Application Note

Methods and Challenges of Variant Annotation in Hereditary Cancer

Introduction

The field of genetic testing for hereditary cancer is rapidly evolving. Identification of BRCA1 and BRCA2 paved the way for personalized medicine and created a new paradigm for hereditary breast and ovarian cancer (HBOC) syndrome diagnosis and prevention. Likewise, discovery of the molecular basis of Lynch syndrome led to a clearer definition of the syndrome's clinical spectrum and improved our ability to identify individuals at high risk of hereditary colon and endometrial cancers. Identification of mutation carriers is critical, as it enables the administration of interventions that are proven to confer significant survival benefits, particularly for highly penetrant genetic mutations.

Beyond these two well-known syndromes, numerous other genes associated with hereditary cancer syndromes have been identified in recent years. Concurrently, advances in next-generation sequencing (NGS) technology have made it possible to test multiple genes simultaneously. High-throughput NGS testing is particularly important in situations where genetic heterogeneity exists, where several genes carry causative mutations, and where it is difficult to predict which gene may be affected on the basis of phenotype and family history alone. Consequently the task of interpretation is time-consuming.

Variant annotation

Variant annotation is an important step in interpreting the clinical significance of the DNA variations detected by NGS. The number of available genetic tests is rapidly increasing, as is the number of genes included in any given test, and clinicians are handling much larger volumes of genetic variants that need clinical classification every day. The process of variant annotation is based on accessing up-to-date information on variants such as their prevalence in healthy people versus those with disease, functional impact on the protein, and results from clinical trials.

Data sources that provide information on variants are numerous, heterogeneous, quickly evolving, and sometimes conflicting, which often makes variant annotation rather a challenging process that relies on probabilistic assessment that the variant is disease-causing. Because of this, a significant discrepancy in classification was shown between different laboratories which might have a tremendous impact on the clinical decision making (1). To work efficiently, clinicians need reliable variant annotation systems that acknowledge existing uncertainty and that will help to collect and aggregate available data from various data sources.

Guidelines for variant annotation

Literature shows that the frequency of disagreements in variant annotation is high, which has initiated changes in the variant annotation process and emphasized the need for greater stringency. American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published variant classification guidelines in 2015 (2) that are applicable to all areas of genetics. They propose a scoring system that gives different weights to various types of evidence, and an algorithm to classify variants into one of the five following classes: pathogenic (class 5), likely pathogenic (class 4), variant of unknown clinical significance (VUS) (class 3), likely benign (class2) and benign (class 1). Pathogenic and likely pathogenic variants are those that have clinical impact, such as making a diagnosis, predicting the course of treatment, and assessing the risk of disease in family members.

These guidelines offer two sets of evidence: one for the classification of pathogenic/likely pathogenic variants, and one for the classification of benign/likely benign variants. Each piece of evidence is given a level of strength that is associated with the particular variant. The pieces of evidence can be found in various data sources, including population data, computational and predictive data, functional data, segregation data, mutation type, and allele data. After gathering all the evidence for a particular variant, the scoring rules should lead to a classification from the five-tier system. Lack of data on the variant may implicate its status as a variant of unknown clinical significance (VUS). When there is conflicting information about the variant, it is difficult to know which classification is the most appropriate.

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B.

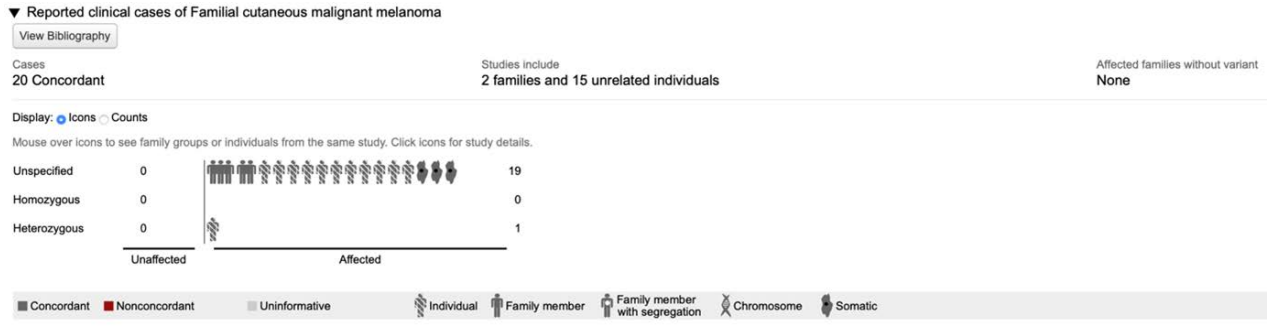

Figure 1. Clinical decision support applications, such as QCI® Interpret, allow for the computation of classification rules based on the available evidence. The visualization of the classification, including the evidence and reported clinical case count, allow laboratory directors to make decisions on the pathogenicity level of a variant in the appropriate disease context. A) QCI Interpret classification result for CDKN2A p.D153Y in the context of cutaneous melanoma. The criteria for classification are based on the ACMG/AMP guidelines. For each of the criteria the definition, strength, and evidence are provided. B) Reported clinical cases from primary literature allow the visualization of the concordance of the variant with disease as well as the type of distribution.

Evidence for variant annotation

Databases

Online databases contain valuable information on a growing number of newly discovered genes and variants. Population databases, such as The Genome Aggregation Database (gnomAD) (4), dbSNP (5), and dbVAR (6), are useful in obtaining information on the frequency of variants in large populations. While population databases usually contain information on healthy individuals, they can also give information on disease cohorts.

For example, if an allele frequency in a control population is higher than 5%, it is strong evidence for a benign interpretation indicating that it could be a common polymorphism. Unlike population databases, disease- and gene-specific databases primarily contain information on patients with a certain disease. The examples are multiples including: ClinVar (7), Leiden Open Variation Database (LOVD) (8), OMIM (9), and Human Gene Mutation Database (HGMD) (10) . They give information on the clinical significance and phenotype relationship of human variations. BRCA Share, BRCA Exchange or BIC (Breast Cancer Information Core) (11-13) are the examples of a gene-specific databases that aim to pool data on BRCA1 and BRCA2 genetic variants with corresponding clinical data from around the world.

Sometimes, databases are not regularly updated or contain variants that are incorrectly classified, so one should use caution when using them. The steps that should be specifically evaluated for each database before its use are the frequency of the database updates, the support for data curation, the use of correct nomenclature, the degree to which the data are validated, and the quality metrics provided to assess the data accuracy.

Literature search

Valuable information on variants may be found in case-control and case-case studies as well as in the studies performing functional *in-vitro* and/or *in-vivo* analysis of detected variants. Results from a segregation analysis can show if the pattern of phenotypes within families is consistent with the transmission of a gene for that phenotype. Functional studies can prove a negative effect of a missense variant on the coding protein giving strong evidence of a damaging effect on the gene or the gene product. This piece of evidence is considered supporting for classifying the variant into either likely-pathogenic or pathogenic. RNA studies can give valuable insight into the effect of the splice-site variants and untranslated regions.

In-silico tools

In-silico algorithms usually perform prediction of the effect that variant has on the resulting protein. Computational evidence makes predictions whether a missense change is located in the conservative region of the protein, whether it is damaging to the protein in terms of structure and function, and whether there is an effect on splicing. The algorithms for missense prediction are numerous, such as Align GVGD, PolyPhen-2, SIFT, MutationTaster, Condel, MutationAssessor, and PANTHER (14-20). Some of the splice-site prediction tools include Human Splicing Finder, GeneSplicer, NetGene2, and MaxEntScan (21-24). These tools are only predictors for variant pathogenicity and their use should be implemented carefully. Since these can sometimes give the opposite prediction, it is recommended that if two out of three tools predict a deleterious effect of a variant, it should be used as a piece of supporting evidence for pathogenicity. But if two out of three tools predict the benign effect, supporting evidence for benign classification should be applied (3).

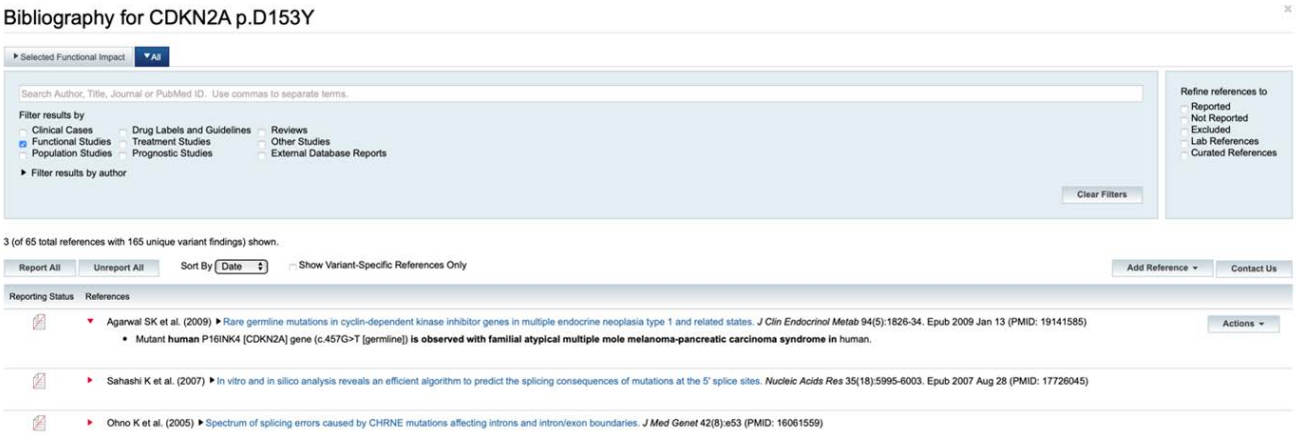

Figure 2. Scientific literature, such as functional impact or functional studies, are keys information. QCI Interpret provides a phenotype-variant bibliography information, allowing quick access to manually curated scientific literatures that can be filtered using different criteria: i.e. clinical case, functional impact, functional studies, populations studies, therapeutics, prognostics. Each line of evidence can be added to the case interpretation report.

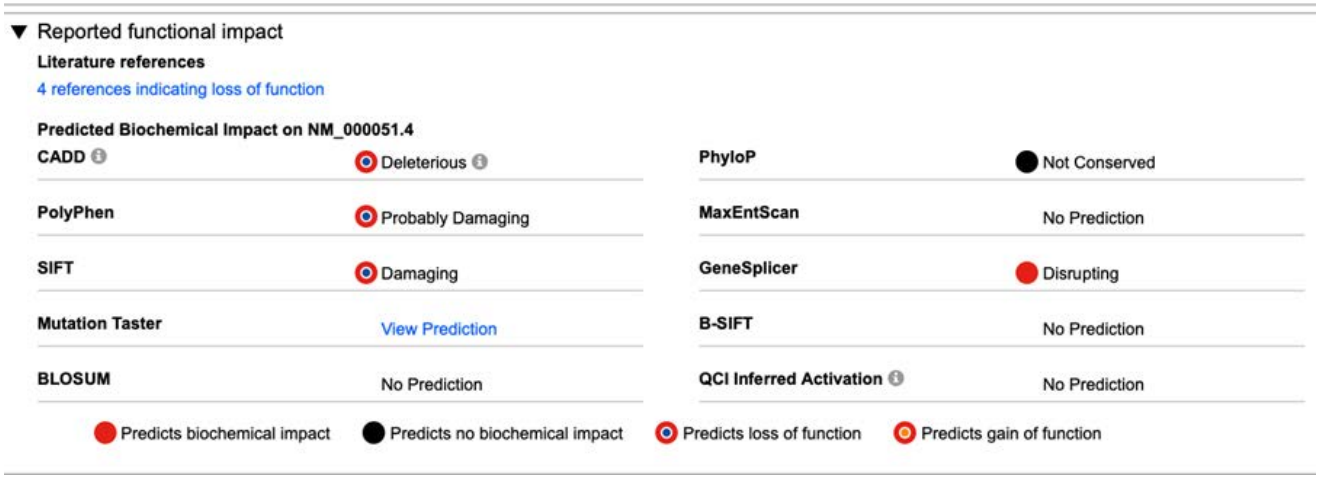

Figure 3. QCI Interpret provides an integrated view of different algorithms predicting the biochemical impact of a variant on the associated transcript. A color-coded diagram allows for quick visualization of the results.

Variants of unknown significance (VUS)

Findings from genetic testing for which the clinical significance is currently unknown are difficult to deal with. Variants are usually classified as VUS when evidence for classification is conflicting or when there is a lack of evidence. Expectedly, multi-gene panel testing has greatly increased the number of VUS encountered in clinical practice. As the number of genes analyzed increases, the higher the likelihood of obtaining uncertain results.

Unlike some other uncertain medical results whose status will not change over time, VUS in genetics can be reclassified as more data are gathered and more evidence appears. Thus, they may be upgraded to pathogenic or likely pathogenic, or downgraded to benign or likely benign in the future. When reclassification occurs, amended reports should be issued and results disclosed to patients.

VUS reclassification

Analytical validity of the test includes sensitivity and specificity of variant detection. NGS technologies, being able to detect variant at low levels (up to 1%) show higher sensitivity than Sanger sequencing (15-20%). In hereditary cancer testing high sensitivity is particularly important for detecting mutations in genes that have high de-novo mutation rate such as TP53 especially when one-fifth of these de-novo variants are mosaics.

Despite the clinical importance of variant reclassification, there is little information on how to handle the changing nature of genetic information in a laboratory setting. How often should the variants be reclassified, who is responsible for making the decision to conduct reinterpretation, who is performing reclassification, in which cases the knowledge has changed enough to warrant reclassification, are just some of the rising issues. As a result, clinicians face limited information about the frequency and implications of VUS reclassification. Since laboratories have different practices, it is important that those providers who order genetic testing understand how each laboratory approaches reclassification. Periodic computational reviews of all variants in the local databases can enable continuous active variant reclassification which some commercial genetic testing laboratories use this approach. In case there is new information on the variant, all providers on the patient`s record should be notified. Another approach requires manual integration of new information by the providers themselves (25).

Recent publications on variant reclassification show that the vast majority of variant reclassifications are downgrades (90.3%), while only 7.5% of variants reclassified from the VUS category are upgraded to pathogenic or likely pathogenic (25-27). Reclassification from VUS to benign in a timely manner may help minimize the risk of misguided management and emotional anxiety on behalf of the patient. VUS reclassification to pathogenic is of particular clinical importance since it may substantially affect patients' medical management in terms of targeted therapy selection, cancer prevention, screening, and surgical decisions. This can also impact family members whom cascade testing should be recommended and whose results may change their personal cancer risk.

Problems for clinicians

Clinicians face many issues receiving VUS results from the laboratory such as how should they counsel the patients, should the VUS effect clinical management, is there an impact to family members, and what happens when the VUS results are reclassified. These issues are challenging for genetic experts but are even more troublesome for clinicians who have little to no training in genetics. The ACMG variant classification guidelines address the issue of VUS management, stating that VUS should not be used in clinical decision making, that the efforts for their reclassification should be made, and that the patients should be monitored until the reclassification happens.

The general consensus suggests that VUS should be disclosed to patients. However, not all clinicians agree. Some say that there are specific circumstances when VUS shouldn't be reported to the patients (28) and some disagree on how much detail should be disclosed. While some of the existing guidelines might be helpful for clinicians, it is still difficult for them to decide how often, when, by whom, and what type of VUS are actually disclosed in practice. The issues with reclassification and patient communication are ongoing since some variants may be reclassified years after the test has been

performed. This question is particularly important in cases when VUS is upgraded to a clinically actionable mutation.

VUS misinterpretation as clinically actionable mutations may lead to serious consequences. Failing to understand the result and acting inappropriately in response to them can lead to unnecessary or even dangerous clinical decision making. For example, a number of breast surgeons indicated that they managed patients with VUS the same way as those with pathogenic variants. Many surgeons performed bilateral mastectomy in VUS carriers, which is recommended as a risk reduction measure for pathogenic/likely pathogenic mutation carriers (29). However, this is not in accordance with official ACMG guidelines and is regarded as patient mismanagement.

Problems for patients

Genetic information can be difficult for patients to understand because of its probabilistic nature. VUS results are particularly difficult to interpret even when time and effort are devoted to their disclosure through pre- and post-test genetic counseling. It can be particularly difficult for patients to understand that a variant could be harmless when it is found in a gene that matches the family history of the disease.

The uncertainty caused by receiving unclear results may cause psychological distress, frustration, and confusion. Genetic counseling that includes expert pre- and post-test sessions is very important for providing patients with all the necessary information. However, uncertain results are rarely discussed when genetic testing has been ordered by non-genetic providers (30), or when there is a shortage of genetic experts and the lack of time for full pre-test genetic counseling even in specialized clinics.

Studies show that even when there is proper counseling, there may still be a gap between what patients are being told about a VUS and what they understand. One study found that 79% of the patients interpreted VUS as a genetic predisposition to cancer even when genetic counselors emphasized the uninformative meaning of the test result (31). It has also been shown that it is very important to explain the information in a way that is adapted to patients' needs since misunderstanding of VUS was more common in those with a lower level of education.

Conclusion

Multigene testing allows for increased detection of hereditary cancer syndromes by utilizing the benefits of high-throughput NGS. Variant interpretation complexities may arise on a more frequent basis with panel testing; however, these challenges are not novel to the field. All health-care professionals who offer hereditary cancer testing must engage in ongoing education as the field is continuously evolving as new data become available. Future research opportunities are many in this field and include analysis of clinical utility for moderate-penetrance genes, delineation of cancer risks and management for individuals positive for mutations in multiple genes, development of robust standards to assess lab quality, and data collection to further refine cancer risks conferred by more newly described genes, especially in diverse populations. While these data will undoubtedly improve upon the usefulness of multigene testing, the current landscape represents an opportunity to expand the number of individuals who can receive timely and appropriate clinical guidance.

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9. OMIM database https://www.omim.org/
10. 1Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/index.php
11. BRCA Share http://www.umd.be/BRCA1/
12. BRCA Exchange https://brcaexchange.org/
13. BIC database https://research.nhgri.nih.gov/bic/
14. Align GVGD http://agvgd.hci.utah.edu/
15. PolyPhen-2 http://genetics.bwh.harvard.edu/pph2/
16. SIFT https://sift.bii.a-star.edu.sg/
17. MutationTaster http://www.mutationtaster.org/
18. Condel https://bbglab.irbbarcelona.org/fannsdb/
19. MutationAssessor http://mutationassessor.org/r3/
20. PANTHER http://www.pantherdb.org/
21. Human Splicing Finder https://www.genomnis.com/
22. GeneSplicer https://ccb.jhu.edu/software/genesplicer/
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QCI Interpret is evidence-based decision support software intended as an aid in the interpretation of variants observed in genomic next-generation sequencing data. The software evaluates genomic variants in the context of published biomedical literature, professional association guidelines, publicly available databases, annotations, drug labels, and clinical trials. Based on this evaluation, the software proposes a classification and bibliographic references to aid in the interpretation of observed variants. The software is NOT intended as a primary diagnostic tool by physicians or to be used as a substitute for professional healthcare advice. Each laboratory is responsible for ensuring compliance with applicable international, national, and local clinical laboratory regulations and other specific accreditations requirements.