Strong functional data for pathogenicity or neutrality classify BRCA2 DNA-binding-domain variants of uncertain significance

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Summary

Determination of the clinical relevance of rare germline variants of uncertain significance (VUSs) in the BRCA2 cancer predisposition gene remains a challenge as a result of limited availability of data for use in classification models. However, laboratory-based functional data derived from validated functional assays of known sensitivity and specificity may influence the interpretation of VUSs. We evaluated 252 missense VUSs from the BRCA2 DNA-binding domain by using a homology-directed DNA repair (HDR) assay and identified 90 as non-functional and 162 as functional. The functional assay results were integrated with other available data sources into an ACMG/AMP rules-based classification framework used by a hereditary cancer testing laboratory. Of the 186 missense variants observed by the testing laboratory, 154 were classified as VUSs without functional data. However, after applying protein functional data, 86% (132/154) of the VUSs were reclassified as either likely pathogenic/pathogenic (39/132) or likely benign/benign (93/132), which impacted testing results for 1,900 individuals. These results indicate that validated functional assay data can have a substantial impact on VUS classification and associated clinical management for many individuals with inherited alterations in BRCA2.

Introduction

Germline pathogenic variants (PVs) in BRCA2 are associated with a significantly increased risk of breast, ovarian, pancreatic, and prostate cancers (MIM: 600185).1 The detection and accurate classification of PVs in BRCA2, among other hereditary cancer genes, proffers a notable benefit to carriers of disease-causing variants and their biological family members found to carry the same PVs who can benefit from enhanced cancer surveillance for the early detection of hereditary cancer and/or pursue prophylactic measures to aid in the prevention of certain cancers. In addition, carriers of PVs with cancer may become eligible and benefit from polyADP-ribose-polymerase (PARP)-inhibitor treatment for advanced/recurrent ovarian cancer and metastatic breast, pancreatic, and prostate cancers (see “NCCN clinical practice guidelines in oncology” in web resources).2,3 However, through the widespread adoption of multi-gene panel testing, the detection of rare BRCA2 variants has outpaced the ability to assess the clinical relevance of the variants, leading to a disproportionate number of variants of uncertain significance (VUSs). Furthermore, independent data and variation in interpretations of classification criteria by genetic testing facilities has led to discordance in variant classification in the public domain.4 Such VUSs and variants with conflicting interpretation pose a challenge to clinical counseling and management of patients and biological relatives.5

In an attempt to harmonize classification criteria, the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) developed variant classification standards and guidelines that provided a five-tiered variant classification framework.6 Although this framework provides a robust starting point for most genes, classification of rare variants remains challenging as a result of limited availability of phenotype and genotype information. However, the guidelines must be tailored for gene-specific considerations because not all rules can be applied uniformly. For instance, interpretation of rare variants in the BRCA1 and BRCA2 cancer predisposition genes is complicated by the limited applicability of phenotype-driven rules that involve assessment of the presence or absence of breast and ovarian cancer given the relatively high frequency of these phenotypes in the general population. Similarly, ACMG/AMP framework rules based on functional assay data are often not effectively used for ACMG/AMP rules-based variant classification because of the absence of validated assays with established sensitivity and specificity for pathogenic variants.7 Classification of missense VUSs in commonly mutated genes, such as BRCA1 and BRCA2, may benefit from development of high-quality functional assays that have been calibrated relative to established pathogenic and benign missense variants. In this study, we characterized missense variants in the BRCA2 DNA-binding domain (DBD) by using a homology-directed DNA repair (HDR) assay with high sensitivity and specificity.8–10 We subsequently combined the results with data elements

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from a clinical diagnostic laboratory (Ambry Genetics) to classify BRCA2 DBD VUSs by using a tailored ACMG/AMP variant classification framework.\textsuperscript{6,11,12}

Material and methods

Variant selection

Missense variants (n = 252) in the BRCA2 DBD (amino acids 2481–3186) were selected for functional analysis based on observation in ClinVar.\textsuperscript{4} These included 20 missense variants established as likely pathogenic/pathogenic (LP/P) and 46 variants established as likely benign/benign (LB/B) via models that were independent of functional data.\textsuperscript{11,13–16} Additional VUSs reported in ClinVar were selected on the basis of BRCA-ML \textit{in silico} sequence-based prediction model scores (>0.551), which are associated with an odds of pathogenicity (sensitivity/[1-specificity]) > 3.0 (data not shown).\textsuperscript{10} Variants predicted by SpliceAI\textsuperscript{17} or known from other studies to have effects on RNA splicing were intentionally excluded from this dataset such that the remaining variants were exclusively evaluated for effects on protein function. Variants are presented according to HGVS recommendations and correspond to the RefSeq transcript ID GenBank: NM_000059.3.

Homology-directed repair (HDR) assay

The BRCA2 homology-directed DNA repair (HDR) assay has been described previously.\textsuperscript{7} In brief, variants were incorporated by site-directed mutagenesis into a mammalian expression construct containing the BRCA2 coding sequence (GenBank: NM_000059.3) that encodes a 3\times\ FLAG-tagged full-length BRCA2 protein (GenBank: NP_000050.2). The presence of variants was verified by plasmid sequencing. BRCA2 expression constructs and the iSce1 expression plasmid were co-transfected into DR-GFP \textit{brca2}-deficient V-C8 cells.\textsuperscript{8–10,14,18,19} All variants were analyzed in duplicate experiments for at least two independently derived clones. BRCA2 expression was verified by immunoblot. For each transfection, the proportion of viable cells displaying GFP expression (GFP\textsuperscript{+}/C) was quantified by flow cytometry. Fold increases in GFP\textsuperscript{+} cells, which are equivalent to HDR-fold changes, were normalized and rescaled to a 1:5 ratio derived from the p.Asp2723His (c.8167G\textsuperscript{>}/C) pathogenic variant control and the benign wild-type BRCA2 control. We used a Bayesian regression model for the log HDR scores to estimate the distribution of HDR scores for all variants.\textsuperscript{9,10} The posterior distribution for the HDR score and the 2.5 and 97.5 percentiles of the posterior distributions were used to assign 95% confidence intervals for all variants. All estimation was done in R with the rstanarm package.\textsuperscript{20} The sensitivity and specificity of the HDR assay for pathogenic missense variants in the DBD of BRCA2 has previously been estimated at 100% (sensitivity, 95% CE: 79%–100%; specificity, 95% CE: 93%–100%) with known neutral and known pathogenic variants.\textsuperscript{9}

ACMG/AMP rule-based evaluation of variants

Among the 252 BRCA2 missense variants with functional data, 186 had previously been observed by the collaborating clinical diagnostic laboratory (Ambry Genetics, Aliso Viejo, CA) and had undergone systematic review as recommended by the ACMG/AMP and the Clinical Genome Resource (ClinGen). In brief, the ACMG/AMP baseline recommendations combine evidence from population, computational, segregation, and functional data into a weighted scheme by applying supporting, moderate, strong, very strong, or stand-alone weights toward a final variant classification of benign (B), likely benign (LB), variant of uncertain significance (VUS), likely pathogenic (LP), or pathogenic (P).\textsuperscript{6,11} An integrated approach based on the ACMG/AMP framework was previously used by this clinical laboratory for BRCA2 variant classification.\textsuperscript{11} The elements of clinical and pathological data used by Ambry Genetics for clinical evaluation of the variants presented in the current study based on a tailored ACMG/AMP model were available for integration with the functional assay data. The study was approved by Mayo Clinic institutional review board and the analysis of the clinical-testing cohort was deemed exempt from review by the Western Institutional Review Board. Modifications to the ACMG/AMP framework used in this study are detailed in the following sections.

\textit{In silico data (PP3/PP4)}

The meta-predictor BayesDel was used for \textit{in silico} interpretation of the variants employing internal, gene-specific cutoffs as previously described.\textsuperscript{21} Variants scoring <0.0560 were considered \textit{in silico} tolerated (BP4), while variants scoring >0.431 were considered \textit{in silico} deleterious (PP3). Variants between these scores were considered \textit{in silico} inconclusive.

General population frequency data (BA1/BS1/PM2)

Empirically derived, general population (gnomAD) allele frequency thresholds of 0.1% for the BA1 (population-based allele frequency for a stand-alone benign classification) and 0.01% for the BS1 (allele frequency for a benign variant greater than expected for the related disorder) rules were applied. In addition, the PM2 rule for rare variants was applied only as a supporting line of evidence (PM2\textsuperscript{supporting}) because many benign variants are also rare in the population. For the purposes of this work, PM2\textsuperscript{supporting} was applied for variants with <0.001% total general population frequency, which equates to ≤1 heterozygote at well-covered positions with a maximum possible allele count of 282,912 in gnomAD v2.1.1.\textsuperscript{22}

Well-studied functional domain (PM1)

PM1 is derived from a computational structural analysis based on protein modeling that incorporates secondary, tertiary, and quaternary structures, protein stability, known clinically important functional domains and motifs, and comparisons to known benign and pathogenic variants within the structure. Results from integrated structural analyses are applied as either PM1 or PM1\textsuperscript{supporting} depending on the amount of data available to inform the analysis.\textsuperscript{11}

ClinVar mining

In order to understand the potential impact of functional data on the classification of variants in the public domain, we analyzed ClinVar data to compare the number of conflicting or VUS assertions for variants within the DBD and how these might be resolved with the uniform application of high-quality protein functional data. Only information from the ClinGen list of clinical laboratories meeting minimum requirements for data sharing to support quality assurance was used. ClinVar variant entries for which the collaborating clinical laboratory was the only submitter or the sole classification outlier were excluded. Final assertions for variants were categorized as “conflicting” or “non-conflicting” based on the ClinVar designation. Conflicting interpretations for variants from this study compared to ClinVar were further binned as either likely pathogenic/pathogenic (conflicting-LP/P) or likely benign/benign (conflicting-LB/B) based on the non-VUS classification.
Results

Functional assessment of missense variants by HDR assay

HDR assay results are provided for 53 variants, along with previously reported data for 199 variants, for a total of 252 missense variants (Figure 1; Table 1; Table S1). Among the 252 variants, 90 were considered non-functional with HDR fold change <1.66, based on the upper 95% confidence interval, which is associated with probabilities of pathogenicity <0.99 (Table 1, Figure 1, Table S1). In addition, 162 variants were considered functional with HDR > 2.25, based on the lower 95% confidence interval, which is the threshold for probabilities of neutrality >0.95 (Table S1, Supplemental references). Non-functional variants ranged spatially from p.Gly2508Arg (c.7522G>C) to p.Ley3180Pro (c.9539T>C) and were evenly distributed in the Helical, OB1, and OB3 globular domains of the DBD. However, only seven variants in OB2 region (residues 2804–3054) and none of the 23 variants in the OB2-associated tower domain of BRCA2 (residues 2838–2962) had non-functional HDR effects (Figure 1). Several amino acids were found to be critical for function and sensitive to substitution. For instance, variants that changed residue 2619 from Trp to Gly, Ser, or Cys all resulted in loss of function. Similarly, variants that change residue 2723 from Asp to Asn, His, Tyr, Ala, Gly, and Val also consistently resulted in loss of function, suggesting that a negatively charged amino acid is required at this position. In contrast, differential effects on function were observed for two alterations in residue Leu3180, where a Pro substitution resulted in loss of function but an Arg substitution resulted in a functional protein (Figure 1, Table S1). Differential impact of substitutions on the same residue was also observed for p.Gly2508Ser/Arg (c.7522G>A/C), p.Ala2603Ser/Pro (c.7807G>T/C), p.Arg2625Lys/Ile (c.7874G>A/T), p.Ile2627Val/Phe/Asn (c.7879A>T/C), and p.Asn3124His/Ile (c.9370A>C, c.9371A>G), suggesting that the PM5 rule, dealing with a missense variant occurring at the same amino acid as another pathogenic missense variant, cannot be applied in an uninformed manner (Figure 1, Table S1).

Application of the HDR assay to the ACMG/AMP protein functional data (PS3/BS3) rule

The HDR assay qualifies as a standardized gold-standard assay on the basis of the updated guidance provided by the ClinGen Sequence Variant Interpretation (SVI) Working Group. Specifically, (1) the V-C8 brc2a deficient cell line is a physiologically relevant model system because it recapitulates many features that are consistent with human BRCA2 deficiency, including chromosomal instability, abnormal centrosomes, reduced nuclear localization of RAD51, and sensitivity to DNA cross-linking agents, (2) the data produced by the HDR assay are highly reproducible, and transient cDNA-based expression is carefully controlled by monitoring protein expression by immunoblot, (3) this assay demonstrates sufficient dynamic range between functionally abnormal and functionally normal; and (4) this assay is calibrated on the basis of a set of 20 known pathogenic and 46 known benign missense variants that were classified with a multifactorial likelihood model and/or an ACMG/AMP-based model that excludes functional data. The ClinGen SVI recommendations for applying weight to functional studies suggest an odds of pathogenicity (odds/path) > 18.7 for the application of both the PS3 and BS3 rules as strong lines of evidence. On the basis of 20 pathogenic and 46 benign standards, the oddspath was calculated as 46.0, and when restricting to 10 pathogenic and 32 benign standards not used for the original identification of HDR thresholds, the oddspath was estimated at 32.0. Thus, strong evidence of pathogenicity (PS3) was applied to any variant with upper 99% confidence interval HDR score < 1.66 and strong evidence for a benign variant (BS3) was applied to any variant with lower 95% confidence interval HDR score > 2.25 (Figure 1; Table 1; Table S1). Because this assay cannot assess possible effects on RNA splicing, splice predictions and splicing data must be considered before classifying a variant as LB or B based on the HDR score. Thus, variants ascribed benign HDR functional weight (BS3) were classified as LB/B only if there was no predicted impact on splicing per the in silico program SpliceAI. Of note, in the original ACMG/AMP guidelines from 2015, strong benign functional evidence (BS3), as a categorical line of evidence, was not sufficient to classify a variant as LB. However, ClinGen SVI now recognizes BS3 as sufficient evidence for LB.

Effect of protein functional data on ACMG/AMP-based variant classification

Among the 186 variants observed by the collaborating clinical laboratory, 154 (83%) were classified as VUSs in the absence of protein functional data. After applying HDR assay protein functional data, 86% (132/154) of these VUSs were reclassified as either LP/P (39/132) (48 after HDR versus 9 before HDR) or LB/B (93/132) (23 before HDR versus 116 after HDR) (Figure 2, Figure 3, Table S1). These variants had previously remained VUSs primarily because of rarity, which limited additional data from other lines of evidence in the ACMG/AMP-like classification framework (Table S1). The 22 variants that remained as VUSs after the application of functional data had either limited or conflicting data on the basis of ACMG/AMP classification guidelines (Table S1).

Concordance with other BRCA2 functional studies

The HDR functional dataset was completely concordant with three other functional studies that evaluated the ability for a human BRCA2 variant to restore survival of Brca2 null mouse embryonic stem cells (Table S1). In another functional study, BRCA2 variants were evaluated for influence on sensitivity to PARP inhibitors in a BRCA2-deficient...
Figure 1. Homology-directed repair (HDR) activity
HDR activity of 252 BRCA2 DNA-binding-domain (DBD) missense variants based on an HDR DR-GFP assay. The HDR fold-change based on proportions of GFP positive cells resulting from HDR activity is displayed on a linear scale between 1 (non-functional, p.Asp2723His) and 5 (functional, wild-type). Functional variants are shown in light gray, and non-functional variants are shown in dark gray. The 95% confidence intervals (CIs) for the HDR scores are included as a measure of the reproducibility of the HDR assay for each variant. Horizontal dotted lines represent 99% probability of pathogenicity (fold increase in GFP [+] cells < 1.66) and 95% probability of neutrality (fold increase in GFP [+] cells > 2.25). BRCA2 DBDs are denoted above each section, with the tower domain in OB2 indicated by a bracket. Different amino acid substitutions at the same position are grouped by brackets.
Table 1. BRCA2 variants with non-functional HDR score

| Variant     | HDR score (upper 95% CI) | ACMG/AMP codes | PM5 (same residue) | PM1 (coseg) | PM3* FA/BP2 (healthy biallelic patients) | PP3/PP4* (BayesDel) | Frequency codes | Before class | Final class |
|-------------|--------------------------|----------------|-------------------|-------------|----------------------------------------|-------------------|----------------|-------------|-------------|
| p.Ala2603Pro| 1.07 (1.25)              | PS3            | –                 | –           | –                                      | –                 | N/O            |             |             |
| p.Ala2730Pro| 1.01 (1.15)              | PS3 PM1_supp  | –                 | PP1         | –                                      | inc              | –              | VUS         | LP          |
| p.Ala2780Asp| 1.04 (1.21)              | PS3            | –                 | –           | –                                      | inc              | PM2_supp       | VUS         | VUS         |
| p.Ala2786Pro| 1.22 (1.43)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Ala3028Pro| 1.18 (1.38)              | PS3 PM1       | –                 | PP1_mod PM3 | BP4                                    | PM2_supp         | LP             | P           |             |
| p.Ala3122Pro| 1.09 (1.24)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Asp2723Ala| 0.99 (1.15)              | PS3            | –                 | PM5         | PP1                                    | –                 | PM2_supp       | LP          | P           |
| p.Asp2723Gly| 0.94 (1.10)              | PS3            | –                 | –           | PP3                                    | PM2_supp         | VUS            | P           |             |
| p.Asp2723His| 1.00 (1.07)              | PS3            | –                 | –           | –                                      | PP3              | VUS            | P           |             |
| p.Asp2723Asn| 0.89 (1.04)              | PS3 PM1       | –                 | –           | inc                                    | PM2_supp         | VUS            | LP          |             |
| p.Asp2723Val| 0.98 (1.11)              | PS3 PM1       | PM5              | PP1         | –                                      | –                 | PM2_supp       | LP          | P           |
| p.Asp2723Tyr| 1.17 (1.36)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Asp2819His| 1.37 (1.60)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Asp2819Val| 1.20 (1.36)              | PS3            | –                 | –           | inc                                    | PM2_supp         | VUS            | VUS         |             |
| p.Asp3073Gly| 1.08 (1.26)              | PS3            | –                 | –           | inc                                    | PM2_supp         | VUS            | VUS         |             |
| p.Asp3073Tyr| 0.98 (1.15)              | PS3            | –                 | –           | inc                                    | PM2_supp         | VUS            | VUS         |             |
| p.Asp3095Glu| 0.95 (1.07)              | PS3 PM1       | –                 | –           | –                                      | inc              | PM2_supp       | VUS         | P           |
| p.Asp3095Gly| 1.04 (1.21)              | PS3            | –                 | PM5         | PP1                                    | –                 | PM2_supp       | VUS         | P           |
| p.Glu2599Gly| 1.46 (1.66)              | PS3            | –                 | –           | inc                                    | PM2_supp         | VUS            | VUS         |             |
| p.Glu2663Lys| 0.83 (0.93)              | PS3 PM1       | –                 | PP1         | –                                      | inc              | PM2_supp       | VUS         | P           |
| p.Phe2562Cys| 1.32 (1.54)              | PS3            | –                 | –           | –                                      | inc              | PM2_supp       | VUS         | VUS         |
| p.Phe2562Val| 1.08 (1.26)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Phe2642Ser| 1.07 (1.25)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Phe3146Ser| 1.06 (1.23)              | PS3            | –                 | –           | inc                                    | PM2_supp         | VUS            | VUS         |             |
| p.Gly2508Arg| 1.08 (1.26)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Gly2596Glu| 1.12 (1.30)              | PS3            | –                 | –           | –                                      | PP3              | PM2_supp       | VUS         | LP          |
| p.Gly2596Arg| 1.28 (1.49)              | PS3            | –                 | –           | –                                      | PP3              | PM2_supp       | VUS         | LP          |
| p.Gly2609Val| 1.07 (1.25)              | PS3 PM1_supp  | –                 | –           | –                                      | inc              | PM2_supp       | VUS         | LP          |
| p.Gly2724Val| 1.07 (1.24)              | PS3            | –                 | –           | –                                      | PP3              | PM2_supp       | VUS         | LP          |
| p.Gly2724Trp| 1.12 (1.27)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Gly2748Asp| 0.95 (1.08)              | PS3            | –                 | PP1         | –                                      | PP3              | –              | VUS         | LP          |
| p.Gly2748Ser| 0.97 (1.13)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |
| p.Gly2793Glu| 1.19 (1.23)              | PS3 PM1       | –                 | –           | PP3                                    | PM2_supp         | VUS            | LP          |             |
| p.Gly2793Arg| 1.18 (1.37)              | PS3 PM1_supp  | PM5              | –           | PP3                                    | –                 | PM2_supp       | VUS         | P           |
| p.Gly2793Val| 1.16 (1.32)              | PS3            | –                 | PM5         | –                                      | PP3              | PM2_supp       | VUS         | P           |
| p.Gly3003Glu| 1.35 (1.57)              | PS3            | –                 | –           | inc                                    | PM2_supp         | VUS            | VUS         |             |
| p.Gly3076Glu| 1.04 (1.18)              | PS3 PM1       | –                 | –           | PP3                                    | PM2_supp         | VUS            | P           |             |
| p.Gly3076Arg| 1.03 (1.21)              | PS3            | –                 | –           | –                                      | –                 | N/O            | N/O         |             |

(Continued on next page)
| Variant       | HDR score (upper 95\% CI) | ACMG/AMP codes | PM5b (same residue) | PM3 FA/BP2 (healthy biallelic patients) | PP3/BP4 frequency codes | Classification data |
|--------------|---------------------------|----------------|---------------------|----------------------------------------|-------------------------|---------------------|
| p.Gly3076Val | 1.16 (1.30)               | PS3 PM1 PM5   | –                   | inc                                    | PM2_supp                | LP P                |
| p.His2623Arg | 0.83 (0.92)               | PS3 PM1       | –                   | PP1                                    | PP3 PM2_supp            | LP P                |
| p.His263Tyr  | 1.15 (1.35)               | PS3 PM5       | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Ile2627Phe | 1.01 (1.18)               | PS3           | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Ile2627Asn | 1.16 (1.32)               | PS3 PM5       | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Ile2751Ser | 1.04 (1.21)               | PS3           | –                   | inc                                    | PM2_supp                | VUS VUS             |
| p.Lys2630Gln | 0.98 (1.15)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Lys2657Thr | 1.05 (1.19)               | PS3           | –                   | inc                                    | PM2_supp                | VUS VUS             |
| p.Leu2510Pro | 1.06 (1.24)               | PS3 PM1_supp  | –                   | PM3_st                                 | PP3 PM2_supp            | LP P                |
| p.Leu2604Pro | 0.98 (1.14)               | PS3 PM1       | –                   | –                                      | PP3 PM2_supp            | VUS P               |
| p.Leu2647Pro | 1.00 (1.13)               | PS3           | –                   | PP1_mod                                 | PP3 PM2_supp            | VUS P               |
| p.Leu2653Pro | 0.92 (1.07)               | PS3           | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Leu2656Pro | 1.12 (1.31)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Leu2686Pro | 0.86 (0.97)               | PS3 PM1       | –                   | PM3_st                                 | inc PM2_supp            | LP P                |
| p.Leu2688Pro | 1.00 (1.13)               | PS3           | –                   | PP1_mod                                 | PP3 PM2_supp            | VUS P               |
| p.Leu2721His | 0.92 (1.07)               | PS3 PM1       | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Leu2753Pro | 1.01 (1.11)               | PS3           | –                   | –                                      | BP4 PM2_supp            | VUS VUS             |
| p.Leu2792Arg | 1.07 (1.24)               | PS3           | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Leu3101Arg | 1.15 (1.30)               | PS3 PM1       | –                   | PP1                                    | PM3_st inc PM2_supp    | P P                  |
| p.Leu3125Phe | 1.08 (1.26)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Leu3125His | 0.96 (1.12)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Leu3125Arg | 0.91 (1.06)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Leu3180Pro | 1.37 (1.59)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Asn2622Asp | 1.20 (1.36)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Asn2622Ser | 1.40 (1.56)               | PS3           | –                   | inc                                    | BS1 (.020\% AFR) VUS    | VUS VUS             |
| p.Asn2781Ile | 1.02 (1.15)               | PS3 PM1_supp  | –                   | inc                                    | PM2_supp                | VUS LP              |
| p.Asn3124Ile | 0.99 (1.11)               | PS3 PM1       | –                   | inc                                    | PM2_supp                | VUS P               |
| p.Gln2561Pro | 1.13 (1.26)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Arg2625Ile | 1.17 (1.37)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Arg2784Trp | 1.35 (1.57)               | PS3 PM1_supp  | –                   | PP1                                    | PM3 inc                 | VUS P               |
| p.Arg2824Thr | 1.36 (1.58)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Arg3052Leu | 1.04 (1.21)               | PS3           | –                   | PM5                                    | inc PM2_supp            | VUS LP              |
| p.Arg3052Trp | 0.97 (1.08)               | PS3           | –                   | –                                      | inc –                   | VUS P               |
| p.Ser2691Tyr | 1.37 (1.60)               | PS3           | –                   | –                                      | –                       | N/O N/O             |
| p.Thr2722Ala | 1.39 (1.62)               | PS3           | –                   | inc                                    | PM2_supp                | VUS VUS             |
| p.Thr2722Ile | 1.24 (1.44)               | PS3           | –                   | inc                                    | PM2_supp                | VUS VUS             |
| p.Thr2722Lys | 1.00 (1.16)               | PS3 PM1 PM5   | –                   | inc                                    | PM2_supp                | LP P                |
| p.Thr2722Arg | 1.08 (1.26)               | PS3 PM1       | –                   | inc                                    | PM2_supp                | VUS P               |

(Continued on next page)
Among the 63 variants that overlapped with the current HDR functional study, all variants that were non-functional by HDR also demonstrated sensitivity to one or more drugs (non-functional-fClass 4 or fClass 5) or were inconclusive (fClass 3). None were considered resistant (functional-fClass 1 or fClass 2) for any of the four drugs in the sensitivity assays. However, among the variants that were functional by HDR assay, six had non-functional scores (fClass 4 or fClass 5) for at least one drug in the drug sensitivity assay (Table S1, red text). Cells carrying variants p.Val2652Gly (c.8723T>G) and p.Val2660Asp (c.8905G>A) were consistently sensitive (non-functional) across all four drugs, but both of these variants have non-conflicting LB assertions in ClinVar on the basis of a multifactorial score influenced by both family history and cosegregation scores, suggesting that further calibration of the drug sensitivity assay is needed.

Concordance with ClinVar classification of variants
To determine the potential impact of high-quality functional data on variant classification in the public domain, we reviewed ClinVar submissions for missense variants in the BRCA2 DBD on January 31, 2020. Among the 987 missense variants in the DBD, 54 were excluded as a result of having no submissions from laboratories meeting minimum requirements for data sharing. A further 169 variants were excluded because the collaborating clinical laboratory provided the sole assertions, was the sole contributor meeting minimum requirements for data sharing, or was the sole classification outlier among the contributors meeting minimum requirements for data sharing. The remaining 764 variants represent ClinVar submissions from at least one contributing laboratory that met minimum requirements for data sharing. Importantly, 93% (709/764) of these variants were classified as VUSs or had unresolved conflicting classifications in ClinVar.

Among the 186 functionally assessed variants in this study that were also observed by Ambry Genetics, 171 had ClinVar assertions that were not dependent on this clinical laboratory (Table S1, Figure 4). Among these, 36 had an LP/P final classification (including functional
data) in this study, of which 39% (14/36) had no-conflict LP/P ClinVar assertions, 30.5% (11/36) had no-conflict VUS ClinVar assertions, and 30.5% (11/36) had conflicting LP/P ClinVar assertions. None of the variants with a final classification of LP/P variants had opposing LB/B assertions in ClinVar (Table S1, Figure 4). Conversely, 99 variants with non-excluded ClinVar assertions had an LB/B final classification. Of these, 22% (22/99) had a no-conflict LB/B ClinVar assertion, 45% (45/99) had no-conflict VUS ClinVar assertions, and 32% (32/99) had conflicting LB/B ClinVar assertions. No internally classified LB/B variants had opposing LP/P assertions (Table S1; Figure 4). The resolution of 132 VUSs by the incorporation of high-quality functional data impacted 1,900 individuals tested at the collaborating clinical laboratory (Figure 3).

Discussion

This study describes the application of a large clinical, pathology, and genetic dataset along with protein functional codes weighted according to ClinGen SVI guidelines for the classification of BRCA2 variants with ACMG/AMP guidelines. Several other studies of missense VUSs in BRCA2 based on multifactorial likelihood classification models incorporating personal and family history of cancer, co-segregation of variants with disease, and in silico sequence-based prediction models have been published.14–16 These quantitative models, that do not incorporate protein functional data for variant classification are useful for comparisons with results from the ACMG/AMP rules-based approach reported here. Among 49 variants that were commonly evaluated by HDR assay and by quantitative classification models in other studies, concordance was observed for 48 variants (98%) (Table S1).14–16 The sole outlier in this comparison was BRCA2 p.Arg2502Cys (c.7504C>T). This variant was classified as LB/B in ClinVar by multiple submitters, and was functional in the HDR assay, but remained as an unclassified VUS in a recent multifactorial analysis.16 Importantly, the overwhelming concordance of HDR functional data with these prior studies, as well as other variants that achieved an LP/P or LB/B classification without the use of functional data, allows for the stringent validation and weighting of this functional assay as a strong line of evidence in an ACMG/AMP-based classification scheme.14–16

Of note, among the 22 variants that remained a VUS for the collaborating clinical laboratory, 13 had limited data from sources other than the functional assay for classification purposes and none of these 13 had resolved ClinVar assertions. Of the variants that remained a VUS after the application of functional weight, eight had conflicting data and two of these eight variants had conflicting-LB/B assertions in ClinVar. Reasons for conflict include in silico BayesDel prediction scores (p.Asp2679Gly (c.8036A>G), p.Gly2812Glu (c.8435G>A), p.Gly2813Glu (c.8438G>A), and p.Leu2753Pro (c.8258T>C)), general population frequency (p.Asn2622Ser (c.7865A>G)), or a line of evidence based on structure or where other variants in the same amino acids were known to be pathogenic or benign (p.Ile2627Val, p.Asn3124His, and p.Tyr2658His [c.7972T>C]).

Interestingly, over 53% (99/186) of the variants observed by the collaborating clinical laboratory had inconclusive scores with the BayesDel in silico method based on stringent internally calibrated thresholds for BRCA2 (Table S1).21 The inconclusive scores did not favor functional or non-functional variants but were spread relatively evenly across the HDR results, suggesting poor sensitivity for the BayesDel method relative to the functional data.10 These data

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**Figure 2. Variant workflow**

Summary of the number of variants subjected to the HDR assay and to variant interpretation. The number of variants within each classification outcome (LP/P, VUS, and LB/B) (first bullet, lower panels) and classification rates for VUS in the absence of protein functional data (second bullet, lower panels) are shown. P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; LB, likely benign; B, benign.

**Figure 3. Influence of HDR functional data on variant classification with an ACMG/AMP-like model**

Classification of variants before (black bars) and after (gray bars) the application of protein functional data. The number of variants is indicated above the bar. P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; LB, likely benign; B, benign.
highlight the need for better gene-specific or even domain-specific in silico predictors for the BRCA2 DBD, such as the BRCA-ML ensemble model. The combination of a high-quality in silico predictor with high-quality functional data to supplement clinical and genetic data is expected to result in the classification of many rare variants in the DBD of BRCA2.

In comparing the HDR functional data and final classifications with non-excluded ClinVar assertions, the majority of variants with previously reported HDR functional data (84/117 = 72%) had unresolved ClinVar assertions of VUSs or conflicting. This suggests that functional data are inconsistently applied in classification schema among ClinVar submitters. The results provided in this study may lead to a more uniform application of a strong line of functional evidence for the BRCA2 HDR assay among all ACMG/AMP-based classification schema for BRCA2 variants.

This study had a high success rate, with 86% (132/154) of observed VUSs classified as LP/P or LB/B because of the application of a strong line of evidence for high-quality protein functional data provided by the BRCA2 HDR functional assay. Assuming a similar resolution rate, an additional 657 of 764 identified missense variants in the BRCA2 DBD with unresolved VUSs or conflicting ClinVar assertions may achieve a classification of LB/B or LP/P through the addition of HDR functional data. These data highlight the substantial impact that high-quality functional data can have on the classification of rare variants with the ACMG/AMP guidelines. The finding that the results of the current study impacted genetic diagnoses of 1,900 probands also highlights the substantial impact that high-quality functional data can have on individuals undergoing hereditary cancer genetic testing. Further incorporation of functional assay data into VUS classification models is expected to result in substantially reduced BRCA2 VUS rates and impact the clinical management of many tested probands and family members.

A limitation of the approach is that the specific assay is only validated for variants in the BRCA2 DBD. However, as no missense variants from other domains have been classified as pathogenic, it remains possible that only missense variants in the DBD increase risk of cancer. The possibility also exists that BRCA2 may have multiple functions associated with cancer risk, although evidence to date shows that all pathogenic missense variants influence HDR activity. Furthermore, as shown in this study, additional classification criteria in combination with functional assay results may always be needed for LP/P classification of VUSs.

Data and code availability
The published article includes all datasets generated or analyzed during this study.

Supplemental Information
Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2021.02.005.

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Declaration of interests
K.F., K.M.D., A.M.D., H.L., M.E.R., E.C., and T.P. are employees of Ambry Genetics. F.J.C. has received consulting fees from Astrazeneca. The remaining authors declare no competing interests.

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Web resources
Clinical laboratories meeting minimum requirements for data sharing to support quality assurance, https://www.clinicalgenome.org/tools/clinical-lab-data-sharing-list/
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