient 8). The variant was observed in 410 (93.2%) of 440 total reads, with a consistent 6.8% of the wild-type c.202G allele (Fig 1, A), which was barely detected by using Sanger sequencing but clearly confirmed by amplicon-based NGS analysis. Additional low levels (2% to 6%) of mosaicism were also observed in other polymorphic loci, supporting the mosaic condition.⁴⁰ This patient was identified by SCID newborn screening with undetectable TRECs.

Novel variants identified. A 4-week-old boy (patient 9) who had few TRECs on SCID newborn screening was found to

have a hemizygous novel c.173C>G (p.P58R) variant in exon 2 of *IL2RG*. The p.P58R change is located at the *IL2RG* C-terminal extracellular domain, where several pathogenic missense mutations have been reported.²⁰

Patient 10, a 3-week-old male infant, had absent TRECs on newborn screening and an abnormal immune phenotype consisting of low CD3⁺ T-cell numbers, increased NK cell numbers, and normal B-cell numbers. He is homozygous for a novel n.*2T>C mutation in *RMRP* (Fig 1, B). The adjacent n.*3T>C mutation has been reported in a patient with Omenn syndrome (previously reported as *5T>C).⁴¹ The n.128G>C variant has been reported in the general population at low frequency (<0.3%) but with a higher frequency of approximately 1% in the Latino ethnic group (Exome Aggregation Consortium) and is thus classified as a polymorphism (reported as g.127G>C).

Deleterious *ATM* mutations in a patient negative for a 46-gene targeted PIDD panel. Patient 11, a 6-month-old boy, had 2 measurements of undetectable TRECs on newborn screening and demonstrated T- and B-cell lymphopenia, neutropenia, and normal NK cell numbers. NGS analysis of the severe PIDD panel identified only the heterozygous unclassified novel variant c.1312A>T (p.M438L) in the IL2 inducible T-cell kinase gene *ITK* (NM_005546), leaving the patient without a diagnosis. Further analyses of all genes captured in the library identified compound heterozygous frameshift mutations in the ataxia telangiectasia mutated gene *ATM* (NM_000051): c.2284_2285delCT (p.V762Vfs*2) from the father and c.3802delG (p.V1268*) from the mother (Fig 1, C). Both are documented pathogenic variants for ataxia telangiectasia (AT), which is consistent with the patient's increased α -fetoprotein level (82 ng/mL) and a molecular diagnosis of AT (case report to be published separately).

TABLE III. Deleterious changes detected in infants by using the severe PID panel

Patient ID	Age	Sex	Clinical presentations	Gene	Deleterious variants*	Zygosity	Inheritance
P1	1 mo	M	Family history consistent with X-linked SCID	<i>IL2RG</i>	c.505C>T (p.Q169*)	Hemizygous	X-linked
P2	5 wk	M	Family history	<i>IL2RG</i>	c.285_286dupTA (p.N96Ifs*52)	Hemizygous	X-linked
P3	2 wk	M	CHARGE syndrome, absent TRECs on NBS	<i>CHD7</i>	c.7282C>T (p.R2428*)	Heterozygous	AD
P4	3 wk	F	SCID	<i>RAG1</i>	c.1648G>A (p.D550N) c.2924G>A (p.R975Q)	Heterozygous Heterozygous	AR
P5	3 wk	M	Absent TRECs on NBS	<i>IL7R</i>	c.354C>A p.C118*	Homozygous	AR
P6	5.5 mo	M	Low TRECs on NBS, severe lymphopenia	<i>FOXN1</i>	c.958C>T (p.R320W)	Heterozygous	AR
P7	3 mo	M	Absent TRECs on NBS	<i>FOXN1</i>	c.1556T>A (p.L519Q)	Heterozygous	AR
				<i>CHD7</i>	c.1275_1278delACTC (p.L426Tfs*123)	Heterozygous	AD
P8	6 wk	M	Undetectable TREC, diarrhea/rash	<i>IL2RG</i>	c.7538G>A (p.R2513Q)†	Heterozygous	AD
P9	4 wk	M	SCID	<i>IL2RG</i>	c.202G>A (p.E68K)	93%, Hemizygous	X-linked
P10	3 wk	M	Absent TRECs on NBS	<i>RMRP</i>	c.173C>G (p.P58R)	Hemizygous	X-linked
					n.128G>C	Homozygous	AR
P11	6 mo	M	Low TRECs on NBS	<i>ATM</i>	n.*2T>C	Homozygous	AR
					c.2284_2285delCT (p.V762Vfs*2)	Heterozygous	AR
					c.3802delG (p.V1268*)	Heterozygous	AR
P12	14 mo	M	Absent T and B cells	<i>DCLRE1C</i>	Exon 1 to 4 deletion	Homozygous	AR
P13	5.5 mo	M	SCID, MHC class II deficiency	<i>CIITA</i>	c.1484T>G (p.L495R),	Hemizygous	AR
					One copy loss of the whole gene	Heterozygous	AR
P14	7 wk	F	Absent TRECs on NBS, Rash	<i>ADA</i>	c.956_960delAAGAG (p.E319Gfs*3)	Heterozygous	AR
					c.34-4257_96-1054dup (exon 2)	Heterozygous	AR

AD, Autosomal dominant; AR, autosomal recessive; F, female; M, male; NBS, newborn screening.

*The previously reported deleterious changes are shown in boldface.

†This variant was also found in the asymptomatic father.

Gross deletions and duplications. Patient 12 is a 14-month-old boy who presented with absent T and B cells and disseminated BCG infection. We observed a complete loss of sequence coverage for exons 1 to 3 of the *DCLRE1C* gene (Fig 2, A). Subsequent PCR and array CGH analyses confirmed this homozygous large deletion. It is a recurrent deletion reported in patients with SCID and Omenn syndrome,⁴²⁻⁴⁵ which is consistent with the affected status of this child.

Patient 13 was well until 5 months of age, when he presented with *Pneumocystis jirovecii* pneumonia. Flow cytometry demonstrated that he did not express MHC class II on T or B lymphocytes. One of his older sisters was similarly given a diagnosis of MHC class II deficiency (ie, bare lymphocyte syndrome type II [OMIM#209920]) at age 19 months but did not survive bone marrow transplantation. NGS analysis detected an apparently homozygous missense variant c.1484T>G (p.L495R) in exon 11 and a deletion involving the entire MHC class II transactivator gene *CIITA* (NM_000246; Fig 2, B), which is consistent with the finding of a maternally inherited 2.7-Mb deletion at 16p13.3-16p13.2, including *CIITA*, separately by means of array CGH analysis because of a family history of intellectual disability. Mutations in *CIITA* are known to cause bare lymphocyte syndrome type II.^{46,47} Detection of the compound heterozygous SNV and CNV in *CIITA* provides a definite diagnosis for patient 13, who is undergoing stem cell transplantation.

Biochemical studies showed *ADA* deficiency in patient 14. A heterozygous frameshift variant, c.956_960delAAGAG (p.E319Gfs*3), and a mild increase in read depth of the 62-bp exon 2 of *ADA* was identified (Fig 2, C). Because of the large size of introns 1 and 2 (approximately 16 and 7 kb, respectively) and insufficient coverage of exon 1, possible duplication in this region was analyzed further by using an exon-targeted array

CGH, which detected an approximately 9-kb copy number gain, including exon 2. Subsequent PCR/sequencing analysis revealed a 10.3-kb tandem duplication spanning from the last quarter of intron 1 to most of intron 2.

Leaky SCID and combined variable immunodeficiencies

In addition to typical SCID, milder immunodeficiencies or other immunologic manifestations (associated features), such as autoimmunity, increased IgE levels, lymphoproliferation, and lymphoma, can also be caused by molecular defects in genes included in our panel.^{48,49} Ten samples from patients from 6 to 20 years of age with suspected immunodeficiencies were analyzed. Patient 15 was shown to harbor a novel homozygous variant, c.1514T>G (p.L514R), in *RAG1* (Table IV). Leucine 514 is located at the catalytic core of the RAG1 protein.^{50,51} The replacement of a hydrophobic amino acid leucine with a positively charged arginine at this position might destabilize the structure and impair function (Fig 3). This patient is a 12-year-old girl presenting with hypogammaglobulinemia, recurrent infections, lymphadenopathy, and hepatosplenomegaly, which is consistent with combined variable immunodeficiency and the diagnosis of atypical SCID.⁵²

Patient 16, a 6-year-old boy exhibiting T-cell deficiency, CD4 lymphopenia, and interstitial lung disease, carried a maternal c.281C>T (p.T94I) variant in compound heterozygous state with a paternal c.6782G>A (p.R2261H) change in *LYST* (NM_000081), leading to a diagnosis of combined immunodeficiency (Table IV). The patient had atypical Chediak-Higashi syndrome with no ocular and hair findings but with toxic granulations observed in neutrophils in a peripheral

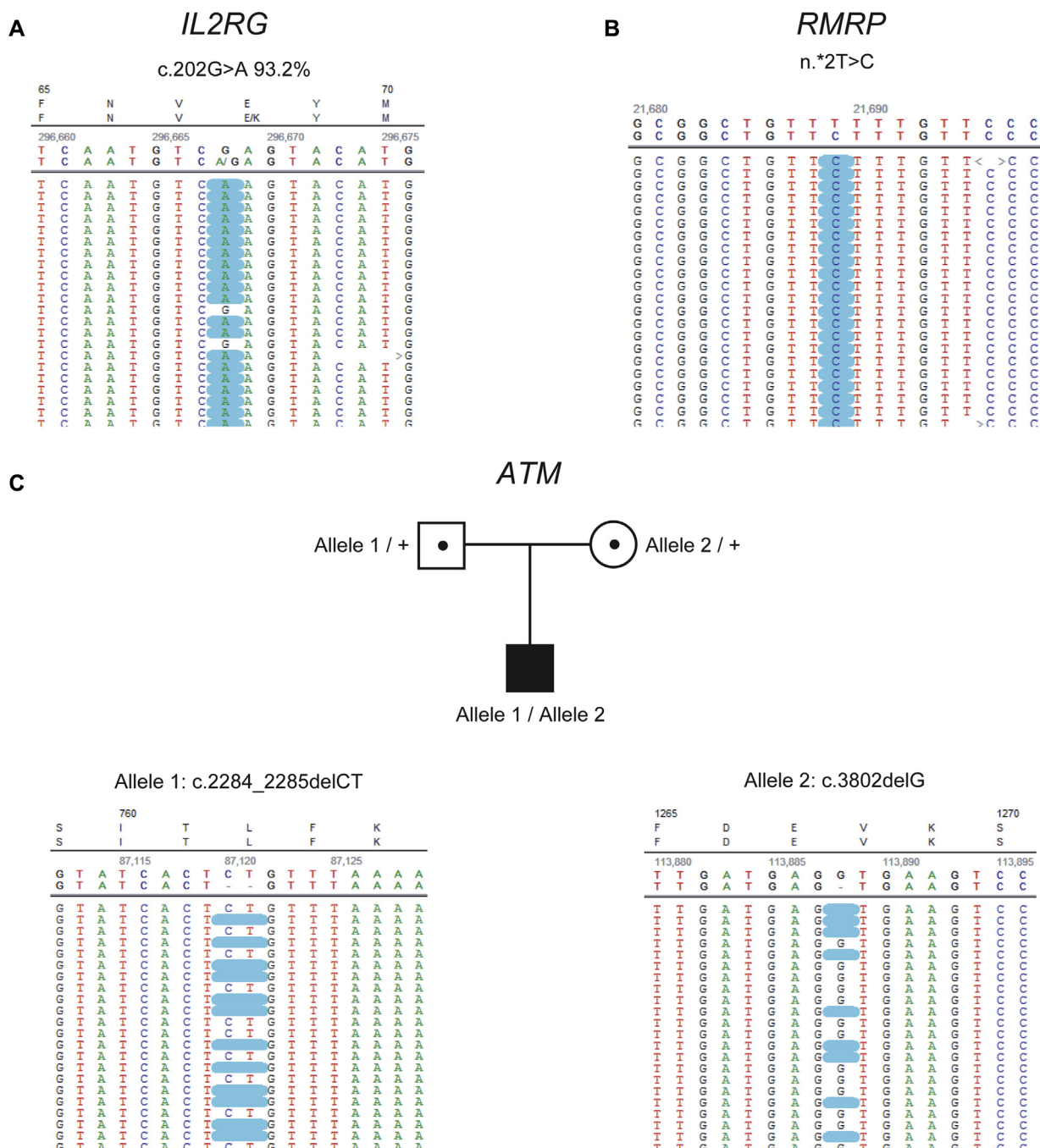


FIG 1. Identification of various pathogenic changes in patients with abnormal SCID newborn screening test results. **A**, A mosaic missense variant c.202G>A (p.E68K) was found in *IL2RG* of patient 9. The wild-type allele c.202G was observed at low frequency (6.8%). **B**, A homozygous novel change (n.*2T>C) was seen in the RNA gene *RMRP* of patient 11. **C**, Compound heterozygous frameshift mutations were identified in the *ATM* gene of patient 12. The c.2284_2285delCT (p.V762Vfs*2) variant came from the father, and the c.3802delG (p.V1268*) variant was from the mother.

blood smear. It remains unclear whether the defects in *LYST* fully explain the decreased T-cell numbers, but no other relevant gene candidates were identified by means of whole-exome sequencing of the proband and his parents. Another 13-year-old male patient (patient 17) with combined variable immunodeficiency was found to harbor the hemizygous pathogenic variant c.1190G>A (p.R397Q) in the forkhead box P3 gene *FOXP3*, which was

reported previously in patients with immune dysregulation, poly-endocrinopathy, enteropathy, X-linked syndrome.^{53,54} However, our patient also carried a heterozygous pathogenic variant, c.616C>T (p.R206*), in the *IL7R* gene (autosomal recessive inheritance) and a heterozygous novel variant of uncertain significance, c.406G>A (p.G136S), in the *TBX1* gene (autosomal dominant inheritance).

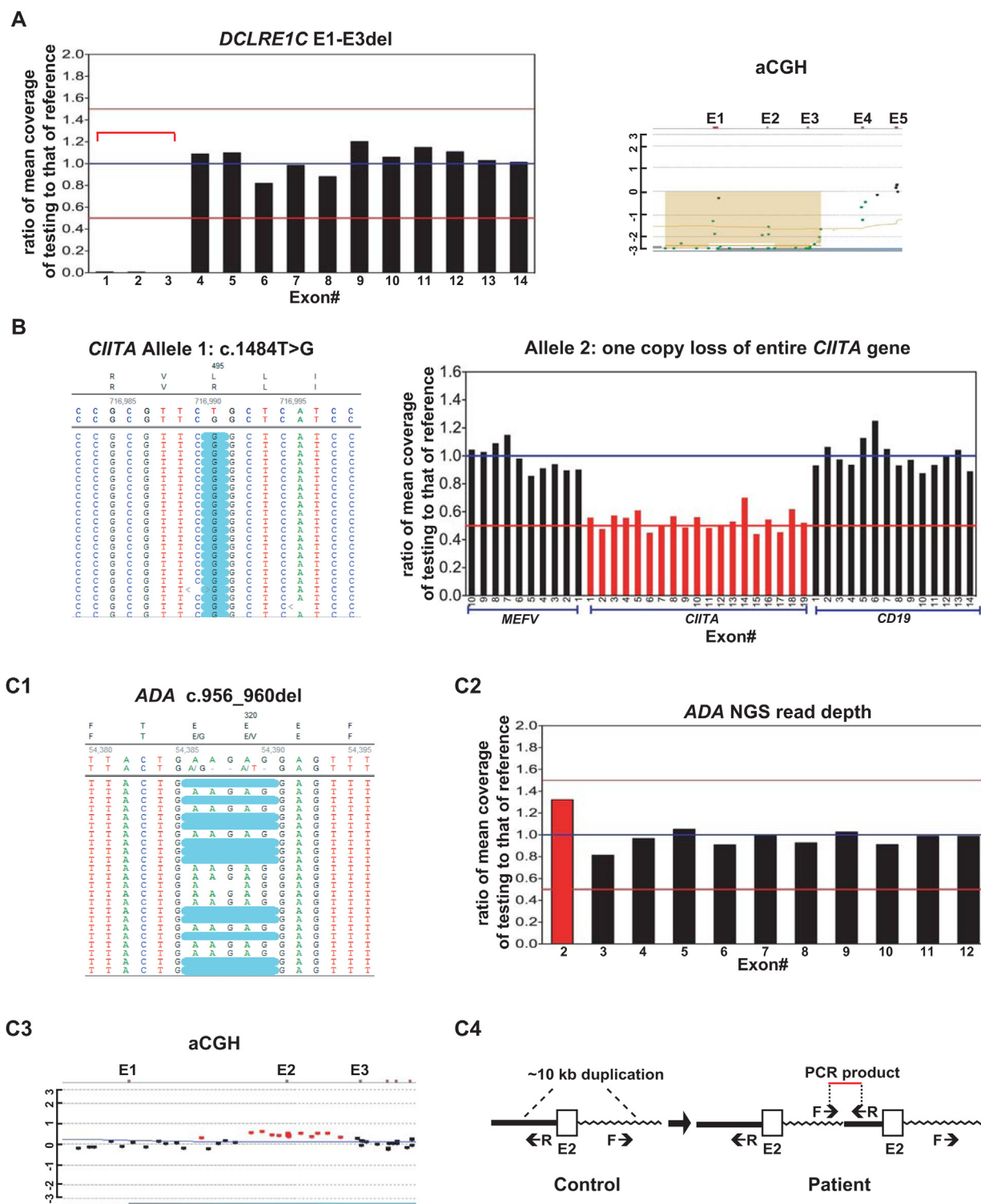


FIG 2. Deleterious genomic structural variants identified in patients with SCID. **A**, Lack of NGS coverage identified a homozygous deletion of the *DCLRE1C* gene involving exons 1 to 3. Targeted array CGH (aCGH) confirmed this deletion. **B**, An apparently homozygous missense variant, c.1484T>G (p.L495R), was observed in the *CIITA* gene of patient 13. This SNV change was actually hemizygous because of loss of the entire *CIITA* gene on the other allele based on CNV analysis using the NGS coverage depth. **C**, In addition to the heterozygous frameshift mutation c.956_960delAAGAG (p.E319Gfs*3) in exon 10 of the *ADA* gene (*C1*), a duplication involving exon 2 was identified by means of combined analysis of NGS read depth (*C2*) and targeted array CGH (*C3*). Subsequent PCR/sequence analysis confirmed that it is a 10.3-kb tandem duplication, c.34-4257_96-1054dup, mediated by 2 *Alu* sequences located in introns 1 and 2, respectively (*C4*).

TABLE IV. Deleterious changes detected in patients with leaky SCID and other combined immunodeficiencies

Patient ID	Age	Sex	Clinical presentations	Gene	Deleterious variants	Zygoty	Inheritance
P15	12 y	F	CVID, lymphoproliferation, recurrent infections	<i>RAG1</i>	c.1514T>G (p.L514R)	Homozygous	AR
P16	6 y	M	T-cell deficiency, CD4 lymphopenia, interstitial lung disease	<i>LYST</i>	c.281C>T (p.T94I)	Heterozygous	AR
P17	13 y	M	CVID		c.6782G>A (p.R2261H)	Heterozygous	
				<i>FOXP3</i>	c.1190G>A (p.R397Q)	Hemizygous	X-linked
				<i>IL7R</i>	c.616C>T (p.R206*)	Heterozygous	AR
				<i>TBX1</i>	c.406G>A (p.G136S)	Heterozygous	AD

CVID, Combined variable immunodeficiency.

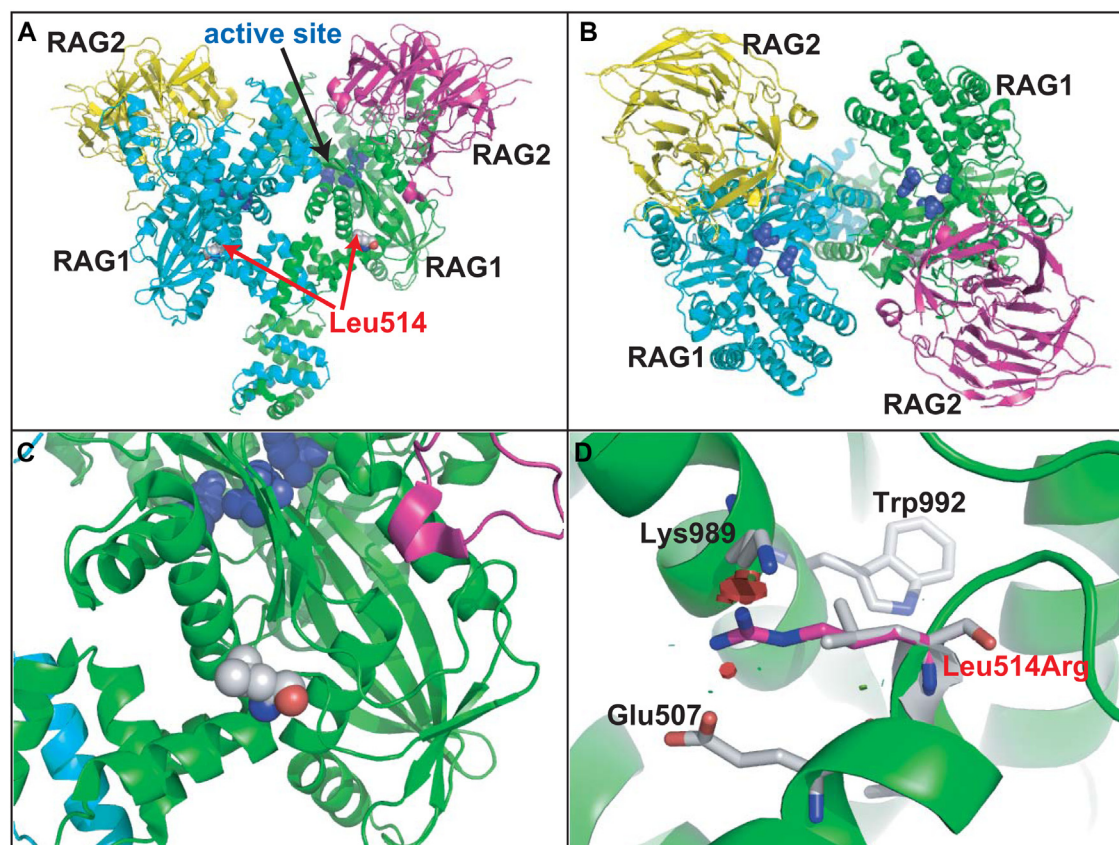


FIG 3. *In silico* analysis and structural prediction of the *RAG1* L514R change. The protein structure and amino acid changes in the 3-dimensional structure of the RAG1-RAG2 complex (PDB# 4WWX) was visualized and modeled by using PyMOL (DeLano Scientific, Palo Alto, Calif). **A**, Cartoon representation of the side view of the RAG1-RAG2 heterotetramer. Active sites in the middle of RAG1 and leucine 514 are represented as spheres. **B**, Top view of the RAG1-RAG2 heterotetramer. **C**, Close view of leucine 514, which is about 22Å away from residues in the active site. **D**, Replacement of leucine with arginine at position 514 creates steric hindrance and hydrophilic residue into the nonpolar environment.

DISCUSSION

A timely and accurate molecular diagnosis of SCID remains important for optimal treatment. Infants with SCID are often clinically silent during the first few weeks of life because of immune protection from transferred maternal antibodies. The latency phase of disease manifestations can delay prompt detection of SCID and other PIDDs. In critical conditions immune reconstitution therapy always proceeds based on immune parameters without definitive molecular diagnoses.⁵⁵ However, an early and accurate molecular diagnosis can greatly assist

providers with management of infants with SCID. For example, congenital athymia can mimic SCID, and thus securing a definitive genetic diagnosis can confirm the need for either bone marrow stem cell transplantation or thymus transplantation.⁵⁶ The diagnosis of radiation-sensitive SCID can directly alter management in terms of minimizing radiation exposure and avoiding the use of alkylating agents during transplantation. In addition, patients with mutations in *ADA* or *IL2RG* can be treated with gene therapy, particularly if appropriate stem cell donors cannot be identified. Clinical symptoms of SCID can surface after

immune challenge, such as live bacterial or viral vaccinations.⁵⁷ Early diagnosis of SCID would permit avoidance of such unnecessary high-risk exposures. The extensive underlying genetic heterogeneity of SCID and PIDDs in general and the interpretation of rare or novel variants with an atypical immune phenotype further challenge the establishment of a definitive diagnosis. The combination of TREC newborn screening and high-throughput NGS analyses of target gene panels enables an effective genotype-based approach to molecular diagnosis in the desired, clinically actionable time frame for all affected infants.

Our NGS approach with 100% coverage of targeted SCID genes confirmed all known molecular defects in the validation samples, including both SNVs and CNVs. The simultaneous detection of SNVs and CNVs by using NGS-based data for clinical diagnosis is unique.³⁰ In addition, the comprehensive targeted gene capture/NGS approach analyzes an extensive list of genes known to cause immune-associated diseases in one test, allowing the identification of underlying molecular defects for overlapping clinical phenotypes and exclusion of certain ambiguous diagnoses.

We identified disease-causing genes in 14 of 20 patients with abnormal TREC newborn screening test results or family history. Four (20%) patients had defects in the *IL2RG* gene, which is consistent with the reported prevalence of X-linked SCID. For autosomal recessive SCID incidences, both compound heterozygous (eg, *RAG1* and *FOXN1*) and homozygous (eg, *IL7R* and *RMRP*) mutations were identified. Detection of exonic CNVs in our patient cohorts in addition to SNVs or small insertions/deletions demonstrates the complexities of genetic defects involved in the pathogenicity of SCID.⁵⁸

T-cell lymphopenia is not restricted to SCID. Patients with DGS, CHARGE syndrome (patient 3), and AT (patient 11) can also have a compromised immune system. Immunologic dysfunction in patients with syndromic disorders is highly variable. Association of AT with T-cell lymphopenia at birth remains poorly recognized. Implementation of SCID newborn screening in the past few years has revealed several AT cases and ours, suggesting newborn T lymphocytopenia as an identifying trait of AT.^{14,59} The clinical neurologic presentation of AT does not manifest until at least 1.5 years of age. Presently, there is no curative or alleviating treatment for AT; however, an international randomized clinical trial with erythrocyte-delivered dexamethasone is underway.⁶⁰ Early recognition of AT might allow for immediate initiation of supportive care and management and might increase the potential for a beneficial overall prognosis. The identification of *ATM* mutations allows prenatal diagnosis for future pregnancies in the family and anticancer monitoring for at-risk family members.

Although T-cell lymphopenia is common in newborns with SCID, some infants with rare forms of PIDDs have normal T-cell numbers and thus cannot be identified by TREC screening.⁶¹⁻⁶³ For example, patient 13 would not have been detected on newborn screening because T cells were present, but without MHC class II expression, he had impaired T-cell function. Therefore access to such high-throughput NGS panels becomes critical for patients with a family history of immunodeficiency regardless of the newborn screening result. Early diagnosis is critical for success after transplantation, and a better outcome is expected for patient 13 than for his older sister, who was given a diagnosis at age

19 months. Thus a comprehensive NGS panel serves as an efficient complementary clinical test for the diagnosis.

Despite its severity, SCID is a disease that can potentially be cured to achieve good quality of life for affected patients. Precise and quick molecular diagnosis by using the NGS panel testing not only facilitates implementation of transplantation within the 3.5-month window and aids care management if transplantation is not necessary or indicated but also calls attention to directed evaluation for unrecognized clinical signs or presentations. Other considerations include avoidance of live viral vaccination, pharmacokinetic monitoring of administered drugs, control of inflammation and proper nutritional support, and prevention of unnecessary exposure to ionizing radiation (eg, defects in *ATM* and genes involved in nonhomologous DNA end-joining).

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Clinical implications: A targeted NGS approach with deep coverage offers simultaneous detection of SNVs and exonic CNVs for molecular diagnosis of severe primary immunodeficiency.

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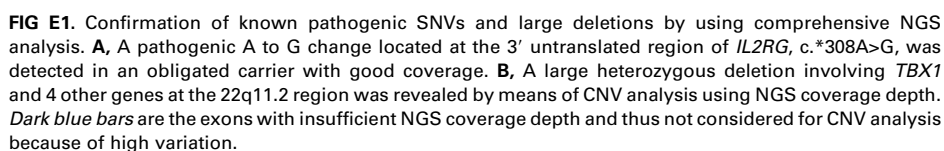


TABLE E1. Genes in the immunodeficiency capture library

Gene	HGMD NM#	Gene	HGMD NM#	Gene	HGMD NM#	Gene	HGMD NM#
<i>ACTB</i>	NM_001101	<i>CHD7</i>	NM_017780	<i>ITCH</i>	NM_031483	<i>RFXANK</i>	NM_003721
<i>ADA</i>	NM_000022	<i>CIITA</i>	NM_000246	<i>ITGB2</i>	NM_000211	<i>RFXAP</i>	NM_000538
<i>AICDA</i>	NM_020661	<i>COLEC11</i>	NM_024027	<i>ITK</i>	NM_005546	<i>RMRP</i>	NR_003051
<i>AIRE</i>	NM_000383	<i>CORO1A</i>	NM_007074	<i>JAK3</i>	NM_000215	<i>RNF168</i>	NM_152617
<i>AK2</i>	NM_001625	<i>CTSC</i>	NM_001814	<i>LAMTOR2</i>	NM_014017	<i>RTKL1</i>	NM_032957
<i>AP3B1</i>	NM_003664	<i>CXCR4</i>	NM_003467	<i>LCK</i>	NM_001042771	<i>SBDS</i>	NM_016038
<i>APOL1</i>	NM_003661	<i>CYBA</i>	NM_000101	<i>LIG4</i>	NM_002312	<i>SEMA3E</i>	NM_012431
<i>ATM</i>	NM_000051	<i>CYBB</i>	NM_000397	<i>LPIN2</i>	NM_014646	<i>SEPT5</i>	NM_002688
<i>BLM</i>	NM_000057	<i>DCLRE1B</i>	NM_022836	<i>LYST</i>	NM_000081	<i>SH2D1A</i>	NM_002351
<i>BLNK</i>	NM_013314	<i>DCLRE1C</i>	NM_001033855	<i>MAGT1</i>	NM_032121	<i>SLC35C1</i>	NM_018389
<i>BTK</i>	NM_000061	<i>DKC1</i>	NM_001363	<i>MASPI</i>	NM_139125	<i>SLC37A4</i>	NM_001164277
<i>C1QA</i>	NM_015991	<i>DNMT3B</i>	NM_006892	<i>MASP2</i>	NM_006610	<i>SMARCAL1</i>	NM_014140
<i>C1QBP</i>	NM_001212	<i>DOCK8</i>	NM_203447	<i>MBL2</i>	NM_000242	<i>SP110</i>	NM_004509
<i>C1QC</i>	NM_172369	<i>ELANE</i>	NM_001972	<i>MED12</i>	NM_005120	<i>SPINK5</i>	NM_006846
<i>C1R</i>	NM_001733	<i>ERCC2</i>	NM_000400	<i>MEFV</i>	NM_000243	<i>STAT1</i>	NM_007315
<i>C1S</i>	NM_201442	<i>ERCC3</i>	NM_000122	<i>MPLKIP</i>	NM_138701	<i>STAT3</i>	NM_139276
<i>C2</i>	NM_000063	<i>FADD</i>	NM_003824	<i>MRE11A</i>	NM_005591	<i>STAT5B</i>	NM_012448
<i>C22orf29</i>	NM_024627	<i>FAS</i>	NM_000043	<i>MS4A1</i>	NM_152866	<i>STIM1</i>	NM_003156
<i>C3</i>	NM_000064	<i>FASLG</i>	NM_000639	<i>MSH5</i>	NM_025259	<i>STK4</i>	NM_006282
<i>C4A</i>	NM_007293	<i>FCN3</i>	NM_003665	<i>MTHFD1</i>	NM_005956	<i>STX11</i>	NM_003764
<i>C4B</i>	NM_001002029	<i>FERMT3</i>	NM_031471	<i>MVK</i>	NM_000431	<i>STXBP2</i>	NM_006949
<i>C5</i>	NM_001735	<i>FOXN1</i>	NM_003593	<i>MYD88</i>	NM_002468	<i>TAP1</i>	NM_000593
<i>C6</i>	NM_000065	<i>FOXP3</i>	NM_014009	<i>NBN</i>	NM_002485	<i>TAP2</i>	NM_000544
<i>C7</i>	NM_000587	<i>FPR1</i>	NM_002029	<i>NCF1</i>	NM_000265	<i>TAPBP</i>	NM_003190
<i>C8A</i>	NM_000562	<i>G6PC3</i>	NM_138387	<i>NCF2</i>	NM_000433	<i>TAZ</i>	NM_000116
<i>C8B</i>	NM_000066	<i>G6PD</i>	NM_001042351	<i>NCF4</i>	NM_013416	<i>TBX1</i>	NM_080647
<i>C9</i>	NM_001737	<i>GFII</i>	NM_005263	<i>NFKBIA</i>	NM_020529	<i>TERF2</i>	NM_005652
<i>CARD11</i>	NM_032415	<i>GNB1L</i>	NM_053004	<i>NHEJ1</i>	NM_024782	<i>TERT</i>	NM_198253
<i>CARD9</i>	NM_052813	<i>GP1BB</i>	NM_000407	<i>NHP2</i>	NM_017838	<i>TINF2</i>	NM_001099274
<i>CASP10</i>	NM_032977	<i>GTF2H5</i>	NM_207118	<i>NLRP12</i>	NM_144687	<i>TLR3</i>	NM_003265
<i>CASP8</i>	NM_001228	<i>HAX1</i>	NM_006118	<i>NLRP3</i>	NM_004895	<i>TMC6</i>	NM_007267
<i>CBL</i>	NM_005188	<i>HMGB1</i>	NM_002128	<i>NOD2</i>	NM_022162	<i>TMC8</i>	NM_152468
<i>CD19</i>	NM_001770	<i>ICOS</i>	NM_012092	<i>NOP10</i>	NM_018648	<i>TNFRSF13B</i>	NM_012452
<i>CD3D</i>	NM_000732	<i>IFNGR1</i>	NM_000416	<i>NRAS</i>	NM_002524	<i>TNFRSF13C</i>	NM_052945
<i>CD3E</i>	NM_000733	<i>IFNGR2</i>	NM_005534	<i>ORAI1</i>	NM_032790	<i>TNFRSF1A</i>	NM_001065
<i>CD3G</i>	NM_000073	<i>IGLL1</i>	NM_020070	<i>PIGA</i>	NM_002641	<i>TRAF3</i>	NM_003300
<i>CD40</i>	NM_001250	<i>IKBKG</i>	NM_001099857	<i>PLCG2</i>	NM_002661	<i>TTC7A</i>	NM_020458
<i>CD40LG</i>	NM_000074	<i>IKZF1</i>	NM_006060	<i>PMS2</i>	NM_000535	<i>TYK2</i>	NM_003331
<i>CD46</i>	NM_002389	<i>IL10</i>	NM_000572	<i>PNP</i>	NM_000270	<i>UNC13D</i>	NM_199242
<i>CD59</i>	NM_203330	<i>IL10RA</i>	NM_001558	<i>PRF1</i>	NM_001083116	<i>UNC93B1</i>	NM_030930
<i>CD79A</i>	NM_001783	<i>IL10RB</i>	NM_000628	<i>PRKDC</i>	NM_006904	<i>UNG</i>	NM_080911
<i>CD79B</i>	NM_000626	<i>IL12B</i>	NM_002187	<i>PSMB8</i>	NM_148919	<i>USB1</i>	NM_024598
<i>CD81</i>	NM_004356	<i>IL17F</i>	NM_052872	<i>PSTPIP1</i>	NM_003978	<i>VPS13B</i>	NM_017890
<i>CD8A</i>	NM_001768	<i>IL17RA</i>	NM_014339	<i>PTPRC</i>	NM_002838	<i>WAS</i>	NM_000377
<i>CEBPPE</i>	NM_001805	<i>IL1RN</i>	NM_173841	<i>RAC2</i>	NM_002872	<i>WRAP53</i>	NM_018081
<i>CFB</i>	NM_001710	<i>IL2RA</i>	NM_000417	<i>RAG1</i>	NM_000448	<i>WRN</i>	NM_000553
<i>CFD</i>	NM_001928	<i>IL2RG</i>	NM_000206	<i>RAG2</i>	NM_000536	<i>XIAP</i>	NM_001167
<i>CFH</i>	NM_000186	<i>IL7R</i>	NM_002185	<i>RECQL4</i>	NM_004260	<i>ZAP70</i>	NM_001079
<i>CFP</i>	NM_002621	<i>IRAK4</i>	NM_016123	<i>RFX5</i>	NM_000449	<i>ZBTB24</i>	NM_014797