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Primary immunodeficiency Diagnosis: Genetic and molecular Assessment

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Abstract: Primary immunodeficiencies (PIDs) encompass a heterogeneous group of inherited disorders characterized by impaired immune function, leading to increased susceptibility to infections and autoimmune complications. Accurate and timely diagnosis is crucial for implementing appropriate treatment strategies and improving patient outcomes. Genetic and molecular assessments have revolutionized PID diagnosis, moving beyond traditional phenotypic characterization based solely on clinical presentation and immunological laboratory tests. Here we outline the current landscape of genetic and molecular approaches used in PID diagnosis, highlighting their strengths and limitations. Next-generation sequencing (NGS) technologies, including whole-exome sequencing (WES) and whole-genome sequencing (WGS), have significantly advanced PID diagnostics. These powerful techniques allow for simultaneous analysis of numerous genes associated with known PIDs, providing a comprehensive assessment of the patient's genome. WES, focusing on protein-coding regions, offers a cost-effective approach with high diagnostic yield, often identifying causal variants in a substantial proportion of patients with suspected PIDs. WGS, while more expensive, provides a complete picture of the genome, enabling identification of non-coding variants and structural variations that might be missed by WES. Targeted gene panels, focusing on a pre-selected set of genes relevant to specific PID subtypes, offer a faster and more cost-effective alternative for patients with a suspected specific diagnosis. However, challenges remain in interpreting NGS data. The identification of variants of uncertain significance (VUS) is common, requiring further functional studies and family segregation analyses for definitive classification. Furthermore, the genetic heterogeneity of PIDs, with many disorders caused by mutations in multiple genes, can complicate the interpretation of NGS results. Advanced bioinformatic analyses and sophisticated data interpretation pipelines are crucial for effectively navigating this complexity. In addition to NGS, other molecular techniques such as quantitative PCR (qPCR), flow cytometry, and functional assays continue to play important roles in characterizing the specific immunological defects and guiding targeted therapies. In conclusion, genetic and molecular assessments are indispensable for the diagnosis of PIDs, providing significant improvements in diagnostic accuracy and speed compared to traditional methods. While NGS technologies have emerged as powerful tools, careful interpretation of results, coupled with a comprehensive clinical and immunological evaluation, remains essential for accurate diagnosis and effective management of these complex disorders. Future advancements in NGS technologies, bioinformatics, and functional assays will further refine our ability to diagnose and treat PIDs, ultimately improving patient outcomes.

Keywords: *Primary immunodeficiency, Diagnosis, Genetic and molecular*

Introduction.

The loss of expression and function (LOF), amorphic or hypomorphic protein encoding primary immune deficiencies (PIDs), or gain of function (GOF), hypermorphic protein encoding PIDs are all outcomes of monogenic germ line mutations in immune system-related genes. Infectious diseases, autoimmune disorders, allergies, and cancer are more common in those with PIDs, which were recently reclassified as Inborn Errors of Immunity (IEI).[1, 2]

The estimated prevalence of PIDs ranged from 1 in 10,000 to 1 in 50,000 live births, making them rare disorders. A better understanding of immuno-genetics has resulted in the identification of the causative genes of phenotypically-known PIDs and numerous additional new PIDs, thanks to recent developments in genetic study platforms and their interface with immunological knowledge..[3, 4] With that said, newer research has demonstrated that PIDs are significantly more common than previously thought, with an estimated 1% of the population suffering from a PID of some kind (including all variants).[5, 6]

The development of next-generation DNA sequencing technologies (NGS, Next-Generation Sequencing) coincided with the Human Genome Project and greatly advanced our understanding of PIDs. These technologies enabled the simultaneous study of numerous genes, including the entire exome or even the human genome. The development of computational analysis platforms allowed for the rapid and adequate analysis of the massive databases produced by genetic sequencing platforms. This field, which combines biology and informatics to a high standard, is now known as bioinformatics or biocomputing. That is to say, the detection and diagnosis of immune system abnormalities that were previously inconclusive have made tremendous strides forward thanks to these novel platforms.[3, 7]

Acquiring a highly-qualified medical team, the International Union of Immunological Societies (IUIS) has organized biannual publications to disseminate information about all advances, new diseases, and genes related to PIDs. Their goal is to monitor, follow, and catalog the latest discoveries published in the medical literature. Up to 406 of these immunological disorders have been reported as of the beginning of 2020, according to the most recent publication. Of these, 430 have been linked to specific genetic abnormalities..[1]

With the goal of shedding light on the various genetic evaluations that are currently available, how to interpret their findings, and the significance of medical knowledge in achieving clinical correlation with these findings, this review article seeks to rescue concepts of medical genetics that are essential for comprehending the advances in the genetic-molecular characterization of primary immunodeficiencies.

Reviewing fundamental ideas in medical genetics

Understanding the pathophysiology and heredity of PID requires a firm grasp of the fundamentals of genetics. To start, keep in mind that each human being possesses a total of 23 sets of chromosomes. One set of these determines our gender and is known as the sex chromosome. The other 22 sets of chromosomes are autosomal, and they contain the genes that are responsible for our complete genetic make-up. Each gene has two pieces of genetic information, one passed down from mothers and one from fathers. These pieces of DNA are called maternal and paternal alleles.[8, 9]

Genes are made up of alternating sections called exons and introns. The exons are responsible for transcription, while the introns have structural and DNA support functions. Transcription involves a structural change of DNA, but only the exon content is utilized to create messenger RNA. Adenine, thymine, guanine, and cytosine are nitrogenous bases that are repeated millions of times in our DNA. They compose the genetic material contained in the exons and introns. Codons are sets of three nitrogenous base sequences found in the exons that direct the gene to add an amino acid to the final product, the protein. Although the majority of people maintain this particular sequence of base pairs in their proteins, genetic variances allow for small changes in certain areas that do not affect the end result. Databases of the human genome have information on these variants, which occur in varying percentages in the population.[8, 9]

However, if this sequence of base pairs is incorrect, it could alter an amino acid that is crucial for a protein's function, resulting in either the protein's inability to function (LoF) or its overactivity (GoF). On the other hand, these alterations can cause a stop codon to be formed, which stops transcription before mRNA is fully formed. This causes the protein synthesis to halt early, which in turn affects its stability and function, and makes it nearly impossible to detect. Another type of mistake that can happen in this nitrogenous base sequence is insertions or minor deletions. These changes affect the entire codon sequence, which in turn affects the structure and function of the protein. Whenever this occurs, the alterations to nitrogenous bases are known as pathogenic variations or mutations.[9]

The concept of inheritance pattern is also of paramount importance in medical genetics. Patients' family histories that include these patterns of inheritance are crucial for genetic counseling when dealing with suspected PID. When a gene's two alleles are different, the resulting phenotype is known as an autosomal recessive (AR) pattern. This is the typical pattern seen in PIDs, and it's associated with the gene's loss of function in most cases. As a result of similarities between the two alleles, the AR pattern is called homozygous, but it can also appear as compound heterozygous, meaning that each allele has its own unique genetic mutation that causes it to lose some of its function.[1, 2]

Under the autosomal dominant (AD) pattern, a change in a single allele of a gene is sufficient to produce a specific clinical manifestation. One kind is a hereditary trait, wherein one or both parents carry the gene variant; another is a de novo mutation, which happens in the gametes and passes the mutation on to future generations regardless of whether either parent carries the variant or not. This pattern is found in a number of PIDs, including Hyper IgE Syndrome, APDS, and CTLA4 haplo insufficiency. The concept of X-linked inheritance, often known as sex-linked inheritance, pertains to genes found on the X chromosome that do not have an identical Y chromosomal counterpart. Hemizygotic refers to the fact that males possess just one copy of chromosome X and that this non-homologous area does not contain any alleles. This inheritance pattern is present in some of the classic immunodeficiencies, including X-linked gammaglobulinemia (XLA), Wiskott-Aldrich syndrome, IPEX syndrome, X-linked dwarfism with gamma chain deficiency, and Severe combined immunodeficiency (SCID) owing to gamma chain deficiency.[1, 2]

Genetic variants

Genetic sequencing compares the analysed DNA's nitrogenous base pair sequence to databases compiled from the Human Genome Project, the majority of which are accessible for free at the moment. Variation in genetic code can be found in both germ cells (eggs and sperm) and somatic cells (bodily tissues and organs). Variation in germ cells is the only kind that can be passed down through generations, impacting both population dynamics and evolution.[10]

The genesis of genetic diversity can be traced back to mutations. An irreversible alteration to the DNA sequence is known as a mutation.De novo mutations happen when repair enzymes miss a mistake during DNA replication and the mistake gets duplicated and mended in the DNA. There are three main types of mutations: those that have no effect on the body, those that have beneficial effects, and those that are harmful and cause pathogenic disorders. Another significant mechanism that contributes to genetic diversity is recombination. Recombination, in which homologous DNA strands align and cross over to generate novel combinations of variations in the germ cells of the progeny, is the process by which each person inherits a portion of their parents' genes.[10]

Genomic areas that deviate from the genomes stored in databases are referred to as genetic variants. Names for variations might be based on the nature of the exchange and the change it brings about..

Silent variant: A single substitution of a nitrogenous base for another happens, changing the codon but leaving the coded amino acid unaltered, so the protein stays the same. Keep in mind that various sequences of nitrogenous bases can encode amino acids for that to happen. In most cases, these variations are deemed benign because they do not pose any harm to patients.

Missense variant: A single substitution of a nitrogenous base for another changes the amino acid's encoding at that specific site. The protein's stability and function could be affected or not by the change to its linear shape. If you want to know if that protein changed in any way, you have to do functional investigations.
Nonsense variant: A stop codon, which is a nitrogenous base exchange, converts the amino acid codon to one that stops translation. The produced protein is typically unstable and/or defective, and no amino acids are added from the point of exchange.
Frameshift variant: Within this set of variations, one can find instances of nitrogenous base insertions or deletions that alter the fundamental codon sequence, hence influencing the subsequent amino acid additions and ultimately producing a whole new protein. In addition to INDEL, they are known as insertion and deletion. Unstable and dysfunctional characterize the majority of them.
Splicing variant: These variations aim to alter the process of making messenger RNA by rearranging the genetic code's exons and introns. In general, they facilitate the production of modified messenger RNA and, by extension, modified proteins. This kind of change is most often linked to variations at bases 1 and 2 in the region where exons and introns transition. The majority of these modified proteins are either inoperable or prone to instability.
At the outset, in order for everyone to be on the same page, genetic sequencing results must be reported using the HGVS-recommended international nomenclature criteria. To avoid a false negative, it is crucial to use the correct nomenclature when screening the patient's relatives for the variant. This will ensure that the same variant that was previously identified is being tested for.At the outset, in order for everyone to be on the same page, genetic sequencing results must be reported using the HGVS-recommended international nomenclature criteria. To avoid a false negative, it is crucial to use the correct nomenclature when screening the patient's relatives for the variant. This will ensure that the same variant that was previously identified is being tested for..[11]
Researchers in the field of human genetics further categorize genetic variants based on the impact that this genome mutation has on the gene's function and, by extension, the likelihood that it will cause or not cause diseases in people. Pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, and benign are the possible names for the variants. For a variant to be considered pathogenic, it must have been previously investigated and proven in both laboratory and clinical settings that the patient suffers from the clinical ailment associated with the researched gene function modification. variations like INDELs, stop codons, and splicing variations, which are known to cause a certain disease, are likely harmful if they are located in genes that are suspected of causing that disease. In contrast, benign variations are ones that have not been found to cause a decrease in gene function and have been confirmed either clinically or in a laboratory setting. Variants with a high frequency in the population—typically greater than 1%—are also thought to be benign, since they wouldn't be there if they were harmful. Variants that do not satisfy all the requirements to be categorized as benign but are not predicted to have any pathogenicity are likely to be benign. This includes silent and intronic variants..
The most challenging group of variations are those with unknown significance. These variations cannot be labeled as benign or harmful based on the current scientific knowledge. Rare variations are those that have never been reported before, aren't commonly discovered in population genetic databases, and have produced contradictory findings when tested using in silico computer programs designed to modify protein functions. This is because these variations necessitate highly specialized labs, which are not available in clinical practice, and further study is needed to confirm the impact of the mutation on the end product and the protein's predicted function..[11]
Genetic sequencing techniques
Analyzing the specific order in which the nitrogen base pairs that compose DNA are studied is known as genetic sequencing. Looking for variations in this sequence that disrupt messenger RNA synthesis and, by extension, the final result of genes—the proteins that carry out various bodily functions—is central to illness research. There are a variety of genetic tests available, each with its own methodology, goals, and maximum number of

genes that may be examined, so it's crucial to have a good idea of what you're hoping to find before requesting one. Consequently, we have decided to conduct a brief evaluation of the ones that deal with the clinical aspect of the existing genetic testing alternatives that aid in patient diagnosis, both for PID and other illnesses..[12]

Sanger technique

Although it is still utilized extensively in several basic genetic studies, the Sanger approach has recently lost a lot of ground in clinical genetics despite being the first to be reported. This method relies on manual genetic sequencing, which is done piecemeal, often exon by exon of each gene. It is exceedingly labor-intensive, has a high cost per investigated gene, and cannot be automated because of these qualities. It becomes a time-consuming and expensive process in PID investigations because multiple genes can cause similar symptoms. In some cases, such as when studying genes with pseudo genes or when using NGS approaches might cause problems, this method is nevertheless highly helpful and is used. This method can also be helpful in cases where it is believed that a single gene is responsible for a disease..[13]

Next-Generation sequencing (NGS)

The idea behind the next-generation sequencing (NGS) technology came from the necessity to automate genetic sequencing in order to evaluate a huge number of genes simultaneously. This method involves the use of primers, which are conventional structures that bind to DNA and enable their examination by amplification. Here, the information derived from nitrogenous bases is transformed into binary sequences and then subjected to computational analysis using a massive amount of data. Consequently, funding for bioinformatics as well as the laboratory is necessary for this process. Genomic sequencing has been incredibly helpful in evaluating primary immunodeficiencies, and it also gives us the option to examine at different depths depending on the focus of current genetic research.[13]

Targeted sequencing or genetic panels

Most clinical genetic laboratories now use targeted sequencing, which is also called genetic panels. Almost every branch of medicine makes use of this sort of sequencing to investigate sets of genes linked to patients' clinical symptoms. The reduced materials utilized and smaller bioinformatics analysis make the panel study more cost-effective due to the limited number of genes.[14] We can confirm the presence of large deletions/common insertions as the genetic cause of several IEI (DOCK8, LRBA, for instance) through a quantitative analysis of the genes' amplification, which is possible with this technique. This analysis is called copy number variation (CNV).[15, 16] Keep in mind that the majority of panels solely examine these genes' exons. It is possible for PID genetic panels to omit genes that aren't actually present in the samples, or genes that are placed in intronic regions, which are non-coding.[13] The genetics lab sets up a different panel, and sometimes the gene (or genes) that one would need to explore might not be on that panel. This is vital information for professionals practicing medicine. It is important to be aware of the institution's panel that will conduct the test and, in particular, whether it takes into account the genes one wishes to study, before submitting a request.[12, 13]

Whole exome sequencing (WES)

The Whole Exon Sequencing Project (WES) generates a mountain of data for bioinformatics research by sequencing every gene in our DNA. The ability to assess all genes simultaneously is, to put it simply, a major benefit of this test. There are a lot of factors to think about, though, including the high cost of the test (much more than the cost of running a specific panel), the large number of variants related to other bodily processes that need to be evaluated using the results, and, worse, the decreased ability to evaluate the CNVs that were mentioned earlier.[12]

Most clinical genetic laboratories now have access to WES, however research has been its primary user due to cost and practical considerations. While some clinical laboratories solely use WES, they tailor their data analysis to each individual patient's needs, which significantly cuts down on both time and money spent on the process. It is possible that in the near future, the cost will be comparable to genetic panels, making it more accessible from a clinical standpoint. This could be due to rising standards in bioinformatics analysis, an expanding global database fueled by the sequencing of various populations, and falling material prices.[13]

Whole genome sequencing (WGS)

Whole genome sequencing (WGS) entails reading every nucleotide and coding sequence from every gene. This method is commonly employed to describe a species and all of its variations. Throughout the COVID-19 pandemic, it has been widely utilized to characterize viruses in various places, which has helped us understand the changes happening with SARS-CoV-2. It has been utilized to investigate novel diseases in the field of IEI research. The expensive price tag and the requirement for extensive bioinformatics equipment to analyze data are two of its major drawbacks. Keep in mind that each person will have billions of letters that need to be examined in an effort to detect a mistake in this sequence that is more than just a deviation from the norm.[13]

Hybridization and micro-array

The term "copy number variation" (CNV) describes the degree to which an individual's gene copy differs from that of another. Gains and losses of genetic material occur in the genome, as shown by the Human Genome Project. These changes can be linked to human diseases. From a practical standpoint, these differences may include substantial deletions; this would mean that sequencing would only amplify one allele, reducing the number of copies produced.17Although bioinformatics methods have been developed to detect these CNVs in NGS, genomic hybridization by microarray is still considered the gold standard for genetic diagnosis. Probes used in comparative genomic hybridization (CGH) are often developed for the detection of copy number variations (CNVs), and the process compares the results of the patient's DNA hybridization with a microarray of these probes to those of competitive DNA hybridization within the same test.[18]

Genetics in inborn errors of immunity

A better knowledge of PIDs was made possible, as mentioned before, by developments in genetic sequencing tools. The clinical field has benefited greatly from the scientific advancements made possible by these investigations, since molecular diagnostics allows for a better understanding of the disease's pathogenesis and its correlation with the patient's clinical situation. As part of the Precision Medicine framework, this knowledge often lets us determine individual treatment options for each patient. To get from the lab to the patient's bedside—or, more accurately, the doctor's office—there are obstacles.

There are already around 430 genes that have been identified as the sole source of primary immunological deficiencies, and this number is anticipated to continue increasing in the years to come. There is a clear need for medical professionals treating patients with PID to have knowledge of genetic sequencing methodologies, as the interface between immunology and genetics has become extremely productive. Only then will we be able to understand the great variation in genetic tests and the great variation in sequencing costs across laboratories. Using this interface, healthcare providers should assess a patient's symptoms and conduct tests based on their clinical suspicions if they suspect PID. If their initial findings indicate that genetic testing can confirm or rule out a diagnosis, offer potential treatments, or even grant access to those treatments, then sequencing should be considered for the patient.[13]

As an example of why genetic sequencing is so important in PID research, a new study indicated that original PID diagnoses were revised in 60 families (or 55%) out of 110 after molecular diagnosis using WES, according to clinical immunological phenol type data. There was a significant shift in the therapy of patients with PID in 25% of cases, or 26 out of 110 families, according to the same study. Following the genetic analysis findings with WES, at least 14 patients had hematopoietic cell transplantation.As an example of why genetic sequencing is so important in PID research, a new study indicated that original PID diagnoses were revised in 60 families (or 55%) out of 110 after molecular diagnosis using WES, according to clinical immunological phenol type data. There was a significant shift in the therapy of patients with PID in 25% of cases, or 26 out of 110 families, according to the same study. Following the genetic analysis findings with WES, at least 14 patients had hematopoietic cell transplantation.[19] A molecular diagnosis can guide a more targeted treatment based on the impacted immune pathway, for example, abatacept for CTLA4 and LRBA deficits, in addition to indicating bone marrow transplantation (BMT) for many PIDs.,[20, 21] the use of Janus-Associated Kinase/STAT inhibitors in conditions of STAT1 and STAT3function gains,[22, 23] the use of AKT-mTOR pathway inhibitors in patients with

PIK3 pathway changes with increased activity,[24] the use of IL1 antagonists in patients with some autoinflammatory diseases,[25] and interferon alfa for defects of the TLR3 pathway,[26] among others.

On the other hand, the same previously mentioned study found no genetic changes that could confirm the molecular diagnosis of PID in 60% of the 278 participating families.[26] The presence of gene regions without adequate coverage, including regulatory regions and polyA, low-grade mosaicisms, small CNV and INDEL, intronic mutations and the presence of pseudogenes may be associated with not finding variants in these cases.[26, 27] As an example, the currently most commonly used WES platforms have less than 100% coverage in94 genes in the IUIS list, less than 99% in at least 26 genes, and five with less than 90% coverage (IKBKB, NCF1, TACI, UNC93B1, and TBX1);in other words, part of the genes are not sequenced and analyzed and, therefore, genetic variants in these regions are not identified.[27]

Table 1 shows a series of genes present in the IUIS list that have intronic pathogenic or probably pathogenic mutations already described in the ClinVar database. Table 2 shows the genes present in the IUIS list that have at least one pseudo gene, which can interfere with the appropriate interpretation of their sequence.

Table 1 Genes related to primary immunodeficiencies present in the list of theInternational Union of Immunological Societies(IUIS) with pathogenic or probably pathogenic variants located in nonexonic regions listed in ClinVar.

ADA	G6PD	NOD2	TA2
ADAR	GATA2	OSTM1	TCN2
ATM	GINS1	PMS2	TERC
BTK	IKBKG	PNP	TERT
CARD14	IL10	POLA1	THBD
RMRP	IL2RG	POLE	TPP1
CD30	IL36RN	PRKDC	TRAC
CFH	KMT2D	PTEN	TRNT1
CFTR	MSH6	RNASEH2B	TT37
CHD7	MVK	SBDS	TTC7A
CTLA4	NBN	SERPING1	UNC13D
CYBB	NCF1	SH2D1A	VPS13B
DNMT3B	NFκb	SPINK5	WAS
ELANE	NLP12	STAT2	ZAP70

Source: Adapted from Henrickson et al.[27]

Table 2 Genes related to primary immunodeficiencies present in the list of the International Union of Immunological Societies(IUIS) that have at least one pseudogene.

IUIS genes with 1 pseudogene	C19orf40, CD46, CDCA7, CSF2Rb, DCLRE1C, FPR1, HAX1, IKBKG, ITCH, MAGT1, MSN, MTHFD1, NBAS, NCSTN, NHP2, NOP10, PIK3CD, PNP, PTEN, RANBP2, RLTBP2, RLTPR, RNASEH2C, RTEL1, TCF3, TMC6, ZBTB24
IUIS genes with more than 1 pseudogene	ACTB, AK2, CFTR, IGLL1, NCF1, PMS2, RAC2, RNF168, RPSA, SBDS, TRNT1, UNG, XIAP

Source: Adapted from Henrickson et al.[27]

Conclusion

Patients with primary immune deficiencies now have access to more information than ever before thanks to genetic testing. In clinical practice, the advantages are clear, including a more precise diagnosis, genetic counseling for families, and the prospect of a more suitable and correct therapy, all of which have the potential to improve patients' quality of life while decreasing their risks of death and complications associated with PID. However, there are a number of obstacles that must be surmounted, including issues with affordability, availability of testing, and understanding of genetic data. If doctors treating PID want to improve the odds of getting useful sequencing results, they need to learn more about the pros and cons of various genetic evaluation methods so they can tailor their investigations to each patient's unique clinical and immune phenotypic profile.

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