Clinically Applicable Models to Characterize *BRCA1* and *BRCA2* Variants of Uncertain Significance

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ABSTRACT

Purpose

Twenty percent of individuals with a strong family and/or personal history of breast and ovarian cancer carry a deleterious mutation in *BRCA1* or *BRCA2*. Identification of mutations in these genes is extremely beneficial for patients pursuing risk reduction strategies. Approximately 7% of individuals who have genetic testing of *BRCA1* and *BRCA2* carry a variant of uncertain significance (VUS), making clinical management less certain. The majority of identified VUS occur only in one to two individuals; these variants are not able to be classified using current classification models with segregation analysis components.

Methods

To develop a clinically applicable method that can predict the pathogenicity of VUS that does not require familial information or segregation analysis, we identified characteristics of breast or ovarian tumors that distinguished sporadic tumors from tumors with *BRCA1* or *BRCA2* mutations. Study participants included individuals with known deleterious mutations in *BRCA1* or *BRCA2* and individuals with classified or unclassified *BRCA* variants.

Results

We applied the models to 57 tumors with 43 different deleterious *BRCA* mutations and 57 tumors with 54 unique classified and unclassified *BRCA* variants. Of the 33 previously unclassified VUS studied, we found evidence of neutrality for 21.

Conclusion

Our models showed 98% sensitivity and 76% specificity for predicting classified DNA changes. We classified 64% of unknown variants as neutral. Classification of VUS as neutral will have immediate benefit for those individuals and their family members. These models are adaptable for the clinic and will be useful for individuals with limited available family history.

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INTRODUCTION

Mutations in *BRCA1* and *BRCA2* confer a greatly increased lifetime risk for breast and ovarian cancer. The number of individuals who have had genetic testing of *BRCA1* and *BRCA2* in the United States now exceeds 70,000.¹ Individuals who carry a mutation in *BRCA1* or *BRCA2* can increase the likelihood of detecting cancers at an earlier stage by more stringent surveillance and can decrease their chance of developing cancer through the use of prophylactic surgery and/or chemopreventive agents. Of the high-risk individuals who have had mutation analyses through Myriad Genetic Laboratories (Salt Lake City, UT), approximately 7% have a variant of uncertain significance (VUS) in *BRCA1* or *BRCA2*.²⁻⁴ VUS are typically missense or potential splice site changes of uncertain biologic or clinical relevance. They are more frequent in the minority ethnic pop-

ulations.³ More than 1,500 VUS have been identified; most have only been reported in one to two individuals.

Through prediction studies, some *BRCA* VUS have been classified as neutral polymorphisms, and a small number of *BRCA* VUS have been classified as likely deleterious. Many of the predicted deleterious VUS are located in highly conserved functional domains or affect transcript start or splice sites. Still, the majority of variants have yet to be classified.⁴⁻¹¹ Several of the algorithms used to characterize VUS use familial segregation analysis or study variants in multiple unrelated individuals to provide evidence for or against causality. One difficulty with this approach is that the vast majority of reported VUS have been observed in only one or two individuals, which makes them ineligible for segregation analysis. Also, population-based studies in *BRCA* carriers have shown that more than 50% of *BRCA*-positive

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individuals may not have a close family member with a *BRCA*-related cancer who would be available for segregation analysis.¹² Finally, some individuals with *BRCA* VUS have *BRCA*-related cancers segregating on both sides of the family. Thus, it is important to develop additional means to classify VUS that do not rely on segregation and can be applied in various clinical settings.

To address this need, we developed four *BRCA* VUS classification models that use clinical information typically available from pathology and mutation testing reports combined with protein prediction model data, cancer type, and tumor loss of heterozygosity (LOH) data. We applied these models to 57 tumors with deleterious *BRCA* mutations and 57 tumors with classified and/or unclassified variants.

METHODS

Human Samples

Studies were approved by local institutional review boards. All study participants signed informed consent for this research. Paired normal and tumor tissues were ascertained through one of the following three sources: The Ohio State University Comprehensive Cancer Center Clinical Cancer Genetics Program, the Mayo Clinic Familial Cancer Program, and the University of California, San Francisco Familial Risk Shared Resource. Eligibility criteria included a diagnosis of breast or ovarian cancer, available normal and tumor DNA, available pathology records, and a *BRCA* testing report showing a *BRCA1* and/or *BRCA2* deleterious mutation, VUS, or a reclassified variant. Hematoxylin and eosin slides from tumors were reviewed by a pathologist, and areas comprising greater than 70% tumor cells were microdissected from sections or were cored. Normal DNA was isolated from blood or histologically normal breast tissue. Lymphoblastoid cell lines were established on a subset of individuals.

Genomic DNA was isolated from fixed archival tissue by removal of paraffin using xylene and ethanol washes. Protein was removed by proteinase K treatment for 48 hours at 55°C in nucleic acid lysis buffer. Samples were phenol/chloroform extracted and ethanol precipitated.

Allele-Specific LOH

We used restriction fragment length polymorphism studies and sequence analysis to conduct allele-specific LOH analysis. When a mutation or variant altered a restriction enzyme site, polymerase chain reaction (PCR) products were digested with the appropriate enzyme, separated on an agarose gel, and stained with ethidium bromide. Bands were visualized on an Alphascreen (Alpha Innotech, San Leandro, CA). For mutations resulting in deletions of more than 20 base pairs, PCR products were run directly on a 1.5% agarose gel. LOH was assessed visually for restriction fragment length polymorphism analysis. When the results were visually ambiguous, ratios of cut to uncut band of normal and tumor DNA were taken using band intensity. Samples were scored as loss if the ratios were greater than 1.5 or less than 0.67 according to criteria used in other studies.^{13,14} To avoid undercalling LOH, we scored samples as uncertain if ratios fell between 0.67 and 0.76 or 1.35 and 1.50. For changes not resulting in an altered restriction site, PCR products were treated with ExoSap-It (USB, Cleveland, OH) and sequenced. Sequence traces in the forward and reverse direction were compared between control DNA and normal DNA and tumor DNA containing the sequence change using DNASTAR 3.0 (DNASTAR, Inc, Madison, WI). Loss was determined visually by two reviewers and consisted of at least 30% difference between the two alleles compared with normal carrier ratios.

Evolutionary Conservation

We used align grantham variation deviation analysis (A-GVGD) to identify missense changes of potential functional significance (<http://agvgd.iarc.fr/alignments.php>).^{6,15} A-GVGD uses a combination of the physical properties of amino acids and protein sequence alignments from multiple species to predict if missense substitutions are more likely to be neutral or affect protein function. Because deleterious missense changes have not been reported out-

side of conserved *BRCA* domains,⁴ we applied A-GVGD only to missense changes occurring in the following three conserved domains: *BRCA1* ring domain (amino acids 1 to 102), *BRCA1* breast cancer C-terminal domain (amino acids 1641 to 1863), or *BRCA2* DNA binding domain (amino acids 2401 to 3200). A-GVGD analysis assigns a probability class to each variant studied ranging from class (C) 0 variants, which are expected to be neutral, to C65 variants, which are expected to affect protein function. Using data from 1,400 VUS,⁴ prior probabilities of a change being deleterious for the A-GVGD classes of variants mapping to conserved regions were as follows: C65 = 0.81; C35, C45, and C55 = 0.66; C15 and C25 = 0.29; and C0 = 0.01.¹⁶ From these likelihood probabilities, we calculated odds of being deleterious for the A-GVGD classes falling into conserved domains as follows: C0 = 0.01; C15 and C25 = 0.41; C35, C45, and C55 = 1.5; and C65 = 4.26. All other missense changes were given a probability of being deleterious of 0.01.⁴

Splicing Studies

To determine whether any VUS affected normal splicing patterns and, therefore, could be functional changes, we used ESEfinder and SpliceSite-Finder to measure the theoretical effect on splicing.^{17,18} These programs are used to identify exonic splice enhancer motifs (ESEfinder) or putative splice sites (SpliceSiteFinder). We isolated RNA from lymphoblastoid cell lines from individuals carrying putative splice site mutations for use in splicing studies. We performed reverse transcriptase PCR using primer sets from the two flanking exons as well as primer sets between the flanking exon and the potentially aberrantly spliced exon using cDNA from patients and wild-type controls. We noted aberrant splicing in cases where bands of sizes other than those seen in 11 wild-type cDNA controls were present. To confirm abnormal splicing, we sequenced any extra bands.

Observation With a Deleterious Mutation

Because homozygous *BRCA1* mutations are expected to be lethal and homozygous *BRCA2* mutations are expected to result in lethality or phenotypes such as Fanconi anemia, the presence of a deleterious mutation in trans with a variant suggests its neutrality.⁶ We used data from mutation reports and the literature to determine whether variants in our study had been observed in trans with a deleterious mutation. For our models, we used the published estimates of the probabilities of two deleterious mutations occurring in trans in *BRCA1* and *BRCA2* of $P = .0001$ and $P = .001$, respectively.⁵

Model Phenotypes

To identify tumor characteristics that would be useful in discriminating deleterious variants from nondeleterious variants, we searched the literature for studies comparing characteristics of breast and ovarian tumors from *BRCA1* and *BRCA2* mutation-positive and mutation-negative individuals. We identified a number of characteristics available in most pathology reports that could be used in a prediction model (Table 1).^{5,19-56} When a considerable range of frequency estimates was observed for a characteristic, we calculated an average frequency based on raw numbers from all of the studies. Not surprisingly, the features predictive of mutation status varied between both breast and ovarian cancer and between *BRCA1* and *BRCA2*. As a result, we created likelihood odds for four predictive models, one for each gene and tumor type, that differ only between gene/cancer site and model-specific likelihood odds (Appendix Table A1, online only; see also Appendix, online only).

Statistical Methods

To derive odds of pathogenicity for each variant, we used a modified multifactorial approach that combines the odds of causation of independent variables.^{4,5,7,9} Odds for each independent variable were determined from frequencies reported in the literature or published odds, with the exception of truncating mutations and splice site alterations for which we calculated odds of being deleterious of 1,000:1 if splicing defects or truncating mutations were identified and odds of 0.01 if splicing defects were not observed. The product of the likelihoods of independent variables was obtained to provide an overall odds of pathogenicity for each sequence alteration (additional details are in Appendix). On the basis of previous studies, we used a cutoff of 1,000:1 in favor of being deleterious and a cutoff of 100:1 in favor of being neutral for final classification status.^{4,5} When two independent tumors with the same sequence alteration were identified, independent factors for both were combined and

Table 1. Frequency of Histopathologic Characteristics of *BRCA1*, *BRCA2*, and Sporadic Tumors

Characteristic	Sporadic Frequency (%)		<i>BRCA1</i> Frequency (%)		<i>BRCA2</i> Frequency (%)	
	Range Reported in Literature	Frequency Used for Weighting	Range Reported in Literature	Frequency Used for Weighting	Range Reported in Literature	Frequency Used for Weighting
Breast						
<i>BRCA1</i> loss	20-40	30	68-100	80	NA	
<i>BRCA2</i> loss	22-50	30	NA		38-100	70
Medullary	1-3	1.5	11-13	12	ND	
Triple negative	10-25	16	80	80	ND	
ER negative	13-37	26	63-90	83	ND	
PR positive	58-68	65	16-21	20	ND	
HER-2 positive	12-35	20	0-3	3	0-3	3
HER-2 negative	70-88	80	97-100	97	97-100	97
ER positive/grade 1	27-42	30	0-2.4	2	5-17	7
ER positive/grade 2	11-28	27	10-17	10	22-45	41
ER positive/grade 3	9-17	13	12-28	13	28-30	28
ER negative/grade 1	3-14	5	0-1	1	1-4	2
ER negative/grade 2	12-13	13	9-13	13	2-17	5
ER negative/grade 3	12-16	16	62-91	65	9-18	16
Grade 1	18-56	22	0-9	2.5	11-22	17
Grade 2	23-49	42	16-26	23	29-49	43
Grade 3	21-49	36	66-100	71	38-64	47
Ovarian						
<i>BRCA1</i> loss	40-66	40	72-100	90	NA	
<i>BRCA2</i> loss	30-42	30	NA		80-100	80
Serous	29-47	34	40-100	50	39-100	60
Mucinous	11-36	17	0-3	2	0-2	1
Endometrioid	5-33	26	NA		0-29	18
Grade 1	10-19	13	1-3	2	0-3	3
Grade 2	21-34	31	22-27	26	13-14	14
Grade 3	50-55	54	72-75	72	81-87	87
Stage I	24		3-17	17	0-6	6
Stage II	17		10-16	10	0-6	6
Stage III	40		69-70	69	82-100	82
Stage IV	19		3-11	6	0-7	5

Abbreviations: NA, not applicable comparison; ND, not calculated because the literature suggests no differences from sporadic rates; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

considered in the final data interpretation. We calculated odds both with and without LOH data because LOH data may not be typically available clinically and the frequency of LOH in neutral *BRCA* variants has not been well documented. If a prediction of variant status was only significant in the combined odds using LOH data, we scored the variant as suspected neutral or suspected deleterious accordingly.

RESULTS

Identification of Study Criteria for the Models

We collected tumor and normal tissue and histopathologic and clinical data from the medical records and mutation reports of 102 individuals with *BRCA* mutations or characterized or uncharacterized variants (Table 2). The study group consisted of 57 tumors with 43 unique deleterious mutations and 57 tumors with 54 unique variants (Tables 2, 3, and 4; Appendix Tables A2 and A3, online only).

LOH

One characteristic of *BRCA1* and *BRCA2* mutation tumors that has been used previously to assess pathogenicity of *BRCA* VUS is tumor LOH.^{5,11,25} LOH occurs more frequently in *BRCA* mutation–

positive tumors than sporadic tumors and is thought to occur predominantly on the nonmutated chromosome (Table 1).^{5,11,25,26,28}

LOH in tumors with a neutral *BRCA1* or *BRCA2* variant is predicted to be random for loss of variant or wild-type allele; however, LOH in this group has not been well studied. To determine whether our observed rates of LOH were comparable to published rates, we successfully conducted allele-specific LOH studies on 56 of 57 tumors from carriers of known deleterious mutations. We detected LOH of the wild-type *BRCA1* allele in 85% of breast tumors (28 of 33 tumors) and 100% of ovarian tumors (three of three tumors; Tables 3 and 4; Appendix Tables A2 and A3). We observed LOH of the wild-type *BRCA2* allele in 63% of breast tumors (10 of 16 tumors) and 25% of ovarian tumors (one of four tumors). We also observed loss of the mutant allele in one *BRCA1* breast tumor and in one *BRCA2* breast tumor (Appendix Table A2). The *BRCA1* frequencies of LOH are similar to published rates. However, the *BRCA2* frequencies of loss were slightly lower than expected and lower than the frequencies used in other studies.⁵ We were also surprised by the high observed frequency of LOH of the mutant allele in breast tumors (one of 33 tumors for *BRCA1* and one of 16 tumors for *BRCA2*). On the basis of our data

Table 2. Study Population

Factor	No. of Mutations			No. of VUS		
	<i>BRCA1</i>	<i>BRCA2</i>	Total	<i>BRCA1</i>	<i>BRCA2</i>	Total
Breast tumors	33	17	50	21	29	50
Ovarian tumors	3	4	7	1	6	7
Unique change	25	18	43	19	35	54
Previously classified	NA	NA	NA	9	12	21
Previously unclassified	NA	NA	NA	10	23	33
Patients*	33	17	50	21	34	55
Patients with two or more changes	2	0		2	4	
Bilateral cancer	3	0	3	1	1	2

Abbreviations: VUS, variant of uncertain significance; NA, not applicable.

*Note that the No. of patients on study (n = 102) does not equal the total No. of tumors studied (n = 114) or the total No. of patients in this table (n = 105). Some individuals have bilateral cancers; other study participants have more than one change identified and are counted in more than one category.

and previous published reports, we used a frequency of LOH to generate likelihood ratios of the wild-type allele of 79% and 69% for *BRCA1*- for *BRCA2*-positive breast tumors, respectively, and 89% and 79% for *BRCA1*- and *BRCA2*-positive ovarian tumors, respectively, in our prediction models (Appendix Table A1). For deleterious mutations, we assumed that 1% of the time there would be loss of the mutant allele, as has been suggested in the literature.^{5,11,25,26,28}

We successfully assessed allele-specific LOH in 55 of 57 tumors with *BRCA* VUS (Tables 2 and 3; Appendix Tables A2 and A3). Twenty-nine percent of tumors (six of 21 tumors) with *BRCA1* VUS showed LOH of the wild-type allele, 14% of tumors (three of 21 tumors) showed LOH of the variant allele, and 57% of tumors (12 of 21 tumors) showed no imbalance. For *BRCA2*, 26% of breast tumors (nine of 34 tumors) showed wild-type allele loss, 23% of tumors (eight of 34 tumors) showed variant allele loss, and 50% of tumors (17 of 34 tumors) showed no imbalance. Variants *BRCA1* IVS2-14 C>T, S127N, and V1804D and *BRCA2* A1170V, E2856A, H1966Y, I2285V, M784V, N1878K, and Y42C were lost in tumors providing evidence for neutrality.

Sequence-Based Methods to Determine Pathogenicity

On the basis of a study of more than 1,433 *BRCA* VUS, pathogenic VUS are most likely to occur in regions that are highly conserved

across species, occur in specific *BRCA* protein domains, and/or affect splicing.⁴ Five *BRCA1* and nine *BRCA2* missense changes fell in or near highly conserved domains. For these variants, we used A-AVG, a statistical algorithm validated and widely used for the study of *BRCA* VUS, to assign classes of C0 (neutral) to C65 (likely deleterious).^{5,6,9,15,16} A-AVG classified 10 of these variants as C0 or neutral (Tables 2 and 3; Appendix Tables A2 and A3). Three VUS and one suspected mutation were assigned a higher class and given an increased likelihood of being deleterious (Tables 5 and 6; Appendix Tables A4 and A5, online only).

Splicing Studies

Using ESEfinder and SpliceSiteFinder, none of the missense variants were predicted to affect splicing (Appendix Tables A4 and A5). To study splicing defects of intronic variants, we isolated RNA from lymphoblastoid cell lines for four individuals with unclassified intronic VUS and from one individual with an intronic variant classified as deleterious. cDNA from 11 individuals not carrying the variants and genomic DNA from the carriers were used as controls. None of the four VUS studied (*BRCA1* IVS2-14 C>T and IVS20-14 C>G and *BRCA2* IVS23+9 C>T and IVS8-12delTA) showed splicing defects in contrast to the intervening sequence variant previously classified as deleterious (*BRCA1* IVS5-11 G>T; data not shown).

Table 3. Examples of Characteristics for Predictive Breast Models

Sequence Change	LOH	Conserved Domain	A-GVGD Class	Splice Defect	In Trans	Age at Diagnosis (years)	Grade	ER Status	PR Status	HER-2 Status	Histology
<i>BRCA1</i>											
1294del40	WT	NA	NA	ND	NA	40	3	—	—	+	IDC
IVS13+1 G>A	None	NA	NA	Yes	No	41	2	—	—	—	IDC
C61G	WT	Yes	C65	ND	No	29	3	—	—	—	IDC
Y856H	None	No	C0	ND	Yes	45	3	+	+	+	DCIS
<i>BRCA2</i>											
2041delA	None	NA	NA	ND	NA	34	3	+	+	—	IDC
IVS8-12delTA	None	NA	NA	No	No	44	1	+	+	—	IDC
N517S	None	No	C0	ND	No	45	1	+	+	ND	IDC
L2106P	None	No	C0	ND	No	57	3	+	+	—	IDC

NOTE. Examples of phenotypes from eight individuals with different types of *BRCA1* and *BRCA2* sequence changes are shown. The full list of tumors and sequence alterations studied is provided in Appendix Table A2.

Abbreviations: LOH, loss of heterozygosity; A-GVGD, align grantham variation, grantham deviation; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; WT, wild-type allele lost; NA, not applicable; ND, no data; —, negative; +, positive; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ.

Table 4. Examples of Characteristics for Predictive Ovarian Models

Gene	Mutation Type	Change	Conserved Domain	LOH	A-GVGD Class	In Trans	Stage	Grade	Age at Diagnosis (years)	Histology
<i>BRCA1</i>	Truncating	1135insA	NA	WT	NA	No	ND	3C	59	Papillary serous
<i>BRCA1</i>	Missense	P334L	No	WT	C0	Yes	ND	ND	39	Unspecified
<i>BRCA2</i>	Truncating	7297delCT	NA	None	NA	NA	ND	3C	22	Serous
<i>BRCA2</i>	Missense	M784V	No	V	C0	No	IIIC	3	45	Unspecified
<i>BRCA2</i>	Missense	V1643A	No	WT	C0	No	IIIC	ND	50	Serous

NOTE. Examples of phenotypes from different *BRCA1* and *BRCA2* sequence alterations seen in ovarian tumors are shown. The full list of phenotypes from all sequence alterations studied is provided in Appendix Table A3.

Abbreviations: LOH, loss of heterozygosity; A-GVGD, align grantham variation, grantham deviation; NA, not applicable; WT, wild-type allele lost; ND, no data; V, variant or mutant allele lost.

Prediction of Deleterious Status

To classify *BRCA* VUS, we developed four weighted models that incorporated allele-specific LOH, tumor histopathology, evolutionary prediction and splicing, and observation with a deleterious mutation (Tables 5 and 6; Appendix Tables A4 and A5). Because LOH may not be as readily available as some of the other parameters used in our models and the frequencies of LOH in tumors with neutral *BRCA* variants have not been well documented, we calculated combined odds with and without LOH status (Tables 5 and 6; Appendix Tables A4 and A5). The combined sensitivity of our four models was 98% for known deleterious mutations (42 of 43 deleterious mutations were predicted as deleterious or suspected deleterious), and the specificity was 76% for previously classified neutral changes (16 of 21 neutral VUS were predicted neutral or suspected neutral).^{4-6,8,9,57-62} The known sequence alterations not classified as deleterious or neutral were all classified as uncertain; none were incorrectly classified. Of previously unclassified variants, 64% (21 of 33 variants) were classified as neutral by our models.

DISCUSSION

We developed four models for the prediction of *BRCA* VUS that do not require family history information or segregation analysis. To our

knowledge, we describe the first models for the characterization of *BRCA* VUS that use ovarian tumor histopathology to characterize VUS. Using these models, we characterized 21 *BRCA* VUS as neutral. Of known deleterious mutations, none were incorrectly classified as neutral, and of predicted neutral variants, none were incorrectly classified as deleterious. For known or predicted deleterious mutations, our models showed 98% sensitivity. No VUS in this study were predicted to be deleterious.

In our study, one known deleterious truncating *BRCA1* mutation was not classified as deleterious or suspected deleterious. The tumor sample was from a 75-year-old woman. It did not show LOH and had pathologic characteristics (estrogen receptor [ER] positive and progesterone receptor [PR] positive) more typical of a non-*BRCA1*, sporadic tumor observed in women of this age. It is possible that this tumor is a phenocopy and not a result of the patient's inherited *BRCA1* mutation.

A few VUS, such as *BRCA2* IVS13+5 G>C and *BRCA1* L1764P, showed odds of greater than 20:1 in favor of being deleterious and warrant additional studies for characterization. L1764P has shown suggestive odds for being deleterious in other studies.^{4,58} A number of VUS were close to a cutoff of 100:1 in favor of a neutral classification. These are likely to be neutral; however, further studies are needed to clarify their status.

Table 5. Examples of Breast Tumor Odds

Sequence Change	Odds												Odds	Odds, No LOH	Interpretation	
	LOH	A-GVGD/ Mutation	Splice	In Trans	Age at Diagnosis	Triple Negative	ER Status	PR Status	HER-2	Grade/ER	Grade	HP				
<i>BRCA1</i>																
1294del40	5.26	1,000	1	1	3.4	1	1	2.29	0.15	4.1	1	1	2.5×10^4	4,788	D	
IVS13+1 G>A	0.285	1	1,000	1	3.4	5	1	1	1	1	0.55	1	2,665	9,350	D*	
C61G	5.26	4.26	1	1	15.3	5	1	1	1	1	1.97	1	3,377	642	DS*	
Y856H	0.285	0.01	1	0.0001	3.4	1	0.23	0.31	0.15	1	1.97	1	2×10^{-8}	7×10^{-8}	N	
<i>BRCA2</i>																
2041delA	0.428	1,000	1	1	4.97	1	1	1	1.2	2.2	1	1	5,616	1.3×10^4	D	
IVS8-12delTA	0.428	1	0.01	1	2.89	1	1	1	1.2	0.23	1	1	0.003	0.008	N	
N517S	0.428	0.01	1	1	2.89	1	1	1	1	0.23	1	1	0.003	0.007	N	
L2106P	0.428	0.01	1	1	2.07	1	1	1	1.2	2.2	1	1	0.023	0.054	U	

NOTE. Examples of odds generated from phenotypic data of Table 3 are shown. The final odds are calculated by combining odds for each of the independent variables. The full list of odds for each sequence variation and tumor studied is provided in Appendix Table A4.

Abbreviations: LOH, loss of heterozygosity; A-GVGD, align grantham variation, grantham deviation; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; HP, histopathology; D, deleterious; DS, suspected deleterious; N, neutral; U, uncertain.

*Reclassified as deleterious by other studies.

Table 6. Examples of Ovarian Tumor Odds

Gene	Change	Odds							Combined Odds	Odds, No LOH	Interpretation
		LOH	A-GVGD/ Mutation	In Trans	Stage	Grade	Histology	Age			
<i>BRCA1</i>	1135insA*	4.45	1,000	1	1	1.33	1.47	11.8	102,662.3	23,070.2	D
<i>BRCA1</i>	P334L	4.45	0.01	0.0001	1	1	1	7.06	0.0000314	0.0000071	N†
<i>BRCA2</i>	7297delCT	0.428	1,000	1	1	1.61	1.76	0.52	630.65	1,473.47	D
<i>BRCA2</i>	M784V	0.067	0.01	1	2.05	1.61	1	4.05	0.009	0.1337	SN
<i>BRCA2</i>	V1643A	4.6	0.01	1	2.05	1	1.76	7.92	1.3145	0.2858	U

NOTE. Examples of odds generated from phenotypes listed in Table 4 are shown. The final odds are calculated by combining odds for each of the independent variables. The full list of odds from all sequence alterations and ovarian tumors studied is provided in Appendix Table A5.

Abbreviations: LOH, loss of heterozygosity; A-GVGD, align grantham variation, grantham deviation; D, deleterious; N, neutral; SN, suspected neutral; U, uncertain.

*Change was also seen in a different individual with breast cancer.

†Reclassified as neutral by other studies.

These models can be readily adapted for use in the clinical setting or for an online clinical prediction tool because all of the weighting factors, except for LOH, are available from mutation and pathology reports, online programs, and published resources. Thus, these models offer a significant advantage over other models currently used to predict VUS status. Recently, a study by the kConFab group proposed a similar model using tumor histopathology as one feature to classify *BRCA* VUS.⁶³ The main differences between our models and the kConFab model are that the kConFab model included segregation analysis, a different protein prediction method, and immunohistochemical staining of basal cytokeratins. Their model also did not evaluate VUS found in ovarian tumors. One advantage of our model over other models is that it does not require segregation analysis or additional laboratory analysis. For example, basal cytokeratin staining is not yet commonly used in many pathology laboratories for breast cancer diagnosis purposes. However, ER, PR, and human epidermal growth factor receptor 2 staining are part of routine practice for breast cancer pathology. In addition, a recent article suggests that, although basal cytokeratins can help to predict *BRCA1* mutation-positive tumors, cytokeratin staining itself was not as good a predictor as age of onset and ER, PR, and human epidermal growth factor receptor 2 status.⁴² Seventy-two percent of the 20 VUS in the kConFab study, including some previously characterized variants, and 64% of the 33 unclassified VUS in our study were classified. Together, these studies demonstrate that inclusion of histopathology can be powerful for the characterization of *BRCA* VUS.

Individuals and their at-risk family members who carry a *BRCA* VUS often delay making decisions about their cancer risk management or make management decisions based on uncertain informa-

tion. These individuals are also not able to take full advantage of prevention and therapeutic strategies typically offered to deleterious mutation carriers. Classification of 21 previously unclassified VUS using the models described here has the potential to help the individuals who carry these variants. Our models should also be useful for the classification of additional *BRCA* VUS in individuals from whom limited family history is available.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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REFERENCES

- Judkins T, Hendrickson BC, Deffenbaugh AM, et al: Single nucleotide polymorphisms in clinical genetic testing: The characterization of the clinical significance of genetic variants and their application in clinical research for *BRCA1*. *Mutat Res* 573:168-179, 2005
- Frank TS, Deffenbaugh AM, Reid JE, et al: Clinical characteristics of individuals with germline mutations in *BRCA1* and *BRCA2*: Analysis of 10,000 individuals. *J Clin Oncol* 20:1480-1490, 2002
- Noll WW, Reid JE, Burbidge LA, et al: Prevalence of *BRCA1* and *BRCA2* mutations is similar in non-Ashkenazi women of difference ethnic back-

grounds undergoing genetic testing. 1st American Society of Clinical Oncology Breast Cancer Symposium, San Francisco, CA, September 7-8, 2007 (abstr 4)

4. Easton DF, Deffenbaugh AM, Pruss D, et al: A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the *BRCA1* and *BRCA2* breast cancer-predisposition genes. *Am J Hum Genet* 81:873-883, 2007

5. Chenevix-Trench G, Healey S, Lakhani S, et al: Genetic and histopathologic evaluation of *BRCA1* and *BRCA2* DNA sequence variants of unknown clinical significance. *Cancer Res* 66:2019-2027, 2006

6. Tavtigian SV, Deffenbaugh AM, Yin L, et al: Comprehensive statistical study of 452 *BRCA1* mis-

sense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 43: 295-305, 2006

7. Goldgar DE, Easton DF, Deffenbaugh AM, et al: Integrated evaluation of DNA sequence variants of unknown clinical significance: Application to *BRCA1* and *BRCA2*. *Am J Hum Genet* 75:535-544, 2004

8. Judkins T, Hendrickson BC, Deffenbaugh AM, et al: Application of embryonic lethal or other obvious phenotypes to characterize the clinical significance of genetic variants found in trans with known deleterious mutations. *Cancer Res* 65: 10096-10103, 2005

9. Wu K, Hinson SR, Ohashi A, et al: Functional evaluation and cancer risk assessment of *BRCA2* unclassified variants. *Cancer Res* 65:417-426, 2005

10. Fleming MA, Potter JD, Ramirez CJ, et al: Understanding missense mutations in the *BRCA1* gene: An evolutionary approach. *Proc Natl Acad Sci U S A* 100:1151-1156, 2003
11. Osorio A, Milne RL, Honrado E, et al: Classification of missense variants of unknown significance in *BRCA1* based on clinical and tumor information. *Hum Mutat* 28:477-485, 2007
12. King MC, Marks JH, Mandell JB, et al: Breast and ovarian cancer risks due to inherited mutations in *BRCA1* and *BRCA2*. *Science* 302:643-646, 2003
13. Weber F, Shen L, Fukino K, et al: Total-genome analysis of *BRCA1/2*-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. *Am J Hum Genet* 78:961-972, 2006
14. Wang C, Horiuchi A, Imai T, et al: Expression of *BRCA1* protein in benign, borderline and malignant epithelial ovarian neoplasms and its relationship to methylation and allelic loss of the *BRCA1* gene. *J Pathol* 202:215-223, 2004
15. Mathe E, Olivier M, Kato S, et al: Computational approaches for predicting the biological effect of p53 missense mutations: A comparison of three sequence analysis based methods. *Nucleic Acids Res* 34:1317-1325, 2006
16. Tavtigian SV, Byrnes GB, Goldgar DE, et al: Classification of rare missense substitutions, using risk surfaces, with genetic- and molecular-epidemiology applications. *Hum Mutat* (in press)
17. Cartegni L, Wang J, Zhu Z, et al: ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res* 31:3568-3571, 2003
18. Houdayer C, Dehainault C, Mattler C, et al: Evaluation of in silico splice tools for decision making in molecular diagnosis. *Hum Mutat* 29:975-982, 2008
19. Katsama A, Sourvinos G, Zachos G, et al: Allelic loss at the *BRCA1*, *BRCA2* and *TP53* loci in human sporadic breast carcinoma. *Cancer Lett* 150:165-170, 2000
20. Janatova M, Zikan M, Dundr P, et al: Novel somatic mutations in the *BRCA1* gene in sporadic breast tumors. *Hum Mutat* 25:319, 2005
21. Meng ZH, Ben Y, Li Z, et al: Aberrations of breast cancer susceptibility genes occur early in sporadic breast tumors and in acquisition of breast epithelial immortalization. *Genes Chromosomes Cancer* 41:214-222, 2004
22. Rio PG, Pernin D, Bay JO, et al: Loss of heterozygosity of *BRCA1*, *BRCA2* and *ATM* genes in sporadic invasive ductal breast carcinoma. *Int J Oncol* 13:849-853, 1998
23. Schmutzler RK, Bierhoff E, Werkhausen T, et al: Genomic deletions in the *BRCA1*, *BRCA2* and *TP53* regions associate with low expression of the estrogen receptor in sporadic breast carcinoma. *Int J Cancer* 74:322-325, 1997
24. Yoshikawa K, Honda K, Inamoto T: Reduction of *BRCA1* protein expression in *BRCA1*-associated cases. *Clin Cancer Res* 5:1249-1261, 1999
25. Osorio A, de la Hoya M, Rodriguez-Lopez R, et al: Loss of heterozygosity analysis at the *BRCA* loci in tumor samples from patients with familial breast cancer. *Int J Cancer* 99:305-309, 2002
26. Cornelis RS, Neuhausen SL, Johansson O, et al: High allele loss rates at 17q12-21 in breast and ovarian tumors from *BRCA1*-linked families: The Breast Cancer Linkage Consortium. *Genes Chromosomes Cancer* 13:203-210, 1995
27. Cleton-Jansen AM, Collins N, Lakhani SR, et al: Loss of heterozygosity in sporadic breast tumors at the *BRCA2* locus on chromosome 13q12-13. *Br J Cancer* 72:1241-1244, 1995
28. Collins N, McManus R, Wooster R, et al: Consistent loss of the wildtype allele in breast cancers from a family linked to the *BRCA2* gene on chromosome 13q12-13. *Oncogene* 10:1673-1675, 1995
29. Gudmundsson J, Johannesdottir G, Bergthorsson JT, et al: Different tumor types from *BRCA2* carriers show wild-type chromosome deletions on 13q12-13. *Cancer Res* 55:4830-4832, 1995
30. Lakhani SR, Gusterson BA, Jacquemier J, et al: The pathology of familial breast cancer: Histological features of cancers in families not attributable to mutations in *BRCA1* or *BRCA2*. *Clin Cancer Res* 6:782-789, 2000
31. Honrado E, Benitez J, Palacios J: Histopathology of *BRCA1*- and *BRCA2*-associated breast cancer. *Crit Rev Oncol Hematol* 59:27-39, 2006
32. Bane AL, Beck JC, Bleiweiss I, et al: *BRCA2* mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. *Am J Surg Pathol* 31:121-128, 2007
33. Haffty BG, Yang Q, Reiss M, et al: Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. *J Clin Oncol* 24:5652-5657, 2006
34. Dent R, Trudeau M, Pritchard KI, et al: Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res* 13:4429-4434, 2007
35. Rakha EA, El-Sayed ME, Green AR, et al: Prognostic markers in triple-negative breast cancer. *Cancer* 109:25-32, 2007
36. Tischkowitz M, Brunet J-S, Begin LR, et al: Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. *BMC Cancer* 7:134, 2007
37. Bauer K, Brown M, Cress RD, et al: Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple negative phenotype. *Cancer* 109:1721-1728, 2007
38. Palacios J, Honrado E, Osorio A, et al: Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to *BRCA1* or *BRCA2* mutations: Differences from breast carcinomas arising in *BRCA1* and *BRCA2* mutation carriers. *Clin Cancer Res* 9:3606-3614, 2003
39. Foulkes WD, Metcalfe K, Sun P, et al: Estrogen receptor status in *BRCA1*- and *BRCA2*-related breast cancer: The influence of age, grade and histological type. *Clin Cancer Res* 10:2029-2034, 2004
40. Owens MA, Horten BC, Da Silva MM: *HER2* amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. *Clin Breast Cancer* 5:63-69, 2004
41. Lakhani SR, van de Vijver MJ, Jacquemier J, et al: The pathology of familial breast cancer: Predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, *HER-2* and p53 in patients with mutations in *BRCA1* and *BRCA2*. *J Clin Oncol* 20:2310-2318, 2002
42. Eerola H, Heinonen M, Kilpivaara O, et al: Basal cytokeratins in breast tumors among *BRCA1*, *BRCA2* and mutation-negative families. *Breast Cancer Res* 10:R17, 2008
43. Armes JE, Trute L, White D, et al: Distinct molecular pathogenesis of early-onset breast cancers in *BRCA1* and *BRCA2* mutation carriers: A population-based study. *Cancer Res* 59:2011-2017, 1999
44. Breast Cancer Linkage Consortium: Pathology of familial breast cancer: Differences between breast cancers of *BRCA1* or *BRCA2* mutations and sporadic cases. *Lancet* 349:1505-1510, 1997
45. Chan KYK, Ozcelik H, Cheung ANY, et al: Epigenetic factors controlling the *BRCA1* and *BRCA2* genes in sporadic ovarian cancer. *Cancer Res* 62:4151-4156, 2002
46. Hilton JL, Geisler JP, Rathe JA, et al: Inactivation of *BRCA1* and *BRCA2* in ovarian cancer. *J Natl Cancer Inst* 94:1396-1406, 2002
47. Tong D, Kucera E, Schuster E, et al: Loss of heterozygosity (LOH) at p53 is correlated with LOH at *BRCA1* and *BRCA2* in various human malignant tumors. *Int J Cancer* 88:319-322, 2000
48. Smith SA, Easton DF, Evans DG, et al: Allele losses in the region 17q13-21 in familial breast and ovarian cancer involve the wildtype chromosome. *Nat Genet* 2:128-131, 1992
49. Kashima K, Oite T, Aoki Y, et al: Screening of *BRCA1* mutation using immunohistochemical staining with C-terminal and N-terminal antibodies in familial ovarian cancers. *Jpn J Cancer Res* 91:399-409, 2000
50. Neuhausen SL, Marshall CJ: Loss of heterozygosity in familial tumors from three *BRCA1*-linked kindreds. *Cancer Res* 54:6069-6072, 1994
51. Gras E, Cortes J, Diez O, et al: Loss of heterozygosity on chromosome 13q12-q14, *BRCA-2* mutations and lack of *BRCA-2* promoter hypermethylation in sporadic epithelial ovarian tumors. *Cancer* 92:787-795, 2001
52. Collins N, Wooster R, Stratton MR: Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene *BRCA2* in normal tissues and in breast and ovarian cancers. *Br J Cancer* 76:1150-1156, 1997
53. Tonin PN, Maugard CM, Perret C, et al: A review of histopathological subtypes of ovarian cancer in *BRCA*-related French Canadian cancer families. *Fam Cancer* 6:491-497, 2007
54. Piek JM, Torrens B, Hermesen B, et al: Histopathological characteristics of *BRCA1*- and *BRCA2*-associated intraperitoneal cancer: A clinic-based study. *Fam Cancer* 2:73-78, 2003
55. Werness BA, Ramus SJ, DiCioccio RA, et al: Histopathology, FIGO stage, and *BRCA* mutation status of ovarian cancers from the Gilda Radner Familial Ovarian Cancer Registry. *Int J Gynecol Pathol* 23:29-34, 2004
56. Lakhani SR, Manek S, Penault-Llorca F, et al: Pathology of ovarian cancer in *BRCA1* and *BRCA2* carriers. *Clin Cancer Res* 10:2473-2481, 2004
57. Tesoriero AA, Wong EM, Jenkins MA, et al: Molecular characterization and cancer risk associated with *BRCA1* and *BRCA2* splice site variants identified in multiple-case breast cancer families. *Hum Mutat* 26:495, 2005
58. Carvalho MA, Marsillac SM, Karchin R, et al: Determination of cancer risk association with germ line *BRCA1* missense variants by functional analysis. *Cancer Res* 67:1494-1501, 2007
59. Wappenschmidt B, Fimmers R, Rhiem K, et al: Strong evidence that the common variant S384F in *BRCA2* has no pathogenic relevance in hereditary breast cancer. *Breast Cancer Res* 7:R775-779, 2005
60. Deffenbaugh AM, Frank TS, Hoffman M, et al: Characterization of common *BRCA1* and *BRCA2* variants. *Genet Test* 6:119-121, 2002
61. Fackenthal JD, Sveen L, Gao Q, et al: Complete allelic analysis of *BRCA1* and *BRCA2* variants in young Nigerian breast cancer patients. *J Med Genet* 42:276-281, 2005

62. National Human Genome Research Institute: Breast Cancer Information Core. <http://research.nhgri.nih.gov/projects/bic/>

63. Spurdle AB, Lakhani SR, Healey S, et al: Clinical classification of BRCA1 and BRCA2 DNA sequence variants: The value of cytokeratin pro-

files and evolutionary analysis—A report from the kConFab Investigators. *J Clin Oncol* 26:1657-1663, 2008

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Acknowledgment

The Acknowledgment is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).