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NEXT GENERATION GENETIC COUNSELING

The Ophthalmic Experience: Unanticipated Primary Findings in the Era of Next Generation Sequencing

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Abstract Next generation sequencing (NGS) technology, with the ability to sequence many genomic regions at once, can provide clinicians with increased information, in the form of more mutations detected. Discussions on broad testing technology have largely been focused on incidental findings, or unanticipated results related to diseases beyond the primary indication for testing. By examining multiple genes that could be responsible for the patient's presentation, however, there is also the possibility of unexpected results that are related to the reason genetic testing was ordered. We present a case study where multiple potentially causative mutations were detected using NGS technology. This case raises questions of scientific uncertainty, and has important implications for medical management and secondary studies. Clinicians and genetic counselors should be aware of the potential for increased information to affect one's understanding of genetic risk, and the pre- and post-testing counseling process.

Keywords Ophthalmology · Retinal dystrophy · Genetic testing · Genetic counseling

Introduction

Next generation sequencing (NGS) is expanding clinicians' ability to search for and identify causative mutations for genetic diseases. These sequencing technologies allow for the simultaneous sequencing of large numbers of genomic regions, and so genetic testing strategies are expanding from examining a single gene, to a panel of genes, to the whole exome, and finally, the whole genome. The wealth of genetic information that can be produced from this technology presents new dilemmas and poses additional risks when NGS is applied in a clinical diagnostic setting.

Much of the discussion surrounding NGS has focused on the implications of secondary, or incidental, findings, defined as genetic variants that are known or suspected to be pathogenic, but that confer risk of diseases unrelated to the reason that genetic testing was ordered (Raffan and Semple 2011). These are genetic results that neither the clinician nor the patient anticipated when the test was ordered (Wolf et al. 2008). However, next generation sequencing also has the possibility to provide unanticipated results that are highly relevant to the patient's clinical diagnosis.

With NGS, multiple genes are sequenced at once, which can result in the identification of pathogenic mutations in more than one gene. Prior to NGS, finding a potentially pathogenic mutation in one gene may have ended the search for a molecular diagnosis. We are reporting here a case where potentially causative variants were detected in two different genes, where mutations in a single gene could be considered sufficient to cause the condition. This case raises questions of scientific uncertainty and has implications for risk counseling. We wanted to bring this case to attention because, as NGS becomes more widely utilized, genetic counseling must adapt to new technologies that generate a broader set of potential etiologies.

Case Study

A 6 year-old male presented to our Retinal Dystrophy Clinic complaining of a rapid decline in his vision in the past year. He reported that he could not see the stars at night, could no longer see the board at school, needed to increase font size for reading, and had a loss of color vision. At his visit, his visual acuity, as measured against a Snellen Chart, was measured as 20/200 in the right eye and 20/150 in the left eye,

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where 20/20 is considered normal visual acuity. A Goldmann Visual Field measurement demonstrated constricted visual fields with a large central scotoma, or blind spot. Color vision testing, using both Ishihara plates and Farnsworth testing, was abnormal. The results of his electroretinogram, which measures the electrical response from the cone and rod photoreceptor cells in the retina, showed a normal cone response but a reduced rod response.

His family history (Fig. 1) was significant for a paternal uncle who was clinically diagnosed with a cone-rod dystrophy around age 7, and had a similar presentation to our patient: reduced visual acuity and large central scotomas in both eyes. An electroretinogram performed at age 32 showed a non-recordable cone response and a reduced rod response. Paternal ancestry is Korean and maternal ancestry is Taiwanese. There was no reported history of consanguinity.

Based on the patient's symptoms, clinical testing, and family history, he was diagnosed with a cone-rod dystrophy.The family was counseled about both autosomal dominant inheritance with reduced penetrance and autosomal recessive inheritance. Clinical genetic testing was ordered for a panel of 25 genes associated with cone-rod dystrophies inherited in an autosomal recessive, autosomal dominant, or X-linked manner (Table 1). Although this panel contains identified genes associated with cone-rod dystrophies, not all genes associated with cone-rod dystrophies are known at this time (Hamel 2007).

Genetic Testing Results

Genetic testing identified three novel variants in two different genes that could potentially provide an explanation for the patient's phenotype. Two variants, IVS23-2A>T and c.2249T>C, were found in the *ABCA4* gene and one variant, c.1282C>G, was found in the *RPGR* gene.

The *ABCA4* gene is located on chromosome 1 and produces an ATP-binding cassette transmembrane protein that is involved in the retinoid visual cycle. Mutations in *ABCA4* are associated with several different autosomal recessive retinal dystrophies, including Stargardt disease, retinitis pigmentosa, and cone-rod dystrophy (McKusick and O'Neill 2011). Novel variants in this gene are not uncommon, as over 600 different variants in *ABCA4* have been identified to date (Allikmets 2007).

RPGR is located on the X chromosome, is expressed in the outer segments and connecting cilia of cone and rod photoreceptor cells, and is essential for photoreceptor maintenance. Mutations in *RPGR* are associated with X-linked retinal dystrophies, including cone-rod dystrophy and retinitis pigmentosa (McKusick and Kelly 2013).

Interpreting Novel Variants of Uncertain Significance

To interpret novel variants of uncertain significance, one uses multiple lines of evidence to determine if a variant is associated with disease. Direct lines of inquiry include co-segregation analyses and case–control studies and indirect evidence includes in vitro assays or bioinformatics modeling.

We routinely obtain parental samples for segregation analysis in our clinic. From an analysis of parental samples, the clinical laboratory determined that the *ABCA4* variant IVS23-2A>G was maternally inherited, and that the *ABCA4* variant c.2249T>C was paternally inherited. The *RPGR* c.1282C>G variant was determined to be maternally inherited, and not *de novo* in our patient.

To determine if the paternally inherited variant is segregating with disease, we recommended that the patient's paternal uncle be tested for mutations in the *ABCA4* gene. It is possible that he possesses two mutations in *ABCA4* - the same variant that our patient inherited from his father, and a different mutation. Determining if *ABCA4* mutations are responsible for the uncle's similar presentation may provide additional evidence for the pathogenicity of the paternal *ABCA4* allele.

None of the variants have been previously reported in the literature or any of the databases that were searched. A different variant at the same position as the maternally inherited variant, IVS23-2A>T, was reported in three siblings with autosomal recessive retinitis pigmentosa, all of whom had a previously reported mutation on the opposite allele (Mandal et al. 2005). In this report, the variant was detected in a heterozygous state in the mother and in another unaffected sibling, and was not detected in another unaffected sibling. Using *in silico* analysis the authors predicted IVS23-2A>T caused the loss of a splice acceptor site.

A bioinformatics approach uses *in silico* analysis to consider evolutionary conservation, the predicted severity of an amino acid substitution, and the predicted effects of exonic or intronic alterations on protein splicing. The maternally inherited variant IVS23-2A>G was predicted to affect a splice site, and was predicted to be damaging by NetGEN2, a web-based splice-site prediction software package. The paternally inherited variant c.2249T>C was predicted to be "probably damaging" using *in silico* analysis programs Polyphen-2, SIFT, and Mutation Taster. Conservation modules in Mutation Taster also suggested that this was a highly conserved amino acid residue. *In silico* analysis of the *RPGR* variant c.1282C>G provided contradictory, and therefore uninformative, results, as it was predicted to be damaging by Polyphen-2 and SIFT, but predicted to be a benign polymorphism by Mutation Taster. *In silico* models are not definitive. Some variants now recognized as pathogenic were initially predicted to be benign, and some variants that were initially predicted to be damaging have later been reclassified as non-pathogenic. Furthermore, these programs are not

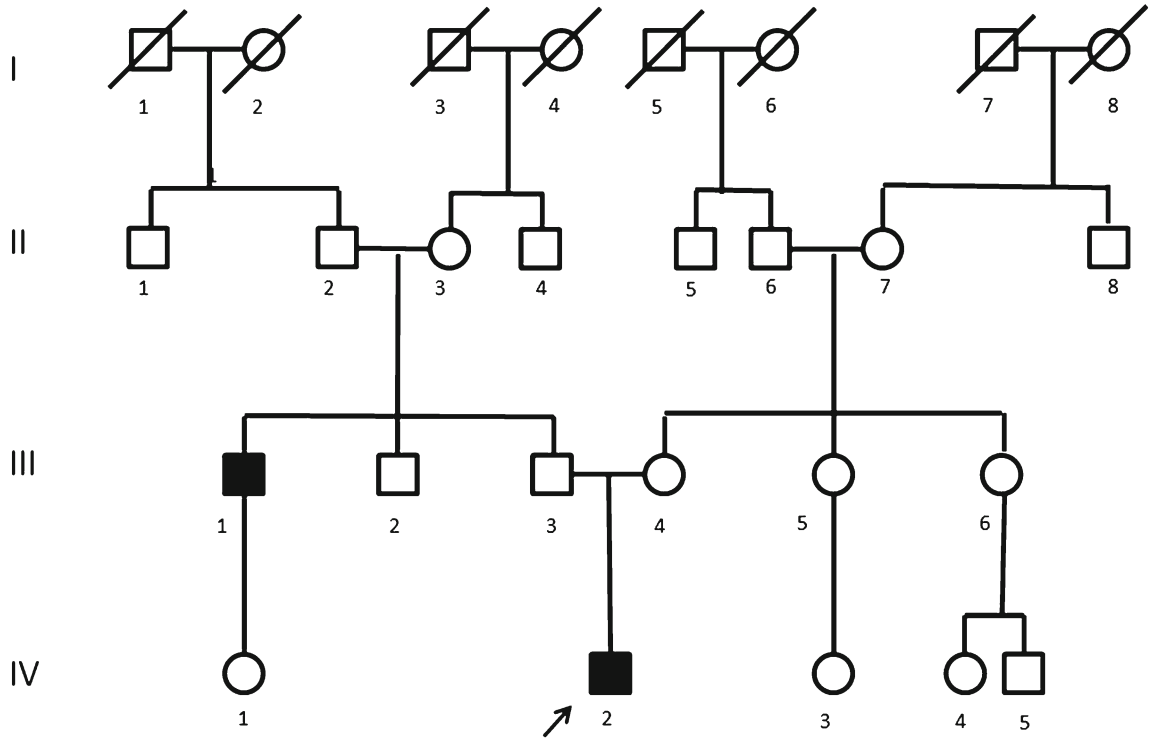

Fig. 1 Our patient is indicated by the arrow (IV-2). The patient's paternal uncle was also diagnosed with a cone-rod dystrophy (Individual III-1)

designed for clinical use and do not provide sufficient evidence for clinical decision-making.

Family Post-Test Counseling

The patient and his family received counseling about the potential implications of the *ABCA4* and *RPGR* variants. The family was counseled that the *ABCA4* variants might provide a genetic explanation for his loss of vision, and autosomal recessive inheritance was again reviewed. We also discussed X-linked inheritance for the *RPGR* variant. Since it has been reported that *RPGR* carrier females can exhibit a range of symptoms, ranging from asymptomatic to retinal disease comparable to males, we recommended that the patient's mother have an electroretinogram and color vision testing performed, have her visual acuity and visual fields measured, and have fundus photographs taken (Churchill et al. 2013). Additional genetic testing for mutations in *ABCA4* for patient's paternal uncle was recommended, because without knowing the uncle's genotype, our ability to interpret our patient's genotype is limited.

Table 1 Testing was performed for these 25 genes using next generation sequencing

Gene	Inheritance mode
AIPL1, c8ORF37, CRX, GUCA1A, GUCY2D[a]*, PITPNM3, PROM1, PRPH2/RDS, RAX2, RIMS1, SEMA4A, UNC119*	Autosomal dominant
ABCA4, ADAM9, CACNA2D4, CDHR1, CERKL, CNGB3, CNNM4, GUCY2D[a]*, KCNV2, PDE6C, RDH5, RPGRIP1*	Autosomal recessive
CACNA1F, RPGR	X-linked recessive

Sanger sequencing was performed to fill in gaps and low coverage regions. Novel variations were confirmed with Sanger sequencing

[a] Mutations in *GUCY2D* have been reported in families exhibiting both autosomal dominant and autosomal recessive modes of inheritance

Discussion

We present this case to highlight that challenges in providing a molecular diagnosis in the age of NGS panels are not only related to incidental findings, but testing may also identify several possible causes for disease. The detection of multiple potential explanations for disease raises questions of uncertainty regarding whether or not any of the variants contribute to the phenotype, and if so, the extent of their contribution. Finding variants of unknown significance underscores the underlying complexity of Mendelian conditions, creates the need for additional studies or analyses to clarify testing results, and raises questions about how this type of information needs to be communicated to patients. Many of these issues are not new to the genetic counseling field, but they are important to

discuss because they will likely become more common as broader testing is performed for more patients.

Uncertainty

Interpreting how even a single variant might influence the onset or progression of disease remains difficult. Any genetic variant needs to be carefully evaluated in the context of the family history. Our patient's family history was most consistent with autosomal dominant inheritance with incomplete penetrance. Cone-rod dystrophy is a rare condition, and would generally not be expected to occur in an autosomal recessive pattern of inheritance in multiple generations of a family without consanguinity. Furthermore, there is a single report in the literature of the pseudodominant inheritance of Stargardt disease in a family with an *ABCA4* variant (Shroyer 2000). The identification of variants in genes associated with autosomal recessive or X-linked inheritance, and negative genetic testing results with regards to known mutations in known genes associated with autosomal dominant cone-rod dystrophies, needs to be considered cautiously.

The detection of multiple genetic variants adds to the uncertainty regarding the contribution of each one to disease. Since each patient represents a unique case, and there have been no other reports in the literature of patients with both *ABCA4* and *RPGR* mutations, it is unclear if the variant(s) in a single gene provide an explanation for the patient's vision loss, or if the effects of multiple variants are additive. In familial cases, more than one mutation may co-segregate with disease, making it difficult to parse out the effects of each mutation (Raffan and Semple 2011). There are also rare reports of digenic inheritance for retinitis pigmentosa (Kajiwara et al. 1994) and Bardet-Biedl syndrome (Fauser et al. 2003). In digenic inheritance, mutations in multiple genes each contribute to disease, and each mutation by itself is thought to be insufficient to explain the condition. This is distinct from the case we present, where variants in a single gene, either *ABCA4* or *RPGR*, could independently provide an explanation for our patient's phenotype.

There are additional challenges for how these types of results may impact medical management. Clinical trials for gene therapy for individuals with Stargardt disease who have molecular confirmation of *ABCA4* mutations are underway (www.clinicaltrials.gov). If at least one of the variants found in our patient can be classified as pathogenic, he might qualify to participate in a future clinical trial. But might the presence of an *RPGR* variant be considered a "concurrent disease" or "laboratory abnormality" that would exclude him from such a trial? It is also unknown how the presence of an *RPGR* variant might affect his response to gene therapy for a different gene.

These testing results also have implications for treatment recommendations. Dietary supplementation with vitamin A is sometimes prescribed for individuals with retinal dystrophies. Vitamin A supplementation should be avoided in patients with *ABCA4* mutations, however, due to the accumulation of toxic pigments in the eye (Radu et al. 2008). At the patient's initial visit, the physician discussed vitamin supplementation with the family. Subsequent to genetic testing, Vitamin A supplementation was not recommended.

Despite increasing the complexity of a management strategy for the patient, NGS provided important information to the clinician that single gene testing may have missed. If, based on the family history, the most likely mode of inheritance was believed to be autosomal dominant inheritance with reduced penetrance, and the patient had only had testing for the *RDS* gene, for example, it would not have been known that Vitamin A was contraindicated. This case highlights how NGS technology can be useful in an era of personalized medicine, where healthcare is customized based on the patient's genetic information.

The experience with genetic forms of cardiovascular disorders provides some insight into how one might utilize and make sense of the increased volume of information on each patient, as panel-based testing has been available for a number of years. In the literature, there are many patients with cardiomyopathies who have multiple disease-causing mutations (Alpert et al. 2005). Because there have been enough cases, studies suggest that having multiple mutations increases an individual's risk to develop end-state disease or experience sudden cardiac death (Girolami et al. 2010). In the cardiovascular field, patients can now be offered more accurate information about their risk of complications. As more patients with retinal dystrophies receive panel-based testing or whole exome testing, it may be possible to establish a large enough patient base to determine how much each mutation contributes to a patient's phenotype.

NGS provides the opportunity to peel back the curtain, to reveal more details about the genetic background of a patient that were previously unseen. The case described above can serve as a reminder that mutations do not exist in a vacuum, but they occur within the context of the patient's entire genome. There are many genes involved in the visual cycle, for example, and variations in each one may play a role in how a patient experiences vision loss. While Mendelian conditions have traditionally been thought of as "one gene, one condition", there are many studies that demonstrate that genetic variants modify disease expression (e.g., Fahim et al. 2011). When one introduces the idea of genetic modifiers, it becomes harder to confidently link a disease to mutations in only one gene.

As more data become available on each newly discovered genetic variant, variants will continue to be reevaluated and reclassified. Researchers and clinicians will also learn more about the interactions between genes and mutations. In the

future, having a more complete picture of an individual's genotype may provide an explanation for some of the variability that is seen in some genetic conditions. Clinicians will continue to struggle with how to translate these findings into meaningful information for patients, however, until they have much more data and better tools to able to understand richer genetic information.

Secondary Studies

Our case example also illustrates that the degree to which additional analyses may need to be performed will likely increase with the use of NGS. For our patient, we requested parental samples to provide confirmation of the segregation pattern of the *ABCA4* and *RPGR* variants. Additional testing, such as whole exome sequencing (WES), which might identify additional variants in our patient and his paternal uncle that would explain the cone-rod dystrophy, should also be considered. Given that there is an affected individual outside the patient's nuclear family, a more traditional type of genetic investigation, in the form of linkage analysis, may also be possible.

The ambiguity resulting from a lack of functional studies of the effect of these variants demonstrates the burden on the clinician for the diagnostic dilemma presented by a novel variant. Functional testing of the effects of each variant could also be conducted. Laboratory or animal studies of the biological effect of the variant may provide evidence that it is causative. These types of studies provide indirect evidence to clarify whether a variant is benign or pathogenic.

Communicating Information to Patients

Using NGS technology increased the amount of information that we needed to provide to our patient to explain the results, and raised some additional questions about the type and amount of information that needs to be provided in pre-test counseling.

While returning the genetic testing results to our patient, we discussed additional modes of inheritance that were not initially suggested based on the pedigree. We initially discussed autosomal dominant inheritance with reduced penetrance. After genetic testing, we discussed both autosomal recessive and X-linked inheritance in more depth. Since we discussed multiple modes of inheritance, we discussed several different risk estimates.

Even in cases where mutations in multiple genes have the same mode of inheritance, the discussion of risk becomes more complex. For an individual with a *BRCA1* mutation, the risk to his or her children of inheriting the mutation is 50 %. For an individual with both a *BRCA1* and a *BRCA2* mutation, the risk to his or her children of inheriting at least one mutation is 75 %. Individuals who have both *BRCA1* and *BRCA2* mutations (Musolino et al. 2005; Steffensen et al. 2010), or both *APC* and *MSH2* mutations (Uhrhammer and Bignon 2008), have been reported in the literature.

For our patient, we ordered panel-based testing that investigated genes associated with conditions with autosomal recessive, autosomal dominant, and X-linked inheritance. Based on the results that were obtained, however, should pre-test counseling have included an in-depth discussion of all three modes of inheritance? Our practice model already includes a discussion of multiple modes of inheritance for simplex cases of retinitis pigmentosa, because one study showed that 15 % of males with no family history of retinitis pigmentosa had a mutation in a gene associated with X-linked retinitis pigmentosa (Branham et al. 2012) and a separate study showed that 10 % of individuals with no family history of retinitis pigmentosa had a mutation in a gene associated with autosomal dominant retinitis pigmentosa (Neveling et al. 2012).

NGS technologies have also raised questions about what needs to be included in the informed consent process, specifically regarding the possibility of incidental findings. Counselors report that this has allowed them to discuss with patients which type of information they would like to receive, but this has also considerably lengthened the informed consent process (Raffan and Semple 2011). In general, in the informed consent process for genetic testing, the clinician may discuss the possibility of positive results (finding a genetic explanation for disease), negative results (not detecting a mutation), or the possibility of finding a variant of unknown significance. If there is also the potential for what amounts to multiple results, should that also be included in the discussion?

Practice Implications and Conclusions

We present this case because the ability to offer more complex genetic diagnostic testing has implications for how to think about what it means to provide comprehensive genetic counseling to patients. Panel-based testing is currently available for a number of conditions, such as cardiomyopathies, retinal dystrophies, and aneurysmal disorders, among others. Due to advances in whole exome and whole genome testing, we may see this scenario even for conditions where panels are not currently established.

Based on our experience in this case, we would stress the importance of clinical support services that help individuals make sense of the entire genetic testing process. Pre-test counseling should include a discussion of the potential benefits and risks of testing, the limitations of testing, the potential for unanticipated or ambiguous results, and that additional testing may be needed to clarify results. If relevant to the

NGS panel, patients should be made aware that testing considers genes associated with conditions with different patterns of inheritance. Post-test counseling should be a discussion of the results, including what is known and unknown about potentially causative variants. Patients may also be encouraged to re-contact the clinic to determine if variants have been reclassified, or to discuss additional testing.

This case is brought to attention so that clinicians and genetic counselors can refine their understanding of genetic risk in light of increased information. Patients may then be counseled about the potential of NGS technology to detect more information about their condition, and understand how this information will be used in their medical care.

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