

Genetic Diagnosis of Primary Immune Deficiencies

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The increasing availability of genetic testing for many disorders is facilitating improvements in patient management [1,2]. Such testing can establish or confirm a suspected diagnosis and also may predict future disease risk in advance of clinical signs and symptoms, inform reproductive decision making, and guide clinicians in selecting the most appropriate therapeutic options. Traditionally, geneticists familiar with the nuances of rare disorders have orchestrated complex genetic testing through intimate relationships with academic laboratories accustomed to low-volume, high-complexity testing. This boutique laboratory approach may be effective for rare “single-gene” disorders but may falter as testing complexity increases. The expanding knowledge of the molecular pathogenesis of many human disorders has revealed new complexities of “nonclassic” single-gene disorders. Moreover, interest in genetic testing has expanded to medical specialties beyond medical genetics [3]. In coming years, knowledge of the complex genetic

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foundation of many common polygenic disorders (eg, diabetes and hypertension) will be used to establish a definitive clinical diagnosis. Thus, genetic medicine is transitioning rapidly from the academic-based genetics clinic to a service routinely used by other medical specialties and community-based primary care providers [1,3].

The field of primary immune deficiencies (PIDs) has seen a similar transition of testing from the research to the clinical setting [4]. Much progress has been made since Bruton [5] presented the first example of human PID in 1952 by using the then-new technology of serum electrophoresis. Since then, many novel laboratory methodologies have been developed and adopted in the clinical diagnosis of PIDs. Although historically the molecular diagnosis of PID has occurred in leading academic centers [6–16], commercial clinical diagnostic laboratories now perform an increasing proportion of genetic testing for PIDs. Moreover, the field of PIDs currently is experiencing a paradigm shift in the definition of PIDs from rare monogenic disorders leading to severe infections in infancy to much more common diseases [17,18]. One in 1200 persons in the United States is diagnosed as having some form of PID [19], and population-wide newborn screening currently is being considered for severe combined immunodeficiency (SCID) [20].

With the emerging understanding that many individuals may suffer from at least one of a multitude of PIDs [21], community-based primary care providers must be empowered to share the responsibility for diagnosis and treatment of PIDs with academic medical centers. To increase awareness of PIDs among health care providers, patients, and families, outreach programs have been established by agencies and organizations such as the US National Institutes of Health, the Mount Sinai Hospital (New York City, NY), the Jeffrey Modell Foundation (<http://www.info4pi.org/>), the Immune Deficiency Foundation (<http://www.primaryimmune.org/>), and the National Organization for Rare Disorders (<http://www.rarediseases.org/>). To facilitate more widespread use of genetic testing as a diagnostic tool, providers must have easy access to testing and know when genetic testing is indicated. Accuracy of test results must be assured, and the interpretations provided by clinical laboratories must be standardized and easy to understand.

In the past, such testing lacked specific national standards for quality assurance, quality control, test accessioning and reporting, or proficiency evaluation. Scientific, clinical, and regulatory efforts are underway to ensure the responsible expansion of genetic testing for PIDs [4]. The Clinical Laboratory Improvement Amendments of 1988 (CLIA; <http://www.fda.gov/cdrh/clia/>), the American College of Medical Genetics (ACMG; <http://www.acmg.net/>), and molecular diagnostics guidelines published by the Clinical and Laboratory Standards Institute (CLSI; <http://www.nccls.org/>) have hastened the transition of genetic testing for PID from academic to clinical laboratories. The Human Variome Project also has recently published guidelines designed to improve genetic testing [22,23]. In this article, some of the advances and challenges currently encountered in the clinical

molecular genetic diagnosis of PIDs are discussed, based on the authors' experience and a review of the published literature.

Clinical utility of genetic testing in the field of primary immune deficiencies

Practice parameters and guidelines for diagnosis and management of PIDs include genetic testing as an integral component [24–26]. Patients who have PID can benefit from early diagnosis and improved clinical management based on the definitive diagnosis that genetic testing can provide [27]. Currently, however, many clinicians view genetic testing more as a confirmatory test than as a screening test. Some of the major impediments to widespread adoption of genetic testing for PIDs include (1) relative costs and related concerns about insurance coverage; (2) difficulties in identifying an appropriate clinical laboratory; (3) the length of time between the request for a test and the delivery of a result; and (4) lack of clinical standardization at the laboratory level. Increased use of genetic testing in clinical practice will be accelerated by educating primary care providers [27] and developing patient-centered screening protocols designed for non-immunologists [26] as well as by the availability of validated clinical genetic testing services. Table 1 provides a nonexhaustive list of PIDs for which a responsible gene has been identified (see Geha and colleagues [28] for more details). A complete list of genetic tests, for both clinical use and research, is provided by the GeneTests Laboratory Directory [2].

Clinical utility of genetic testing for severe combined immunodeficiency

SCID subtypes often are classified based on counts of individual cell populations. This information is a valuable guide to clinicians attempting to select the most appropriate genetic test [24,29]. Because distinct mutations within the same gene can lead to different immunologic phenotypes [30], a pitfall of this method of classification is that a clinical diagnosis based on an immunologic phenotype could obscure a definitive genetic diagnosis. Of note, genetic testing can provide a definitive diagnosis of SCID and SCID subtype, especially when clinical and immunologic diagnosis based on counts of individual lymphocyte populations is complicated by engraftment of maternally derived lymphocytes. Muller and colleagues [31] detected maternal T cells in 40% of infants who had SCID. In eight patients who had prominent skin graft-versus-host disease (GVHD), the underlying SCID presentation was characterized only by the absence of B cells, although maternal T-cell levels did not correlate well with GVHD intensity and skin manifestations [31], and maternal engraftment might not occur in certain SCID subtypes [31]. Consequently, the significant heterogeneity of clinical and immunologic findings present in SCID must be considered when attempting a diagnosis. Testing peripheral blood for maternal T cells should be a routine part of both the pretransplantation compatibility

Table 1
Primary immune deficiencies for which a responsible gene has been identified

Disease	Inheritance	Genetic defects	Gene symbol
Combined T-cell and B-cell immunodeficiencies			
T ⁺ B ⁺ severe combined immunodeficiency			
γ c deficiency	XL	Defect in γ chain of receptors for IL-2, -4, -7, -9, -15, -21	<i>IL2RG</i>
JAK3 deficiency	AR	Defect in Janus-associated signaling kinase 3	<i>JAK3</i>
IL7R α deficiency	AR	Defect in IL-7 receptor α chain	<i>IL7RA</i>
CD45 deficiency	AR	Defect in CD45	<i>CD45</i>
CD3 δ /CD3 ϵ deficiency	AR	Defect in CD3 δ or CD3 ϵ chains of T-cell antigen receptor	<i>CD3D, CD3E</i>
T ⁺ B ⁻ severe combined immunodeficiency			
RAG 1/2 deficiency	AR	Complete defect of Recombinase activating gene 1 or 2	<i>RAG1, RAG2</i>
DCLRE1C (Artemis) deficiency	AR	Defect in Artemis DNA recombinase-repair protein	<i>DCLRE1C</i>
ADA deficiency	AR	Mutations in the Adenosine deaminase gene	<i>ADA</i>
Omenn syndrome	AR	Missense mutations allowing residual activity	<i>RAG1, RAG2, DCLRE1C, IL7RA</i>
CD40 ligand deficiency	XL	Defects in CD40 ligand (CD40L) protein	<i>CD40L/TNFSF5</i>
CD40 deficiency	AR	Defects in CD40 protein	<i>CD40/TNFRSF5</i>
ZAP-70 deficiency	AR	Defects in the Zeta-associated protein of 70-kD signaling kinase	<i>ZAP70</i>
Predominantly antibody deficiencies			
Severe reduction in all serum Ig isotypes with severely decreased or absent B cells			
BTK deficiency	XL	Mutations in the Bruton tyrosine kinase gene	<i>BTK</i>
Other gene deficiencies	AR	Mutations in μ heavy chain, λ 5, Ig α , IgB, the BLNK adapter	<i>IGHM, λ5, Igα, IgB, BLNK</i>

Severe reduction in serum IgG and IgA with normal, low or very low numbers of B cells			
ICOS deficiency	AR	Mutation in the Inducible costimulator gene	<i>ICOS</i>
CVID	AD (AR)	Mutation in the Transmembrane activator and calcium-modulator and cyclophilin ligand interactor gene, and in other genes	<i>TNFRSF13B, other genes</i>
X-linked lymphoproliferative syndrome 1	XL	Mutations in the SH2 domain protein 1A gene	<i>SH2D1A</i>
Severe reduction in serum IgG and IgA with normal or elevated IgM and normal numbers of B cells			
CD40 ligand deficiency	XL	Defects in CD40 ligand (CD40L) protein	<i>CD40L/TNFSF5</i>
CD40 deficiency	AR	Defects in CD40 protein	<i>CD40/TNFRSF5</i>
AICDA deficiency	AR	Mutation in the Activation-Induced Cytidine Deaminase gene	<i>AICDA</i>
UNG deficiency	AR	Mutation in the Uracil-DNA glycosylase gene	<i>UNG</i>
Other well-defined PIDs			
Wiskott-Aldrich syndrome	XL	Mutations in the Wiskott-Aldrich syndrome protein gene	<i>WASP</i>
Other DNA repair defects			
Ataxia-telangiectasia	AR	Mutation in the Ataxia-telangiectasia gene	<i>ATM</i>
Nijmegen breakage syndrome	AR	Mutation in the Nibrin gene	<i>NBS1</i>
Bloom syndrome	AR	Mutation in Helicase gene	<i>BLM</i>
Hyper-IgE syndrome (HIES)			
Job syndrome	AD	Mutations in the Signal transducer and activator of transcription 3 gene	<i>STAT3</i>
AR HIES with mycobacterial and viral infections	AR	Mutation in Tyrosine kinase 2 gene	<i>TYK2</i>

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Table 1 (*continued*)

Disease	Inheritance	Genetic defects	Gene symbol
Diseases of immune dysregulation			
Immunodeficiency with hypopigmentation			
Chediak-Higashi syndrome	AR	Mutations in the Lysosomal trafficking regulator gene	<i>LYST</i>
Griscelli Syndrome, type 2	AR	Mutations in the Rab protein 27A gene	<i>RAB27A</i>
Hermansky-Pudlak syndrome, type 2	AR	Mutations in the B subunit of the Adaptor protein-3 complex gene	<i>AP3B1</i>
Familial hemophagocytic lymphohistiocytosis syndromes			
Perforin deficiency	AR	Mutations in the Perforin 1 gene	<i>PRF1</i>
Munc 13-D deficiency	AR	Defects in MUNC13D	<i>MUNC13D</i>
X-linked lymphoproliferative syndrome (XLP)			
XLP1	XL	Mutations in the SH2 domain protein 1A gene	<i>SH2D1A</i>
XLP2	XL	Mutations in X-linked inhibitor of apoptosis protein (XIAP)	<i>XIAP</i>
Syndromes with autoimmunity			
Autoimmune lymphoproliferative syndrome			
type 1a	AD (AR)	Mutations in the CD95 (Fas) gene	<i>TNFRSF6</i>
type 1b	AD, AR	Mutations in the CD95L (Fas ligand) gene	<i>TNFSF6</i>
type 2a	AD	Mutations in the Caspase 10 gene	<i>CASP10</i>
type 2b	AD	Mutations in the Caspase 8 gene	<i>CASP8</i>
Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED)	AR	Mutations in the Autoimmune regulator gene	<i>AIRE</i>
Immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX)	XL	Mutations in the Forkhead box protein 3 gene	<i>FOXP3</i>
Congenital defects of phagocyte number, function, or both			
Severe congenital neutropenias	AD	Multiple gene defects	<i>ELA2, GFII, G-CSFR</i>
Kostmann disease	AR	Mutations in the HCLS1-associated protein x1 gene	<i>HAX1</i>
Cyclic neutropenia	AD	Mutations in the Elastase 2 gene	<i>ELA2</i>

Leukocyte adhesion deficiency (LAD)			
type 1	AR	Mutations in the adhesion protein INTG2	<i>ITGB2</i>
type 2	AR	Mutations in the Fucose transporter 1 gene	<i>FUCT1</i>
Rac 2 deficiency	AD	Mutations in the Regulation of actin cytoskeleton gene	<i>RAC2</i>
B-Actin deficiency	AD	Mutations in the cytoplasmic actin B gene	<i>ACTB</i>
Localized juvenile periodontitis	AR	Mutations in the Chemokine receptor FPR1	<i>FPR1</i>
Papillon-Lefevre syndrome	AR	Mutations in the Cathepsin C activation of serine proteases gene	<i>CTSC</i>
Shwachman-Diamond syndrome	AR	Mutations in the Schwachman-Bodan-Diamond syndrome gene	<i>SBDS</i>
Chronic granulomatous disease			
X-linked	XL	Mutations in the Electron transport protein (gp91phox) gene	<i>CYBB</i>
Autosomal	AR	Multiple gene defects	<i>CYBA, NCF1, NCF2</i>
IL-12 and IL-23 receptor B1	AR	Mutations in the IL-12 and IL-23 receptor B1-chain gene	<i>IL-12RB1</i>
IL-12p40 deficiency	AR	Mutations in the gene of the IL-12p40 subunit of IL12/IL23	<i>IL-12p40</i>
IFN- γ receptor 1 deficiency	AR/AD	Mutations in the IFN- γ receptor 1 gene	<i>IFN-γR1</i>
IFN- γ receptor 2 deficiency	AR	Mutations in the IFN- γ receptor 2 gene	<i>IFN-γR2</i>
STAT1 deficiency	AR/AD	Mutations in the signal transducer activator of transcription 1 gene	<i>STAT1</i>
Defects in innate immunity			
Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID)	XL	Mutations in the NF- κ B essential modulator (NEMO) gene	<i>IKBKG</i>
	AD	Mutations in the NF- κ B inhibitor, alpha ($\text{I}\kappa\text{B}\alpha$) gene	<i>IKBA</i>
IL-1 receptor-associated kinase 4 (IRAK4) deficiency	AR	Mutations in the IL-1 receptor-associated kinase 4 gene	<i>IRAK4</i>
WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) syndrome	AD	Mutations in the Chemokine receptor CXCR4 gene	<i>CXCR4</i>

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Table 1 (continued)

Disease	Inheritance	Genetic defects	Gene symbol
Epidermodyplasia verruciformis	AR	Mutations in the Epidermodyplasia verruciformis genes 1 or 2	<i>EVER1, EVER2</i>
Herpes simplex encephalitis	AR	Mutations in the <i>UNC93B1</i> gene	<i>UNC93B1</i>
	AD	Mutations in the Toll-like receptor 3 gene	<i>TLR3</i>
Autoinflammatory disorders			
Familial Mediterranean fever	AR	Mutations in the Mediterranean fever gene	<i>MEFV</i>
TNF receptor-associated periodic syndrome (TRAPS)	AD	Mutations of the Tumor necrosis factor receptor soluble factor 1A gene	<i>TNFRSF1A</i>
Hyper-IgD syndrome	AR	Mutations in the Mevalonate kinase gene	<i>MVK</i>
Muckle-Wells syndrome	AD	Mutations in the cryopyrin (PYPAF1/NALP3/CIAS1) gene	<i>CIAS1</i>
Familial cold autoinflammatory syndrome	AD	Mutations in the cryopyrin (PYPAF1/NALP3/CIAS1) gene	<i>CIAS1</i>
Neonatal-onset multisystem inflammatory disease (NOMID)	AD	Mutations in the cryopyrin (PYPAF1/NALP3/CIAS1) gene	<i>CIAS1</i>
Pyogenic sterile arthritis, pyoderma gangrenosum, acne (PAPA) syndrome	AD	Mutations in the Proline/serine/threonine phosphatase-interacting protein 1 gene	<i>PSTPIP1</i>
Blau syndrome	AD	Mutations of the Nucleotide-binding oligomerization domain protein 2 gene	<i>NOD2 (CARD15)</i>
Majeed syndrome	AR	Mutations in the Lipin-2 gene	<i>LPIN2</i>
Complement deficiencies			
Complement gene deficiencies	AR/AD/XL	Multiple gene defects	

Table 1 is based on the PID classification of the 2007 Update from the International Union of Immunologic Societies Primary Immunodeficiency Diseases Classification Committee, which contains further details and a complete list of PIDs. A full list of genetic testing available for PIDs and laboratories contact information also is available at: www.genetest.org.

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CVID, common variable immunodeficiency; IL, interleukin; IFN, interferon; NF- κ B, nuclear factor-kappa B; SCID, severe combined immunodeficiency; XL, X-linked.

From Geha RS, Notarangelo LD, Casanova JL, et al. Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. *J Allergy Clin Immunol* 2007;120(4):779; with permission.

assessment [32] and genetic test selection and results interpretation for patients who have SCID.

Use of full-gene sequencing to detect mutations

Full-gene sequencing is considered the reference standard in molecular genetic diagnostics, because it can identify both previously characterized and novel sequence variations with little or no ambiguity. Some diseases are caused by only a few common mutations in a gene and therefore can be detected by targeted screening for these particular mutations. Such cases are unusual, however, and often are dependent on the ethnicity of the individuals tested. For most genes, searching for previously characterized sequence variation may not be informative. For example, about half of all SCID-associated alleles of the adenosine deaminase (*ADA*) gene deficiency are unique to single families, and most patients are compound heterozygotes with a different mutation on each allele [33]. This observation also is supported by data derived from the authors' genetic testing service across the spectrum of PIDs and other disorders.

Among the currently available sequencing methods, dideoxy chain termination sequencing (Sanger sequencing) [34] remains the standard in sensitivity and specificity. Its initially high cost has been reduced significantly through the introduction of automation, so that cost no longer justifies the use of alternative mutation scanning methods such as single-stranded conformation polymorphism, heteroduplex analysis, denaturing gradient gel electrophoresis, or denaturing high-performance liquid chromatography, which are less sensitive methods of mutation detection [35]. Because direct sequencing of complementary DNA (cDNA) is limited by messenger RNA (mRNA) instability and inability to detect intronic variation, sequencing usually is performed on polymerase chain reaction (PCR)-amplified genomic DNA. Using genomic DNA as template allows determination of both protein-coding regions (exons) and noncoding regions (introns and 5' and 3' untranslated regions). Typically only 20 to 50 nucleotides flanking each exonic region are sequenced, because known functional elements generally are located within this interval and the clinical significance of mutations deeper into the introns usually is unclear. Therefore, testing of additional noncoding sequences would be considered only when the functional significance of these sequences has been demonstrated previously.

Because of the average size and number of exons per gene [36], the development of affordable high-throughput sequencing technology is necessary. Several alternatives to standard dideoxy chain termination sequencing [34] now are available, including pyrosequencing [37], combinatorial sequencing by hybridization [38], oligonucleotide resequencing arrays [39], multiplex polymerase colony or polony sequencing [40], and sequencing-by-synthesis [41]. These alternatives may revolutionize genetic testing further by drastically reducing cost and turnaround time and allowing a full-genomic sequencing

approach. In current clinical practice, dideoxy chain termination sequencing remains the most sensitive and is still considered the standard.

Limitations of full-gene sequencing for mutation detection

Mutation detection by gene sequencing, although highly sensitive and specific, is subject to a number of limitations. For example, gene sequencing cannot distinguish if two heterozygous mutations are located on the same or on different alleles. For a recessive disease such as *ADA*-related SCID, the former case would be a carrier, and the latter would be affected. These possibilities can be determined by testing the proband's biologic parents. If the two heterozygous variants are located on the same allele, they have been inherited from the same parent.

Another important limitation of gene sequencing is its inability to detect certain types of pathogenic mutations such as large deletions, insertions, duplications, and inversions. For example, failure to amplify an X-linked gene in a male may be the result of a gene deletion, and large deletions of a single allele for autosomal genes may go undetected, leading to false-negative results. Genomic copy number variation is widespread in healthy individuals [42] and may complicate the interpretation of sequence-based results. Because failure to amplify one allele of an autosomal gene will lead to apparent homozygosity of all variants located in the corresponding region of the amplified allele, homozygosity for a rare variant in a non-consanguineous family always should raise suspicion. In this case, true homozygosity can be distinguished from hemizygosy caused by non-amplification of one allele by testing the proband's biologic parents for the presence of the rare variant. Finally, variants in a PCR primer site may lead to PCR failure of the variant-containing allele, a phenomenon known as "allele drop-out" [43]. This technical failure can result in erroneous interpretation of a disease variant's zygosity or erroneous reporting of sequence data reflective of only one copy of a given chromosome. In diseases in which a significant proportion of cases may be caused by deletions or other types of mutations not detectable by Sanger sequencing, complementary methods such as Southern blot, fluorescent in situ hybridization, real-time quantitative PCR, multiplex ligation and probe amplification, or comparative genomic hybridization may be required. The details of these techniques are reviewed elsewhere [44].

Other limitations of genetic testing may depend on the sample type. When testing patients who have SCID, for example, the use of DNA derived from peripheral blood could lead to contamination of proband sequences with maternal sequences. Maternal-fetal transfusion occurs commonly among patients who have SCID [31] and can lead to maternally derived engrafted cells. Sequence data preferentially derived from the maternal DNA could obscure a pathogenic variant or lead to improper interpretation of a variant's zygosity. Sequencing of DNA isolated from a buccal swab can avoid this pitfall. Detection of variations that are present only in some

cell types obviously depends on the sample type being tested and is another important consideration, because variants present at low levels of somatic mosaicism may be very hard to detect. Selective expansion of rare revertant lymphoid cells also has been described [45,46]. In at least one published case [47], a “second site” mutation restoring normal *ADA* mRNA splicing seems to have occurred in a common progenitor of T and B cells but not in non-lymphoid cells. Thus, the possibility of mosaicism must be considered when interpreting gene-sequencing results, particularly when test results do not correlate with the clinical picture.

In general, the technical limitations of each genetic test must be brought to the attention of ordering physicians and genetic counselors. These providers also must be aware that a negative genetic test result cannot exclude a diagnosis and must be prepared to inform the patient of these limitations (both technical and scientific), ideally through a process of informed consent initiated before the test is performed. Thus, gene-sequencing results always should be considered carefully in the context of the clinical presentation and should not be considered an isolated diagnostic determinant.

Gene testing and quality assurance

Clinical mutation detection requires a comprehensive approach to provide the highest level of accuracy and reliability. A single genetic test performed once on a patient can lead to a life-long diagnosis. Clinical decisions made as a result of genetic testing may be irrevocable. Both false-positive and false-negative results could have a devastating impact on the lives of patients. A false-positive result would lead to an inappropriate intervention, whereas a false-negative result would lead to false reassurance and potential missed intervention.

The analytic validity of a gene test is determined by a number of elements, such as analytic sensitivity, analytic specificity, and overall assay robustness. In the authors’ practice, all three parameters are validated before clinical implementation. Robustness and specificity of the assay are demonstrated by reproducibly obtaining sequences for the desired gene regions from a defined number of normal samples. Sensitivity and specificity of mutation detection are ascertained by detecting known mutations in a group of blinded samples, in which all mutations must be detected on both DNA strands. Importantly, the assay must be able to discriminate the target gene sequences from pseudogenes and functional homologous genes with highly similar or even identical sequences. Also, because DNA sequencing has an inherent error rate, it is important that pathogenic mutations are reported only when found by two sequencing reactions (ie, sequencing of both forward and reverse DNA strands).

Laboratory quality control protocols also are essential in maintaining assay reliability. Periodic internal proficiency testing should be performed, consisting of unannounced inclusion of blinded samples of known genotype

into the workflow. Continuous monitoring of processes and results serves to identify developing issues, such as primer degradation or unanticipated occurrence of normal variation underneath the primer site, preventing amplification of alleles containing such variation (allele drop-out) [48]. Well-documented laboratory quality control procedures, including personnel training and periodic proficiency evaluations, are essential elements for laboratories performing clinical genetic testing.

An additional key factor in minimizing human error during clinical performance is automation, which facilitates reproducibility of results and tracking of errors in processes that otherwise are susceptible to error (eg, setting up reactions for PCR amplification or registering sequence analysis data). Records of automated processes facilitate monitoring and quality assurance. For example, the authors' laboratory has developed an integrated laboratory-information management system for genetic testing that automates large portions of the genetic testing process from test performance through result reporting. As the availability and awareness of genetic testing continues to expand, genetic testing laboratories will depend increasingly on automation and informatics to support high test volume with short turn-around times, low costs, and, most importantly, accurate and reliable results.

Clinical interpretation of gene-sequencing results

Advances in molecular genetic techniques have made gene testing for PIDs increasingly available in clinical practice with more timely results. At the same time, such testing detects more and more variants that do not obviously alter the gene structure and function and are difficult to interpret in the clinical setting. Nonsense mutations, frame-shifting mutations, deletions, gene rearrangements, and obvious splice-junction mutation are relatively easy to interpret. In contrast, missense amino acid changes and most intronic and promoter variants often are difficult to interpret.

Interpretation of genetic testing results must consider each individual variant as well as the interpretation of the overall set of variants identified. Genetic variants are interpreted based on predictions of the effect on protein structure and function and potentially on published descriptions of the functional effect of a variant in an experimental system. The frequency of a variant in the population may be a clue to pathogenicity. Common variants are less likely to be pathogenic.

Standardization is highly necessary, considering the complexity of gene testing results. Because sequence variations are described best at the DNA level, the adoption by all clinical laboratories of the gene variant syntax and nomenclature recommended by the Human Variation Genome Society is an important step [49]. Importantly, the use of the appropriate cDNA reference sequence for naming mutations avoids dangerous confusion by

guarding against historical changes in the numbering of introns and communication errors. In contrast, the phenotypic classification of genetic variants is not currently unified. Different laboratories apply different variant classification algorithms. Moreover, the clinical interpretation and the threshold criteria for classifying variants of truly unknown significance versus pathogenic and normal variants largely reflect the protocols and specific experience of the clinical laboratory that has performed the PID genetic test. This lack of standardization ultimately represents a major impediment to the effective communication of genetic test results to physicians and genetic counselors. The following sections provide a brief overview of some principles guiding the clinical interpretation of gene variants.

Interpretation of novel variants

A novel variant is defined commonly as a variant not previously described in the published literature in association with a disease phenotype. Identification of a novel genetic variant in a patient is of no clinical use unless it is “clinically classified” based on available biochemical and genetic factors. In the absence of empiric data, the clinical laboratory may attempt to predict a variant’s biochemical and functional consequences by considering (1) mutation type; and (2) location within the gene (in a coding sequence versus a noncoding sequence). A missense mutation causes an amino acid change, but not all missense changes are pathogenic. The laboratory considers whether the amino acid change is conserved across species and whether it affects known functional domains or motifs within the protein. Even without considering functional implications, the laboratory may infer the likelihood of pathogenicity based on the allele’s frequency in a control population of healthy individuals. Variants occurring commonly in healthy individuals are very unlikely to be pathogenic. Cosegregation of the variant in families with disease, as quantified by the overall number of genotype–phenotype concordant versus discordant individuals, is another consideration. The exact weighting of each factor in the ultimate equation is debatable but can be incorporated into a rational and reproducible approach to predict pathogenicity.

The ultimate goal of any clinical laboratory is classification of variants into defined categories ranging from disease-causing mutations to normal variants (Fig. 1). A variant classification system must be flexible enough to assimilate novel scientific knowledge and data. A substantial proportion of identified variants will be of uncertain clinical significance but may be defined more easily in the future. To address all of these needs, the authors’ laboratory has developed its own standardized algorithm to classify each variant for probability of association with disease and to store the results in an adaptable database (GeneExplorer, Correlagen Diagnostics, Waltham, MA). A number of integrated algorithms and statistical approaches also have been reported previously [50–53]. In general, many algorithms

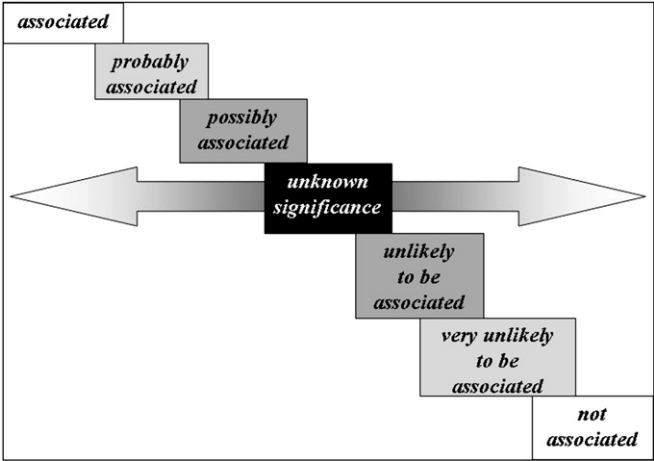


Fig. 1. Clinical classification of gene variants into defined categories. After its identification in a patient’s DNA, a gene variant is evaluated for its clinical significance. A standardized algorithm based on both genetic and biochemical information is applied. This approach allows the classification of each variant for probability of association with disease, ranging from disease-causing mutations (*left side of the panel*) to normal variants (*right side of the panel*). A certain proportion of identified variants will be of unknown significance (*center of the panel*) but may be defined more easily in the future when additional information could become available.

incorporate segregation data, species conservation, biochemical analysis, and functional data to allow the most accurate and efficient classification of genetic variants.

Interpretation of previously published variants

Clinical testing often is performed on isolated individuals or small families, and the classification of typically rare variants in small pedigrees presents a substantial challenge. Although predictive biochemical and genetic tools can help, empiric data from previous patients often are very helpful. This information usually is catalogued in locus-specific databases or reported in peer-reviewed publications. The compendium on genetic disorders and genes “The Mendelian Inheritance in Man (MIM)” [54] represented the first attempt at summarizing and cataloguing the clinical effects of genetic variation. Highly curated databases of published and unpublished mutations in PID genes are publicly available [55,56]. Although typically accurate, such online databases may be incomplete or only sporadically updated with the most recent peer-reviewed literature. Databases with direct links to electronic copies of supporting literature are desirable but virtually nonexistent.

To meet these needs, the authors’ laboratory has attempted to capture and classify such published information into comprehensive and dynamic

gene-specific databases, as described previously. Sophisticated data-mining algorithms and monitoring mechanisms are used to guarantee database curation through constant integration of the latest scientific information. The interfacing of this variant repository with a rules-based reporting system (RightReport, Correlagen Diagnostics, Waltham, MA) is key in assuring consistency of reporting and interpretation of variants, based on the most updated variant information. Of note, although ideally the wealth of published genetic variant information would be incorporated into public databases [57], much key data currently reside in proprietary laboratory databases or individual patient records around the world. Impediments to well-curated databases include lack of available resources, privacy concerns, and the lack of generally acknowledged means to report or publish these data [22,23].

Several publicly available general resources, such as the Single Nucleotide Polymorphism Database (dbSNP; <http://www.ncbi.nlm.nih.gov/projects/SNP/>) [58], the HAPMAP Data Coordination Center (<http://www.hapmap.org/>) [59], and the Human Genome Variation Database (HGVbase; <http://hgvbase.cgb.ki.se>) [60] also may assist in variant classification. Such databases do not attempt to determine explicit causative relationships between genotype and phenotype but are helpful in determining allele frequencies in individuals from different ethnic groups. In conclusion, complete, accurate, and standardized variant repositories are a prerequisite for variant classification algorithms seeking to classify correctly variants identified in an isolated patient, variants that otherwise would, improperly, be considered “novel” variations.

Interpretation of variants that affect messenger RNA splicing

The proper clinical classification of previously uncharacterized single-nucleotide intronic variants may be particularly challenging. Intronic variants at the consensus splice donor and acceptor sites can be classified definitively as pathogenic, but outside those positions the interpretation typically is unclear. Within coding regions, synonymous mutations that do not change an amino acid may be clinically relevant because of their effects on mRNA stability or because they create cryptic splice sites that alter the mRNA sequence [61]. Although a number of predictive tools are available [62,63], in vitro mRNA transcription studies often are the only way to determine the effect of these variants. Although molecular assays can determine the mutation's impact on mRNA message splicing [64], because causative intronic variants usually are removed from the mRNA message by splicing, it is important to associate the mutation to the allele that does not contribute to the normal mRNA, which can only be done using haplotyping [53]. In clinical practice, the allele frequency of a sequence variant in a population of unaffected individuals often is the only factor suggesting that an intronic variant is more or less likely to be pathogenic. Allele frequencies vary

across ethnic groups, however, necessitating variant prevalence studies in specific populations (eg, the Human Population Collection samples obtained from the Coriell Institute for Medical Research, Camden, NJ). The Single Nucleotide Polymorphism Database [58] and the HAPMAP Data Coordination Center [59] often are very helpful in this process, although data accuracy and validation always must be taken into consideration, particularly when data contamination by pseudogenes may be an issue.

Interpretation of missense variants

The clinical classification of novel, previously unreported missense variants also can be particularly challenging [65]. A number of biochemical algorithms and software tools designed to predict *in silico* the biochemical effect of amino acid changes may be helpful but must be used cautiously. Some examples are (1) BLOSUM (Blocks Substitution Matrix) [66]; (2) the GRANTHAM matrix [67]; (3). SNPper Amino Acid Variation [68]; (4) SIFT (Sorting Intolerant From Tolerant) [69]; and (5) PolyPhen (Polymorphism Phenotyping) [70]. Many of these predictive algorithms are based on the analysis of multiple protein sequence alignments and comparative interspecies analysis in combination with chemical differences.

Predictive methods are not as reliable as empiric data in the interpretation of clinical genetic test results, and when these algorithms are tested in parallel a high false-positive prediction rate generally is seen [71]. In one large study [72] the authors concluded that position of deleterious missense substitutions is biased strongly toward invariant residues and tends to have relatively high chemical difference index scores. Indeed, the majority of non-synonymous single-nucleotide polymorphisms probably are neutral and do not have an effect on molecular function, phenotype, or fitness [70].

As indicated by comparative genomics studies, approximately 10% of deviations of a nonhuman protein from its human orthologue are compensated pathogenic deviations caused by a missense mutation that, at the given site, would be pathogenic to humans [73]. The Encyclopedia of DNA Elements (ENCODE) project [74] recently has suggested the possibility of a large genomic pool of neutral elements that are biochemically active and act as a “warehouse” for natural selection. Therefore, because certain missense changes in conserved positions could help by creating functional specificity for diverging gene paralogues, analyses of sequence alignments is best performed if restricted to true gene orthologues. Notably, by comparing computational methods that use evolutionary conservation alone, chemical change, or a combination of the two, Chan and colleagues [75] has shown a high predictive value for methods that use evolutionary sequence conservation, with or without considering protein structural change. Moreover, a higher biochemical predictive value can be achieved if different computational biology tools are used simultaneously [75].

Interpretation of genetic variants based on data from functional assays

Biochemical, functional, or in vitro cellular assays with informative phenotype readouts are available for many PID genes, often based on flow cytometry or Western blotting techniques [24,25,76–80]. Ideally, high-throughput assays would be designed to characterize any genetic variant identified within a PID gene, especially when variants of uncertain clinical significance are encountered. In actual practice, biochemical assay data may be available from peer-reviewed publications and can be taken into consideration when providing a clinical interpretation of genetic test results. In vitro experimental follow-up, if available, generally is recommended when novel gene variation of uncertain significance is identified by gene testing. Of note, in vitro tests are not immune to pitfalls and limitations. Many proteins related to PID lie at the cell surface, and many amino acid substitutions with a clinical consequence are located in the protein's cytoplasmic domain, thus affecting intracellular signaling but not cell membrane expression. Thus, mutated cell receptors may be detected as normal by flow cytometry [77]. Also, missense mutations of cytoplasmic adapters may affect signal transduction without impairing a protein's stability and expression [81,82]. For example, in a biochemical functional study of 10 different *SH2D1A* missense mutations identified in patients who have X-linked lymphoproliferative (XLP) syndrome [83], selected mutants (eg, p.Thr53Ile and p.Thr68Ile) impaired the *SH2D1A* ability to bind to receptors of members of the signaling lymphocytic activation molecule (SLAM; CD150) family, although they did not affect protein stability or half-life. Gene sequencing may obviate certain pitfalls of biochemical studies or aid by strengthening the interpretation of such studies.

Standardization of criteria for interpretation and reporting of genetic variants

In conclusion, clinical testing may identify genetic variants of uncertain significance, either novel coding mutations or intronic variation. Nonsense mutations, specific missense mutations, and splice-site mutations are recognized as deleterious based on empiric observations in high-risk families, the absence of such variants in control populations (except at carrier frequency), comparative genomics predictive algorithms, and/or functional molecular or biochemical evidence. The optimization of computational algorithms integrating this information is sorely needed; because of advances in technology, the detection of mutations has outpaced the ability to interpret them. Although generic American College of Medical Genetics guidelines for sequence variant interpretation are helpful [84], standardized algorithms and approaches for clinical interpretation of the gene variants identified by gene sequencing are a requirement for a cost-effective use of genetic testing in patient management [22,23]. Moreover, result reports for genetic tests should include all test results and also should provide clear and standardized

interpretations, to be consistent across patients and over time and also to allow revisions as new data on variant significance emerge.

Genetic variants and phenotypic manifestations of primary immune deficiencies

Genotype–phenotype correlations

Establishing a correlation between a specific pathogenic variant and disease severity or selective PID subphenotypes often is impossible. Allelic heterogeneity (distinct mutations within the same gene) can lead to different phenotypes, and distinct phenotypes can arise from a single mutation [30]. The same PID phenotype also may arise through locus heterogeneity (ie, mutations in different causative genes) [85,86]. Few examples are known of strict genotype–phenotype correlation for PID genes [33,56], although some weaker correlations seem to be more common [87]. The inability to correlate genotype with phenotype may arise from interaction between disease-causing mutations and genetic variants elsewhere in the genome [88]. Additionally, host–environment interactions could account, at least in part, for the variable penetrance of autosomal dominant PIDs [10]. For example, individuals who have single heterozygous mutations in the tumor necrosis factor receptor superfamily member 13B (*TNFRSF13B*; *TACI*) have variable phenotypes ranging from unaffected to overt common variable immunodeficiency [89–91], suggesting a role for unidentified genetic modifiers and redundant signaling pathways. Complex host–environment interactions play an additional role, as demonstrated by some PIDs that arise in infancy and resolve spontaneously in adulthood, probably because of compensation by adaptive immunity [17,21].

Clinical, immunologic, and genetic diagnosis of primary immune deficiencies

The weak genotype–phenotype correlation for PIDs ultimately translates into difficulties in clinical classification and diagnosis. Historically, classification of PIDs has evolved as an “immunologic” classification as discoveries of these disorders has shaped and furthered the understanding of many immune system functions [6,27,92–94]. In particular, a combination of immunologic phenotypes and modes of inheritance has been the ruling principle for the International Union of Immunological Societies Committee Classifications of PIDs [28,94]. Because of the recent definition at the molecular genetic level of selective susceptibility to a number of specific pathogens, an updated clinical criteria-based classification of PIDs also has been proposed [21].

Ultimately, any classification system serving clinical purposes must be primarily phenotypic [17,21]. Because clinical presentation can vary and is dependent on environmental effects, deciphering the complexity of mutations

and genes involved in PIDs ultimately will allow the best operative approach to diagnosis, prognosis, and patient management. For example, in the case of XLP, identifying a pathogenic mutation in the *SH2D1A* gene provides a definitive diagnosis, overcoming clinical diagnostic difficulties caused by the pleomorphic clinical presentations of this disorder (Fig. 2) [82,95] and allowing therapeutic intervention before the patient contracts a potentially fatal Epstein-Barr virus infection. Additionally, among B-cell deficiencies, nomenclature of the hyper-IgM syndromes recently has been amended with a recommendation to reference the gene defect directly [85]. With respect to the immunologic diagnosis, gene testing offers the most definitive diagnosis in immunoglobulin-deficient patients, overcoming the variability of serum IgM levels in these gene diseases. Continued improvements in understanding the complex genetic basis of PIDs will lead to operational changes in the immunologic and phenotypic classification.

Role of hypomorphic mutations

Milder or atypical mutations, also known as “hypomorphic mutations,” in PID genes or related genes in a pathway also could be responsible for more common conditions, such as autoimmune, allergic, or inflammatory disorders. Supporting this notion, different experimental studies have linked the XLP1 gene *SH2D1A* and its receptors, the *SLAM/CD150* family member genes, with selective susceptibility to infections and autoimmune disorders [96–99]. Because of the key role that *SH2D1A* plays in host immune defenses [82,100,101] and the striking homology between *SH2D1A* and the EAT-2 (EWS/FLI1 activated transcript 2) adapter [99,102,103], it is tempting to speculate that mutations in the *EAT-2* gene and its ligands also could be involved in autoimmune disorders or human PIDs. Notably, these genes map within the lupus susceptibility *Sle1b* locus [98,102].

Genetic diagnosis by gene sequencing identifies benign and disease-causing genetic variations and variants of uncertain significance. Variants that currently are difficult to interpret may be understood more clearly in the future. New information may lead to reclassification of genetic variants, and patients must be made aware of these changes through updated results reports. Therefore, the authors’ laboratory reports all genetic variants identified by gene testing, because patients may only be tested once, and because clinical genetic test reports should be as informative as possible. Because of the exponential increase in genetic and functional data acquired both experimentally and in the context of the clinical genetic practice, flexible and adaptable databases are needed to collect the wealth of knowledge produced by clinical genetic testing [57]. At the present time, variant interpretation criteria primarily are relevant only to monogenic disorders. As time goes on, genetic test interpretation will be able to consider variable penetrance caused by modifier genes and hypomorphic genomic variation. A complete understanding of PIDs will require combined analysis of Mendelian and common

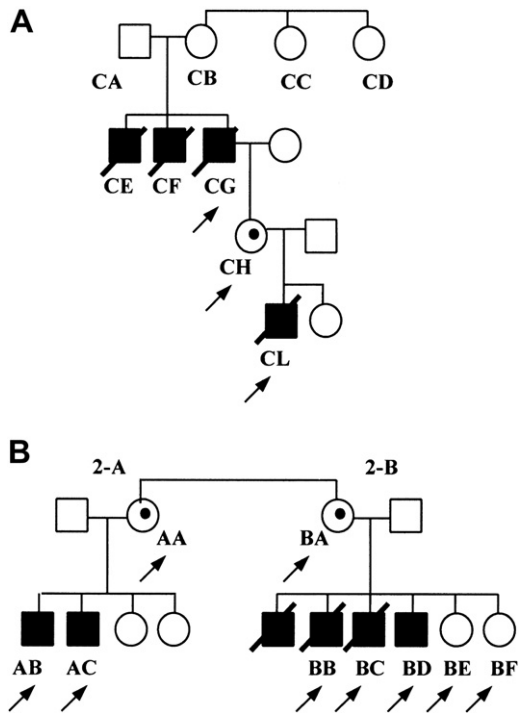


Fig. 2. Variability of clinical phenotypic manifestations in family members affected by X-linked lymphoproliferative-1 syndrome (XLP1) carrying the same *SH2D1A* mutation. Three main clinical phenotypes can characterize the clinical presentation of XLP1: fatal infectious mononucleosis (FIM), lymphomas, and immunoglobulin deficiencies. Defects in the XLP1 gene, *SH2D1A* were investigated in two unrelated families in which male members were affected by common variable immune deficiency (CVID). Arrows indicate patients analyzed for the presence of *SH2D1A* gene mutations. The genetic trees of the two families are shown in panels A and B, respectively. Patients are labeled with numbers and letters; boxes indicate males; circles indicate females. A line through the box or circle indicates that the patient is deceased. A solid box indicates an affected male; a dot within a circle indicates a carrier; open boxes and circles indicate unaffected subjects. (A) Three brothers (CE, CF, and CG) were examined for recurrent respiratory infections. A grandson (CL) died of a severe *Aspergillus* infection secondary to progressive immunoglobulin deficiency, FIM, aplastic anemia, and B-cell lymphoma. (B) Two brothers had B lymphocytopenia and immunoglobulin deficiencies (AB and AC). The presentation of FIM in another male relative (BC) led to the investigation of the *SH2D1A* gene. Pathogenic *SH2D1A* mutations were detected in both branches of the family. These results highlight the difficulties in establishing a diagnosis of X-linked lymphoproliferative disease (XLP) based on a clinical presentation and the relevance of gene testing in establishing a definitive diagnosis. Notably, the genetic diagnosis of XLP allows the selection of appropriate therapy (such as bone marrow transplantation) because the prognosis for XLP1 is much worse than for CVID syndrome in general. (From Morra M, Silander O, Calpe S, et al. Alterations of the X-linked lymphoproliferative disease gene *SH2D1A* in common variable immunodeficiency syndrome. Blood 2001;98(5):1323; with permission. Copyright © 2001, American Society of Hematology.)

Box 1. Key points

1. When ordering a genetic test, one should be aware of the testing methodology and its limitations and properly inform and counsel the patient in advance.
2. Gene sequencing, although the reference standard for molecular diagnostics, has a number of technical limitations, as described in the text.
3. Some genetic variants currently identified by complete gene sequencing are difficult or impossible to interpret and are called “variants of unknown significance.”
4. The clinical understanding of variants of unknown significance will improve as new genetic information becomes available, requiring updated clinical reports and communication with patients and their families.
5. Genetic testing of family members is recommended in many cases because it might help provide a better understanding of a novel gene variation identified in an affected proband.
6. One always should test for the presence of maternal cells in the blood of a patient who has SCID. Detection of DNA sequences from maternal cells might obscure the identification of genetic variants in DNA isolated from peripheral blood of such a patient.
7. Although genetic testing can be performed on samples other than blood (eg, buccal swabs), one always should archive a blood sample from a patient undergoing bone marrow transplantation.
8. One always should discuss genetic testing results with the clinical laboratory that has performed the test if there are any uncertainties.

genetic variation. Eventually the utility of genetic testing will extend beyond highly penetrant disorders to predictive tests for low-penetrance and genetically heterogeneous disorders. Because the clinical presentation of PIDs often is nonspecific, with sometimes mild or absent clinical signs, the majority of PID patients may be undiagnosed [87]. Many mild disease-causing mutations probably are involved in these cases, and the definitive diagnosis will depend on genetic testing.

Summary

Box 1 lists key points in the clinical use of gene testing.

Clinical genetic testing laboratories are growing contributors to the field of PIDs, both in terms of knowledge being accumulated and patient care.

PID gene testing now is performed outside the traditional domain of academic, research-oriented laboratories and is being used by physicians who have a range of clinical expertise. The traditional separation between PIDs and more common infectious, autoimmune, and inflammatory diseases is fading as knowledge accumulates. In the future, genetic testing will become an increasingly important diagnostic tool in immunology clinics. Clinical diagnostic laboratories must adhere to the highest practice standards to ensure the quality of genetic testing for PIDs. Adoption of automation, laboratory informatics interfaces, and quality assurance protocols will improve the reliability of genetic test results. High-throughput gene-sequencing approaches will speed the process while maintaining quality. Optimization and standardization of approaches to variant interpretation will increase further the usefulness of genetic testing results by minimizing the reporting of variants of unknown significance. Clinical diagnostic laboratories are playing an increasing role in providing genetic health care and personalized medicine to patients who have PID.

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