Sultan Qaboos University Med J, May 2015, Vol. 15, Iss. 2 pp. e218–225, Epub. 28 May 15
Submitted 11 Sep 14
Revision Req. 4 Nov 14; Revision Recd. 13 Nov 14
Accepted 3 Dec 14

CLINICAL & BASIC RESEARCH

Predicting the Pathogenic Potential of *BRCA1* and *BRCA2* Gene Variants Identified in Clinical Genetic Testing

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توقع امكانية حدوث الأمراض للمتغيرات الجينية ل *BRCA1* و *BRCA2* المحددة في الفحص الجيني السريري

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abstract: ***Objectives:*** Missense variants are very commonly detected when screening for mutations in the *BRCA1* and *BRCA2* genes. Pathogenic mutations in the *BRCA1* and *BRCA2* genes lead to an increased risk of developing breast, ovarian, prostate and/or pancreatic cancer. This study aimed to assess the predictive capability of *in silico* programmes and mutation databases in assisting diagnostic laboratories to determine the pathogenicity of sequence-detectable mutations. ***Methods:*** Between July 2011 and April 2013, an analysis was undertaken of 13 missense *BRCA* gene variants that had been detected in patients referred to the Genetic Health Services New Zealand (Northern Hub) for *BRCA* gene analysis. The analysis involved the use of 13 *in silico* protein prediction programmes, two *in silico* transcript analysis programmes and the examination of three *BRCA* gene databases. ***Results:*** In most of the variants, the analysis showed different *in silico* interpretations. This illustrates the interpretation challenges faced by diagnostic laboratories. ***Conclusion:*** Unfortunately, when using online mutation databases and carrying out *in silico* analyses, there is significant discordance in the classification of some missense variants in the *BRCA* genes. This discordance leads to complexities in interpreting and reporting these variants in a clinical context. The authors have developed a simple procedure for analysing variants; however, those of unknown significance largely remain unknown. As a consequence, the clinical value of some reports may be negligible.

Keywords: Genes, BRCA1; Genes, BRCA2; HBOC Syndrome; In Silico.

الملخص: الهدف: تكتشف المتغيرات المغلوطة بصورة شائعة عند فحص الطفرات في جينات *BRCA1* و *BRCA2*. والطفرات المسببة للأمراض في جيني *BRCA1* و *BRCA2* تؤدي الي زيادة خطورة حدوث سرطانات الثدي والمبايض والبروستاتا والبنكرياس. وهدفت هذه الدراسة الي تقييم القدرة التنبؤية لقواعد بيانات وبرامج "انسيليكو" لمساعدة المعامل التشخيصية في تحيد قدرة الطفرات التسلسلية علي التسبب في الأمراض. **الطريقة:** تم تحليل 13 متغير مغلوط في جينات ال *BRCA* في الفترة مابين يوليو 2011 الي ابريل 2013 في عينات المرضي المحولة الي الخدمات الصحية النيوزيلاندية (Northern Hub) لتحليل جينات ال *BRCA*. واستخدم في التحليل 13 برنامج "انسيليكو" التنبؤى للبروتين، واثنين برنامج "انسيليكو" للتحليل النصي وفحص ثلاثة قواعد بيانات لجين ال *BRCA*. **النتائج:** أظهرت نتائج التحليل اختلافا ملحوظا في تفسير "انسيليكو" لمعظم المتغيرات. وهذا يوضح التحديات التي تواجه المعامل التشخيصية في تفسير هذه النتائج. **الخلاصة:** للأسف فانه يحدث الكثير من الأختلاف عند تصنيف المتغيرات المغلوطة في جين ال *BRCA* بأستخدام قواعد بيانات الشبكة العنكبوتية لتنفيذ تحليل "انسيليكو". وقد أدي هذا الأختلاف الي حدوث تعقيدات كبيرة في تفسير هذه المتغيرات في السياق السريري. وقد طور الباحثون طريقة بسيطة لتحليل المتغيرات ولكن بالرغم من ذلك فانه لايزال هناك الكثير منها غير معلوم الدلالة. وبالتالي فأن القيمة السريرية لبعض هذه التقارير يمكن إهمالها.

مفتاح الكلمات: الجينات، *BRCA1*؛ والجين، *BRCA2*؛ متلازمة HBOC؛ "انسيليكو".

Advances in Knowledge

- *The analysis of sequence-detectable variants in the BRCA1 and BRCA2 (BRCA1/2) genes is critical in establishing if these variants are disease-causing.*
- *The analysis presented here shows the challenges posed by in silico programmes.*
- *Diagnostic laboratories may therefore have to rely on familial segregation studies or the development of better in silico programmes possibly based on advanced neural network modelling requiring phenotypic as well as genotypic data.*

Appication to Patient Care

- *The analysis in this study shows the advantages and disadvantages of database searching and in silico analyses in predicting the pathogenicity of gene variants.*
- *In the case of BRCA1/2 gene variants, evolving analytical tools offer an improved outcome for guiding counselling of patients at risk of hereditary breast and ovarian cancer.*

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Pathogenic mutations in the *BRCA1* and *BRCA2* (*BRCA1/2*) genes predispose patients to an increased risk of developing breast, ovarian, prostate and/or pancreatic cancer; these genes are two of the genes most commonly tested for cancer predisposition. In the USA, a known pathogenic mutation is detected in approximately 10–15% of patients who undergo sequencing of the entire coding regions of the *BRCA* genes.[1] However, a variant of uncertain significance (VUS) is detected in more than 5% of patients, with higher frequencies seen in less commonly tested ethnic groups.[2]

Patients with known pathogenic *BRCA* gene mutations are offered preventative strategies including enhanced surveillance, chemoprevention and irreversible surgical interventions. A study of patients in the USA, surveyed two years after being given either an uninformative (UN) *BRCA* gene-negative or VUS result by trained genetic counsellors, found that a VUS result did not result in excessive surgeries, exaggerated distress or increased risk perception compared to patients with a UN result.[3] The risk-reducing mastectomy rate was 7% in both groups and the oophorectomy rate was 5% for VUS patients and 3% for UN patients.[3]

A pathogenic mutation refers to a genetic variant that has been shown to cause or contribute to disease. A benign variant does not significantly impact on the function of the protein or increase disease risk, and it includes polymorphisms which are seen in over 1% of the general population. A VUS is a variant where the effect on protein function and disease risk is unknown.[4] In the case of the *BRCA* genes, VUS are largely missense substitutions where a single nucleotide change results in an altered amino acid. The terms, VUS and unclassified variant (UV) are often used interchangeably in the literature; however, they have slightly different interpretations. The term UV is suggestive of an unstudied variant, whereas a VUS may or may not have been studied but still has unknown clinical relevance.[5]

Providing a clear interpretation of a VUS is a complex challenge for a diagnostic laboratory. Common methods used to predict pathogenicity can include literature and database searches, *in silico* analyses, segregation analyses and functional studies. The requesting clinician may be faced with the difficult task of deciphering the ambiguity of the VUS and communicating the result to the patient along with clinical recommendations. Furthermore, it is imperative that the classifications of VUS are regularly checked and any changes to their classifications are relayed to the patients and their family.

The majority of missense mutations in the *BRCA* genes are classified as VUS. The exceptions include missense mutations that lie within the highly conserved *BRCA1* RING and the *BRCA1* carboxyl-terminal domains.[6] Known pathogenic missense mutations in the *BRCA2* gene are less common but may occur in the DNA-binding domain.[7]

The difficulty in the interpretation of missense variants in the *BRCA* genes arises due to the discordance in the classification of variants in the breast cancer databases and the variety of predictions based on *in silico* analyses. Recently, Lindor *et al*. used a quantitative posterior probability model to reclassify VUS in the *BRCA1/2* genes into five classes as defined by the International Agency for Research on Cancer (IARC) Working Group on Unclassified Genetic Variants.[8] These classes range from class 1 (not pathogenic) through to class 5 (definitely pathogenic). This reclassification attempts to combine a range of information regarding each VUS in the literature and convert this into a useful posterior probability.

This study analysed nine *BRCA1* and four *BRCA2* gene missense variants identified in the Diagnostic Genetics LabPLUS, Auckland City Hospital, Auckland, New Zealand, where the interpretation was hampered by the diversity of classifications in international databases and online *in silico* predictions.

Methods

This study was carried out between July 2011 and April 2013 and included 20 patients referred to Genetic Health Services New Zealand (Northern Hub) for *BRCA1/2* gene mutation screening. DNA was extracted from peripheral blood samples in ethylenediaminetetraacetic acid (EDTA) using the Gentra Puregene DNA Extraction kit (Qiagen GmbH, Hilden, Germany).

Genomic DNA from 20 patients were subjected to *BRCA1/2* gene sequencing as described elsewhere;[9] any identified variants were subsequently confirmed by exon-targeted polymerase chain reaction amplification and bi-directional Sanger-based sequencing.[10] Sequence traces were analysed using KB Basecaller Version 1.4 (Applied Biosystems Inc., Foster City, California, USA), on Variant Reporter™ Software Version 1.0 (Applied Biosystems Inc.), with a minimum trace score of 35, which corresponds to an average false base-call frequency of 0.031%. The analysis of sequence data and the subsequent investigation of databases and bioinformatic programmes used the relevant Reference Sequence (RefSeq) transcript, RefSeq protein and Uniprot accession numbers for the *BRCA1* (NM_007294.3; NP_009225.1; P38398) and

Table 1: Missense *BRCA* gene mutations identified in the DNA of 20 patients

Mutation	Predicted amino acid change	Detection frequency
BRCA1		
c.140G>A	p.Cys47Tyr	0.05
c.1067A>G	p.Gln356Arg	0.14
c.2077G>A	p.Asp693Asn	0.10
c.2315T>C	p.Val772Ala	0.05
c.2612C>T	p.Pro871Leu	0.38
c.3113A>G	p.Glu1038Gly	0.48
c.3119G>A	p.Ser1040Asn	0.05
c.3548A>G	p.Lys1183Arg	0.43
c.4837A>G	p.Ser1613Gly	0.43
BRCA2		
c.865A>C	p.Asn289His	0.05
c.1114A>C	p.Asn372His	0.52
c.2971A>G	p.Asn991Asp	0.05
c.8149G>T	p.Ala2717Ser	0.05

BRCA2 (NM_000059.3; NP_000050.2; P51587) genes.

All variants were checked for splicing effects using two *in silico* splice prediction programmes: the Splice Site Prediction by Neural Network online tool of the Berkeley Drosophilia Genome Project and the Alternative Splice Site Predictor (ASSP) tool.[11–14]

All of the patients included in the study gave informed consent. The New Zealand Multi-Region Ethics Committee has ruled that cases of patient management do not require formal ethical approval from a committee.

Results

The missense *BRCA* gene variants identified are shown in Table 1. These variants were checked for pathogenicity in six databases (three of which were specific to the *BRCA* genes)[Figure 1 and Table 2].[8,15–19]

The missense variants were also scored for predicted pathogenicity using 13 online *in silico* protein analysis programmes [Figure 2 and Table 3].[20–43] When all variants were checked for splicing effects using the two aforementioned *in silico* splice prediction programmes, both of the programmes predicted that each variant would have no effect on splicing (data not shown).

Apart from four of the variants, the results of the *in silico* protein analysis programmes varied depending on which programme was used. The frequency with which variants were predicted to be pathogenic varied significantly between programmes [Table 4].[22–43]

A total of 13 missense *BRCA* gene mutations were identified and only one was identified as probably pathogenic (*BRCA1*: c.140G>A) based on the combined results achieved from databases and *in silico* programmes. However, this variant was predicted to be benign using the Polymorphism Phenotyping, Version 2 (PolyPhen-2), HumVar database and the Protein Variation Effect Analyzer (PROVEAN).[20,37] In addition, a further three variants appeared to be probably benign (*BRCA1*: c.2612C>T, c.3548A>G and *BRCA2*: c.2971A>G). However, the remaining nine variants could not be interpreted even though minor allele frequencies on the Database of Single Nucleotide Polymorphisms (dbSNP) ranged from 0.01 to 0.327.[19]

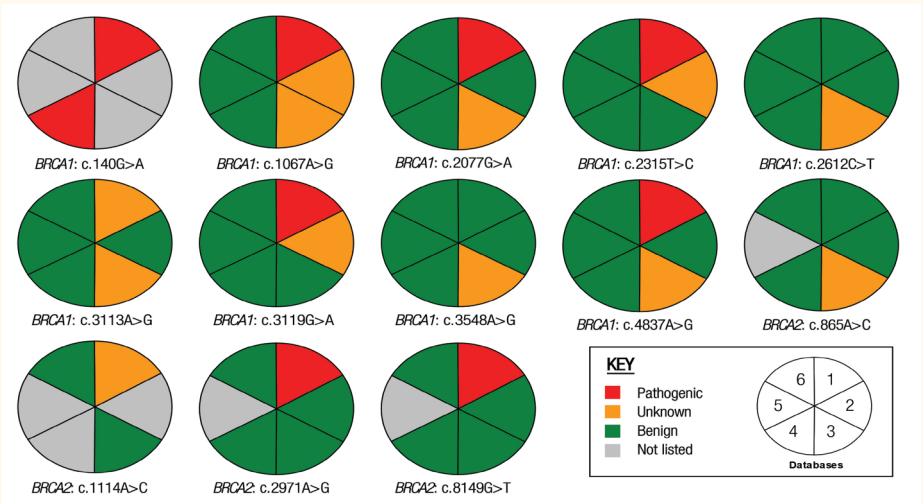

Figure 1: Diagrammatic representation of the pathogenicity calls of 13 *BRCA* gene missense variants in six databases, including the (1) Human Gene Mutation Database Professional 2013;[15] (2) Breast Cancer Information Core;[16] (3) Universal Mutation Database;[17] (4) Leiden Open Variation Database;[18] (5) International Agency for Research on Cancer,[8] and (6) Database of Single Nucleotide Polymorphisms.[19]

Table 2: Database listings for *BRCA* gene missense mutations

Variant	HGMD class	BIC clinically important	LOVD summary	UMD biological significance	IARC class	dbSNP	dbSNP MAF
BRCA1							
c.140G>A	DM	Not listed	Not listed	5 – Causal	Not listed	Not listed	-
c.1067A>G	DP	Unknown	Mixed	1 – Neutral	1 - not path	rs1799950	C = 0.028
c.2077G>A	DP	No	Mixed	1 – Neutral	1 - not path	rs4986850	T = 0.039
c.2315T>C	DM	Unknown	Neutral	1 – Neutral	1 - not path	rs80357467	-
c.2612C>T	DFP1	No	Mixed	1 – Neutral	1 - not path	rs799917	A = 0.483
c.3113A>G	DP2	No	Mixed	1 – Neutral	1 - not path	rs16941	C = 0.303
c.3119G>A	DM?	Unknown	Neutral	1 – Neutral	1 - not path	rs4986852	T = 0.012
c.3548A>G	DP1	No	Mixed	1 – Neutral	1 - not path	rs16942	C = 0.324
c.4837A>G	DM?	No	Mixed	1 – Neutral	1 - not path	rs1799966	C = 0.327
BRCA2							
c.865A>C	DP1	No	Mixed	1 - Neutral	Not listed	rs766173	C = 0.058
c.1114A>C	DFP	Listed as C>A	Neutral	Listed as C>A	Listed as C>A	rs144848	C = 0.240
c.2971A>G	DM?	No	Neutral	Polymorphism	Not listed	rs1799944	G = 0.062
c.8149G>T	DM?	No	Neutral	1 - Neutral	1 - not path	rs28897747	T = 0.001

HGMD = Human Gene Mutation Database Professional 2013;[15] BIC = Breast Cancer Information Core database;[16] LOVD = Leiden Open Variation Database;[18] UMD = Universal Mutation Database;[17] IARC = International Agency for Research on Cancer;[8] dbSNP = Database of Single Nucleotide Polymorphisms;[19] MAF = minor allele frequency; DM = disease-causing mutation; DP = disease-associated polymorphism; path = pathogenic; DFP = disease-associated polymorphism with additional supporting functional evidence; 1 = associated with a decreased risk; 2 = comments included "polymorphism"; DM? = potential disease-causing mutation.

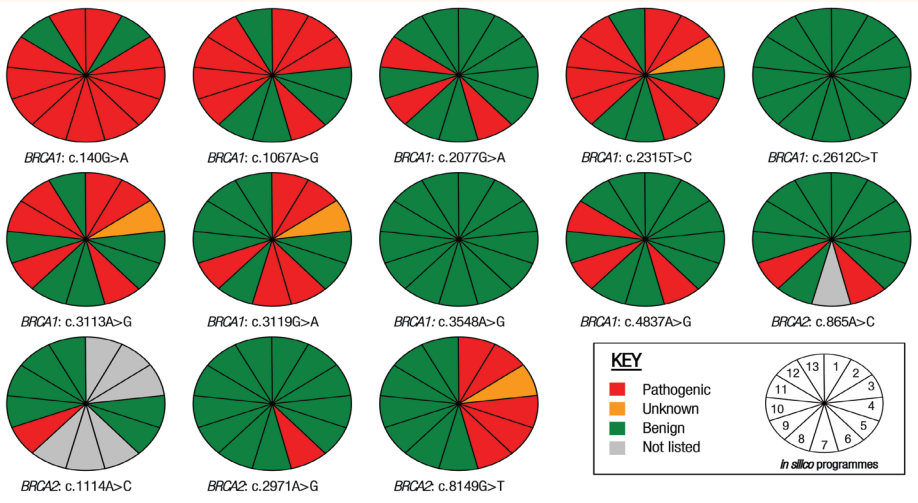

Figure 2: Diagrammatic representation of the pathogenicity calls of 13 *BRCA* gene missense variants using 13 online *in silico* analysis programmes (all used in default online mode). These prediction programmes included: both the (1) HumDiv and (2) HumVar predictions of Polymorphism Phenotyping, Version 2;[20,21] (3) Mutation Assessor, release 2;[22,23] (4) I-Mutant, Version 3.0, for the prediction of disease-associated single point mutations from protein sequence;[24,25] (5) MutPred, Version 1.2;[26,27] (6) SNPs&GO;[28,29] (7) Protein Analysis Through Evolutionary Relationships Evolutionary Analysis of Coding SNPs, Version 6.1;[30,31] (8) Align-Grantham Variation Grantham Deviation used with the supplied *BRCA1* and *BRCA2* alignments;[32,33] (9) SNAP;[34,35] (10) Predictor of Human Deleterious Single Nucleotide Polymorphisms;36 (11) Protein Variation Effect Analyzer, Version 1.1.3, and Sorting Intolerant from Tolerant;[37–39] (12) Sorting Intolerant from Tolerant BLink,[40,41] and (13) Mutation Taster.[42,43]

Table 3: Online *in silico* predictions* regarding the pathogenicity of *BRCA* gene missense mutations

Variant	PolyPhen2 HumDiv	PolyPhen2 HumVar	MutAss	I-Mutant	MutPred	SNPs&GO	PANTHER	Align-GVGD	SNAP	PhD-SNP	SIFT	PROVEAN	MutTas
	Score	Score	FI score	RI	Score	RI	subSPEC	Class	RI, Accuracy	RI	SeqRep	Score	Probability
BRCA1													
c.140G>A	Possibly 0.677	Benign 0.223	High 4.025	D 2	D 0.989	D 10	-4.6203	C65	Non-N 6, 93%	D 7	Not tolerated 0.23	N -1.588	Disease 0.975
c.1067A>G	Probably 0.998	Probably 0.988	High 3.635	N 6	N 0.232	D 7	-1.91524	C0	Non-N 3, 78%	D 4	Not tolerated 0.98	D -3.302	Poly 0.999
c.2077G>A	Benign 0.000	Benign 0.010	Neutral 0.55	N 5	N 0.135	D 3	-1.63602	C0	Non-N 2, 70%	N 6	Not tolerated 1.00	N 0.032	Poly 0.999
c.2315T>C	Possibly 0.848	Probably 0.928	Medium 2.63	N 4	D 0.847	D 8	-2.25807	C0	Non-N 4, 82%	D 3	Not tolerated 1.00	D -3.612	Poly 0.999
c.2612C>T	Benign 0.000	Benign 0.000	Neutral -3.395	N 4	N 0.232	N 6	-2.71872	C0	N 0, 53%	N 8	Tolerated 1.00	N 5.742	Poly 0.996
c.3113A>G	Possibly 0.936	Possibly 0.606	Medium 2.54	N 3	N 0.192	D 2	-2.4878	C0	Non-N 4, 82%	N 3	Not tolerated 0.98	D -5.687	Poly 0.999
c.3119G>A	Probably 0.974	Possibly 0.831	Medium 2.785	N 6	N 0.123	D 7	-3.46389	C0	Non-N 4, 82%	N 3	Tolerated 0.98	N -1.683	Poly 0.999
c.3548A>G	Benign 0.000	Benign 0.001	Neutral -1.085	N 8	N 0.096	N 6	-0.82666	C0	N 4, 85%	N 7	Tolerated 1.00	N 0.398	Poly 1
c.4837A>G	Benign 0.255	Benign 0.038	Low 1.04	N 5	N 0.127	D 2	-1.57816	C0	Non-N 2, 70%	N 6	Not tolerated 0.26	N -0.509	Poly 0.999
BRCA2													
c.865A>C	Benign 0.278	Benign 0.034	Low 1.445	N 0	N 0.194	D 10	Error	C0	Non-N 4, 82%	N 4	Tolerated 0.37	N -0.509	Poly 0.999
c.1114A>C	Called as H at 372		H residue	N 4	N 0.058	Error	Error	Error	Non-N 0, 58%	N 9	Tolerated 0.33	N -0.599	Poly 0.999
c.2971A>G	Benign 0.000	Benign 0.000	Neutral -1.15	N 0	N 0.325	D 7	-0.45225	C0	N 3, 78%	N 9	Tolerated 0.99	N 2.127	Poly 0.999
c.8149G>T	Possibly 0.955	Possibly 0.763	Medium 1.965	D 1	D 0.783	D 9	-2.3929	C0	N 1, 60%	N 8	Tolerated 0.37	N -0.441	Poly 0.961

*PolyPhen2 = Polymorphism Phenotyping, Version 2, prediction and score shown;[20,21] MutAss = Mutation Assessor, release 2, predicted FI (high or medium) or non-FI (low or neutral) and combined score is shown;[22,23] I-Mutant = Version 3.0, prediction of disease-associated single point mutation from protein sequence and RI shown;[24,25] MutPred = Version 1.2, prediction of an amino acid substitution as D or N and score shown;[26,27] SNPs&GO = prediction of human disease-related mutations in proteins with functional annotations, predicted effect and RI score from 0 (unreliable) to 10 (reliable) shown;[28,29] PANTHER = Protein Analysis Through Evolutionary Relationships, Version 6.1, evolutionary analysis of coding single nucleotide polymorphisms, subSPEC scores of continuous values from 0 (neutral) to -10 (most likely to be deleterious) shown**;[30,31] Align-GVGD = Align-Grantham Variation Grantham Deviation, using supplied alignments, class shown (class C65 most likely and C0 less likely;[32,33] SNAP = prediction of effect of non-synonymous polymorphisms on function, RI and expected accuracy shown;[34,35] PhD-SNP = Predictor of Human Deleterious Single Nucleotide Polymorphisms, prediction and RI shown;[36] SIFT = Sorting Intolerant from Tolerant BLink, tolerated or not tolerated prediction and SeqRep score shown; [40,41] PROVEAN = Protein Variation Effect Analyzer, Version 1.1.3, using the Human Protein Batch tool, prediction and score (score of ≤-2.5 signified a "deleterious" effect and score of >-2.5 signified a "neutral" effect) shown;[37–39] MutTast = Mutation Taster, prediction and probability (using P values, with a value close to 1 indicating the high security of the prediction; the P value used here is not the probability of error as used in t-test statistics) shown;[42,43] FI = functional impact; RI = reliability index; SeqRep = fraction of sequences that contain one of the basic amino acids, where a low fraction indicates the position is either severely gapped or unalignable and has little information, a poor prediction is expected at these positions; D = disease-associated; N = neutral; Poly = polymorphism; H = histidine.*

**All programmes were used in default online mode. **A score of -3 is the previously identified cutoff point for functional significance.*

Table 4: Percentage of *BRCA1* and *BRCA2* gene missense variants predicted to be pathogenic using online *in silico* analysis programmes

Programme	% predicted to be pathogenic
SNPs&GO	83
SNAP	69
PolyPhen - HumDiv	50
SIFT	46
PolyPhen - HumVar	42
MutPred	23
PhD-SNP	23
PROVEAN	23
PANTHER	18
MutAss	17
I-Mutant	15
Align-GVGD	8
MutTas	8

SNP&GO = predicts human disease-related mutations in proteins with functional annotations;[28,29] SNAP = predicts effect of non-synonymous polymorphisms on function;[34,35] PolyPhen = Polymorphism Phenotyping, Version 2;[20,21] SIFT = Sorting Intolerant from Tolerant BLink;[40,41] MutPred = Version 1.2, classifies an amino acid substitution as disease-associated or neutral;[26,27] PhD-SNP = Predictor of Human Deleterious Single Nucleotide Polymorphisms;[36] PROVEAN = Protein Variation Effect Analyzer, Version 1.1.3;[37–39] PANTHER = Protein Analysis Through Evolutionary Relationships, Version 6.1;[30,31] MutAss = Mutation Assessor programme, release 2;[22,23] I-Mutant = Version 3.0;[24,25] Align-GVGD = Align-Grantham Variation Grantham Deviation;[32,33] MutTast = Mutation Taster.[42,43]

Discussion

The results presented here illustrate a major problem in interpreting missense *BRCA1/2* gene variants. The classifications from various databases and the predictions from a variety of online *in silico* analysis programmes can vary widely. This highlights the risk of relying on information obtained from just one database or from using only a few *in silico* programmes when reporting missense variants, as the outcome can affect clinical surveillance and prevention decisions.

Of the 13 missense *BRCA* gene mutations identified, only one was shown to be probably pathogenic, although the same variant was predicted to be benign by the PolyPhen-2 HumVar database and PROVEAN.[20,37] Lindor *et al.* classified nine of the variants in their study as IARC class 1 (not pathogenic).[8] Their reclassification uses a model based on prior probabilities derived from evolutionary predictions combined with a likelihood component from segregation information, co-occurrence in 'trans', personal and family history and a histopathology profile to give a posterior probability of causality. The outcome of this analysis is based on combining a wide range of information, which is clearly different from the predictions made from individual databases and single *in silico* programmes, and again highlights the importance of an over-reliance on one source of information to determine the disease causality of a variant.

The Clinical Molecular Genetics Society (CMGS) in the UK states in their 2007 guidelines for interpreting and reporting UVs that it is unacceptable to rely solely on *in silico* predictions to assign pathogenicity to a previously unclassified variant.[44] Furthermore, the Association for Clinical Genetic Science states in their 2013 practice guidelines for the reporting of sequence variants in clinical molecular genetics that "the classification generated from the prediction tools must not be considered definitive".[45] The American College of Medical Genetics (ACMG) guidelines state that all variants of unknown clinical significance must be included in a laboratory's report and be followed by an interpretation of their likely clinical significance.[46] ACMG recommend categorising uncertain sequence variants as either "previously unreported and of the type which may or may not be causative of the disorder" or "previously unreported and probably not causative of disease".[46] The CMGS 2007 guidelines also state that it is "essential to report all UVs where the clinical significance is uncertain" and furthermore that it is "essential that reports of UVs should be issued to appropriately trained clinicians".[44] The European Molecular Genetics Quality Network's best practice guidelines for genetic analysis in hereditary breast ovarian cancer recommend that the identification of *BRCA* gene VUS do not "provide a basis for changing the clinical management of the patient or for offering predictive testing to at risk relatives".[6]

The protocol which the authors have established for interpreting *BRCA* gene missense variants includes: (1) Checking the Breast Cancer Information Core (BIC)and IARC databases;[8,16] (2) Checking the dbSNP for classification and minor allele frequency;[19] (3) Undertaking splice site predictions using the online Splice Site Prediction by Neural Network and ASSP tools,[11,13] and (4) Undertaking *in silico* protein analysis using the Grantham score PolyPhen SIFT BLink, SNPs&GO and PROVEAN.[2,20,28,32,40]

In the event that a database search is conflicting, or there is no entry, the authors recommend that dbSNP and splice site/*in silico* protein analysis programmes are also used. Apart from the BIC and IARC databases, other databases are not as comprehensive, or provide little value in assigning benign/disease-causing status to a missense variant.

The *in silico* programmes use a variety of approaches to achieve a prediction: sequence and

evolutionary conservation-based methods, protein sequence and structure-based methods and machine learning methods. The data from this study support using a number of programmes to achieve a consensus prediction rather than relying on only one programme. The authors suggest that, when results are uncertain, a report of the cascade approach used should be recorded and a detailed work-up should be archived for the clinician to refer to if necessary.

The authors recommend that their conclusions are reviewed by clinicians to determine their continuing validity. The predictions have varying levels of confidence, but are considered as an aid to clinical interpretation, although the work described here shows that the value of these predictions may be largely ambivalent at best, or misleading at worst. The authors recommend that the testing of additional family members and a correlation with clinical findings would be helpful to determine the significance of the result.

This recommendation for segregation analysis is not entirely fool-proof, especially in light of the predominance of breast cancer in families with *BRCA1/2* gene mutations, and that cancer risk may involve an appreciation of familial context rather than a population-based calculation.[47] Critically, the authors suggest only accepting referrals from trained genetic counsellors or clinicians with a sufficient understanding of interpreting complicated genetic results. In the event of *BRCA* gene missense mutations that are reliably benign (stated as such in BIC/IARC databases or when all *in silico* predictions agree), then these should be relegated to an ancillary table in the report with a footnote indicating how the benign status was determined.

This study highlights the complexity of interpreting and reporting missense *BRCA1/2* gene variants where the results will be used in genetic counselling, screening and disease prevention. It demonstrates that some *BRCA* gene missense variants cannot be clearly interpreted with the tools and data available today; however, these variants must be included in laboratory reports so that if future information becomes available regarding their classification then this can be passed on to the patient and their family. This future information could be provided by international developments under the auspices of the Enhancing Neuro Imaging Genetics through Meta Analysis Consortium which is involved in coordinating the development of algorithms for the classification of variants in the *BRCA1/2* genes.[48] Recent work reported by this consortium has embraced functional assays of *BRCA2* gene variants and has attempted to translate functional outcomes into a probability of pathogenicity.[49]

Conclusion

The findings of this study show that there is significant discordance in the classification of some missense variants in the *BRCA* genes when using online mutation databases and carrying out *in silico* analyses. This discordance leads to complexities in interpreting and reporting these variants in a clinical context. As such, it is vital that laboratories have agreed guidelines for determining the pathogenicity of a given variant based on a wide range of information and for reporting an uncertain result to the referring clinician. Importantly, the complexity of interpreting and communicating VUS findings highlights the importance of sequencing results being conveyed to patients in a specialist genetic counselling environment.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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