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Editorial

Using Molecular Diagnostics for Inherited Retinal Dystrophies: The 6 "I"s That Are Necessary to Diagnose 2 Eyes Genetically

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This editorial presents clinicians with the critical questions and information to assist them in the appropriate use of molecular genetic testing for patients with inherited retinal dystrophies (IRDs). Clear and thoughtful guidelines and indications for genetic testing have been published by the American Academy of Ophthalmology (https://www.aao.org/clinical-statement/recommendations-genetic-testing-of-inherited-eye-d), including the role of genetic testing for research.

To aid in the discussion of the molecular genetic testing for IRDs, we have divided the genetic testing process into 6 "I"s: indications, input, informed test selection, interpretation, initiative, and instructions. It is important to remember that the clinician ordering the genetic tests, whether an ophthalmologist or another health care provider who is licensed to prescribe such testing, ultimately is responsible for ensuring that all facets of molecular genetic testing for their patients are addressed.

Two programs currently offer free IRD molecular genetic testing and genetic test counseling after the results are available at no direct costs to patients (United States residents only). The first is the My Retina Tracker program sponsored by the Foundation Fighting Blindness in collaboration with Blueprint Genetics and InformedDNA (https://www.fightingblindness.org/open-access-genetic-testing-program). The second program is the ID YOUR IRD sponsored by Spark Therapeutics with Invitae Genetics (https://www.invitae.com/en/idyourird/). The My Retina Tracker panel includes the entire mitochondrial genome, high-resolution copy number variant detection, and coverage of the full *RPGR* gene, as well as IRD-related noncoding variants. The ID YOUR IRD panel is able to detect insertions and deletions < 15 bp in length and determines copy number at a single exon resolution. Patient information is shared with Spark Therapeutics, a for-profit company, and the panel does not include the *RPGR* gene, accounting for most (approximately 70%−90%) of X-linked rod−cone dystrophy, which could be an issue for male IRD patients.[1] It is essential for the ordering clinician to understand and assess the potential benefits and risk of such testing and to ensure that proper counseling and follow-up logistics are carried out before and after the testing has been completed.

Indications: Who Should You Be Testing?

Genetic testing of infants can be invaluable, especially to look for syndromic disease as well as stationary versus progressive disease before degenerative changes become evident. In many cases, molecular genetic testing of infants and small children can be accomplished more easily and safely, rather than having them undergo diagnostic studies under additional anesthesia or sedation. For children who clearly have an IRD that is nonsyndromic or has been identified with known syndromic features, molecular genetic testing may or may not be appropriate. If one is only going to carry out testing on the proband, then it may be prudent to wait until the individual is older or when potential treatments or clinical trials become available, the genetic testing technology has advanced both in detection methods and reporting the pathogenicity of the variants, or both. However, the urgency of testing may be increased by family planning concerns, anxiety within the family, the potential availability of key family members for follow-up genetic analyses, or a combination thereof.

Molecular genetic diagnostics may be appropriate when the presence of the IRD is having a psychological impact on the individual's ability and willingness to engage in some interpersonal relationships such as dating. Genetic testing may establish causation better and may provide clarification of risk of transmission, which can be therapeutic. Such psychological impact may or may not be applicable to children and teenagers, and documentation of behavioral or psychological disturbances relative to the IRD can be helpful.

One frequently encounters adults with late-onset vision symptoms resulting from bilateral symmetric retinopathies that can arise from multiple causes (Table 1),[2] including IRD, that potentially may warrant specific treatments. Although the abrupt onset and rapid progression of visual symptoms suggests an acquired condition, in some instances, patients unknowingly have adapted to a slow, life-long progressive condition until their vision impairment or symptoms have become evident. A subset of autoimmune retinopathy cases is paraneoplastic, and thus a prompt search for prior or occult malignancy, or both, may be warranted. Genetic testing can be indicated if no identifiable cause is found and if the result either will establish or lower the probability of an IRD and potentially will affect clinical care. More extensive testing (such as anti-retinal antibody testing and cancer surveillance) can be undertaken if the genetic test results are inconclusive, negative, or both.

Despite the accepted indications for molecular genetic testing for IRDs, a substantial number of individuals remain for whom a direct impact on clinical care is not yet achievable.

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Table 1. Possible Causes for Bilateral Symmetric Retinopathies Other Than Inherited Retinal Dystrophy

Infectious	Rubella
	Syphilis
	Lyme
Metabolic	Vitamin A deficiency
Toxic	Deferoxamine
	Pentosan polysulfate
	Hydroxychloroquine
	Phenothiazines
Toxin	Heavy metals
Autoimmune/paraneoplastic	Autoimmune retinopathy
	Cancer-associated retinopathy
	Melanoma-associated retinopathy

Although some patients are willing to bear the costs of the genetic testing personally, one must try to establish realistic expectations of obtaining definitive or inconclusive results from the test. Thus, the free programs previously described may be a valuable resource. However, these testing programs should not be misused such as for screening at-risk, asymptomatic individuals, unless those individuals are key to interpreting the test results of the proband, if a potential treatment or a current or planned clinical research trial exists for which they would be eligible, or both.

Input: What You Need to Know before You Order Molecular Genetic Testing

As in all patients, gathering clinical information is the first step before ordering molecular genetic tests (Table 2). This information both will justify the molecular genetic testing and will aid your ability to understand and interpret confidently the results that will be obtained. Is it essential to carry out exhaustive phenotyping before genetic testing? Studies such as fluorescein angiography, autofluorescence imaging, OCT, and electroretinography rarely impact the selection of the genetic panel for testing, but they can identify features and comorbidities that may warrant monitoring and treatment. Certain retinal features may heighten one's suspicions for specific causative genes and may reinforce the certainty of the molecular diagnosis, but these features should not be the only determinants of the gene selection for testing. Nonocular phenotyping (neurological, metabolic, skeletal, and systemic) can be invaluable for refining the interpretation of the molecular genetic results and helping to identify syndromic IRDs that may necessitate additional medical attention.

Informed Selection of the Genetic Test

A Clinical Laboratory Improvement Amendments-approved laboratory is necessary for the molecular genetic testing. One can find this information at https://www.ncbi.nlm.nih.gov/gtr/. It is best to know whether the laboratory will cover the testing of variants that are required for follow-up analyses, as well as the costs for both the basic tests and testing of other family members. Ideally, one should be able to obtain reports that provide clear interpretations of the test results, including the explanation of the allele described as variant of unknown significance (VUS) findings. One should be able to obtain the supplemental data on the variants that were identified. As much as possible, the ordering physician should be aware of the methods that are being used by the laboratory and their strengths and weaknesses.

One can test single genes; gene panels that are specific for certain phenotypes, methods of inheritance, or both; gene panels that are inclusive of a wide range of conditions (such as retinal conditions or ocular conditions); extensive clinical exome panels (up to 22 000 genes); whole genome sequencing; as well as comparative genomic hybridization to look for insertions or deletions of DNA sequence that are too large to be detected by next-generation sequencing methods and newer methods for long-range sequencing that can detect a broader range of structural variants in the genome. It is best to avoid single gene tests except in special cases, for example, an at-risk individual in a family with a known genetic mutation or a condition that is known to be caused only by a single gene (such as Von Hippel-Lindau disease or retinoblastoma). One should select a gene panel that has been validated and can provide effective coverage of potential genes for the phenotype. It is important to recognize that we are still learning phenotype–genotype correlations, so it is advantageous to try to avoid limiting the gene selection based on one's belief that certain features are pathognomonic. It is helpful if you know the extent to which mutations in certain genes are the result of insertions or deletions of DNA sequence that are generally overlooked by next-generation sequencing methods and if additional testing may be warranted to ensure that these are evaluated. A higher probability exists that a VUS is pathogenic in a particular gene if one can exclude pathogenic variants in other genes that can give rise to overlapping phenotypes. The identification of a single pathogenic variant, VUS, or both in a causative gene that is known to require both alleles to be defective is not considered to be a definitive result, even if one has a clinical suspicion that that is the causative gene. One group recently reported finding IRD-causative pathogenic mutations in 2 genes in 4% of their testing cohort.[3] In several cases, they found that the combination of these genes led to differences in the phenotypes of other affected family members. The fact that some individuals may have more than 1 gene (autosomal recessive, autosomal dominant, or X-linked) as a potential contributor to the IRD is another reason why extensive gene panels that include all hereditary forms of disease are the preferred strategy for testing.

A strong motivation exists for considering clinical (or whole) exome sequencing and whole genome sequencing as another approach, but one should keep in mind that they will not provide uniform or complete coverage of all known IRD genes (*RPGR* is a particularly noteworthy example). The density of sequencing allows more information on copy number variants, insertions, and deletions

Table 2. Checklist of Data to Gather before Ordering, as Well as after Receiving the Results of, Molecular Genetic Tests

Clinical symptoms	• Be specific and assess the onset for each symptom.
Clinical findings	• Functional and structural. • Presence or absence of ocular symmetry. • Note distinctive pathognomonic features, but try to avoid trying to use those features to limit your testing. • Associated ocular and nonocular clinical findings: neurological, facial, body habitus, orthopedic, renal. • Nongenetic causes or exposures. - Infections, autoimmune, paraneoplastic, drug exposures (pentosan polysulfate, phenothiazines, hydroxychloroquine, canthaxanthin), toxic exposures (heavy metals), nutritional deficiencies (vitamin A deficiency) that may be associated with prior gastrointestinal surgery, eating disorders, inflammatory bowel disease, malabsorption syndromes. • Family history. - Try to identify all first-degree (parents, siblings, children) and second-degree relatives (aunts, uncles, grandparents, nieces and nephews, grandchildren), and possibly third-degree relatives (cousins, great grandparents; these are less reliable). - It can be helpful to obtain information about fourth-degree relatives, although it is less reliable, especially if one suspects autosomal dominant, X-linked, or mitochondrial inheritance. - It is important to inquire as to possible consanguinity, including if the parents are known to be distantly related, both born within a region that may have limited migration or high rates of intermarriage (such as from an island or a culturally isolated group within the population), or both. - Remember to ask about the age at onset, severity of the disease, or both for each person when multiple individuals within a family are affected. - A negative family history from an adopted individual with no knowledge of their family history is not the same as a negative family history when the person knows all of their paternal and maternal extended family members. - X-linked disease also can appear as autosomal dominant, but the women generally have later age at onset and a milder course of disease. - Identify relevant family members whose clinical or molecular assessments or both can help to resolve some or all of the uncertainties associated with the genetic testing results. - Additional family outreach is not necessary when the person has a known pathogenic variant in an autosomal dominant or X-linked gene with a clear family history, if there are known pathogenic variants that are homozygous in an autosomal recessive gene, or if there are compound heterozygous pathogenic variants in a gene that can be established to be on separate alleles by the testing itself. - If one intends to perform molecular genetic testing in an affected family member (especially one who is as distant as a second-degree relative or more), it is prudent not to test just for the expected pathogenic variant, but also to test the entire IRD gene panel to ensure that other pathogenic variants are identified. - Although segregation analysis is indicated and informative for many IRD patients, additional scenarios (presented in the Supplemental Materials) highlight how additional family ascertainment can alter the interpretation of the molecular genetic test results of the proband and can alter both our clinical and genetic understandings of an IRD.
Clinical diagnostic tests	• Additional laboratory testing may be indicated to address potential acquired causes of the retinopathy, especially when the diagnosis of an IRD has not been established, even after genetic testing. • Full-field electroretinography may be useful to help to distinguish an acquired, postinfectious retinopathy from an IRD and, in some instances, can help to distinguish whether the primary defect is either in the rod or cone photoreceptor pathways or to identify a negative B-wave pattern that can be indicative of an inner retinal genetic defect (such as for congenital stationary night blindness or X-linked retinoschisis). • Multifocal electroretinography generally is not a useful diagnostic tool for IRDs. • Blood work to test for syphilis, Lyme disease, vitamin A, iron toxicity, or autoimmune retinopathies should be considered. • It is not uncommon to have patients for whom an autoimmune or paraneoplastic retinopathy is difficult to distinguish from an IRD, although a later onset of symptoms and the relatively rapid progression of symptoms can differentiate these conditions in part, but it is reasonable to undertake IRD genetic testing for these individuals. In a small series of 6 such patients, 2 individuals were identified with causative mutations in either *USH2A* or *RHO*. Antiretinal antibodies are seen commonly (although not universally) in patients with IRDs, so the presence of these antibodies does not aid in distinguishing the IRDs from these acquired conditions. Several authors have suggested that these antiretinal antibodies may contribute to the retinal degeneration seen in IRDs, but plenty examples exist of patients with an IRD who experience degenerative changes with no evidence of serum antiretinal antibodies. • Serial high-quality ocular phenotyping, which may include functional testing with visual acuity, visual field, microperimetry, color vision testing, mobility maze, full stimulus thresholds (if available and appropriate), or a combination thereof, as well as structural assessments with retinal imaging (OCT, OCT angiography, multispectral imaging, autofluorescence, or a combination thereof) can be invaluable for the assessment of disease severity and rate of progression. These studies also can document the severity of visual disability. Eligibility for clinical trials should be considered on a regular basis.

and also can identify structural variants and genomic rearrangements, but they are not necessarily optimized to provide complete coverage of all of the exons in genes that are sequenced. It is unlikely that exome sequencing of an individual alone will be informative if one has failed to identify a causative gene using one of the extensive and well-designed gene panels, unless it is analyzed specifically to evaluate copy number and structural variants in the known genes. Generally, whole genome sequencing is more informative of these types of DNA alterations. Again, at the current level of expertise, one should only use whole genome sequencing if one has additional

informative individuals within the family whose DNA results could help to identify causative variants.

Interpretation of Genetic Test Results

What is missed or potentially overlooked by the tests that you will be ordering? One needs to be aware not only of the limitations of the laboratory performing the tests, but also the genotyping methods that are used (Supplemental Material). In addition, one of the biggest challenges in molecular genetic testing is determining whether a gene sequence variant is pathogenic. In some cases, the evidence is extremely strong based on its presence in other affected individuals, the evidence that the protein encoded by the gene or the expression of that gene are disrupted, or both. However, we more frequently encounter variants for which the determination of pathogenicity is much more uncertain, hence the term "variant of unknown significance." Although standards for variant interpretation have been developed by the American College of Medical Genetics and Association for Molecular Pathology, the application of these standards has some subjectivity as well as variable adoption (Table 3).[4] This leads to a situation in which laboratories can offer different classifications of the same variants.[5,6] Multiple programs exist for analyzing coding region variants, including specific programs for protein structure and function such as PolyPhen-2, SIFT, and Align-GVGD.[7–9] Other programs such as ANNOVAR integrate multiple scoring approaches and databases.[10] Databases

Table 3. Standards for Variant Interpretation Obtained from Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology

Pathogenic Variants		Benign Variants	
Very strong	PVS1: null variant (nonsense, frameshift, canonical ± 1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where loss of function is a known mechanism of disease	Stand alone	BA1: allele frequency is > 5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium
Strong	PS1: same amino acid change as a previously established pathogenic variant regardless of nucleotide change PS2: de novo in a patient with the disease and no family history PS3: well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product PS4: the prevalence of the variant in affected individuals is increased significantly compared with the prevalence in control participants	Strong	BS1: allele frequency is greater than expected for disorder BS2: observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age BS3: well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing BS4: lack of segregation in affected members of a family
Moderate	PM1: located in a mutational hot spot, critical and well-established functional domain (e.g., active site of an enzyme) without benign variation, or both PM2: absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium PM3: for recessive disorders, detected in *trans* with a pathogenic variant PM4: protein length changes as a result of in-frame deletions or insertions in a nonrepeat region or stop-loss variants PM5: Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before PM6: Assumed de novo, but without confirmation of paternity and maternity	Supporting	BP1: missense variant in a gene for which primarily truncating variants are known to cause disease BP2: observed in *trans* with a pathogenic variant for a fully penetrant dominant gene or disorder or observed in *cis* with a pathogenic variant in any inheritance pattern BP3: in-frame deletions or insertions in a repetitive region without a known function BP4: multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) BP5: variant found in a patient with an alternate molecular basis for disease BP6: reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation BP7: a synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence, nor the creation of a new splice site, and the nucleotide is not highly conserved
Supporting	PP1: cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease PP2: missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease PP3: multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) PP4: patient's phenotype or family history is highly specific for a disease with a single genetic cause PP5: reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation		

of reported clinically relevant variants such as ClinVar, HuVarBase, CLINVITAE, as well as variant databases that are disease specific, for instance the *ABCA4* mutations within the LOVD3 database or region-specific databases such as DPV, a Japanese pathogenic variant database, also are used.

Some laboratories provide extensive annotation of their VUS designations (see, for example, Invitae); others provide specific details of some of the programs used to predict pathogenicity (for example, Blueprint, MVL, Prevention Genetics, Fulgent, GeneDx). Given the enormous amount of data, nearly all laboratories will exclude reporting variants that are considered to be benign, whereas other laboratories will provide VUS designations with little or no annotation. The Carver laboratory at the University of Iowa stands out as fairly unique in its use of the estimating pathogenic probability classification system, which was developed and validated in house.[11]

Variant of unknown significance interpretation within molecular genetic test results will remain an area of controversy and evolution for some time, and clinicians should not be surprised to see that variants may be reclassified as additional data become available. Also, it is important to remember that even negative or uncertain test results have meaning (Supplemental Material). Clinical assessments and follow-up genetic studies of family members play a crucial role in determining if some variants are causative for disease, and it is essential that every clinician who cares for patients with an IRD assumes an ongoing, active role in this process.

Initiative: What We Should Do after We Obtain the Genetic Test Results

As noted previously, one may have elected to perform the molecular genetic testing with a comprehensive gene panel before extensive phenotyping of the patient or with limited diagnostic testing for the causes of an acquired retinopathy. With the molecular genetic test results in hand, one can decide the relative value of additional functional and imaging studies, as well as laboratory investigations. In this context, one also can include selective systemic, nonocular evaluations that may be indicated by variants in genes that are known to cause syndromic IRDs, as well as what additional information may inform and reinforce the molecular genetic results (Table 2).[12–15]

Prenatal genetic testing is becoming increasingly common and often includes detection of potential pathogenic variants in IRD genes. This raises the question of what to do when the penetrance of the gene in question is unknown (which is often the case) and the pathogenicity of the variants also may be uncertain. As noted previously, the genetic variants often are poor predictors for the age at onset, progression, and severity of the condition. Parents seeking answers to these questions for a fetus or asymptomatic infant can find this uncertainty extremely upsetting. Under these circumstances, counseling is critical and the efforts of a trained genetic counselor can be invaluable.

Instruction: Providing Genetic Information to the Patient and Family

Are you going to provide the genetic counseling personally, do it in collaboration with a genetic counselor, or completely delegate the process to a genetic counselor? If you rely on a genetic counselor, consider your obligations as the eye provider and clinician—see "Initiative." Genetic counseling is intended to convey information and provide education that empowers the patient (and their family members) to make informed choices as to how they will use that information for their care and family planning (Supplemental Material). It is not intended to dictate a specific course of action for the individual or their family members (that is still part of the discussion that they may choose to have with their physician). Stay informed as to the current clinical trials (and, if possible, current research that will lead to future clinical trials) that are specific for the patient's specific condition. ClinicalTrials.gov is a valuable reference site for past, current, and upcoming clinical trials, but be aware that listing on that site is not an endorsement as to the scientific validity or appropriateness of any particular clinical trial. One also should be knowledgeable of the potential IRD therapies that are not gene specific. Be careful to advise patients to avoid scams and fraudulent scientific claims, because many are desperate to find any possible treatment. Patients need to be counseled that clinical trials that require payment by the participants should be viewed with particular scrutiny and caution.

In summary, molecular genetic testing is a relatively unique and complex clinical undertaking compared with the diagnostic testing that is the mainstay of traditional medicine and ophthalmology. The frequent uncertainty of results (which may change over time), the need for follow-up investigations (both clinical and genetic), and the implications of the findings both for the patient and their family members (present and future) need to be appreciated and addressed. It is critical that we abide by the Hippocratic challenge to "do no harm" by ensuring that we interpret the results accurately and convey those findings in a manner that is compassionate and beneficial for our patients. We all have a responsibility to make sure that we take the necessary steps to maximize the knowledge that we gain from molecular genetic testing and to refine the process as we move forward. Genetic-based therapies are on the horizon and bring the promise of a major advancement in the treatment of IRDs. The more we understand the genetic foundations of IRDs, the better we will be able to treat our patients now and be capable of bringing that promise to fruition.

Footnotes and Disclosures

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