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CLINICAL & BASIC RESEARCH

Determination of Pathogenicity of *Breast Cancer 1* Gene Variants using the American College of Medical Genetics and Genomics and the Association for Molecular Pathology Guidelines

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تقدير إمْراضِيَّة المتغيرات الجينية لمورث سرطان الثدي (1) باستخدام الدلائل الارشادية التي أصدرتها الكلية الأمريكية لعلم الجينوم الطبي وجمعية علم الأمراض الجزيئي

أنجيلا براون، منصور زمانبور، دونالد لوف، وديبرا بروسر

ABSTRACT: ***Objectives:*** Molecular diagnostic laboratories screen for mutations in disease-causing genes in order to confirm a clinical diagnosis. The classification of DNA variants as 'pathogenic' or 'likely pathogenic' mutations creates a workflow bottleneck, which becomes increasingly challenging as greater number of genes are screened. The classification challenge is also acute if there are conflicting reports regarding pathogenicity and differing classification criteria between laboratories. This study aimed to compare two procedures for the classification of variants in the *breast cancer* (*BRCA*)*1* gene. ***Methods:*** This bioinformatic study was conducted at LabPLUS, Auckland, New Zealand, from February to June 2017. DNA was extracted from peripheral blood samples of 30 patients and gene library construction was carried out using a commercially available targeted panel for the *BRCA1* and *BRCA2* genes. The genes were subsequently sequenced and the sequence data analysed. The guidelines published by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) provides a comprehensive framework for the interpretation of variants in genes that are associated with Mendelian disorders. The use of these guidelines were compared to the variant classifications that were achieved by reference to those reported in the BRCA Exchange database. ***Results:*** The results showed concordance between the two classification protocols for a panel of 30 *BRCA1* gene variants, although the transparency in following the ACMG/AMP guidelines provides a diagnostic laboratory with a generalisable approach that allows laboratory-directed revisions to be undertaken in light of new information. ***Conclusion:*** The ACMG/AMP-based guidelines were applied to a cohort of patients with *BRCA1* gene variants. The use of these guidelines provides a system which creates consistency in variant interpretation and supports subsequent clinical management.

Keywords: BRCA1 Gene; Bioinformatics; DNA Sequencing; Nonsense Codon; Splice Donor Site; New Zealand.

الملخص: الهدف: تقوم معامل التشخيص الجزيئي بالتحري عن وجود طفرات في المورثات المحدثة للمرض بغرض تأكيد التشخيص الاكلينيكي. ويفضي تصنيف متغيرات الحمض الريبي النووي المنزوع الأوكسجين (دنا) إلى كونها متغيرات (ممرضة) أو (يحتمل أن تكون ممرضة) لاختناق سير العمل. وهو تحد يزداد صعوبة مع زيادة عدد المورثات التي ينبغي فحصها. وكذلك يغدو عسر التصنيف أكثر حدةً إذا كانت هنالك تقارير متضاربة من مختلف المعامل حول تحديد الإمراضية ومعايير التصنيف نفسها. وتهدف هذه الدراسة لمقارنة طريقتين لتصنيف المتغيرات الجينية لمورث براكا 1. **الطريقة:** أجريت هذه الدراسة المعلوماتية الحيوية في مختبرات (لاب بلس) في اوكلاند بنيوزيلندا بين فبراير ويونيو من عام 7102م. وتم في هذه الدراسة استخلاص دنا من عينات دم طرفية لثلاثين مريضا، وانشأت مكتبة مورثات باستخدام لوحة مستهدفة لمورثات السرطان براكا 1 وبراكا 2 متوفرة تجاريا. وتم عمل تسلسل تتابعي لها وتحليل المعلومات المستقاة. تقدم الدلائل الارشادية التي أصدرتها الكلية الأمريكية لعلم الجينوم الطبي وجمعية علم الأمراض الجزيئي إطارا شاملا لتفسير المتغيرات الجينية المرتبطة بالأمراض المندليَّة. تمت مقارنة نتائج استخدام النوعين من الدلائل الارشادية لتصنيف المتغيرات الجينية المتحصل عليها في قاعدة بيانات (براكا) التبادليةَ. **النتائج:** أثبتت النتائج وجود اتفاق بين التصنيفين في لوحة مكونة من ثلاثين متغيرا جينيا لبراكا 1. غير أن شفافية الالتزام بالدلائل الارشادية التي أصدرتها الكلية الأمريكي لعلم الجينوم الطبي وجمعية علم الأمراض الجزيئي توفر لمعمل التشخيص نهجا معمما تتيح إمكانية المراجعة والتعديل في النتائج حين ظهور معلومات جديدة. **الخلاصة:** استخدمت الدلائل الارشادية التي أصدرتها الكلية الأمريكية لعلم الجينوم الطبي وجمعية علم الأمراض الجزيئي في تشخيص المتغيرات الجينية لبراك 1 عند فئة من المرضى. يوفر استخدام تلك الدلائل الارشادية اتساقا في تفسير المتغيرات الجينية، ويؤازر التدابير العلاجية الإكلينيكية اللاحقة.

الكلمات المفتاحية: مورث براك 1؛ المعلوماتية الحيوية؛ تسلل دانا التتابعي؛ رامِزَةٌ هُرَائِيَّة؛ موقع مانح الوصلة؛ نيوزيلندا.

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Advances in Knowledge

- *This study reports the use of internationally-accepted criteria for the classification of DNA variants in the context of the breast cancer 1 gene.*

Application to Patient Care

- *This study emphasises that universally adopted criteria for classifying DNA variants assists in the consistent confirmation of clinical diagnoses and the genetic counselling of family members regarding their risks.*

Breast cancer is the most common malignancy among women, both in developed and developing countries, accounting for one in ten of all new cancers diagnosed worldwide.[1] Approximately 5% of breast and ovarian cancers occurs as a result of variants in the *breast cancer* (*BRCA*)*1* gene (MIM# 113705; NM_007294.3; Locus Reference Genomic [LRG]_292), which is localised to the long arm of chromosome 17 at band q21 and comprises 23 exons. Variants within the *BRCA1* gene are associated with an 80–90% lifetime risk of breast cancer.[2] Patients with pathogenic variants in the *BRCA1* gene frequently have a positive family history of breast and ovarian cancer and are often diagnosed at a young age. They may also have a higher incidence of double or multiple primary breast tumours than breast cancer patients in general.[3] Another gene implicated in breast cancer, *BRCA2* (MIM# 600185; NM_000059.3; LRG_293), is localised to the long arm of chromosome 13 at band q13.1 and comprises 27 exons.[4] Patients who carry a mutation in the *BRCA2* gene have an average risk of 45% for breast cancer and 11% for ovarian cancer by the age of 70 years.[5] There is an elevated risk for prostate cancer in *BRCA2* gene carriers and an increased risk of pancreatic, head and neck, stomach and other cancers in patients with a mutation in either of these genes.[5] Germline pathogenic variants in both the *BRCA1* and *BRCA2* genes are expressed in an autosomal dominant manner.[6]

Most *BRCA1* or *BRCA2* gene variants are point mutations or small insertions or deletions.[7] In 2–12% of high-risk families, there may be a large genomic rearrangement.[5] Therefore, it is important to identify patients with disease-causing variants within the *BRCA1* or *BRCA2* genes as this may enable early treatment options such as surgery, including bilateral prophylactic mastectomy or bilateral prophylactic oophorectomy. The correct classification of variants is paramount in order to avoid invasive outcomes for patients who are not at a high risk of developing cancer. Accurate classification also determines whether predictive testing for other 'at risk' family members would be of value.

Standardisation, in terms of variant classification, is a complex topic characterised by several challenges such as the failure to develop standards, a lack of consensus and incompatible implementation of agreed standards.[8] These issues should be addressed in order to provide a consistent framework for reporting variants. Identifying a causative variant allows patients and families to be aware of the risks and therefore pursue appropriate management. If the significance of a variant is unknown, then the clinician should decide how to deal with this finding for their patient and the patient's relatives. The heightened uncertainty resulting from a variant of unknown significance can be distressing for patients.[9] In order to manage and enable consistent and reproducible reporting of these variants, the establishment of universal guidelines is necessary.

To address the need for internationally standardised guidelines for variant nomenclature, a working group was set-up to initiate this process.[10,11] Currently the responsibility for the maintenance and refinement of these guidelines is carried out by the Human Genome Variation Society (HGVS), and an online tool, termed Mutalyzer, has been developed for laboratories to check the description of sequence variants.[12]

Many online tools have become available to assist in variant interpretation such as ClinVar (www.ncbi.nlm.nih.gov/clinvar/), which aggregates information about genomic variation and its relationship with human health. In addition, the 1000 Genomes Project (www.1000genomes.org) and the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/), the latter being superseded by GnomAD (https://gnomad.broadinstitute.org/), are useful in obtaining the frequencies of variants in large populations. Disease-specific databases are also available in order to aid standardisation.

In the case of variants detected in the *BRCA1*/*2* genes, useful online resources include the Breast Cancer Information Core (BIC; https://research.nhgri.nih.gov/bic/) and the ENIGMA consortium (http://enigma.ini.usc.edu/). An ENIGMA member works collaboratively towards the classification of variants and contributes data from families with unclassified sequence variants and/or conducts statistical analysis or laboratory-based assays. Critically, a more global and gene-targeted effort in gathering and collating data, termed the BRCA Challenge, was launched by the Global Alliance for Genomics and Health. As a result of the BRCA Challenge, the BRCA Exchange (https://

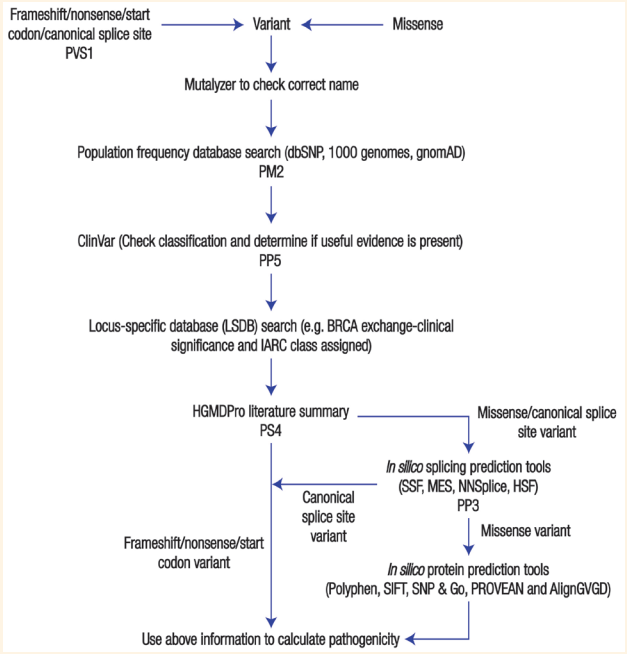

Figure 1: Workflow for classification of variants used in the current study (N = 30).

PVS = very strong evidence of pathogenicity; PM = moderate evidence of pathogenicity; PP = supporting evidence of pathogenicity; LSDB = locus-specific database; BRCA = breast cancer; IARC =International Agency for Research on Cancer; PS = strong evidence of pathogenicity.

brcaexchange.org/) was launched. This initiative combines *BRCA1/2* genes variant data from many resources such as ClinVar, LOVD (www.lovd.nl/), BIC, ExAC (and GnomAD), 1000 Genomes, ESP (http://evs.gs.washington.edu/EVS), exLOVD and ENIGMA into the world's largest source of non-proprietary BRCA variant-level data, which has enabled the expert review of thousands of variants.

Adding to this gene-specific effort, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) published standards and guidelines for the interpretation of sequence variants in 2015.[13] These guidelines describe a framework for classifying Mendelian variants using a list of 28 criteria for determining the impact of a variant. With these guidelines, variants are classified as 'pathogenic', 'likely pathogenic', 'uncertain significance', 'likely benign' or 'benign.'

In the authors' laboratory, next-generation sequencing (NGS) analysis using targeted *BRCA1* and *BRCA2* gene panels was validated for diagnostic testing in 2014. The ACMG/AMP guidelines were published and incorporated into testing at the facility as soon as they were published in 2015. This study aimed to classify *BRCA1* gene variants according to ACMG/AMP guidelines and compare the classifications to those reported in the BRCA Exchange.

Methods

This bioinformatic study was conducted at LabPLUS, Auckland, New Zealand, from February to June 2017. DNA was extracted from peripheral blood samples of 30 patients referred to the Northern hub of the Genetic Health Services New Zealand for *BRCA1/2* gene mutation screening. The diagnostic referrals were forwarded for testing based on the Cancer Institute New South Wales guidelines for genetic testing for heritable mutations in the *BRCA1* and *BRCA2* genes.

Library construction was carried out using the commercially available targeted panel for the *BRCA1* and *BRCA2* genes from Multiplicom (Agilent Technologies, Inc, The Netherlands). Sequencing was performed using a GS-Junior (Roche Holding AG, Basel, Switzerland) platform. Data analysis was undertaken using JSI SeqNext software (JSI medical systems GmbH, Ettenheim, Germany). In accordance with the AMP and College of American Pathologists NGS validation guidelines, a minimum of 30 reads was considered sufficient for minimum depth of coverage.[14] If fewer than 30 reads were obtained, Sanger-based sequencing was performed to ensure a reliable result. Analysis included evaluating the coding regions and exon-intron boundaries (±20 base pairs) of the entire *BRCA1* gene. Alamut® Interactive Biosoftware (www.interactive-biosoftware.com/doc/alamut-visual/2.6/splicing.html)

Table 1: Criteria used in the investigators' laboratory for variant classifications based on American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines

Criteria	Description
PVS1	Frameshift/nonsense/start codon/splice junction ± 2 base pairs/exonic deletion.
PS1	For missense: the same amino acid change at the same codon is known to be pathogenic (different nucleotide position). The same amino acid change to be counted as strong evidence must also, by ACMG definition, be a class 5. If the "other variant" is a class 4, then this evidence become "moderate" rather than "strong" and can only be used to count as supporting evidence for the variant of interest to be a class 4 (likely pathogenic).
PS2	*De novo* (paternity confirmed) in a patient with the disease, no family history and phenotype reasonably specific for the disorder.
PS3	Functional study supports damaging effect.
PS4	Prevalence in affected Pts > prevalence in controls–OR is statistically significant. Where a significant number of cases have been reported carrying the variant and showed similar phenotype, it can be used as a "moderate" evidence without OR.
PM1	In mutational hot spot/critical domain (without benign variations).
PM2	Absent from population databases or extremely low MAF. Slightly higher is acceptable for recessive conditions.
PM3	For recessive disorders: in trans with known mutation.
PM4	In-frame insertion/deletion in non-repeat region or stop-loss variant that results in protein length changes.
PM5	For missense: a known mutation occurs at same codon (different amino acid residue). The "known" mutation occurred at the same codon must be either a class 4 or 5 by ACMG definition.
PM6	Not present in parents (i.e. assumed *de novo*) and phenotype reasonably specific for the disorder (without confirmation of paternity and maternity).
PP1	Co-segregation with disease (increased weighting may be applied if stronger evidence available).
PP2	For missense: in this gene missense variants are usually pathogenic.
PP3	Bioinformatic analysis predicts pathogenic (protein and/or splice).
PP4	Phenotype is highly specific for this single genetic aetiology.
PP5	A reputable source lists variant as pathogenic but doesn't state how the classification was determined (reputable sources are ClinVar at three or four stars).
BA1	MAF ≥5% in any of the population databases.
BS1	Allele frequency above expected for disorder.
BS2	Found in a healthy individual when full penetrance is expected at that age (in correct zygosity for disorder).
BS3	Functional study supports benign effect.
BS4	Doesn't co-segregate with disease (beware: penetrance).
BP1	For missense: in this gene missense variants are usually benign.
BP2	Observed in trans with a pathogenic variant (for dominant inheritance) or in cis with a pathogenic variant (any inheritance pattern).
BP3	In-frame insertion/deletion in a repetitive region without a known function.
BP4	Bioinformatic analysis predicts benign (protein and splice).
BP5	Variant found in a case with an alternative molecular basis for disease.
BP6	A reputable source lists variant as benign (reputable sources are ClinVar at three or four stars): the reputable source must be experts in this disease e.g. a LSDB or a lab that has longstanding experience. "No evidence" means no evidence of their classification details and does not mean no reference at all. Where the classification is present but no details on their individual criteria, it is considered "no evidence". The reference or paper it cites can still be investigated. However, if the detailed criteria is provided, then each evidence must be weighted and this option is no longer viable.
BP7	For synonymous/intronic: splice predictions are benign. At present, if a synonymous/intronic variant is given benign prediction by splice programmes it can be classified as class 2 without the need to investigate conservation.

PVS = very strong evidence of pathogenicity; PS = strong evidence of pathogenicity; ACMG = American College of Medical Genetics and Genomics; OR = odds ratio; PM = moderate evidence of pathogenicity; PP = supporting evidence of pathogenicity; BA = stand-alone evidence of benign impact; BS = strong evidence of benign impact; BP = supporting evidence of benign impact; LSDB = locus-specific database.

Table 2: Criteria used to allocate variants into the five American College of Medical Genetics and Genomics and the Association for Molecular Pathology classes

Class	Requirement	
1. Benign	BA1 MAF ≥5% or 2 BS	
2. Likely benign	1 BS + 1 BP or 2 BS. Only BP7 needed for synonymous	
3. Uncertain	Conflicting information or doesn't meet criteria of another class	
4. Likely pathogenic	1 PVS + 1 PM; 1 PS + 1 PM; 1 PS + 2 PP	3 PM; 2 PM + 2 PP; 1 PM + 4 PP
5. Pathogenic	1 PVS + 1 PS; 1 PVS + 2 PM; 1 PVS + 1 PM + 1 PP; 1 PVS + 2 PP	2 PS; 1 PS + 3 PM; 1 PS + 2 PM + 2 PP; 1 PS + 1 PM + 4 PP

BA = stand-alone evidence of benign impact; BS = strong evidence of benign impact; BP = supporting evidence of benign impact; PVS = very strong evidence of pathogenicity; PM = moderate evidence of pathogenicity; PS = strong evidence of pathogenicity; PP = supporting evidence of pathogenicity.

was used to aid in the interpretation of the variants. As recommended by the ACMG/AMP guidelines, the nomenclature used to describe the variants is based on Human Genome Variation Society guidelines. The reference sequence for this gene was derived from the LRG database. An outline of the approach taken to classify the 30 *BRCA1* gene variants according to the ACMG/AMP guidelines is shown in Figure 1.

The classification categories were assigned by applying a series of criteria. The criteria were divided into seven attributes based on the ACMG/AMP guidelines. In terms of evidence of pathogenicity these criteria were: very strong (PVS1), strong (PS1-4), moderate (PM1-6) and supporting (PP1-5). In terms of benign impact, the criteria were: stand-alone evidence (BA1), strong evidence (BS1-BS4) and supporting evidence (BP1-BP7) [Table 1]. The outcome of each call for a particular variant was incorporated to give a final score, which was used to determine the final classification from the five-tiered system [Table 2].

Table 3: Summary of the patients classified in this study (N = 30)

No.	Nucleotide change based on HGVS nomenclature	Amino acid change based on HGVS nomenclature	*BRCA1* gene (LRG_292) exon number	LSDB classification (BRCA Exchange)	Classification based on the ACMG/AMP Guidelines
1	c.66dup	p.(Glu23Argfs*18)	2	Pathogenic	class 5
2	c.117_118del	p.(Cys39*)	3	Pathogenic	class 5
3	c.212+1G>T	p.?	-	Not reviewed	class 5
4	c.212+2T>C	p.?	-	Not reviewed	class 5
5	c.220C>T	p.(Gln74*)	5	Pathogenic	class 5
6	c.427G>T	p.(Glu143*)	6	Pathogenic	class 5
7	c.1018del	p.(Val340*)	10	Pathogenic	class 5
8	c.1298_1299dup	p.(Ser434Profs*8)	10	Not reviewed	class 4
9	c.1374del	p.(Asp458Glufs*17)	10	Not reviewed	class 5
10	c.1953dup	p.(Lys652Glufs*21)	10	Pathogenic	class 5
11	c.1961dup	p.(Tyr655Valfs*18)	10	Pathogenic	class 5
12	c.2071del	p.(Arg691Aspfs*10)	10	Pathogenic	class 5
13	c.2074del	p.(His692Metfs*9)	10	Pathogenic	class 5
14	c.2188_2201del	p.(Glu730Thrfs*5)	10	Pathogenic	class 5
15	c.2280_2281del	p.(Glu761Lysfs*6)	10	Not reviewed	class 4
16	c.2447A>G	p.(His816Arg)	10	Not reviewed	class 3
17	c.2475del	p.(Asp825Glufs*21)	10	Pathogenic	class 5
18	c.2681_2682del	p.(Lys894Thrfs*8)	10	Pathogenic	class 5
19	c.3143del	p.(Gly1048Valfs*14)	10	Pathogenic	class 5
20	c.3254_3255dup	p.(Leu1086Aspfs*2)	10	Pathogenic	class 5
21	c.3400G>T	p.(Glu1134*)	10	Pathogenic	class 5
22	c.3607C>T	p.(Arg1203*)	10	Pathogenic	class 5
23	c.3706_3707del	p.(Asn1236Tyrfs*7)	10	Pathogenic	class 5
24	c.3718C>T	p.(Gln1240*)	10	Pathogenic	class 5
25	c.3756_3759del	p.(Ser1253Argfs*10)	10	Pathogenic	class 5
26	c.3759dup	p.(Lys1254*)	10	Pathogenic	class 5
27	c.4065_4068del	p.(Asn1355Lysfs*10)	10	Pathogenic	class 5
28	c.4113del	p.(Cys1372Valfs*21)	11	Pathogenic	class 5
29	c.4327C>T	p.(Arg1443*)	12	Pathogenic	class 5
30	c.5152+1G>T	p.?	-	Pathogenic	class 5

HGVS = Human Genome Variation Society; BRCA = breast cancer; LRG = locus reference genomic; LSDB = locus-specific database; ACMG/AMP = American College of Medical Genetics and Genomics and the Association for Molecular Pathology.

All diagnostic referrals gave informed consent. The national multi-region ethics committee of New Zealand has ruled that cases of patient management do not require formal ethics committee approval.

Results

A total of 30 variants were classified in this study, of which 27 were classified as pathogenic (class 5), two variants were classified as likely pathogenic (class 4) and one variant was classified as unknown significance

Table 4: Criteria used to classify *breast cancer 1* gene variants

No.	Nucleotide change	Type	Summary of criteria	Evidence used for classification
1	c.66dupA	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars. BRCA exchange pathogenic)
2	c.117_118delTG	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars. BRCA exchange pathogenic)
3	c.212+1G>T	Canonical splice site	PVS1, PM2, PP3	PVS1 as splice junction ± 2 base pairs PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP3 as *in silico* evidence predicts splicing to be affected (agreement from all four algorithms used in the investigators' laboratory)†
4	c.212+2T>C	Canonical splice site	PVS1, PM2, PP3	PVS1 as splice junction ± 2 base pairs PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP3 as *in silico* evidence predicts splicing to be affected (agreement from 3/4 algorithms used in the investigators' laboratory: MES, NNSplice and HSF)†
5	c.220C>T	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar)
6	c.427G>T	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as MAF = 0.00010/12 (ExAC); absent from ESP PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
7	c.1018delG	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
8	c.1298_1299dupCC	Frameshift	PVS1, PM2	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP)
9	c.1374delC	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange not yet reviewed)
10	c.1953dupG	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
11	c.1961dupA	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as MAF = 0.000008/1 (ExAC); absent from 1000 genomes and ExAC PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
12	c.2071delA	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
13	c.2074delC	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
14	c.2188_2201del14	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
15	c.2280_2281delTG	Frameshift	PVS1, PM2	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP)
16	c.2447A>G	Missense	PM2	PM2 as MAF = 0.000008/1 (ExAC); 0.0116/0.0/0.0077 (ESP); MAF = 0.00008/1 (GO-ESP)
17	c.2475delC	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
18	c.2681_2682delAA	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)

*PVS = very strong evidence of pathogenicity; PM = moderate evidence of pathogenicity; PP = supporting evidence of pathogenicity; NMD = nonsense-mediated decay; BRCA = breast cancer *NMD prediction based on the premature termination codon not occurring in the 3' most exon or the 3'-most 50bp of the penultimate exon; †Using Splice site prediction programme (www.interactive-biosoftware.com/doc/alamut-visual/2.6/splicing.html), MES (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSplice (www.fruitfly.org/seq_tools/splice.html) and HSF (www.umd.be/HSF3/).*

Table 4 (cont.): Criteria used to classify *breast cancer* 1 gene variants

No.	Nucleotide change	Type	Summary of criteria	Evidence used for classification
19	c.3143delG	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
20	c.3254_3255dupGA	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
21	c.3400G>T	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
22	c.3607C>T	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as MAF = 0.00008/1 (GO-ESP), 0.0/0.0227/0.0077. Absent from 1000 Genomes and ExAC PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
23	c.3706_3707delAA	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
24	c.3718C>T	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
25	c.3756_3759delGTCT	Frameshift	PVS1, PS4, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PS4 as this variant has been reported in multiple patients with breast and ovarian cancer PM2 as MAF = 0.00002/3 (ExAC), MAF = 0.0210/263 (GO-ESP), 2.1934/1.9231/2.1013 (ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
26	c.3759dupT	Nonsense	PVS1, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
27	c.4065_4068delTCAA	Frameshift	PVS1, PS4, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PS4 as this variant is reported in multiple patients with breast and ovarian cancer PM2 as MAF = 0.00002/2 (ExAC). Absent from population databases (1000 Genomes and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
28	c.4113delG	Frameshift	PVS1, PM2, PP5	PVS1 as frameshift (predicted to undergo NMD)* PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
29	c.4327C>T	Nonsense	PVS1, PS4, PM2, PP5	PVS1 as nonsense (predicted to undergo NMD)* PS4 as this variant is reported to be a common cause of breast and ovarian cancer in the French Canandian population but also observed in individuals from other ethnicities. PM2 as MAF = 0.00002/3 (ExAC), absent from ESP PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)
30	c.5152+1G>T	Canonical splice site	PVS1, PM2, PP3, PP5	PVS1 as splice junction ± 2 base pairs PM2 as absent from population databases (1000 Genomes, ExAC and ESP) PP3 as *in silico* evidence predicts splicing to be affected (agreement from all four algorithms used in the investigators' laboratory)† PP5 as listed as pathogenic by a reputable source (ClinVar-Pathogenic at three stars; BRCA exchange pathogenic)

*PVS = very strong evidence of pathogenicity; PM = moderate evidence of pathogenicity; PP = supporting evidence of pathogenicity; NMD = nonsense-mediated decay; BRCA = breast cancer *NMD prediction based on the premature termination codon not occurring in the 3' most exon or the 3'-most 50bp of the penultimate exon; †Using Splice site prediction programme (www.interactive-biosoftware.com/doc/alamut-visual/2.6/splicing.html), MES (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSplice (www.fruitfly.org/seq_tools/splice.html) and HSF (www.umd.be/HSF3/).*

(class 3) [Table 3]. The details of evidence used to classify each variant are shown in Table 4.

Other than the missense mutation (c.2447A>G), the remaining variants were assigned PVS1. The rationale for this classification was that all of these variants were either nonsense (variants 2, 5–7, 21, 22, 24, 26 and 29), frameshift (variants 1, 8–15, 17–20, 23, 25, 27 and 28) or canonical ±1 or 2 splice site (variants 3, 4 and 30) variants. The ACMG/AMP guidelines assign these variants as PVS1 as they can often be assumed to disrupt gene function by leading to complete absence of the gene product by lack of transcription or nonsense-mediated decay (NMD) of the altered transcript.[13] Variants predicted not to

Table 5: Summary of the implications and consequences of variant classifications

Class	Further revision	Reported as	Predictive testing	Familial segregation analysis
1	No	Not reported	No	No
2	Bi-annually	Not reported	No	No
3	Annual	Uncertain clinical significance	No	Yes
4	Annual	Likely pathogenic	Yes (counselling required)	No
5	No	Pathogenic	Yes (counselling required)	No

trigger NMD have been defined as those that lead to a stop codon 50 nucleotides before or within the last exon.[15] It is also suggested that variants in close proximity to the translation initiation codon can also escape NMD due to downstream reinitiation.[16] In this study, the most 5' variant was c.66dupA, which is in exon 2, 66 nucleotides downstream from the first nucleotide; it is therefore not considered very close to the translation initiation codon. The most 3' exon with a variant in this gene was in exon 17. Given that exon 18–23 contain 436 nucleotides, it was concluded that none of the current detected variants would prevent NMD from occurring. Therefore, PVS1 was assigned to each frameshift and nonsense variant. For the three splice site variants (variants 3, 4 and 30), Alamut Visual software (Interactive Biosoftware, Rouen, France) predicted that skipping of exons 4 and 17 was very likely. Both exons 4 and 17 are 78 base pairs or 26 amino acids long. Skipping of these exons would lead to an in-frame deletion of this protein, which would normally lead to assigning criterion PM4. However, exons 4 and 17 encode part of the Really Interesting New Gene and BRCA1 C-Terminus domains, which are essential for the proteins role as a tumour suppressor.[17] Therefore, the investigators decided that they had sufficient evidence to assign criterion PVS1 to these three variants.

Variant frequencies were assessed in population databases including 1000 genomes, ExAC and ESP. Variants were assigned the PM2 criterion if the variant was absent or had an extremely low frequency in population databases. The databases examined for each variant are described in Table 4. In the investigators' laboratory, ClinVar was considered a reputable disease database and the PP5 criterion was assigned to any variant with a pathogenic interpretation in ClinVar that had expert panel review status. Variants designated PP3 were those in which *in silico* data had been obtained [Table 4]. The PS4 criterion can be assigned to variants with an increased prevalence in affected individuals compared to the controls. In this study, PS4-assigned variants were those that had been reported to be seen in multiple patients with breast and ovarian cancer.

The investigators' variant classification approach led to one variant being classified as class 3; two as class 4 and 27 as class 5. In the case of the class 3 variant (variant 16), it satisfied criterion PM2 as it was present at an extremely low frequency in population databases. This variant had conflicting evidence for pathogenicity in ClinVar as did *in silico* prediction software. In addition, there was an absence of publications describing this variant. The two class 4 variants (variants 8 and 15) were classified as likely pathogenic as there was a lack of supporting ClinVar evidence and no literature was found describing these variants. Table 3 provides a summary of the classification together with those present in the BRCA Exchange. The classification of variants in the BRCA Exchange were concordant with the ACMG/AMP-based classifications.

Discussion

The purpose of this study was to classify 30 variants detected in the *BRCA1* gene according to the ACMG/AMP guidelines. The variants described in the current study were detected prior to the implementation of these guidelines, and as a result they had not been curated consistently. For the initial classification, a variety of online resources had been used such as: the BIC database; their ClinVar status and their International Agency for Research on Cancer class. Included in the study were seventeen frameshift, nine nonsense, three intronic variants and a single missense variant.

The classification of the variants was weighted heavily on the type of variant, the frequency of the variant and the ClinVar classification. *BRCA1* exhibits an autosomal dominant pattern of inheritance.[18] Therefore, a heterozygous variant has potential to cause disease. The majority of clinically significant deleterious mutations within the *BRCA1* and *BRCA2* genes have been reported to be protein-truncating mutations, while a small number are missense mutations.[5] The majority of variants in this study were either frameshift or nonsense mutations. The investigators' interpretation of the ACMG/AMP guidelines enabled the majority (97%) of the variants to be assigned PVS1.

The criteria were sufficient to enable all but one of the variants to be classified as either 'likely pathogenic' or 'pathogenic', thus providing an informative result for clinicians who could focus on the clinical management

of patients with these variants in the future. Use of the current methodology based on ACMG/AMP variant classification guidelines gave final calls with a high level of concordance to well-curated locus-specific databases. All 24 variants in this study that were listed as pathogenic by the BRCA Exchange were also classified as class 5 pathogenic variants.

Recent evaluations of the ACMG/AMP guidelines have been published.[19,20] One study examined the classification findings across nine laboratories in the USA.[19] Although these findings showed that the use of the ACMG/AMP guidelines did not initially improve inter-laboratory concordance, they did provide a common framework to facilitate resolution of the differences when discussed subsequently.[19] In addition, Maxwell *et al*. classified 1,640 variants and summarised that their findings supported the clinical utility of ACMG/AMP variant-classification guidelines.[20] Outside of the USA, other countries are adopting these guidelines. In November 2016, a consensus statement was issued by the Association for Clinical Genomic Science (ACGS). It recommended use of the ACMG/AMP guidelines for germline variant classification and interpretation in UK diagnostic genetic laboratories performing testing of rare disease and familial cancers.[21] This development highlights that there is an international effort in place to standardise variant interpretation. These guidelines were recognised to provide a starting point for further refinements and extensions in the future.[22] The Sequence Variant Interpretation (SVI) Working Group has begun this process, taking on the task of improving the current ACMG/AMP recommendations in order to develop quantitative approaches to variant interpretation. As a result of this, the ACGS published best practice guidelines for variant classification in 2018.[21]

According to the ACMG/AMP guidelines, the PVS1 criterion provides a very strong level of confidence in assigning pathogenicity; however, there are certain caveats to this criterion. The factors to take into consideration when assigning PVS1 include: ensuring that loss of function is a known disease mechanism; using caution when interpreting loss of function variants at the extreme 3' end of a gene; attention to splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact; the presence of multiple transcripts and assuming that a null variant will lead to disease if found in an exon where no other pathogenic variants have been described. Due to concerns that inappropriate use of this criterion may have a significant impact on a patient's well-being, the SVI working group has produced recommendations for interpreting the loss of function PVS1 ACMG/AMP variant criterion.[23] These recommendations provide a detailed description of the types of variants that can be assigned this classification, as well as a decision tree to aid in variant interpretation.

The PP5 criterion was frequently used in the current study. This criterion requires that a reputable source lists the variant as pathogenic but doesn't state how the classification was determined. This criterion is appropriate when information is obtained from a clinical laboratory that has long-standing expertise in the disease area. Within the current laboratory, it was thought appropriate to apply PP5 when there was a ClinVar review with three stars supporting pathogenicity. The SVI working group suggested that PP5 (along with its benign equivalent, BP6) be removed from the criteria. This is due to the fact that ClinVar has become so successful that submissions with 'assertion criteria provided' account for the majority of cases uploaded and that sufficient primary evidence is now available.[22]

The PP3 criterion was used in this study alongside PVS1 for intronic variants. Although not stated in the ACMG/AMP guidelines, the SVI working group has suggested avoiding using PP3 together with PVS1 as the evidence is based on the same set of data and has the potential to lead to errors in classification.[23] If that suggestion had been followed in the current study, two of the three canonical splice site variants (variants 3 and 4) would be reclassified as class 4 (from class 5) but the remaining variant (variant 30) would remain a class 5.

Table 5 summarises the implications to the laboratory and to the clinic regarding the consequences of variant classification. Having the ACMG/AMP guidelines in place allows the laboratory to assign an appropriate frequency of revisions.

As discussed above, refinement of the ACMG/AMP guidelines has already began and will continue to evolve over time. Within the investigators' laboratory, the development of a work-up document for internal use following the ACMG/AMP guidelines has enabled a more systematic approach to variant interpretation and proved a useful resource in achieving rigour regarding variant classification. The refinement of these guidelines will continue to improve the interpretation of variants, ultimately leading to an improvement in patient care.

For the classification of variants in this study the following criteria were not used: PS2 (*de novo*-paternity confirmed); PM6 (assumed *de novo*, but without confirmation); PP1 (co-segregation with disease); BS4 (lack of segregation in affected members) and PP4 (patient's phenotype or family history is highly specific for a disease with a single genetic aetiology). Information

was not available regarding family history, the inheritance of the variants or the patients' clinical status. Although the majority of the variants described here were classified as pathogenic without this additional information, this is not always going to be the case and the availability of as much information as possible is essential to provide accurate variant classification.

The advantage of an approach that adheres to international guidelines is the acknowledgement that variant classification requires objectivity but recognising that best practice is aspirational. While the BCRA Exchange classifications were used as a benchmark, recent work of Cusin *et al.* provides a salutary lesson.[24] These authors highlight the importance of including information regarding the molecular and cellular impacts of variants, which need to be retrieved in a computationally accessible format.[24] This is especially so for class 3 variants that currently suffer from a paucity of functional data, as well as limited clinical impact data due to the logistic challenges of performing familial segregation analysis. Diagnostic laboratories should play a larger role in addressing the latter, while research laboratories should address the former.

Conclusion

The use of ACMG/AMP guidelines for the classification of DNA variants allows a diagnostic laboratory to benchmark their classifications against other laboratories so as to achieve consistency in outcomes. This benchmarking also enables clinicians to have confidence in the veracity of diagnostic reports and provides a transparent process that is subject to objective evidence.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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