AIMS Genetics, 2(4): 281-292.
DOI: 10.3934/genet.2015.4.281
Received date 8 October 2015,
Accepted date 14 December 2015,
Published date 21 December 2015

http://www.aimspress.com/

Review

Rare genetic variants: making the connection with breast cancer susceptibility

Tú Nguyen-Dumont[1], Jenna Stewart[1], Ingrid Winship[2], Melissa C. Southey[1,*]

[1] Genetic Epidemiology Laboratory, Department of Pathology, The University of Melbourne, Victoria, 3010, Australia and The Royal Melbourne Hospital, Parkville, Victoria, 3050, Australia
[2] Department of Medicine, The University of Melbourne, Victoria, 3010, Australia and The Royal Melbourne Hospital, Parkville, Victoria, 3050, Australia

* **Correspondence:** Email: msouthey@unimelb.edu.au; Tel: +61 03 8344 4895.

Abstract: The practice of clinical genetics in the context of breast cancer predisposition has reached another critical point in its evolution. For the past two decades, genetic testing offered to women attending clinics has been limited to *BRCA1* and *BRCA2* unless other syndromic indicators have been evident (e.g. *PTEN* and *TP53* for Cowden and Li-Fraumeni syndrome, respectively). Women (and their families) who are concerned about their personal and/or family history of breast and ovarian cancer have enthusiastically engaged with clinical genetics services, anticipating a genetic cause for their cancer predisposition will be identified and to receive clinical guidance for their risk management and treatment options. Genetic testing laboratories have demonstrated similar enthusiasm for transitioning from single gene to gene panel testing that now provide opportunities for the large number of women found not to carry mutations in *BRCA1* and *BRCA2*, enabling them to undergo additional genetic testing. However, these panel tests have limited clinical utility until more is understood about the cancer risks (if any) associated with the genetic variation observed in the genes included on these panels. New data is urgently needed to improve the interpretation of the genetic variation data that is already reported from these panels and to inform the selection of genes included in gene panel tests in the future. To address this issue, large internationally coordinated research studies are required to provide the evidence-base from which clinical genetics for breast cancer susceptibility can be practiced in the era of gene panel testing and oncogenetic practice.

Two significant steps associated with this process include i) validating the genes on these panels (and those likely to be added in the future) as *bona fide*[1] breast cancer predisposition genes and ii)

[1] *Bona fide* breast cancer predisposition gene defined as a gene in which genetic mutation(s) have validated association with breast cancer risk (of any magnitude).

interpreting the variation, on a variant-by-variant basis in terms of their likely "pathogenicity" — a process commonly referred to as "variant classification" that will enable this new genetic information to be used at an individual level in clinical genetics services. Neither of these fundamental steps have been achieved for the majority of genes included on the panels.

We are thus at a critical point for translational research in breast cancer clinical genetics — how can rare genetic variants be interpreted such that they can be used in clinical genetics services and oncogenetic practice to identify and to inform the management of families that carry these variants?

Keywords: Rare variants; breast cancer; clinical genetics; clinical translation; *in silico* prediction

1. Introduction

Since 1997, genetic testing of *BRCA1* and *BRCA2* has been offered to selected women in various clinical contexts, internationally. For the vast majority of women (~80%), these tests are uninformative as they do not identify pathogenic mutation in either of these genes. Today commercial and public diagnostic testing facilities are including a larger number of "breast cancer susceptibility genes" identified by continued research, in a single test, at considerably reduced cost [1,2].

The transition of massively parallel sequencing (or next generation sequencing) into molecular diagnostics has enabled a technical revolution in the way that molecular diagnostic testing can be performed. It is now a reality that a gene-panel test can be applied at the same, or lower, laboratory cost than the single-gene analyses that have been conducted for the past two decades.

Today, many public and commercial (including direct-to-the-public) testing facilities are using these panels to provide tests for genetic susceptibility to breast (and other) cancer(s). In several areas of clinical genetics, such as Sudden Cardiac Death, panel testing is now considered standard of care [3].

The genes currently included in commercial breast cancer susceptibility gene panels (in addition to *BRCA1* and *BRCA2*) vary between laboratory/company, are numerous, and range from breast cancer predisposition genes that are now well characterised (e.g. *PALB2, ATM, CHEK2*) to genes that are putative breast cancer predisposition genes and lack extensive (sometimes any) validation (e.g. *BARD1, BRIP1*) [1]. It is not infrequent that women obtain these tests before attending genetic clinic, and come to clinic seeking advice. These gene-panel tests pose considerable challenge to clinical genetic services, as very little is known about the cancer risks associated with almost all of the observed genetic variation [1,4]. How can this data currently be interpreted and what initiatives are likely to improve these interpretations in the future?

2. Rare variants and *BRCA1* and *BRCA2*

Clinical genetics services embraced testing for rare genetic variants (minor allele frequency <1%) in *BRCA1* and *BRCA2* very shortly after these genes were identified and genetic tests have demonstrated clinical validity and utility. Research has since defined the (average) penetrance of protein truncating mutations in these genes (*BRCA1*, 67% (95% CI = 36–83); *BRCA2*, 43% (95% CI = 14–62) to age 70 years) [5]. More recent international collaboration has enabled a broader appreciation of the spectrum of risks associated with some of the genetic variation occurring in *BRCA1* and *BRCA2* that has challenged and changed some of the assumptions made nearly two

decades ago. Most notable observations are the characterisation of genetic variants associated with more moderate risk of breast cancer (e.g. *BRCA1,* R1699Q, 25% (95% CI=10–40%) to age 70 years [6,7]) and variants associated with very small increase in risk of breast cancer (e.g. *BRCA2*, K3326*, OR 1.26 (95% CI=1.14–1.39) $p = 5.7 \times 10^{-6}$) [8,9].

3. Additional genes included on gene panel tests

At the time of writing, the definition of, or at least the clinical, diagnostic and research community's interpretation of, what is and what is not a breast cancer susceptibility gene is somewhat variable. A review of the numerous breast cancer susceptibility gene panels currently available reveals that 18 genes are consistently included. In addition to *BRCA1* and *BRCA2,* these are *ATM, BARD1, BRIP1, CDH1, CHEK2, FANCM, MRE11A, MUTYH, NBN, NF1, PALB2, PTEN, RAD50, RAD51C, STK11* and *TP53* (Table 1). The evidence for mutations in these genes being associated with breast cancer risk is variable and has been extensively reviewed elsewhere (e.g. [1]). Although the reasons for the inclusion of some of these genes are inconsistent, they are the genes for which women are being tested and more information is required, both related to breast cancer risk and the risk for a range of other cancers which may not have been considered by testing clinicians.

PALB2 is now regarded as a *bona fide* breast cancer predisposition gene and is justifiably included on current breast cancer predisposition gene testing panels [10]. *PALB2* is an exemplar gene for the acquisition of appropriate data, from which new clinical guidelines were written and genetic information was translated into clinical genetics practice, as described below.

4. PALB2: demonstrating the capacity of international collaborations

Mutations in *PALB2* make a small contribution to heritable breast cancer susceptibility in most populations [10-18]. To consider penetrance of a larger number of *PALB2* genetic variants and a larger number of families, the *PALB2* Interest Group embarked on a collaborative effort that collected data from 362 members of 154 families who had deleterious truncating, splice, or deletion mutations in *PALB2*. The average cumulative risk of breast cancer was estimated to be 14% (95% CI = 9%–20%) by age 50 and 35% (95% CI = 26%–46%) by age 70 [10]. Thus, all published estimates of penetrance of *PALB2* mutations are comparable to the risk associated with *BRCA2* mutations: 45% (95% CI = 31–56%) [5].

However, unlike *BRCA1* and *BRCA2*, there is no evidence that missense variants in *PALB2* as a group are associated with risk of breast cancer [19]. On a variant-by-variant basis, it is difficult to assess this category of variants and provide any information for use in clinical genetics for the individual.

It is apparent that large international collaborative studies are required to provide the evidence on which two fundamental criteria can be met for the majority of genes included in breast cancer susceptibility panel tests – that is, i) validating the genes as *bona fide* breast cancer predisposition genes, with clear clinical utility and ii) interpreting the variation, on a variant-by-variant basis in terms of their likely "pathogenicity". Neither of these fundamental steps have been achieved for the majority of genes included on the panels. There is therefore a need to extend international efforts that are currently trying to classify rare variants identified in *BRCA1* and *BRCA2* to include rare variants identified in *PALB2* and other susceptibility genes, to assist the clinical management of the individuals who carry them.

Table 1. Breast cancer susceptibility genes commonly included in gene panel testing.

Gene Name	Protein Function	Magnitude of Breast Cancer Risk	Ref
PALB2	Homologous recombination DNA repair	Average cumulative risk 35% (95% CI = 26%–46%) by age 70	[10]
ATM	Homologous recombination DNA repair	Average cumulative risk 52% (95% CI = 28–80%) by age 70 *ATM* c.7271T>G only	[50]
BARD1	Essential to BRCA1 stability Homologous recombination DNA repair	Unknown	[51]
BRIP1	Binds to BRCA1 Homologous recombination DNA repair	2-fold (not replicated)	[52]
CDH1	E-Cadherin, cell to cell adhesion	Unknown	[53]
CHEK2	Responds to DNA damage Involved in cell cycle arrest	Approx. 2-fold (based on one mutation *1100delC)	[23]
NBN/ NBS1	Part of MRE11/RAD50 double strand break repair complex	3-fold (population specific studies)	[54]
MRE11A	Part of MRE11/RAD50 double strand break repair complex	Unknown	[55]
RAD50	Part of MRE11/RAD50 double strand break repair complex	Unknown	[55]
MUTYH	Base excision repair	<2	[56]
NF1	Control of cell division	5-fold under age 50 years (95% CI = 2.4–8.8)	[57]
PTEN	A hydrolases, acting on phosphoric monoester bonds	Unknown	[58,59]
RAD51C	Recombination DNA repair and meiotic recombination.	Unknown	[60,61]
STK11	Serine/Threonine kinase Regulates cell polarity	Relative risk 20.3	[62]
TP53	DNA stability	Unknown	[63,64]
XRCC2	Homologous recombination DNA repair	Unknown	[65]
RINT1	RAD50-interacting protein involved in G_2–M checkpoint	OR 3.24; 95% CI = 1.29–8.17; P = 0.013	[66]
FANCM	Repairs DNA at stalled replication forks	OR 1.86, 95% CI = 1.26–2.75 *FANCM* c.5101C>T only	[67]

OR: Odds Ratio

CI: Confidence Interval

5. The challenge of interpreting missense variants

It is now generally accepted that protein-truncating variants in tumour suppressor genes, with the exception of those occurring in the last coding exon, are associated with loss of function and pathogenicity. This type of variant dominates the spectra of *BRCA1* and *BRCA2* mutations that are

classified as pathogenic. This attitude is reflected in variant classification schemas that generally require evidence against pathogenicity to move any protein-truncating genetic variant from a pathogenic class allocation. Conversely, classification schemas generally require several lines of evidence for pathogenicity before a missense variant can be classified (that is, outside of the "default" unclassified variant category) [20].

Early work in the context of estimating breast cancer risk associated with protein truncating mutations in *BRCA1* and *BRCA2* demonstrated that protein-truncating mutations (as a group, on average) and missense mutations (as a group, on average) are associated with at least as high risk for breast cancer (83% (95% CI = 40–100%) to age 70 years) [21]. Similarly, it has been demonstrated that for genes such as *ATM* and *CHEK2*, the breast cancer risk fraction contributed by missense variants is as high, if not higher, than protein-truncating variants [22,23]. Missense substitutions may result in a variant protein with function that is entirely normal through to completely disrupted, thus these groups of missense variants are highly likely to be made up of mutations with differing levels of associated risks (some probably have no associated breast cancer risk) — but which ones are associated with high, perhaps very high, risk? This is a critical question and one that is not limited to the classification of *BRCA1* and *BRCA2* genetic variants when attempting to personalise genetic information for women attending clinical genetics services seeking advice for risk management.

To personalise heritable risk due to breast cancer predisposition gene mutations, average risk across a group of variants is insufficient, and the provision of variant specific risk estimation is required. Currently we rely on assumptions that have enabled generalisations across mutation types that have proven validity and utility for some groups of mutations (e.g. protein-truncating mutations) but the classification of missense variants poses a great challenge to this process. As a consequence, in clinical genetic testing, missense variants are generally reported as unclassified variants (UVs) or variants of unknown significance (VUSs) and are not informative for the patients. Clinicians seek clarity, since the use of variants in practice demands certainty.

6. *In-silico* assessment of missense variants

A large number of methodologies and tools are available to analyse missense variants and their effects on cellular localisation, aggregation, folding aggregation, stability and functional effects. These methods include sequence- and structure-based analyses and are reviewed in Thusberg and Vihinen, 2009 [24].

In regards to *in-silico* pathogenicity prediction of missense substitutions, it has been demonstrated since the 1960s that conservation of specific sequences during evolution is indicative of functional constraints and that pathogenic missense substitutions tend to occur at evolutionary-conserved positions [25,26]. *In-silico* pathogenicity prediction methods use sequence and/or structural information to assess the functional consequence of a missense substitution on protein function. All evolutionary history-based methods rely on i) the construction of a multiple sequence alignment (MSA), preferably including orthologous sequences for increased accuracy [27] and ii) the assessment of the fitness of the missense variants relative to the pattern observed in the evolutionary history, using positional conservation measures and/or probabilistic scoring algorithms. Some methods such as SIFT and PMUT (a web-based tool for the annotation of pathological mutations on proteins) are based on phylogenic information computed along with sequence weights [28,29]. Other methods rely on a pre-calculated phylogenic tree (LRT, [30]) or combine structural information with the MSA to increase prediction accuracy (PolyPhen2 [31]). Align-GVGD

and SNAP (screening for non-acceptable polymorphisms) combine information about the biophysical characteristics of the wild-type and substituted residue with evolutionary information [32,33]. Most tools return a prediction on whether a missense variant is pathogenic or not; Align-GVGD provides a ranking of missense substitutions into seven classes, from least likely (C0) to most likely (C65) pathogenic.

7. The integrated evaluation of unclassified variants

The first quantitative classification model for UVs was described by Goldgar et al. (2004) and relied on personal and family cancer history to calculate a likelihood ratio in favour of pathogenicity [34]. The current integrated evaluation approach (or multifactorial method), developed by the Breast cancer Information Core (BIC) over the last decade, relies on a Bayesian framework combining a sequence analysis-based prior probability in favour of pathogenicity with likelihood ratios in favour of pathogenicity derived from four types of observational data: co-segregation of UVs with cancer phenotype, with known pathogenic mutations, and tumour immuhistochemistry and histological grade [35-37]. The prior probability in favour of pathogenicity is computed for each class of variants output by the *in-silico* program Align-GVGD calibrated using personal and family history data from Myriad Genetics Laboratory. The result of the integrated evaluation is a posterior probability in favour of pathogenicity, which is a continuous variable ranging from 0.00 to 1.00 supplemented by a five-category classification table that provides a clinically useful translation from the posterior probability to a qualitative system meant for use by clinical cancer geneticists and genetic counsellors [20]. At the third Human Variome Project (HVP) meeting (UNESCO, Paris 2010), this classification table, also referred to as the "IARC 5-class" table, was accepted as an HVP standard.

8. ENIGMA: The Evidence-based Network for the Interpretation of Germline Mutant Alleles

Although the classification of *BRCA1* and *BRCA2* variants has greatly benefited from empirical data obtained from ~90,000 patients screened at Myriad Genetics Laboratories, the interpretation of UVs in *BRCA1* and *BRCA2* remains complex. The Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) is an international consortium that was established to address the challenge of classifying UVs in *BRCA1* and *BRCA2* [38]. Reclassification efforts are more advanced for *BRCA1* and *BRCA2* than for other genes. Vallee et al. (2012) have extracted 248 *BRCA1* and *BRCA2* missense substitutions and have created a database of former UVs (Ex-UVs) following re-assessment of all the missense substitutions under the updated multifactorial evaluation model [39].

However, with testing of other breast cancer susceptibility genes becoming more widespread, ENIGMA and other similar international collaborations will have to try to address the issue of accumulation of UVs in the expended set of genes included in gene panels. Application of the integrated evaluation approach will be more problematic for intermediate-risk genes/mutations because they are likely to have weaker segregation patterns and summary family cancer history than high-risk genes/mutations.

In addition, variants are individually rare and the magnitude of risk conferred by variant alleles can vary widely. The assessment of UVs in these genes will require a classification model that integrates functional data into the multifactorial models.

9. Functional assays

Functional assays revolve around the concept that since inherited sequence alterations causing the loss of function of tumour suppressor genes are usually associated with cancer predisposition, detecting a decrease of tumour suppressor activity likely indicates elevated cancer predisposition. Consequently, laboratory tests are designed to quantify any alterations in the activity of a tumour suppressor variant [40]. A number of functional assays have been developed for *BRCA1* and *BRCA2* [6,41], and for *ATM, CHEK2* and *TP53* missense variants [42-44]. Iversen et al. (2011) and Guidugli et al. (2013) have developed functional assays that include recalibration with neutral and pathogenic mutations in the BRCT and DNA binding domains of BRCA1 and BRCA2, respectively [45,46].

These assays can be used to classify UVs occurring in these regions and will assist in classifying variants when little family history is available for the integrated evaluation. However, calibrated assays do not exist for most domains or functions of most genes. Variant classification models will only be enhanced by the integration of functional assays once i) these assays achieve sufficient accuracy, reproducibility and efficiency to contribute to UVs classification and ii) the assay outputs can properly be recalibrated into odds ratios in favour of pathogenicity. In the absence of robust missense classification models, a large fraction of the genetic variants identified during gene panel testing will remain in the UVs category, which might lead to missing a considerable fraction of *bona fide* genetic risk.

10. Classification for clinical reporting and consistent clinical interpretation and management

Efforts to classify rare variants identified in cancer predisposition genes have been ongoing for some time. Some increased consistency of interpretation and reporting has been achieved by efforts to align with other standardised reporting systems used in oncology [37]. Indeed, it is anticipated that in parallel to a shift from single-gene to panel-gene testing, there will be necessary changes to the testing process that will involve an increased clinical contribution from non-geneticists. This oncogenetic model of genetic testing may see genetic testing conducted as routine by multidisciplinary teams that only refer individuals with “actionable” mutations to clinical genetic specialists [47]. This new model can only be successful with consistent interpretation and reporting of genetic variation observed in panel testing but presently still lacks the evidence-base from which this can be done.

This brings us back to the two key elements of this discussion: the need to validate the genes on these panels as *bona fide* breast cancer predisposition genes with known clinical utility, and identify and develop methods to interpret the variation, on a variant-by-variant basis in terms of their likely “pathogenicity”.

11. What is being done now?

A number of large translational research initiatives are currently addressing the key questions raised in this discussion. There is well-founded optimism within these multi-disciplinary studies that data will be generated quickly on the necessary scale to start to make definitive analyses related to the assessment of these genes as breast cancer predisposition genes. This is in part due to the established networks of researchers that have track records of successful collaboration within other

related networks such as The *PALB2* interest group [12] and The Breast Cancer Association Consortium [48], the recognition of the importance of this challenge by a number of key funding bodies and the developing engagement with commercial testing facilities. Key initiatives in this area have been recently funded by the European Commission (BRIDGES; Horizon 2020), The National Institute of Health (USA), The National Health and Medical Research Council (Australia) and the National Breast Cancer Foundation (Australia). Sequence analysis of (what will shortly be) hundreds of thousands of women (from across the breast cancer risk spectrum) will provide data to enable these genes to be assessed as breast cancer susceptibility genes and to greatly advance our capacity to assess variants on a variant-by-variant basis to increase the precision of personal risk estimation and risk management.

Large international collaborations have also identified a large number of common genetic variants individually associated with small increments in breast cancer risk, both for carriers and for non-carriers of high-risk variants (e.g. [8]). These discoveries have illustrated the complexity and diversity of factors involved in creating the genetic breast cancer risk spectrum. While these variants are not necessarily causal, they are many, and so can be used to identify women at different absolute risk. This is important information to incorporate into penetrance estimations and personalised breast cancer risk modeling. Extensive research effort is being put to characterising the biological pathways influenced by these common genetic variants [49]. However, this discussion has focused on the efforts being brought to the issues around classification of rare genetic variants in breast cancer predisposition genes rather than common genetic variants, which have some complementary but also differing issues around clinical translatability/utility.

12. Conclusion

Currently available gene-panel tests pose considerable challenge to clinical genetic services, as very little is known about the cancer risk associated with almost all of the observed variation in these genes. Large internationally set and linked studies are responding to produce the data required to i) validate the genes on these panels (and those likely to be added in the future) as *bona fide* breast cancer predisposition genes and ii) interpret the variation, on a variant-by-variant basis, in terms of their likely pathogenicity. This information can then be linked to established system for classification of variant for clinical reporting to enable consistent interpretation and clinical management.

This information is required urgently as clinicians must have robust data with proven utility before using genetic test results as the basis of personalised risk assessment and often irreversible risk management (that includes mastectomy and bilateral salpingo-oophorectomy). The potential for gene-panel testing to improve the health outcomes of those at high risk of breast cancer can not be realised until the magnitude of the risks (penetrance) associated with variants in these genes are understood and incorporated into current personalised risk assessment models.

Acknowledgements

M.C.S is a National Health and Medical Research Council Senior Research Fellow. J.S. is a recipient of a Department of Pathology, The University of Melbourne, Honours student scholarship.

Conflict of interest

The authors have no conflict of interest to declare.

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