



# More is not always better: clinical genetics of familial breast cancer in the era of massively enhanced sequencing capacities

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In the past decade, DNA sequencing has vastly increased in throughput, as well as vastly declined in cost per base pair with the emergence of massively parallel next-generation sequencing technology. This rapid technological progress has made clinical genetic testing of multigene panels and even whole exomes instead of single genes technically feasible, and is widespread practice today. This shift in genetic testing has increased the complexity and challenges in sequence interpretation, and the rate at which new sequence variants are being discovered continues to outpace the rate at which these data can be understood and turned into biological and clinical insight. Moreover, methods for classification of these variants differ between clinical laboratories, and are not as well studied and developed as the sequencing methods to identify them. To address these challenges, the American College of Medical Genetics and Genomics (ACMG) has released updated standards and guidelines for the classification of sequence variants (1). Recently, these guidelines were successfully applied to the classification of variants identified by multigene panel sequencing in families affected by hereditary breast cancer (2).

## The ACMG guidelines for the interpretation of sequence variants

The ACMG, the Association for Molecular Pathology (AMP), and the College of American Pathologists have recently published a joint consensus recommendation for the interpretation of sequence variants identified by genetic tests in clinical laboratories (1). This recommendation

was elaborated by a workgroup of members of the three societies based on expert opinion, workgroup consensus, a literature survey of recommendations from other professional societies, and input from the clinical laboratory community in the United States and Canada. Following evaluation in several clinical laboratories, the final ACMG standards and guidelines were agreed on in a workshop held at the AMP meeting in November 2013. In addition to providing a set of 28 detailed criteria for the classification of sequence variants, this guideline also recommends the use of specific standard terminology (1).

First, the term “variant” instead of both “mutation” and “polymorphism” is recommended (1). The problem with the latter is the widespread, yet incorrect assumption that mutation is synonymous to “pathogenic variant”, and polymorphism to “benign variant”. Second, a five-tier system for classification of variants with the following terms is recommended (1): pathogenic variant (P), likely pathogenic variant (LP), variant of uncertain significance (VUS), likely benign variant (LB), or benign variant (B). P/LP comprise the potentially clinically actionable variants because they could be considered in screening, treatment and reproductive risk-reduction recommendations, whereas VUS, LB and B variants are not acted upon clinically. The ACMG guidelines propose that the terms “LP” and “LB” should mean a certainty of greater than 90% that a variant is pathogenic or benign, respectively. Although that definition is somewhat arbitrary, the workgroup felt that clinicians and patients were willing to tolerate a 10% chance of error (1).

First and foremost, the ACMG guidelines provide a process of how to classify variants into these five

categories, by applying a set of 16 detailed criteria based on population, segregation, functional, predictive, allelic, *de novo* occurrence, computational and other data for classification as P/LP, and another set of 12 similar criteria for classification as B/LB (1). Moreover, a set of scoring rules how to combine these criteria to classify a variant as P, LP, VUS, LB, or B is also provided. The criteria are weighed, such that a larger number of e.g., moderate than strong criteria have to be fulfilled to classify a variant as e.g., P. The guidelines specify one very strong (PVS1), four strong (PS1-PS4), six moderate (PM1-PM6), and five supporting (PP1-PP5) criteria to classify a variant as P/LP, as well as one stand-alone (BA1), four strong (BS1-BS4), and seven supporting (BP1-BP7) criteria to classify a variant as B/LB. For example, PVS1 is met if a variant is predicted to be null in a gene for which loss-of-function is a known mechanism of disease, PM2 is met if a variant is absent in a control or general population (which can be analysed by searching publicly available population databases), and BS4 is met if a variant does not segregate with the disease under investigation (1). Obviously, the criteria for classification as P or B are often highly related. The default classification is “uncertain significance”, which is applied to all variants that do not fulfill enough of the weighed criteria for classification as (likely) pathogenic or benign, and to those variants for which the evidence for P *vs.* B is conflicting (1).

The guidelines allow for some necessary flexibility to variant classification. Accordingly, the weight assigned to some of the criteria may be altered using professional judgment and depending on the evidence available. Various algorithms for *in silico* variant classification such as PolyPhen2, SIFT and MutationTaster (3-5) are implemented in most variant analysis software that is supplied together with next-generation sequencing instruments. However, the results of such *in silico* analyses are considered to be of only supporting evidence by the ACMG guidelines. Importantly, the results provided by different software packages is not truly independent evidence since similar algorithms and/or basic assumptions are most likely used by all of the software used (1).

### **Evaluation of the ACMG guidelines in hereditary breast cancer**

Recently, these ACMG guidelines for standardized variant classification were successfully applied in families affected by hereditary breast cancer by Maxwell *et al.* (2). Whole-exome sequencing was performed in 404 individuals from

253 families that were at high risk for breast and/or ovarian cancer. In total, 180 medically relevant genes were further analyzed and subjected to variant classification. These 180 genes included all 32 non-cancer associated genes listed by the ACMG as reportable incidental findings, 135 genes listed as cancer susceptibility genes by the Institute of Cancer Research and/or in Rahmann (6), and 13 additional genes included in commercially available cancer gene panels. Of the 148 cancer-associated genes, 7 were associated with a well-established high relative risk of breast cancer (BRCA1, BRCA2, TP53, CDH1, PALB2, STK11 and PTEN), 4 with a well-established moderate risk of breast cancer (ATM, CHEK2, NBN and NF1), and 14 with a proposed, but less well-established risk of breast cancer.

A total of 1,640 different non-silent exonic germline variants in total were identified in 166 of the 180 genes. No non-silent mutations were found in ten cancer associated and in four non-cancer-associated genes. These 1,640 variants were next classified as P, LP, VUS, LP, or B by following the ACMG guidelines. The ACMG guidelines allow some flexibility with respect to the applicability and weight assigned to the specific criteria, since they may vary by gene and disease (1). Maxwell *et al.* did not alter the weight assigned to the different criteria, however, some of the criteria were omitted (2). For example, PP4 was not used since breast cancer susceptibility is not considered as disease with a single genetic etiology. Additional criteria left out from their analysis were PS2 and PM6 (*de novo* occurrence), since systematic analysis of parents and confirmation of paternity and maternity was not attempted, and BP7. Classification of variants as P/LP relied most heavily on PVS1 scores (null variant in a gene where loss of function is a known mechanism of disease), which is the predominant mechanism by which pathogenic variants lead to breast cancer susceptibility, and PM2 (absent in a control or general population, which was defined as absent from both EVS6500 and 1000 Genomes Project databases in this case). Quite a few of these variants also had a BS4 score (lack of segregation with the relevant disease), particularly in genes associated with autosomal recessive cancer susceptibility, but were classified as P/LP based on other criteria (2).

Following this raw classification of variants based on ACMG guidelines, all raw calls were manually re-assessed by two of the authors based on detailed reviews of the literature and locus-specific databases (LSDBs). Particularly, all variants with a P/LP raw classification, all truncating variants, and all variants with raw classifications that differed

from those in LSDBs and/or ClinVar were manually re-evaluated in detail. This applied to 306 variants in total, and the initial classification was manually overridden for 182 of them. Mostly, VUS raw classifications were manually re-classified as either LB (n=88) or LP (n=33), and another 58 variants initially classified as benign were re-classified as VUS (2).

The results of this final variant classification were compared to appropriate LSDBs (available for 28 of the studied genes/219 of the identified variants), as well as to ClinVar (482 variants) and Human Gene Mutation Database (HGMD; 245 variants overlapping with ClinVar plus 110 non-overlapping variants). The absolute concordance of the ACMG guideline-based classifications with LSDBs and ClinVar was in the order of 70–80%, and considerably lower with HGMD. When the five ACMG categories were grouped into clinically actionable (LP and P) *vs.* non-actionable (VUS, LB and B), much higher concordance rates of 95% with LSDBs and ClinVar were achieved (2). In all of these comparisons, the manually re-evaluated variant classification performed considerably better than the raw classification, demonstrating that professional judgment is imperative in variant classification. Thus, although the ACMG guidelines are amenable to (semi-)automated application, such approaches may not be ready for use in clinical practice yet. The study of Maxwell *et al.* thus demonstrates the clinical utility of the ACMG guidelines of variant classification plus expert review in the assessment of hereditary breast and ovarian cancer risk (2). This is quite an achievement, since the ACMG guidelines are intended primarily for interpretation of variants in (monogenic) Mendelian disorders in a clinical diagnostic laboratory setting. Classifying variants associated with cancer predisposition identified via whole-exome sequencing in a research setting is more complex, in large part because of its variable, incomplete penetrance, and it was not clear from the outset that the ACMG guidelines would perform so well in this setting.

Next, Maxwell *et al.* took a close look at the number and types of variants identified and the genes affected in their study population (2). P/LP variants in BRCA1 and BRCA2 were found in 15 individuals from 10 families. Of the remaining 243 families, 6 (2.5%) had P/LP variants in high-risk breast cancer susceptibility genes (4 in TP53, 1 each in CDH1 and PALB2), and 12 families (4.9%) had such variants in moderate-risk breast cancer susceptibility genes (7 in ATM, 5 in CHEK2). In another 8 families (3.3%), P/LP variants were found among the 14 genes associated

with a proposed, but less well-established risk of breast cancer analysed in this study. When all the remaining 74 genes associated with autosomal dominant cancer susceptibility were analysed, P/LP variants were identified in two additional families (0.8%). Thus, no P/LP variants in any known autosomal dominant cancer risk gene were found in 215 of these 243 families (88%), leaving the genetic etiology associated with their breast cancer susceptibility unexplained in the large majority (2).

As the number of analysed genes increased, so did the number of VUSs. A reasonable ratio of non-BRCA1/2 families with at least one VUS (n=30) to families with clinically actionable LP/P variants (n=18) was observed when only the 11 best-established breast cancer genes were analysed. 8 families had LP/P variants, but 43 families had at least one VUS in the 14 additional proposed breast cancer susceptibility genes, and when all the remaining 74 genes associated with autosomal dominant cancer susceptibility were analysed, P/LP variants were identified in only two additional families, but VUSs in 189 families (77% of all families). Accordingly, adding these 74 genes to the analysis delivered almost no increase in clinical benefit, but lead to increased clinical complexity due to a large increase in the number of VUSs identified. There seems to be a threshold for the number of genes to be analysed, after which the clinical benefit does not increase appreciably any more, but the number of identified VUSs (and also incidental/secondary findings) keeps growing at an at least linear rate. This threshold was in the range of 11–25 genes in the study by Maxwell *et al.*, which is substantially lower than the number of genes typically contained in commercially available multigene sequencing panels (2). Thus, although technically feasible as sequencing becomes ever faster and cheaper, the decision to include additional genes in genetic testing of hereditary breast cancer should not be taken lightly, as the burden of additional VUSs and incidental findings can be substantial (2). The ACMG recommends re-classifications of VUSs to P/LP (based on additional evidence not available at the time of the initial report) in genes related to the primary indication to be communicated to health-care providers, either proactively or by encouraging regular inquiry (1). Accordingly, the more genes are analysed in multigene panels, the longer the list of VUSs will grow that have to be kept an eye on for potential future re-classification. Moreover, it is of course highly unsatisfactory if the unknowns (VUSs) greatly outnumber the variants that can be classified with confidence.

The number of VUSs obviously increased with the number of genes analysed, but the higher rate of VUS calls in less-well studied genes also played a role. In BRCA1/2, less than 10% of all variants identified by Maxwell *et al.* were classified as VUS after manual review (2). In all 11 well-established breast cancer susceptibility genes combined, the rate of VUS was 31%, in the 78 other genes associated with autosomal dominant cancer susceptibility it was 54%, in genes associated with autosomal recessive cancer susceptibility 58%, and in non-cancer-associated genes 71%. One likely reason for this gradient is an underlying gradient of the level of characterization and understanding of each of the genes. In fact, the ten ACMG guideline criteria that required literature or database evaluation were fulfilled significantly more often by the variants identified in the best-established breast cancer genes (2), simply because more population, segregation, functional, and other data were available for these genes and variants. As stipulated by the ACMG guidelines, all variants default to the classification “uncertain significance” if they do not fulfill enough of the weighed criteria for classification as (likely) pathogenic or benign. In light of the fact that the genetic etiology associated with their breast cancer susceptibility remained unexplained in 88% of the families, it seems likely that some of the variants classified as VUS may in fact be pathogenic, but could not be classified as P/LP due to the lack of sufficient supporting evidence, particularly in the less well-characterized genes.

## Outlook

In conclusion, a major future goal should be to conduct additional research to allow reclassification of VUSs as B or P. With sequencing technologies being that fast, efficient and cost-effective, the discovery of new variants currently outpaces their classification and understanding, but that should change eventually (7). In recent years, the availability of variant frequency data among large populations has allowed many VUSs to be reclassified as B, and these efforts will likely continue as more sequencing results become available. Testing additional members of families identified to carry specific variants should also help to reclassify those variants. Another worthwhile goal would be to come to universally agreed standards on which genes to test, perhaps in a stepwise process, and how to interpret and classify the variants. The work of Maxwell *et al.* has shown that the ACMG guidelines provide good starting point, but perhaps the details could be better defined and tailored to

the application to hereditary breast and ovarian cancer (1,2). For example, it could be defined and standardized what exactly constitutes a “well-established functional study” for the key breast cancer susceptibility genes (ACMG-criteria PS3 and BS3), additional locus-specific databases could be generated and the existing ones expanded, and the evidence leading to the classification of variants could be documented in detail, as exemplified by the study by Maxwell *et al.* (2). The high concordance of ACMG guideline-based variant classification with current practice allows some optimism that these goals are not out of reach entirely.

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