Original Article

Exceptions to the rule: Case studies in the prediction of pathogenicity for genetic variants in hereditary cancer genes

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Based on current consensus guidelines and standard practice, many genetic variants detected in clinical testing are classified as disease causing based on their predicted impact on the normal expression or function of the gene in the absence of additional data. However, our laboratory has identified a subset of such variants in hereditary cancer genes for which compelling contradictory evidence emerged after the initial evaluation following the first observation of the variant. Three representative examples of variants in BRCA1, BRCA2 and MSH2 that are predicted to disrupt splicing, prematurely truncate the protein, or remove the start codon were evaluated for pathogenicity by analyzing clinical data with multiple classification algorithms. Available clinical data for all three variants contradicts the expected pathogenic classification. These variants illustrate potential pitfalls associated with standard approaches to variant classification as well as the challenges associated with monitoring data, updating classifications, and reporting potentially contradictory interpretations to the clinicians responsible for translating test outcomes to appropriate clinical action. It is important to address these challenges now as the model for clinical testing moves toward the use of large multi-gene panels and whole exome/genome analysis, which will dramatically increase the number of genetic variants identified.

Conflict of interest

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

Establishing the clinical significance of genetic variation detected in the laboratory presents challenges that increase proportionally with the amount of genetic material analyzed. This is a frequently cited concern as the clinical testing strategy for many inherited conditions transitions to large multi-gene panels or whole exome/genome analysis. However, standard practice holds that many genetic variants can immediately be presumed clinically significant based on their predicted impact on protein function, most often when it is known that loss of gene function (LOF) is a cause of a disorder. Current practice, supported by existing professional society guidelines, states that variants in these genes should be classified as pathogenic or suspected pathogenic if they result in premature truncation of a protein, disrupt the normal reading frame, alter the start codon, alter the sequence of a consensus splice junction in a manner that is predicted to disrupt normal mRNA processing, or delete a protein segment containing a critical functional domain (1). Results including variants with
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these characteristics are considered to be ‘positive’ for the associated genetic condition.

Examples of potentially benign variants include those which are not predicted to alter the amino acid sequence or impact mRNA splicing, transcription or protein translation, or those which are shown to be very common within a normal control population (1). A genetic test result is considered to be ‘negative’ when the only variants detected are classified as benign. A variant which cannot immediately be assigned a classification using the above criteria will likely be classified as a ‘variant of uncertain clinical significance’ (VUS) until additional data are gathered. The majority of VUSs are missense changes that alter a single amino acid in the protein, nucleotide changes for which the impact on splicing cannot be confidently predicted, or in-frame deletions or insertions of small numbers of amino acids.

Our laboratory has extensive experience with clinical testing for inherited cancer genes, most notably BRCA1 (OMIM #604370) and BRCA2 (OMIM #612555), the genes responsible for hereditary breast and ovarian cancer syndrome (HBOC), but also genes for other cancer-related conditions, such as Lynch syndrome (OMIM #120435). Patients found to have pathogenic or suspected pathogenic variants in BRCA1 and BRCA2 are candidates for medical management strategies including intensive surveillance aimed at the early detection of cancer, chemoprevention and consideration of risk-reducing surgeries, including mastectomies and oophorectomies. To date, we have identified thousands of variants in BRCA1, BRCA2, and other genes which could be immediately classified as pathogenic or suspected pathogenic consistent with the guidelines described above.

In order to provide more definitive clinical classifications for variants initially classified as VUSs, we maintain a comprehensive variant classification program (VCP) (2). This program utilizes clinical data obtained from test requisition forms as well as co-segregation data from testing offered at no charge to selected relatives of patients with VUSs. The availability of powerful data-driven classification methodologies has resulted in a drop in the percentage of BRCA1 and BRCA2 clinical reports containing a VUS from 12.8% to 2.1% over the past decade (2).

In addition to reclassifying VUSs, data from the VCP also allows us to more closely scrutinize variants initially presumed to be pathogenic based on the criteria described previously. Unexpectedly, we have found compelling evidence contradicting the predicted classification for a very small fraction of specific variants. This report provides three representative examples of genetic variants which were initially classified as pathogenic based on predicted LOF, but for which new information triggered an evaluation of clinical data from subsequent observations of the variants in multiple families. These data contradict the expected classification, creating significant challenges for the laboratory, providers and patients. These examples illustrate the need for caution, particularly in regard to testing strategies based on the analysis of larger amounts of genetic data from multigene panels or whole exome/genome assays, which increases the likelihood that a patient could be impacted.

Materials and methods

Initial assessments of pathogenicity

In accordance with current guidelines from the American College of Medical Genetics and Genomics, our laboratory classifies newly observed variants as pathogenic or suspected pathogenic if they are of a type ‘that is expected to cause the disorder’ (1). Classification criteria strongly indicative of pathogenicity include, but are not limited to, the following:

1. A premature stop codon is created prior to the most 3’ known pathogenic variant in a gene, due to either a nonsense variant or a frame-shift resulting from insertion/deletion of one or more nucleotides. Premature stop codons close to the gene’s 3’ end are possible exceptions because loss of terminal amino acids may not significantly impair protein function. Therefore, our laboratory does not classify variants as pathogenic if they truncate the protein after the last known pathogenic variant in the gene.

2. An invariant consensus nucleotide at a splice junction is altered and is predicted to result in the production of abnormally spliced mRNA unable to code for a functional protein. In cases where the loss of an exon or exons creates an in-frame deletion of a portion of the protein with no known function, the variant may not immediately be presumed to be pathogenic.

3. The initiation codon is altered.

Variants meeting any of these criteria are not classified as pathogenic or suspected pathogenic if there is significant contradictory evidence identified. However, this is unlikely when a variant is being observed for the first time.

Data-based models for evaluation of variants

Data are gathered and evaluated as part of a structured VCP, the details of which have been described previously (2). These data consist of clinical information submitted on the test request form, test results, and information from detailed family histories obtained in connection with targeted offers of testing at no charge to potentially informative relatives in families where variants have been identified. A brief overview of the types of data utilized and the applied statistical models follows.

The history weighting algorithm (HWA) compares the aggregated clinical histories of patients with specified variants to large numbers of matched controls positive or negative for known pathogenic variants in the same gene. This model is based on the principle that pathogenic variants will be more common in patients with high-risk personal and family histories while the presence of clinically benign variants will not be correlated with clinical history (3, 4). A detailed description of the
The expected classification of c.594-2A > C in trans BRCA1 status of the three variants presented, one each in BRCA1, BRCA2 and MSH2. Based on standard guidelines for the interpretation of sequence changes, each of these variants would initially be classified and reported as pathogenic or suspected pathogenic. However, in each case, there is contradictory data available as a result of monitoring the published literature and internal laboratory data.

**Results**

Table 1 summarizes the available information and current status of the three variants presented, one each in BRCA1, BRCA2 and MSH2. Based on standard guidelines for the interpretation of sequence changes, each of these variants would initially be classified and reported as pathogenic or suspected pathogenic. However, in each case, there is contradictory data available as a result of monitoring the published literature and internal laboratory data.

**BRCA1 c.594-2A > C**

The expected classification of BRCA1 c.594-2A > C as pathogenic is based on the predicted impact on mRNA splicing at the intron 9/exon 10 boundary. Splicing analysis algorithms predict that this change disrupts normal mRNA splicing because the consensus splice acceptor sequence at this position is highly conserved (8). Biochemical studies confirm skipping of exon 10 in transcripts from the BRCA1 c.594-2A > C allele, resulting in the loss of 77 nucleotides and premature truncation of the BRCA1 protein (9).

We re-evaluated BRCA1 c.594-2A > C following published studies of a BRCA1 variant located at a different splice junction, c.591C > T (p.C197C) (10). This variant is predicted to disrupt splicing at the exon 9/intron 9 boundary, but it has long been classified as clinically insignificant based on multiple lines of evidence, including a high frequency in control populations. Given this benign designation, it is somewhat surprising that this variant results in a significant reduction in the production of full length BRCA1 transcripts and the production of abnormal transcripts missing exon 9. However, this is accompanied by an increase in the proportion of an alternatively spliced transcript lacking both exons 9 and 10. In fact, this Δ(9,10) isoform is normally present at relatively high levels in breast tissue containing only wild-type BRCA1 and may retain at least some BRCA1 function as the deletion of both exons 9 and 10 results in an in-frame deletion of 41 amino acids in a portion of the protein unrelated to homologous repair (Fig. 1) (11). These findings raise the possibility that this alternative transcript can ‘rescue’ variants that disrupt splicing for exons 9 and 10, or possibly even sequence changes within those exons.

We subsequently performed a systematic review of available data for c.594-2A > C in our clinical database along with a concerted effort to perform testing at no charge for relatives of patients in whom this variant had been identified (Table 1). As of September 23, 2013, we had detected this variant in 110 apparently unrelated individuals as an outcome of comprehensive sequencing and large arrangement analysis. It has been observed in multiple patients from six families who also have deleterious variants in BRCA2, placing this variant in the benign category using the MCO model. BRCA1 c.594-2A > C also falls within the benign range using the HWA (Fig. 2a). The family testing outcomes do not demonstrate consistent segregation of the variant with breast or ovarian cancer, although we can not exclude some contribution of the variant to the familial aggregation of cancer (Table 2).

In the past, it has been believed that embryonic lethality was inevitably associated with homozygosity or compound heterozygosity for pathogenic BRCA1 variants, but recent reports of two patients with two deleterious BRCA1 variants in trans has demonstrated that in rare cases this can lead to a clinical condition similar to FA (12). We have observed a patient with variant c.594-2A > C in trans with the BRCA1 founder variant exon13ins6kb. This patient does not have reported symptoms consistent with FA, which further contradicts a pathogenic interpretation for this variant.

Myriad currently reports BRCA1 c.594-2A > C as a ‘special interpretation’ variant based on the as yet unresolved conflict between the predicted impact on protein sequence and function and the available data.

**BRCA2 c.9699_9702del (p.Cys3233Trpfs*15)**

The four nucleotide deletion variant BRCA2 c.9699_9702del (p.Cys3233Trpfs*15) results in a frame-shift leading to 14 novel amino acids at position...
### Table 1. Summary of data for variants

| Variant | Presumed classification and rationale | Basis to question presumed classification | Plausible biological mechanism for alternative classification | In trans (same gene) co-occurrence data (Myriad) | Mutation co-occurrence model (Myriad) | Family history weighting (Myriad) | Co-segregation analysis (Myriad) |
|---------|-------------------------------------|------------------------------------------|---------------------------------------------------------------|--------------------------------------------------|-----------------------------------|---------------------------------|-------------------------------|
| **BRCA1** c.594-2A>C \( ^a \) (aka BRCA1 IVS9-2A>C) | Pathogenic, based on predicted impact of base change on splicing and biochemical evidence that exon 10 is lost from transcripts | Published work for BRCA1 c.591C>T, a variant at a different splice junction | Formation of Δ(9,10) transcript, which likely retains partial function | One in trans observation with another BRCA1 pathogenic variant in a patient with no reported features of FA | Benign | Falls in benign range | Does not support pathogenicity |
| **BRCA2** c.9699_9702del (aka BRCA2 9927del4) | Pathogenic, based on predicted premature truncation of the protein | Observation of variant in trans with other BRCA2 deleterious variants in two families in which patients did not display classical clinical symptoms of FA | None | In trans observations with one BRCA2 pathogenic variant and one BRCA2 suspected pathogenic variant in patients with mild features of Fanconi anemia | Indeterminate | Falls in benign range | Does not support pathogenicity |
| **MSH2** c.1A>C \( ^b \) (aka MSH2 M1L (1A>C)) | Pathogenic, based on change in start codon, expected to significantly reduce translation | A series of publications providing evidence that this variant is substantially less penetrant than other pathogenic MSH2 variants | Re-initiation of translation at a methionine 26 codons downstream from the start codon | Published case of two siblings with this variant in trans with a deletion of exons 1–6 in MSH2, and no reported features of CMMR-D. Additional observation at Myriad in trans with the pathogenic variant MSH2 c.2038C>T \( ^c \) (p.Arg680*) in patient without reported features of CMMR-D | Indeterminate | Indeterminate | Limited data |

CMMR-D, constitutional mismatch repair deficiency syndrome; FA, Fanconi anemia.

\( ^a \)NCBI Ref: NM_007294.3.

\( ^b \)NCBI Ref: NM_000059.3.

\( ^c \)NCBI Ref: NM_000251.2.
possibly functional, because loss of both exons leads to an in-frame deletion of 41 amino acids (11).

This isoform is normally present at low levels in cells with only wild-type lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10). This isoform is normally present at low levels in cells with only wild-type lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10). This isoform is normally present at low levels in cells with only wild-type lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10). This isoform is normally present at low levels in cells with only wild-type lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10).

Fig. 1. Wild-type and alternatively spliced transcripts of BRCA1. The variant BRCA1 c.594-2A>C is expected to produce a non-functional transcript lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10). This isoform is normally present at low levels in cells with only wild-type lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10). This isoform is normally present at low levels in cells with only wild-type lacking exon 9. Studies of another variant known to produce transcripts lacking exon 9 have shown that this is accompanied by increased production of an isoform lacking both exons 9 and 10 (10).

3233, followed by premature truncation. Although this variant is near the 3’ end of the BRCA2 gene, there are known pathogenic variants downstream of this position, i.e. c.9924C>G (p.Tyr3308*) (13). Therefore, as with BRCA1 c.594-2A>C described above, this variant is expected to cause HBOC and would typically be classified as pathogenic.

Questions about the clinical significance of c.9699_9702del arose following observations of the variant in trans with other deleterious BRCA2 variants in two sets of patients with no reported symptoms of FA, which is a known outcome of compound heterozygosity for pathogenic variants in BRCA2 (14). Family 1 was ascertained when a female diagnosed with breast cancer at age 22 was found to carry both BRCA2 c.9699_2702del and BRCA2 c.145G>T (p.Glu49*), most likely in trans, although this could not be confirmed. Her brother, with no reported FA symptoms at 15, was also found to have both variants. These siblings were both reported to have had abnormal chromosome mitomycin C stress testing (15). Family 2 was ascertained when the proband, who had a past history of acute myelogenous leukemia at age 9, was diagnosed with breast cancer at age 27 and was found to have both BRCA2 c.9699_9702del and c.8009C>T (p.Ser2670Leu) (16). Although this patient does have some clinical features consistent with FA, the two variants were also seen in trans in her healthy 29-year-old brother with no reported symptoms. This suggests that this variant is potentially pathogenic for a relatively mild form of FA in comparison with other BRCA2 pathogenic variants.

As of September 27, 2013, we have detected this variant in 67 apparently unrelated individuals undergoing comprehensive analysis of BRCA1 and BRCA2 (Table 1). In addition to the two observations in patients who have other pathogenic variants in BRCA2, it has been seen in two individuals who also carry a pathogenic variant in BRCA1, but these data have not yielded a conclusive MCO result. However, this variant currently meets the threshold for a benign designation using the HWA.

As with the previous variant, family testing outcomes (Table 2) do not demonstrate consistent segregation of the variant with breast or ovarian cancer, although some contribution of the variant to the familial aggregation of cancer cannot be excluded.

BRCA2 c.9699_9702del is currently reported as a ‘special interpretation variant’, similar to BRCA1 c.594-2A>C.

MSH2 c.1A>C (p.Met1_Gly25del)

MSH2 c.1A>C (p.Met1_Gly25del) is predicted to adversely impact production of the MSH2 protein due to alteration of the consensus translation start codon. Therefore, this variant was regarded as causative of Lynch syndrome when initially described in a patient diagnosed with colon cancer under age 30, although the authors of that publication noted that a potential alternative start codon is present at codon 26 (17). Our laboratory first reported this variant as likely pathogenic, but the classification was changed to ‘special interpretation’ based on published studies demonstrating that colorectal tumors from patients with c.1A>C lacked high levels of microsatellite instability or loss of MSH2 detectable by immunohistochemistry (18). Additionally, two siblings were identified with MSH2 c.1A>C in trans with a deletion of exons 1–6 in MSH2, neither with clinical features of constitutional mismatch repair deficiency syndrome (CMMR-D) (19).

As of September 27, 2013, Myriad has observed MSH2 c.1A>C in 14 individuals from eight families, including one patient with another in trans MSH2 pathogenic variant, c.2038C>T (p.Arg680*). Similar to previously described patients, this individual does not have any reported features of CMMR-D. This variant does not yet meet the threshold to be classified as benign using the HWA, although it is trending in that direction (Fig. 2c). Our laboratory has never observed this variant in a patient or family with a pathogenic variant in a different Lynch syndrome gene. We have
perform only five single-site tests for relatives of probands (Table 2). The interpretation of available data for MSH2 c.1A>C is consistent with previous indications of substantially lower penetrance than other Lynch gene pathogenic variants. In fact, the available data indicate such reduced penetrance of MSH2 c.1A>C that it is doubtful that its presence is diagnostic of Lynch syndrome.

**Discussion**

Each of the three variants described here would normally be reported as pathogenic based on widely accepted guidelines for interpretation of sequence variation ‘expected to cause the disorder’ in the absence of additional data (1). However, in each case, empiric data contradict this assessment. These variants demonstrate that the challenges associated with establishing the clinical relevance of genetic variation apply not only to the missense variants that make up the bulk of VUS results today, but also to variants predicted to be pathogenic as a result of presumed LOF. It is imperative that we address these challenges now, as testing strategies move to the analysis of large gene panels or even entire exomes and genomes. It is widely accepted that with this transition increased numbers of uncertain variants will be reported. The variants described here demonstrate it will also be important to ensure that we do not return ‘false positive’ results for a subset of variants classified as pathogenic based on current guidelines and practice.

Each of the cases described here requires resolution of a conflict between expectations based on our current understanding of molecular biology and what might actually be happening in patients. The situation for MSH2 c.1A>C is fairly straightforward, as the mechanism for production of functional protein through use of an alternative start codon was predicted and has now been demonstrated in vitro (20). However, for BRCA1 c.594-2A>C, it is only a hypothesis that the adverse impact of the loss of exon 10 can be ‘rescued’ by an alternative transcript, and there is as yet no compelling hypothetical mechanism as to why BRCA2 c.9699_9702del is not causative of HBOC. Without a plausible biological mechanism, clinicians may balk at suggestions that these variants are not associated with high risks for cancer.

It is important to note that none of the three variants described here are yet proven to have absolutely no impact on cancer risk, although the available data in each case suggest that the risk falls far short of what is considered to be diagnostic of the associated clinical syndrome. Therefore, patients with these variants are unlikely candidates for the aggressive medical management strategies prescribed by professional societies for individuals with a diagnosis of HBOC or Lynch syndrome. These recommendations encompass aggressive surveillance as well as the consideration of irreversible surgical interventions, such as oophorectomies, hysterectomies and mastectomies. The boundaries for considering a variant as causative for a defined syndrome, vs an intermediate or even small/negligible increase in cancer risk, have not been defined.

This also illuminates the limitations of current classification schemes, which are focused on assigning discrete classifications to high penetrance variants. Systematically deviating from this premise will be challenging given the limitations of current classification models.
### Table 2. Outcomes of family testing

| Sex, age | Positively aff | Positively unaff | Negatively aff | Negatively unaff |
|----------|----------------|------------------|----------------|-----------------|
| BRCA1 c.594-2A>C |                |                  |                |                 |
| F, 18–29 | 3              | 2                | 6              |                 |
| M, 18–100 | 9              |                  |                |                 |
| BRCA2 c.9699_9702del (p.Cys3233Trpfs*15) |                |                  |                |                 |
| F, 18–29 | 2              | 6                |                |                 |
| M, 18–100 | 9              |                  |                |                 |
| MSH2 c.1A>C (p.Met1_Gly25del) |                |                  |                |                 |
| F, 18–29 | 1              |                  |                |                 |
| M, 18–100 | 1              |                  |                |                 |

*Results shown for affected and unaffected relatives of probands for each of the three variants. Information is provided for female (F) and male (M) relatives by age of cancer diagnosis for affected (aff) patients and by age at which testing occurred for unaffected (unaff) relatives. Relatives tested for BRCA1 c.594-2A>C and BRCA2 c.9699_9702del were marked as affected or unaffected based on diagnoses of breast, ovarian cancer (including fallopian and peritoneal cancer) and pancreatic cancer (for BRCA2 c.9699_9702del only). Among the affected relatives, the only relative affected with a cancer other than breast was diagnosed at age 71 with fallopian tube cancer and tested positive for BRCA1 c.594-2A>C. Relatives tested for MSH2 c.1A>C were considered unaffected if they did not have a diagnosis of any of the following cancers: colorectal, uterine, gastric, ovarian, small intestine, and urinary tract. No male relatives are included in the table for MSH2 c.1A>C because no testing was submitted for this variant in men.*
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At this time, there is no reason to doubt the clinical significance of the vast majority of BRCA1, BRCA2, and mismatch repair variants identified as pathogenic by standard criteria for predicting LOF. We do not present these cases to suggest that these conventions be abandoned, as that would be a disservice to the tens of thousands of individuals who have received life-saving information through the identification of variants which put them at a high risk for preventable cancers. However, these cases illustrate the need for caution in the interpretation of whole exome/genome tests and large multi-gene panels. While the number of patients impacted by possible misdiagnoses as a result of variants like those described here constitutes only a fraction of the tests performed for HBOC and Lynch syndrome, the risk increases significantly with the number of genes analyzed, and it may be of particular concern when testing identifies variants in genes unrelated to a patient’s personal and family history.

These cases illustrate the need for discussions about responsibility and expectations surrounding the ongoing evaluation of data obtained through clinical testing, which must encompass ongoing monitoring for variants already thought to have definitive classifications as well as those initially deemed to be of uncertain significance. In the examples here, we were able to target specific pathogenic variants for re-evaluation based on publications and/or monitoring of internal data. The results of these re-evaluations have led us to also investigate variants suspected of being subject to the same phenomena, i.e. other splice site changes where there may be alternative transcripts, truncating variants within exons 9 and 10 of BRCA1, and other frame-shift variants close to BRCA2 c.9699_9702del which lead to truncation at the same amino acid. Out of caution, we are reporting many of these other variants in the Special Interpretation format, even though in most cases there is insufficient data for the types of analyses that were performed for the three variants described here.

Finally, these cases illustrate the need to further consider where responsibility resides for notifying clinicians and patients about changes in the interpretation of genetic testing results. Although there is consensus that the laboratory has some responsibility for this process, it is not required by regulatory agencies and laboratories currently have different policies and protocols for providing updates (1, 25, 26). Clinicians receiving patient reports containing a VUS result are already aware that a change in the clinical interpretation is possible. However, their vigilance is less assured when a variant has already received a definitive classification as either pathogenic or benign. We believe that our experience with these variants illustrates the importance of laboratory policies that emphasize ongoing efforts to gather information on variants and a commitment to notify providers about changes in interpretation.

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