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EPIDEMIOLOGY

Characterization of *BRCA1* ring finger variants of uncertain significance

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Abstract The majority of pathogenic mutations in *BRCA1* result in a truncated protein. Although most missense changes in *BRCA1* are of unknown functional significance, a handful of deleterious missense mutations have been identified. The majority of these occur in splice sites or highly conserved protein domains. Previously, we developed a predictive model, VUS Predict, to classify *BRCA* variants of uncertain significance as neutral or deleterious. It uses evolutionary prediction algorithms together with clinical information from cancer pathology reports and *BRCA* genetic testing results. Because of the higher probability that missense changes occurring in conserved BRCA1 domains are of pathogenic significance, we identified all individuals in our cohort who had been tested for *BRCA*1 and *BRCA2* mutations who had missense changes in the *BRCA1* ring finger domain and sought to classify those changes. We applied VUS Predict to three previously uncharacterized variants and four missense changes known to be deleterious. Two variants, L22S and T37K, were predicted to be deleterious and one variant, K45Q, was predicted to be neutral by VUS Predict. The mutations C39R, C44Y, C44S, and C61G were confirmed as deleterious.

Keywords *BRCA1* · Variants of uncertain significance · Ring finger domain · Mutation characterization

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Introduction

Mutations in *BRCA1* confer an increased risk of early onset breast and ovarian cancer. Individuals with a mutation in *BRCA1* can increase the likelihood of surviving cancer through more rigorous surveillance and can reduce cancer risk through prophylactic surgery or chemopreventative agents. During the decade that clinical testing for *BRCA1* has been available, hundreds of different sequence alterations have been reported (http://research.nhgri.nih.gov/bic/) [1]. The majority of deleterious *BRCA1* mutations are large deletions, rearrangements, splice-site, frameshift, or nonsense changes that result in a truncated protein. However, a handful of proven deleterious *BRCA1* mutations are missense changes that occur in key conserved protein domains such as the ring finger domain and the breast cancer C-terminal domains (BRCT; [1, 2]). The BRCA1 ring finger domain is critical for the ubiquitin E3 ligase activity of BRCA1 through its binding of partner BARD1. The BRCT domains modulate transcriptional activity [3]. About 7.6% of individuals who have clinical testing in the United States for *BRCA1* or *BRCA2* are found to have a variant of uncertain significance (VUS), a change in the gene whose consequence for cancer risk is unknown [1]; about 35% of VUSs are in *BRCA1*. VUSs in both

BRCA genes have been reclassified as neutral or deleterious using multiple methods that combine data from segregation analyses, co-occurrence rates with deleterious mutations, histopathology, evolutionary protein alignments, and functional studies [4–7].

We developed a predictive algorithm, VUS Predict, to characterize VUSs that utilizes clinically available data from personal history, mutation testing reports, and pathology reports in combination with evolutionary conservation comparisons [8]. Based on previous data suggesting that VUSs in conserved domains are more likely to have functional consequences, we scanned our cohort of individuals who were tested for *BRCA1* mutations to identify any with missense changes in the *BRCA1* ring finger domain. We hypothesized that variants in this domain would have a fairly high likelihood of being pathogenic. We applied VUS Predict to seven missense changes in the *BRCA1* ring finger domain—three uncharacterized VUSs in the *BRCA1* ring finger domain and four deleterious *BRCA1* ring finger domain missense changes.

Materials and methods

Human subjects

Studies were approved by the local institutional review board according to the Helsinki guidelines. All study participants or their next of kin signed informed consent for this research. Study participants were ascertained from individuals having clinical genetic testing of *BRCA1* and *BRCA2* at the Ohio State University Clinical Cancer Genetics Program due to a personal and/or family history of breast and ovarian cancer. Individuals were eligible for this study if they were found to carry a missense change in the *BRCA1* ring finger domain, a copy of their test results was available, and they had a diagnosis of breast or ovarian cancer. BRCAPro risk assessments were done on all probands prior to genetic testing (http://astor.som.jhmi.edu/BayesMendel/brcapro.html).

Information utilized in predictive model

Clinical information utilized for prediction of VUS status using VUS Predict is as described [8] and includes the age of diagnosis of cancer, histopathology, grade, estrogen receptor (ER) status, progesterone receptor (PR) status, and Her2Neu amplification status. For ovarian cancer, grade, stage, and histopathology were used. We used align-grantham variation deviation analysis (A-GVGD) to determine whether missense changes were evolutionarily conserved and therefore likely to have functional consequences (http://agvgd.iarc.fr/alignments/php; [5]). By A-GVGD analysis, mutations in this study fell into two classes, Class 65 (likely deleterious) and Class 0 (likely neutral). Based on prior calculations, these were assigned odds of 4.26 and 0.01 for being deleterious, respectively.

Application of the model

Using VUS Predict, we determined the odds of pathogenicity of each variant using a modified multifactorial approach that combines the odds of causation of independent variables [8]. We included odds for evolutionary conservation, age, and tumor characteristics. As no missense changes in this study were observed with a deleterious mutation, we did not incorporate these odds into our calculations. Odds for each feature are described previously [1, 8]. On the basis of previous studies, we used an odds cutoff of 1,000:1 in favor of deleterious to definitively call a variant a pathogenic mutation, a cutoff of 100:1 in favor of deleterious to call a variant likely pathogenic and a cutoff of 1:100 to call a variant neutral. When combining data from multiple individuals or tumors with the same variant, only independent variables were used. For each variant, the youngest age at diagnosis of breast or ovarian cancer was used.

Results

Since much of the ring finger domain in the *BRCA1* gene is highly conserved and is known to contain deleterious mutations, we hypothesized that a VUS mapping to this domain had a high probability of being deleterious. To study this further, we scanned our database of individuals who had undergone clinical genetic testing of *BRCA1* and *BRCA2* in order to classify these for pathogenicity. We identified three individuals in our cohort who were found to carry missense changes of uncertain significance in the *BRCA1* ring finger domain (amino acids 1–102). These VUSs included L22S (184T > C), T37K (229C > A), and K45Q (252A > C). To serve as controls, we scanned our patient population for individuals with known neutral or known deleterious changes in the BRCA1 ring finger domain. We identified one individual each with the C39R (234T > C), C44Y (250G > A), and C44S (249T > A) mutations and four individuals from two families with the C61G (300T > G) mutation.

We obtained available medical records on cancer diagnoses, genetic testing reports, and family history information from these ten individuals (Table 1). We utilized these data in VUS Predict, a VUS predictive algorithm that we previously developed, and compared our results to those from the five individuals with deleterious ring finger domain mutations (Table 2) [8].

Table 1 Clinical information from individuals on study

Individual	Mutation	Age	Histopathology	ER	PR	Her2	Grade	BRCAPRO	A-GVGD	Fam Hx:
45683	L22S	Br = 38	ND	ND	ND	ND	ND	49.7%	Class 65	M. Aunt: Br 40's
		Ov = 48	ND	N/A	N/A	N/A	ND			
183040	T37K	Br = 27	Invasive ductal	Neg	Neg	Neg	3	99.7%	Class 65	P. Aunt: Ov 59
		Br = 33	Invasive ductal	Neg	Neg	Neg	3			P Cou: Ov 38;
										P. GM: Ov 80;
										M. Aunt: Br 40
132	C39R	Br = 35	Intraductal	Neg	Neg	ND	3	42%	Class 65	Limited structure
		Br = 35	Invasive ductal	Neg	Neg	ND	3			
		Br = 38	Invasive ductal	Neg	Neg	ND	3			
		Br = 38	Invasive ductal	Pos	Neg	ND	2/3			
159546	C44S	Ov = 43	Papillary serous	N/A	N/A	N/A	ND	95.7%	Class 65	Mother: Br 47;
										M. Aunt Br 30's,
										M. Aunt Ov 40's;
										M. Aunt Br 45
33200	C44Y	Br = 41	Invasive ductal with DCIS	Neg	Neg	Neg	3	87%	Class 65	Mother: Br 32, 52;
										M GM Br 46, 47;
										M. Aunt: St or Ov 70's
169479	K45Q	Br = 60	Invasive ductal	Pos	Pos	Neg	2	5%	Class 0	Father Br 74
		Br = 60	Lobular	Pos	Neg	Neg	2			Mother Br 83;
										M. Aunt Br 60, 78
65175	C61G	Br = 32	Invasive ductal	Neg	Neg	Pos	3	91%	Class 65	
73575	C61G	Br = 25	ND	ND	ND	ND	ND	N/A	Class 65	
67367	C61G	Br = 38	Invasive ductal	Neg	Neg	ND	3	N/A	Class 65	
19882	C61G	Br = 29	Medullary	Neg	Neg	ND	1	ND	Class 65	Brother Pr 50
		Br = 48	Invasive ductal	Neg	Neg	Neg	3			P. Aunt Br 34
										P. Cousin Br 30's

Age, age of cancer diagnosis; ER, estrogen receptor status; PR, progesterone receptor status; Her2, Her2Neu amplification; Br, breast cancer; Ov, ovarian cancer; Pr, prostate cancer; St, stomach cancer; ND, no data; N/A, not applicable; Neg, negative; Pos, positive; DCIS, ductal carcinoma in situ; M., maternal; P, paternal; GM, grandmother; Cou, cousin

Table 2 Odds of pathogenicity

Change	Age Br Ca	Age Ov Ca	A-GVGD	HP	Grade 3	Triple neg	ER status	ER+ grade 2	ER− grade 3	PR status	Her2 status	Odds	Call
L22S	9.65	18	4.26	N/A	ND	ND	ND	ND	ND	ND	ND	740:1	LD
T37K	15.3	N/A	4.26	N/A	3.88[a]	25[a]	N/A	N/A	N/A	N/A	N/A	6,322:1	D
C39S	9.65	N/A	4.26	N/A	1.97	N/A	0.23	N/A	68.9[a]	27.5[a]	0.15	5,294:1	D
C44Y	3.40	N/A	4.26	N/A	1.97	5	N/A	N/A	N/A	N/A	N/A	143:1	LD[b]
C44S	N/A	18	4.26	1.47	ND	N/A	N/A	N/A	N/A	N/A	N/A	113:1	LD[b]
K45Q	1.25	N/A	0.01	N/A	N/A	N/A	N/A	0.14[a]	N/A	0.71[a]	1.44[a]	0.002:1	N
C61G	15.3	N/A	4.26	8.0	1.97	5	3.2	N/A	16.8a	12[a]	0.15	5×10^5:1	D

Age Br Ca, age of breast cancer diagnosis; Age Ov Ca, age of ovarian cancer diagnosis; HP, histopathology; Triple Neg, triple negative (ER, PR, Her2Neu negative); ER+, estrogen receptor positive; ER−, estrogen receptor negative; PR, progesterone receptor status; Her2, Her2Neu amplification status; ND, no data; N/A, not applicable; D, deleterious mutation; LD, likely deleterious; N, neutral

[a] Odds by combining multiple tumors with same feature

[b] Classified previously as deleterious mutation

Unclassified variants

L22S

L22S was identified in a woman of Ashkenazi Jewish ancestry whose tumor block was first tested for the three common Ashkenazi Jewish mutations (185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2*). Testing was negative for the presence of these mutations, but she was subsequently found to carry the L22S mutation. According to the genetic testing report, this mutation has been observed nine times, never with a deleterious mutation, and segregation analyses have not been performed. The L22S variant was observed in one Japanese family in two individuals with early onset breast and breast/ovarian cancers [9]. Our proband was diagnosed with breast cancer at age 38 and ovarian cancer at age 48. By report, her maternal aunt was diagnosed with breast cancer in her 40s. Pretest BRCAPro analysis gave the proband a 49.7% likelihood of having a *BRCA* mutation. We were unable to conduct loss of heterozygosity analysis in this individual as only limited, poor quality tumor DNA was available.

By evolutionary alignment, the L22S amino acid is highly conserved (observed in 13 of 13 species), and A-GVGD assigns the missense change as a class 65 mutation, which is consistent with pathogenicity. If we consider the ovarian and breast cancer as independent variables, VUS Predict assigns an odds of being deleterious as 740:1. This is highly suggestive of a mutation, but does not meet the stringent criteria of 1,000:1 recommended by Easton et al. (2007) [1].

T37K

T37K was identified in a woman of African-American ancestry with bilateral breast cancer diagnosed at ages 27 and 33. Both tumors were grade 3 and estrogen receptor (ER), progesterone receptor (PR), and Her2-neu (Her2) amplification negative (triple negative). She has a strong family history of breast and ovarian cancer including a paternal aunt with ovarian cancer diagnosed at 59 years, a paternal cousin diagnosed with ovarian cancer at 38 years, and a paternal grandmother reported to have breast cancer diagnosed in her 50's. A paternal great grandmother was reported to have ovarian cancer diagnosed in her 80s. There is a possibility that the genetic cause of the cancer seen in the proband is of maternal origin given that she has a maternal half-aunt with breast cancer diagnosed at the age of 40. Segregation analysis has not been possible in this family, so it is not known from which side of the family the T37K change comes. Pretest BRCAPro analysis gave the proband a 99.7% likelihood of having a *BRCA* mutation.

By evolutionary alignment, the T37K position is highly conserved (observed in 13 of 13 species), and A-GVGD assigns a mutation class of 65, which is consistent with a deleterious mutation. Using her age of diagnosis of 27 and the histopathology of each tumor as independent events, VUS Predict assigns her mutation an odds of being deleterious of 6,200:1. This meets the stringent criteria of 1,000:1 odds to classify a mutation as deleterious.

K45Q

K45Q was observed in a woman with bilateral breast cancers, both diagnosed at the age of 60. One breast cancer was of lobular histology. Her family history is significant for a male breast cancer in her father at 74 years, a female breast cancer in her mother at 83 years, and a maternal aunt with bilateral breast cancer diagnosed at 60 and 78 years. Pretest BRCAPro analysis gave the proband a 5% chance of having a *BRCA* mutation.

By single-site mutational analysis, her father was not found to carry the K45Q variant indicating that it comes from the maternal side. According to the genetic testing report, this variant was observed seven times, never with a deleterious mutation. It did not track with cancer in one family tested. By evolutionary conservation, the K45 residue is not conserved, and the K45Q change is assigned a class 0 status, suggesting that it is nonpathogenic. Using histopathology from both tumors, VUS Predict assigns this change an odd of being deleterious of 0.002:1. This meets the criteria for assignment of a variant as neutral.

Deleterious missense changes in the ring finger domain

C39R

C39R was identified in a woman who has been described to have two separate primary breast cancers diagnosed at 35 years, two separate primary breast cancers diagnosed at 38 years, and a melanoma diagnosed at 31 years. Pretest BRCAPro analysis gave the proband a 42% chance of having a *BRCA* mutation. The family history is lacking for *BRCA*-related cancers, although there is limited family structure on the paternal side [10]. By A-GVGD, this mutation is assigned a class 65 (predicted deleterious), consistent with functional data for this mutation [11]. Assuming each of the breast cancers represents a new primary, VUS Predict assigns an odds of 5,294:1 in favor of deleterious.

C44S and C44Y

Two individuals with deleterious mutations at the C44 position were identified. The proband with the C44Y mutation had a personal and family history consistent with a *BRCA* mutation including a diagnosis of breast cancer at age 41, a mother with bilateral breast cancers diagnosed at ages 32 and 52, a maternal grandmother with bilateral breast cancer diagnosed at age 46 and 47, and a maternal aunt with stomach or ovarian cancer diagnosed in her 70s. Her breast cancer was ER−, PR−, Her2−, and high grade, all features observed at much higher incidence in individuals with a *BRCA1* germline mutation [12]. Pretest BRCAPro analysis gave the proband an 87% likelihood of having a *BRCA* mutation. The proband with the C44S mutation had a diagnosis of papillary serous ovarian cancer at 44 years. By report, her family history is significant for a mother diagnosed with breast cancer at 47 years, twin maternal aunts, one with breast cancer in her 30s and one with ovarian cancer diagnosed in her 40s, and a third maternal aunt with breast cancer diagnosed at 45 years. Pretest BRCAPro analysis gave her a 95.7% chance of having a *BRCA* mutation.

Both C44S and C44Y are assigned a class 65 status by A-GVGD. VUS Predict assigns the C44Y and the C44S missense changes odds of 143:1 and 113:1 in favor of deleterious, respectively. Inclusion of additional information from family members would likely improve prediction of both variants.

C61G

The C61G mutation was identified in four women from two different families. The proband for one of the C61G families was diagnosed with an ER−, PR−, Her2+, grade 3 breast carcinoma at age 32. Her sister who also carries the C61G mutation had an ER−, PR−, grade 3 breast tumor diagnosed at 38, and her daughter had a breast cancer diagnosed at the age of 25. BRCAPro analysis gave the proband a 91% likelihood of having a *BRCA* mutation. The proband from a second family was diagnosed with bilateral breast cancers at 29 and 48 years of age. Her first cancer was ER− and PR−, and her second breast cancer was a grade 3, triple negative, medullary carcinoma. The proband's brother was diagnosed with prostate cancer at 50 years of age. She also had a paternal aunt diagnosed with breast cancer at age 34 and a paternal first cousin diagnosed with breast cancer in her 30s. By A-GVGD, this mutation is assigned a class 65 which fits with functional data generated by others [11, 13–15]. Based on the large number of individuals with *BRCA1*-like tumor histopathology, VUS Predict generates an odd of 497,000:1 in favor of being deleterious.

Family history method to predict variant status

As there are a number of available models to predict variant status, we wished to utilize a second model to establish the reproducibility of our results. Gomez Garcia et al. (2009) [16] developed a logistic regression model to predict *BRCA1* variant status primarily based on family history information. Specifically, the model utilizes the BRCAPro score, the total number of ovarian tumors, the age at diagnosis of the proband, and the interaction between BRCAPro and the age at diagnosis. A cutoff point was provided based on the specificity analysis [minimized the receiver operation characteristic (ROC) distance]. In the case of a variant within a single family, if the predicted probability is above the cutoff point of 0.675, the variant is classified as being deleterious. For the three uncharacterized missense changes in our study, we calculated the probability of being deleterious from the family history logistic regression model built by Gomez Garcia et al. (2009) [16] and compared to the cutoff point (0.675). From this model, L22S and T37K were given high probabilities (99.7 and 100%, respectively) of being deleterious, and the K45Q variant was given a low

Table 3 Confirmation of results by family history prediction method

Change	BRCAPro score	# Ovarian tumors	Dx age	Predicted probability of being deleterious	Classification[a]
L22S	0.497	1	38	0.9993	Deleterious
T37K	0.997	3	27	1.0000	Deleterious
K45Q	0.05	0	60	0.1135	Neutral

#, Number; Dx Age, Age proband was first diagnosed with cancer

[a] Classification using the Gomez Garcia model as described [16]

probability (11%) of being deleterious (Table 3). Thus, an independent prediction tool confirms the results of our model VUS Predict.

Discussion

As missense changes in the *BRCA1* ring finger domain have a higher likelihood of being deleterious than mutations in other regions of this gene, we tested seven missense changes mapping to this domain using VUS Predict, a model developed to classify *BRCA* variants of uncertain significance. Three of the changes were classified by the testing laboratories as variants of unknown significance, and four were previously predicted to be deleterious. We found with our model and the model by Gomez Garcia et al. that the L22S and the T37K variants are likely to be pathogenic. We also classified the K45Q variant as neutral by both models. In our study, the known mutations serving as positive controls were confirmed as likely deleterious or deleterious.

A comparison of some of the clinical features such as family history and BRCAPro pretest analyses revealed striking differences between probands with deleterious changes and likely neutral changes. The families with the C44S, C44Y, and C61G mutations have strong histories of early onset and bilateral breast cancers classic for individuals with known *BRCA1* mutations [17]. The woman with the previously unclassified T37K mutation has a very strong family history of early onset breast and ovarian cancer. Conversely, the woman who was found to have a neutral *BRCA1* change, K45Q, also had some relatives with *BRCA*-like cancers, but most were of later ages of onset. Limited family history was available for the proband with the L22S mutation. However, the L22S mutation was observed in one Japanese family whose proband had breast cancer diagnosed at 45 years of age and another relative with the variant had breast and ovarian cancer diagnosed at 39 and 40 years of age, respectively [9]. This family history is consistent with our classification of L22S as deleterious. These data suggest that the family history, even in the absence of mutation testing, may be another feature that could be added to predictive models as has been recently described [16].

A number of *BRCA1* deleterious missense mutations have been identified in the ring finger domain. These include a number of changes in highly conserved cysteines at positions 39, 44, 47, 61, and 64 [15]. The ring finger domain of *BRCA1* is characterized by eight highly conserved cysteine and histidine residues in a C3HC4 motif [18]. This region is essential for ubiquitin protein ligase E3 (E3) activity likely through binding to its partner BARD1 [14]. Functional studies of the C61G mutation show that it disrupts binding of a Zn^{2+} binding site of the RING domain, which results in inactivation of *BRCA1* E3 activity [13–15].

Several groups have developed different in vitro functional assays to measure BRCA1 activity. As BRCA1 has many roles and functions, it can be difficult to determine which in vitro functional assays are most predictive of mutation status. Despite the complexity, results from Ubiquitin E3 ligase activity/BARD1 binding studies are thought to be the most robust in prediction of status of missense changes in the ring finger domain [19]. Morris et al. (2006) assessed 44 ring finger variants for BARD1 binding, E2 binding and E3 ligase activity [11]. Based on those studies, the C39R, C44F, and the C61G known mutations were confirmed to not bind BARD1 or E2, nor have E3 ligase activity. Two changes at position K45 (K45N and K45T) were tested; both were predicted to be deleterious based on lack of or reduced U2 binding and E3 activity. The K45T change is assigned a Class 25 by A-GVGD rating indicating possible functional consequence attributed to this change, unlike the K45Q variant in our patient. Interestingly, an N at the equivalent position to 45 is observed in other species (A-GVGD), indicating that this method of prediction may not be perfect. A T37R missense change was assessed functionally. The change retained BARD1 binding, but did not show E2 binding or E3 ligase activity and was predicted to be deleterious. Like the T37K variant, the T37R variant is predicted to be deleterious by evolutionary conservation studies. The L22S variant was not tested functionally, nor do we have LOH data that would provide additional insight into the pathogenicity of

the variant. These data indicate that although BRCA1 functional studies may provide some insight into the pathogenesis of missense changes, they should not be the sole factor in determining pathogenicity.

In conclusion, we used a predictive model, VUS Predict, to assess the clinical significance of missense changes in the *BRCA1* ring finger domain. Two of three unclassified *BRCA1* ring finger missense variants in our study are likely pathogenic. This information has immediate clinical relevance for cancer prevention and decision making for family members or other carriers of these rare changes.

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