Original Article

A comprehensive laboratory-based program for classification of variants of uncertain significance in hereditary cancer genes

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Genetic testing has the potential to guide the prevention and treatment of disease in a variety of settings, and recent technical advances have greatly increased our ability to acquire large amounts of genetic data. The interpretation of this data remains challenging, as the clinical significance of genetic variation detected in the laboratory is not always clear. Although regulatory agencies and professional societies provide some guidance regarding the classification, reporting, and long-term follow-up of variants, few protocols for the implementation of these guidelines have been described. Because the primary aim of clinical testing is to provide results to inform medical management, a variant classification program that offers timely, accurate, confident and cost-effective interpretation of variants should be an integral component of the laboratory process. Here we describe the components of our laboratory’s current variant classification program (VCP), based on 20 years of experience and over one million samples tested, using the BRCA1/2 genes as a model. Our VCP has lowered the percentage of tests in which one or more BRCA1/2 variants of uncertain significance (VUSs) are detected to 2.1% in the absence of a pathogenic mutation, demonstrating how the coordinated application of resources toward classification and reclassification significantly impacts the clinical utility of testing.

Conflict of interest

All authors are employees of Myriad Genetics, Inc. and Myriad Genetic Laboratories, Inc. and receive salaries and stock options as compensation.

Sequencing and large rearrangement analyses detect DNA changes within hereditary cancer genes and are offered to individuals with a personal and/or family history of cancer to identify pathogenic mutation carriers. Early identification of mutation carriers allows for increased clinical surveillance and early detection, and may prompt more aggressive prevention strategies, such as prophylactic surgery or chemoprevention, to reduce risk. For example, patients with pathogenic mutations in the genes BRCA1 and BRCA2 have a diagnosis of hereditary breast and ovarian cancer syndrome (HBOC), a condition for which there are extensive medical management guidelines aimed at the prevention and early detection of breast and ovarian cancer (1).

Once a genetic variant is detected in the laboratory, its clinical significance must be determined. Guidelines for the classification of variants have been proposed by the American College of Medical Genetics (2), the
International Agency for Research on Cancer (IARC) Unclassified Genetic Variants Working Group (3) and other researchers (4). These guidelines recommend a multi-tier classification system, grouping variants based upon perceived risk of disease association. Our laboratory has developed and currently utilizes a similar five-tier variant classification system composed of the following variant classification categories: ‘deleterious’ (pathogenic), ‘suspected deleterious’ (likely pathogenic), ‘variant of uncertain clinical significance’ (VUS), ‘genetic variant, favor polymorphism’ (likely not pathogenic), and ‘polymorphism’ (not pathogenic).

In a small proportion of patients, genetic testing will identify a VUS, which confounds the clinical interpretation of the result. VUSs consist primarily of missense substitutions that result in single amino acid changes, but also include variants that have the potential to alter RNA splicing (5) and other changes that have the potential to alter the production of fully functional protein (2). VUSs present a diagnostic challenge to the clinician. Similar to other non-informative results – for example, a ‘no mutation detected’ result in an individual with no family history of a specific mutation – clinical management of individuals carrying a VUS should be based upon personal and family history and not the presence or absence of the variant itself (6). However, non-informative results including VUSs often increase anxiety among patients, family members, and providers who cannot take advantage of the risk assessment, prevention, and therapeutic measures that are available to carriers of known deleterious mutations to modify behavior or lifestyle, or to make important clinical decisions that may, in many cases, involve prophylactic surgery (7). In addition, all first-degree relatives including non-carriers are considered at risk as long as the contribution of the variant to disease cannot be assessed, resulting in frequent unnecessary anxiety and prophylactic screening. However, unlike other non-informative results, the presence of a VUS may provoke anxiety that testing is not complete until the pathogenicity of the variant is determined (8).

The overall interpretation of VUS is currently reported in 2.1% of patients undergoing genetic analysis for HBOC at Myriad Genetic Laboratories (9, 10). This represents a decline from around 13% over the past decade. The dramatic decline in the percentage of patients receiving a VUS result reflects both the impact of targeted efforts directed at determining the pathogenicity of variants, as well as the availability of data from an increased number of individuals undergoing testing for HBOC (10).

The primary aim of clinical genetic testing is to provide results that inform medical management, so it is vital that diagnostic laboratories have in place a robust variant classification program that offers timely, accurate, confident and cost-effective interpretation of variants as an integral part of their testing services. Here we describe our current laboratory-based variant classification program which integrates multiple sources of both passively and proactively ascertained data in a coordinated fashion for use in a clinical setting. We use BRCA1/2 here as a model, but similar techniques for variant classification can be applied to other genes.

### Materials and methods

Novel variant interpretation

Myriad’s New Mutations Committee (NMC) consists of American Board of Medical Genetics (ABMG)-certified laboratory directors, the chief medical officer, clinical variant specialists, genetic counselors, and other representatives with expertise in clinical care, statistical genetics, biochemistry and structural biology. The NMC is responsible for the initial classification of new variants on a daily basis. The initial classification of new variants follows the guidelines set forth by the American College of Medical Genetics (2) in addition to a set of internal guidelines developed based on NMC.

### Table 1. Myriad Genetic Laboratories’ current categorization of variant reclassification methods

| Variant reclassification | ACMG guidelines | in trans | Mutation co-occurrence | Segregation | History-weighting algorithm | Evolutionary conservation | Functional or mRNA splice-site assays | Population frequency |
|--------------------------|-----------------|----------|-------------------------|-------------|-----------------------------|--------------------------|-------------------------------------|----------------------|
| Initial variant classification | X               | –        | –                       | X           | X                           | X                        | X                                   | X                    |
| Variant reclassification | –               | X        | –                       | –           | –                           | –                        | –                                   | –                    |
| Primary upgrade           | –               | –        | –                       | –           | –                           | –                        | –                                   | –                    |
| Secondary upgrade         | –               | –        | –                       | –           | X                           | X                        | X                                   | –                    |
| Primary downgrade         | –               | –        | –                       | –           | –                           | –                        | –                                   | X                    |
| Secondary downgrade       | –               | –        | –                       | –           | –                           | –                        | X                                   | –                    |

ACMG, American College of Medical Genetics and Genomics.

aNota: this table represents a framework and does not replace the expert review process that is needed before implementing any particular methodology. See the main text for clarification.

bIn trans: identification of homozygous and compound heterozygous individuals.

cInfrequently these data are available in the literature or through publicly accessible population frequency databases at the time of a variant’s initial classification.
Variant reclassification

The monitoring of variant data is embedded in our laboratory operations. Automation protocols alert the NMC when there is sufficient statistical evidence to consider reclassification of a variant. The literature and public databases are also continuously monitored to determine whether additional data have been made available that would inform the reclassification of a variant. The NMC meets to discuss reclassification data, and variants may be reclassified following a thorough evaluation of all relevant data. Table 1 outlines the different reclassification methods that are currently employed in our laboratory. Amended reports are sent weekly to healthcare providers who have patients for whom a variant reclassification affects their report.

Our laboratory VCP utilizes multiple lines of evidence, described below and in Table 1, for reclassification of a variant’s disease status. Statistical methods must reach an acceptable level of certainty before they can be used. This level of certainty currently exceeds 99% positive and 99% negative predictive values in our laboratory. Semi-quantitative and qualitative methods are thoroughly evaluated by experts in the relevant field prior to use. Independent methods are used only once to obtain a single step in reclassification (e.g. from ‘VUS’ to ‘suspected deleterious’). Figure 1 represents a simplified flow chart for the use of primary and secondary evidence in VUS reclassification (see also Table 1). Primary evidence is data that can be used by itself to upgrade or downgrade a variant by one classification step (e.g. from ‘VUS’ to ‘variant favor polymorphism’) as long as no significant contradictory evidence exists. Secondary evidence is data that can be used in conjunction with primary evidence for a full two-step upgrade or downgrade of a variant. Additional supporting evidence should be considered when variants are reclassified, but it is not considered strong enough to alter a classification. Unless otherwise specified, the methods described below can be used for primary lines of evidence for a variant reclassification. The reclassification process for any particular variant is initiated when new evidence is either generated in the production laboratory or made available in the public literature. All lines of evidence available at that time are then considered, whether they are independently sufficient for a reclassification (primary evidence), are supportive (secondary evidence) or are contradictory.

Literature review

Our laboratory employs scientists in a variety of fields who continually evaluate the literature to determine if there is sufficient evidence to reclassify a variant. In some instances, information from the literature is sufficient to be used on its own as primary evidence.
for a one- or two-step upgrade or downgrade of a variant. If the literature were to be used for a full two-step upgrade or downgrade of a variant, two separate methodologies with independent and significant findings would be required. Some of the reclassification methods described below can be found in the published literature.

Population frequency

Databases containing whole-exome sequencing data of control populations have recently become publicly available (11, 12). Our laboratory classifies variants as benign polymorphisms if they are present in >2% of a control population with a sample size >200 individuals without significant evidence to the contrary. These populations primarily consist of families in which hereditary cancer-predisposing syndromes are not indicated. Our laboratory also uses a comparative approach to statistically evaluate the affected population tested at our laboratory against control populations. A variant present in statistically equal frequencies in the two populations is considered benign. A variant enriched in the affected population is evaluated further for potential causality but is not reclassified on these data alone.

mRNA splice-site assays

The general mechanisms of RNA splicing are well understood (13), so novel genetic variants occurring at canonical splice acceptor and donor sites can be classified at their first observation based upon this knowledge. However, other variants not immediately at the splice-site junction may also impair RNA splicing. Biochemical analysis of potential mRNA splicing variants can provide evidence for variant reclassification. Analysis of patient mRNA or a minigene assay demonstrating a particular variant results in abnormal mRNA splicing may provide evidence that the variant is deleterious (14, 15). In our laboratory, evidence from splice-site assays in the literature is primarily used, after expert review, to upgrade a variant provided the assay clearly shows complete loss of the functional mRNA isoform(s) transcribed from the variant allele. Because of this requirement, the application of this method is limited.

Functional assays

Because cancer predisposition in HBOC results from the inheritance of alterations that result in loss of function of tumor suppressor genes, in vitro detection of a decrease in activity of a tumor suppressor may correspond to increased cancer predisposition. Functional assays assess the effects of BRCA1/2 missense variants on known protein function. Existing assays include, but are not limited to, those designed to measure variant effects on centrosome number control (16), homologous recombination (17), transcription, protein-folding, and phosphopeptide binding (18). These techniques include homologous recombination and centrosome amplification assays (19). In addition, analysis of the solved portion of the BRCA2 crystal structure can sometimes be utilized to determine the effect that a variant may have on BRCA2 function (20). Because functional assays have only been tested on a limited number of variants, our laboratory may use published data from these assays as supporting evidence in conjunction with primary data for the upgrade or downgrade of a variant.

Evolutionary conservation

Evaluation of species conservation may provide supportive evidence for variant reclassification. Phylogenetic conservation of protein sequence throughout evolution often reflects the requirement for certain amino acids for protein activity. Multiple computational algorithms, including Sorting Intolerant from Tolerant (SIFT), Polymorphism Phenotyping (Poly Phen) and Align-GVGD (21–24), have been designed to evaluate the evolutionaryfunctional significance of an amino acid change through analysis of multiple species protein alignments. However, because of high false positive and false negative rates (4), our laboratory does not currently use these algorithms to reclassify a variant to a ‘deleterious’ or ‘suspected deleterious’ category. Identification of a particular amino acid change in multiple species does provide supportive evidence that a variant is benign; therefore, in our laboratory, we currently use conservation analysis as secondary evidence to support a downgrade if the exact missense change seen in a patient sample exists in multiple reference sequences of higher species (primarily vertebrates). Protein sequences for different species are added to the alignments as they become publicly available. No fewer than 15 species are used. It is important to note that these methods are subject to the quality of the species alignment used and the context of the particular missense mutation in question. Regions of poorly aligned sequences are considered insufficient for this analysis. Once a missense variant has achieved a classification of favor polymorphism (FP, that is, a variant with one significant line of evidence in favor of benign) based upon an independent line of evidence, if that particular missense variant is seen repeatedly in other species in well-aligned and conserved regions of the protein, the variant may be downgraded to ‘polymorphism’.

Segregation analysis

Segregation analysis measures whether or not a variant segregates with cancer in one or more families. It has traditionally relied upon obtaining one or more large pedigrees with multiple affected family members available for analysis. Myriad uses a modified approach to segregation analysis that allows for analysis of small families, similar to that described by Thompson, Easton, and Goldgar (25). A 500:1 likelihood ratio for deleterious or benign is considered sufficient for a reclassification of a VUS to suspected deleterious or favor
polymorphism. Statistical data obtained from a series of small families sharing the same variant are combined to assess the clinical significance of the specific variant. Almost without exception, this approach requires active participation of multiple families before a variant can be reclassified.

Variant test results from our laboratory are frequently accompanied by an offer of no-cost testing after evaluation of each proband’s pedigree. Our protocol directs that testing offers be typically made to the most informative individuals in the pedigree, such as older unaffected females and younger affected women who are 1° or 2° relatives. Results for multiple families with the same variant are combined. Owing to the relative high phenocopy rates for HBOC, this method’s primary limitation is the large amount of data required to statistically overcome the phenocopy observations.

Identification of homozygous and compound heterozygous individuals (in trans)

Given the severe phenotypes associated with homozygosity for a BRCA1 or BRCA2 deleterious mutation (26–29), observation of a homozygous variant or a variant in trans with a deleterious mutation (i.e. compound heterozygosity) in a healthy individual or an individual with later-onset cancer provides significant evidence that the variant itself does not represent a deleterious mutation. For example, with few exceptions (30), biallelic BRCA1 mutations are embryonic lethal; therefore, if a known pathogenic mutation is present, a VUS is highly unlikely to be deleterious. Conversely, biallelic BRCA2 mutations result in Fanconi anemia, an autosomal recessive syndrome characterized by congenital anomalies, bone marrow failure, cellular sensitivity to DNA cross-linking agents, and predisposition to cancer. Therefore, the non-pathogenicity of a novel BRCA2 variant co-occurring in trans with a known pathogenic BRCA2 mutation can be assessed by the absence of features of the Fanconi anemia phenotype. A limitation of this method is that attenuated biallelic disease states can exist (30). For example, in rare cases of BRCA2 biallelic states, Fanconi anemia is not obvious in the patient. To address this limitation, where attenuated forms of Fanconi anemia may exist, Myriad facilitates the Chromosome Breakage Analysis test, at no-cost to the patient, in order to obtain a definitive diagnosis.

Mutation co-occurrence

Mutation co-occurrence (MCO), similar to ‘ascertainment ratio’, is a statistical technique based on the empirical observation that if a pathogenic mutation is identified in a family, that mutation is usually found to be the primary cause of disease in the family (31). Therefore, the presence of a known pathogenic mutation in a biochemical pathway reduces the likelihood that a VUS in the same pathway is clinically relevant (24, 31). The known deleterious mutation can either be in the same gene (in cis or in trans) or in other genes in the same pathway (e.g. a BRCA1 mutation and a BRCA2 VUS). One limitation of this method is that the development of MCO for a particular gene and pathway requires large sets of empirical data to account for the clinical consequences of carrying two pathogenic mutations of the gene(s) in question and to account for ascertainment bias. To ensure that each reclassification methodology used for a particular variant is independent of any other reclassification method already used for a variant, care must be taken in the use of MCO such that compound heterozygous observations which have been used previously to reclassify a variant by one step are not used again for MCO to obtain a second reclassification downgrade.

History-weighting algorithm

Our laboratory’s history-weighting algorithm is based on the premise that individuals with deleterious mutations are expected to have more severe personal and family histories than individuals with benign polymorphisms (32). The technique is an advance, facilitated by the quantity of data analyzed since its publication, on the method described by Easton et al. (33) in which the probability of deleterious mutation is calculated for each proband based upon their personal and family history. The combined history-weighting scores for unrelated probands carrying a particular VUS are compared against the observed clinical population tested by the laboratory which accounts for patient ascertainment bias.

The history-weighting score is validated for both single steps in upgrades and downgrades of a classification. One limitation of this method is that vanishingly rare variants cannot be reclassified using this method. However, owing to the large volume of testing at our laboratory, this method accounts for the majority of variant reclassifications (Fig. 4, Table 2).

Review of a genetic variant classification program

Table 2. Relative effectiveness of reclassification methods by proband count for HBOC (October 2011–November 2012)

| Reclassification method              | Total reclassification events | Average number of probands per downgrade (minimum²) | Average number of probands per upgrade (minimum³) |
|--------------------------------------|------------------------------|----------------------------------------------------|--------------------------------------------------|
| in trans                             | 84 (16%)                     | 17.5 (1)                                            | NA                                               |
| Mutation co-occurrence               | 24 (4.7%)                    | 31.2 (2)                                            | NA                                               |
| Segregation                          | 18 (3.5%)                    | 41.8 (9)                                            | 32 (13)                                          |
| History-weighting algorithm          | 290 (56%)                    | 19.4 (6)                                            | 33.7 (28)                                        |
| Evolutionary conservation            | 90 (18%)                     | 15.4 (NA)                                           | NA                                               |
| Functional or mRNA splice-site assays| 6 (1.0%)                     | 7 (NA)                                              | 12.6 (NA)                                        |
| Population frequency                 | 2 (0.4%)                     | 8.5 (NA)                                            | NA                                               |
| Total                                | 514                          | –                                                   | –                                                |

HBOC, hereditary breast and ovarian cancer syndrome; NA, not applicable.

²The minimum numbers are representative of the empirical data during the specified time period and are not necessarily theoretical minimums.
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Fig. 2. Decline in rate of HBOC variants of uncertain significance. Myriad’s data was analyzed periodically from 2002 to 2013 to establish the percentage of tests reported with an overall interpretation of VUS and subdivided by ancestry. Inclusion criteria consisted of individuals who were referred for clinical genetic testing of BRCA1/2. Patients for which no ancestry was selected or for which multiple ancestries were selected were excluded from the ancestry breakdown but were included in the total count.

Results

Overall outcomes of variant reclassification process

Myriad’s mutation data set was analyzed periodically from 2002 to 2013 to establish the percentage of overall tests reported with an interpretation of VUS and subdivided by ancestry (Fig. 2). Inclusion criteria consisted of individuals who were referred for clinical genetic testing of BRCA1/2. Patients for whom no ancestry was selected or for whom multiple ancestries were selected were excluded from the ancestry breakdown but were included in the total count.

The VUS rate is defined as the percentage of BRCA1/2 patients comprehensively tested in the entire Myriad test history that have an overall test report of VUS at the time point specified. From 2002 to 2013, the VUS rate declined from 12.8% of all BRCA1/2 test results to 2.1% of all results (84% decline). During this time period there was also a decline in the VUS rate across all ancestries. It is important to note that a patient who has a suspected deleterious or deleterious mutation accompanying a VUS will have an overall test result of suspected deleterious or deleterious, respectively. A patient who has a favor polymorphism or polymorphism (benign) in addition to a VUS will have an overall test result of VUS. More than one million BRCA1/2 test reports were evaluated to attain the 2013 VUS rate.

VCP segregation analysis/family testing uptake

Between October 2011 and August 2012, Myriad recorded family history submissions from 16.8% of the VUS/FP results (Fig. 3). Family testing was offered to an average of 2.3 relatives per family history submission, with a 24.1% response rate.

Relative contribution of variant reclassification techniques

During October 2011 to November 2012, segregation analysis resulted in only 3.5% of reclassification events (Table 2). The history-weighting algorithm and MCO achieved 61% of all reclassification events. Both techniques are powered by our laboratory’s data set and do not require any additional follow-up family testing beyond the proband’s results. Of reclassification events, 16% were a result of in trans observations,
which occasionally require follow-up testing of family members to determine phase. Of reclassifications, 19% were achieved through methods using publicly available data: evolutionary conservation evaluation (18%), functional and mRNA splice-site assays published in literature (1%) and population frequency data (<1%).

Historically, most VUSs are determined to be benign (33), representing not only a true bias, but also the greater number of methods to discover benign variants compared to deleterious mutations (Table 2 and Fig. 4). Of the reclassification events that were upgrades to deleterious or suspected deleterious in the 13-month time period reported here, 20% were based on segregation data, 30% were based on the history-weighting algorithm, and 50% were based upon functional or mRNA splice-site assays reported in the literature (Fig. 4). The history-weighting algorithm is dependent on the total number of probands carrying a particular variant. For segregation analysis, the likelihood of obtaining sufficient participating families to achieve a reclassification increases as the number of probands for a variant increases. Of the upgrades to ‘suspected deleterious’ or ‘deleterious’, the history-weighting algorithm and segregation analysis had similar averages of the number of proband carriers at time of reclassification (Table 2).

Discussion

On the basis of analysis of our dataset of more than one million patients, our laboratory has developed an extensive variant classification program which utilizes multiple variant classification techniques; new classification methods are continually being assessed by our scientists. Although variations of some of these techniques have been utilized by geneticists for many years, others have only become feasible within recent years following the accumulation of the large data set required for their development and use, and they are unique to our variant classification process. The program is particularly effective for autosomal dominant genes such as BRCA1/2. The techniques described here would need to be altered to account for sex-linked or autosomal recessive genes.

From 2002 to 2013, the VUS rate declined from 12.8% of all BRCA1/2 test results to 2.1% of all results (84% decline, Fig. 2). The substantial decline in the VUS rate in these ancestries is a result of improved methods for establishing the clinical significance of variants and increased utilization of testing in these populations, which provides more data for analysis.

Uncertainty remains about the clinical relevance of VUS. Although our laboratory recommends that clinical management of VUS carriers should be based upon personal and family history and not the presence or absence of the variant itself, some healthcare providers increase surveillance or pursue treatment options beyond that recommended for such variants (34). However, our results show the majority of BRCA1/2 VUSs are discovered to be benign through a variety of methods, with history-weighting analysis the most robust method. Segregation analysis shows particular power in identifying deleterious variants rather than benign variants. As shown by the minimums in Table 2, segregation analysis is able to achieve a reclassification with fewer probands per variant compared with the history-weighting algorithm. Although segregation analysis is labor intensive, its success rate is limited; only 3.5% of variant reclassifications are achieved using this technique. Such limitations emphasize the utility of a variant classification program that can weigh other forms of evidence in addition to segregation data. Considering laboratories and community research centers have finite resources, these data therefore suggest that the tailoring of family analysis to specific families with higher likelihoods of having a deleterious mutation may be the most productive use of resources. These data show...
that while segregation analysis is a comparatively poor tool for discovering benign variants, it is a powerful tool for discovering deleterious mutations (Fig. 4).

The decrease in our laboratory’s VUS rate over time is a function of increased data sets and increased expertise in evaluating variants. Our laboratory has classified thousands of VUS and continues to identify dozens of novel VUS every week (9). Thus, the importance of experience and technical acumen of the laboratory classifying the variants cannot be overstated. Because of both the need for timely test interpretation and the pace at which new variants are identified and existing variants reclassified, we believe that variant classification is an integral part of the testing process to be performed by a CLIA-approved (for USA laboratories), quality-assured laboratory as standard operating procedure.

New technologies, with their attendant increases in tests ordered, and, thus, variants identified, will challenge variant classification programs – for example, in reporting VUSs and sending amended reports when variants are reclassified. The amount of BRCA1/2 testing is likely to increase if therapies specifically targeting BRCA1/2 mutated tumors, such as poly-(ADP-ribose) polymerase (PARP) inhibitors, move out of clinical trials and into practice. In addition, the advent of next-generation massively parallel sequencing will necessitate reliable, high-throughput variant classification programs. As costs decrease for whole-genome sequencing experiments, it is likely that a larger number of individuals show genetic variation beyond the exons and intron/exon boundaries routinely covered by current clinical tests (35). One value of our model-based approach is that it provides a quantitative output that can be used to categorize variants into defined classification categories and so minimizes subjectivity and decreases turnaround time (3).

On the basis of 20 years of experience, our laboratory has developed a robust program for classification of variants. Our variant classification program has lowered the percentage of tests in which one or more BRCA1/2 variants of uncertain significance (VUSs) are detected to 2.1%, showing how the coordinated application of resources toward classification and reclassification significantly impacts the clinical utility of testing.

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