

Genetic and Histopathologic Evaluation of *BRCA1* and *BRCA2* DNA Sequence Variants of Unknown Clinical Significance

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Abstract

Classification of rare missense variants as neutral or disease causing is a challenge and has important implications for genetic counseling. A multifactorial likelihood model for classification of unclassified variants in *BRCA1* and *BRCA2* has previously been developed, which uses data on co-occurrence of the unclassified variant with pathogenic mutations in the same gene, cosegregation of the unclassified variant with affected status, and Grantham analysis of the fit between the missense substitution and the evolutionary range of variation observed at its position in the protein. We have further developed this model to take into account relevant features of *BRCA1*- and *BRCA2*-associated tumors, such as the characteristic histopathology and immunochemical profiles associated with pathogenic mutations in *BRCA1*, and the fact that ~80% of tumors from *BRCA1* and *BRCA2* carriers undergo inactivation of the wild-type allele by loss of heterozygosity. We examined 10 *BRCA1* and 15 *BRCA2* unclassified variants identified in Australian, multiple-case breast cancer families. By a combination of genetic, *in silico*, and histopathologic analyses, we were able to classify one *BRCA1* variant as pathogenic and six *BRCA1* and seven *BRCA2* variants as neutral. Five of these neutral variants were also found in at least 1 of 180 healthy controls, suggesting that screening a large number of appropriate controls might be a useful adjunct to other methods for evaluation of unclassified variants. (Cancer Res 2006; 66(4): 2019-27)

Introduction

Approximately 7% of breast cancer and 10% of ovarian cancer are hereditary (1). The majority (~84%) of hereditary breast and ovarian cancer results from inherited mutations in *BRCA1* (MIM 113705) and *BRCA2* (MIM 600185; ref. 2). The pathogenicity of many changes in *BRCA1* and *BRCA2* and other disease-associated

genes is easily predicted from the nature of the mutation. Large deletions and frame shifts that cause loss of important functional domains or result in unstable transcripts can be classified with reasonable confidence as loss-of-function mutations. However, classification of rare missense changes can present a challenge. According to the Breast Cancer Information Core database (<http://research.nhgri.nih.gov/projects/bic/>), approximately half of the unique *BRCA1* and *BRCA2* variants detected (excluding common polymorphisms) are protein-truncating or known deleterious missense mutations, whereas the remaining are missense variants of unknown pathogenic potential, termed “unclassified variants.” However, this statistic does not take into account the frequency with which these variants are found in a given population undergoing testing. In testing done on a very large population of probands ($N = 60,000$) by Myriad Genetic Laboratories, Inc., only 8.7% of these individuals received a “variant of uncertain clinical significance” result due to a missense variant. “Unclassified variants” of *BRCA1* or *BRCA2* have been found in 20% (72 of 358) of breast cancer families ascertained through the Kathleen Cuninghame Consortium for Research into Familial Breast Cancer (kConFab) that carry a variant (excluding known polymorphisms) in *BRCA1* or *BRCA2*. However, the fraction of individuals receiving results of “uncertain clinical significance” is decreasing over time as more scientific information emerges. An extensive ongoing program of reclassification of such variants by Myriad Genetic Laboratories and other groups is providing important information that is used in the clinical management of individuals carrying these variants. Given that accurate prediction of pathogenicity is clearly important in genetic counseling of individuals carrying these missense changes, there is a growing interest in developing additional efficient and reliable ways to classify unclassified variants.

A variety of approaches for classification of *BRCA1* and *BRCA2* amino acid substitution variants has been reported (3). These include family studies analyzing patterns of cosegregation of unclassified variants with disease (4); loss of heterozygosity (LOH) in tumors (5), where loss of the variant allele suggests that the unclassified variant is not pathogenic, whereas loss of the wild-type allele particularly or no loss is consistent with pathogenicity; the frequency of occurrence in unaffected controls (6, 7), where variants detected in unaffected controls are considered likely benign; *in silico* predictions based on the conservation, position, and nature of the amino acid change (8–13), the validity of which

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relies on availability of gene sequence from other species; and an increasing number of biochemical and functional assays, including assessment of transcription, translation, and protein stability (14–19), as well as mouse models (20). In addition, there are reports of splicing defects associated with nucleotide substitutions outside of conserved splice regions in *BRCA1* exons (20, 21). These are presumed to disrupt exonic splice enhancers (ESE), sequences embedded within exons that promote and regulate the splicing of the transcript in which they are located. ESE prediction software is available and can be used to try to identify substitutions that may alter ESE function. No single functional assay, or set of assays, has thus far been shown to predict pathogenicity with absolute certainty, and *in silico* predictions are largely unverified. Until recently, classification was dependent largely on genetic approaches and was thus hampered by the rarity of most unclassified variants and the poor availability of material from family members outside of research consortia, such as the kConFab.

An integrated approach to classification of unclassified variants in *BRCA1* and *BRCA2* into high-risk mutations and neutral variants was recently developed by Goldgar et al. (22). This multifactorial likelihood model used data on co-occurrence of the unclassified variant with pathogenic mutations in the same gene (under the assumption that homozygotes for pathogenic variants are embryonic lethal in the case of *BRCA1* and are either lethal or produce a recognizable phenotype of Fanconi anemia in the case of *BRCA2*), cosegregation of the unclassified variant with affected status, the Grantham Matrix Score, and information on the evolutionary conservation of the amino acid. Subsequently, the Grantham analysis component of the model was modified to take better account of the fit between the missense substitution and the evolutionary range of variation observed at its position in the protein (13). The model was used to estimate the odds of causality, a ratio of the likelihood of the observed data under the hypothesis of causality to that under the hypothesis of neutrality. Unclassified variants with overall odds of causality of >1,000:1 were considered pathogenic, and those with odds of causality of <1:100 were considered to be neutral, although the choice of these thresholds is somewhat arbitrary and may depend on the particular clinical or research context. However, this model did not take into account relevant features of *BRCA1*- and *BRCA2*-associated tumors, such as the characteristic histopathology and immunochemical profiles associated with pathogenic mutations in *BRCA1* (23–25), nor the fact that ~80% of tumors from *BRCA1* and *BRCA2* carriers undergo inactivation of the wild-type allele by LOH (26, 27). It is likely that extension of this model to include this information will facilitate classification of missense variants of *BRCA1* and *BRCA2*.

The purpose of our study was therefore to examine a number of *BRCA1* and *BRCA2* unclassified variants identified in Australian, multiple-case breast cancer families and to classify them as pathogenic or neutral using an enhanced version of the model previously developed by Goldgar et al. (22), which takes into account the joint effects of histopathology and immunohistochemistry and inactivation of the wild-type or variant allele by LOH.

Materials and Methods

Ascertainment of pedigrees with unclassified variants. Pedigrees with unclassified variants in *BRCA1* and *BRCA2* were ascertained by the kConFab according to eligibility criteria established by the organization (<http://www.kconfab.org/epidemiology/eligibility.asp>). The kConFab currently defines as pathogenic all missense variants that are well charac-

terized in family studies of multigenerations, are not described in the literature in controls, result in a nonconservative amino acid substitution, and occur in a functional domain. Missense variants that do not fulfill all these criteria are called “unclassified variants” by the kConFab. Some unclassified variants were identified in more than one kConFab pedigree, and some pedigrees carried more than one unclassified variant. In addition, we analyzed two pedigrees from Greece with *BRCA1* 5331 G > A (G1738R). Analysis of *BRCA1* and *BRCA2* was carried out by sequencing ($n = 29$), chemical cleavage of mismatch ($n = 1$), or denaturing high-performance liquid chromatography (DHPLC; $n = 3$) in the index cases from each pedigree.

***In silico* analyses.** Extensive protein multiple sequence alignments were made with the online alignment engines Toffee and 3Dcoffee (28). The *BRCA1* alignment contained 13 full-length sequences, the most divergent of which was from the tunicate *Ciona intestinalis*; the *BRCA2* alignment contained six full-length sequences, the most divergent of which was from the pufferfish, *Tetraodon nigroviridis*. The degree of sequence variation present overall and at each position in the alignment was used to calculate the number of positions that are under strong functional constraint or not (9), and the likelihood ratio for whether a substitution at any particular position will be deleterious or not (13, 22). The Grantham variation (GV), which is a quantitative measure of sequence variation within a protein multiple sequence alignment, was calculated for each position in the alignments. Similarly, the Grantham deviation (GD), which is a quantitative measure of the fit between a missense substitution and variation observed at its position in a protein multiple sequence alignment, was calculated for each missense substitution (13). The alignment-based likelihood expression, GV, and GD were combined as follows. If, in the alignment from human to frog, the position of interest is variable ($GV > 0$) and the missense substitution falls within that range of variation ($GD = 0$), then the substitution is a member of an alignment-GVGD (A-GVGD) set of substitutions that is enriched for neutral variants and we consider the alignment likelihood expression “valid, enriched neutral.” If, in the alignment from human to pufferfish, the position of interest shows only conservative sequence variation ($GV < 62$) and the missense substitution falls outside that range of variation ($GD > 0$), then the substitution is a member of an A-GVGD set of substitutions that is enriched for deleterious variants and we consider the alignment likelihood expression “valid, enriched deleterious.” These two conditions define a nonoverlapping set of sequence variants. For substitutions falling into the gap between these conditions ($GV \geq 62$, $GD > 0$), we consider the analysis to be uninformative (A-GVGD = not valid); therefore, there is no contribution from sequence conservation to the overall multifactorial likelihood for these variants.

The variant sequences were also screened by SpliceSiteFinder to determine whether any unclassified variant altered or created a consensus splice site (<http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html>) and by ESEfinder (<http://rulai.cshl.edu/tools/ESE/>) to determine whether the unclassified variant is likely to affect an ESE (29). For ESE analysis, default threshold values of 2 for SR protein ASF and 3 for the other SR proteins were used.

Protein modeling. Molecular modeling was carried out on an SGI work station using the Insight II software package (Accelrys, San Diego, CA). Modeling was carried out on the crystal structure of the ring domain (1JM7.pdb; ref. 30) and BRCT repeat region of *BRCA1* (1JNX.pdb; ref. 31).

Frequency of the variants and coexistence with pathogenic mutations of *BRCA1* and *BRCA2*. For each variant identified in the kConFab resource, we queried the Myriad Genetic Laboratories database of ~60,000 full-sequence tests to determine the number of times that variant was observed, and the number of times that variant was observed in an individual who also was found to have a known deleterious mutation in the same gene. In particular, we noted the number of *different* deleterious mutations that were observed to co-occur with each variant because it is important to determine the number of times the unclassified variant is seen *in trans* with a deleterious mutation. Without direct long-range molecular haplotyping, or segregation data, this cannot be known with certainty. Because this information was only available in a limited number of cases, in the absence of these data, we made the assumption that if an unclassified

variant is seen with n different deleterious variants, that at least $n - 1$ of these are *in trans*. Note that this implies that if a variant is observed a single time in an individual with a deleterious mutation, we essentially assume that it is *in cis* with the variant. As in Goldgar et al. (22), we assumed a frequency of two deleterious mutations occurring *in trans* in the same individual of 0.0001 in *BRCA1* and 0.001 in *BRCA2*.

DHPLC analysis of controls and family members. Unaffected females over the age of 45 with no family history of breast cancer ($n = 180$), ascertained by the Australian Breast Cancer Family Study (ABCFS; ref. 32) were screened by DHPLC for all the unclassified variants, using DNA from a known carrier as a positive control. The underlying sequence changes for all variant peaks were identified by sequencing. DHPLC was also used to screen genomic DNA samples, extracted from blood, from the kConFab for the relevant unclassified variants from all available relatives of the index case with reamplification and sequencing to clarify genotypes where DHPLC genotype was equivocal. PCR primers were designed for DHPLC using the web based program, Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Table 1). Population control samples that produced a profile similar to the positive variant control, an aberrant shift in retention time, or unusual peak shape were reamplified for sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 377 sequencer.

Causality analysis. Bayes factor analysis was done by computing the likelihood ratio of the pedigree and genotype data under causality compared with neutrality using the approach described in Thompson et al. (4). For causality, the assumed age- and site-specific penetrance values

for *BRCA1* and *BRCA2* were those estimated from the meta-analysis of 22 population-based studies by Antoniou et al. (33). For families in which both a *BRCA1* pathogenic mutation and a *BRCA2* unclassified variant were segregating, we did the Bayes factor analysis through use of two-locus linkage analysis as implemented in the program TMLINK (34) to model the disease phenotype in terms of the two underlying disease genes and the genotypes of the two sequence variants.

LOH analysis at the BRCA1 and BRCA2 loci. Tumor blocks were available from the kConFab for some family members for LOH analysis. One slide stained with H&E was reviewed by a pathologist, and material from an adjacent section containing at least 70% tumor cells was then collected for DNA isolation (35). PCR, using primers that spanned the relevant unclassified variant, and subsequent sequence analysis were then done as described above. In some cases, we were unable to amplify the tumor DNA using the original primer pair; thus, primers were redesigned to reduce the size of the PCR product (Table 1). LOH was scored by the absence of the heterozygous peak seen in the germ line control. To use the LOH data in our assessment of causality, we assumed that LOH for *BRCA1* and *BRCA2* occurs in 30% of sporadic tumors (36, 37) compared with 80% of *BRCA1/BRCA2* tumors (5, 27, 38, 39). Among *BRCA1/BRCA2* tumors that exhibit LOH, we assumed further that almost all (79 of 80; refs. 5, 26, 27, 38, 40) lose the wild-type allele, whereas the allele loss is random in non-*BRCA* tumors with LOH. Thus, a tumor from an unclassified variant carrier that shows loss of the variant allele contributes odds of 15:1 against causality of this variant.

Histopathology. Available tumor sections from each of the pedigrees were analyzed for *BRCA1*-like histology by a pathologist (S.L., P.W., or M.C.)

Table 1. Primer sequences for DHPLC and sequencing

Variant	Forward sequence (5' > 3')	Reverse sequence (5' > 3')	Melt temperature (°C)
DHPLC primers			
BRCA1 544 C > A (P142H)	GGGTTTCTCTTGTTTCTTTGA	CAAATGGTTTTACCAAGGAAGG	55
BRCA1 4719 G > A (V1534M)	GCTGCCAGCAAGTATGATT	TGTTTCCGTCAAATCGTGTG	58 and 59
BRCA1 1225 del 3 (D369del)	GGACTCCCAGCACAGAAAAA	TCCCATCATGTGAGTCATC	57
BRCA1 1605 C > T (R496C)	TGGGAAAACCTATCGGAAGA	CCGTTTGGTTAGTTCCTGA	57
BRCA1 2531 G > C (Q804H),	GGCACTCAGGAAAGTATCTCG	AATGACTGGCGCTTTGAAAC	58
BRCA1 2640 C > T (R841W)			
BRCA1 3582 G > C (D1155H)	CCTGGAAGTAATTGTAAGCATCC	GCTTTTGCTAAAAACAGCAGA	56
BRCA1 5215 G > A (R1699Q),	GAGGCTCTTTAGCTTCTTAGGAC	CCAGCATCACCAGCTTATCT	56
BRCA1 5242 C > T (A1708V)			
BRCA1 5331 G > A (G1738R)	TGACGTGTCTGCTCCACTTC	CAGAGTGGTGGGGTGAGATT	59
BRCA2 353 A > G (Y42C)	TCTTAACTGTTCTGGGTCA	TGGTTTCCTTTGTGGAGTTT	54
BRCA2 1379 C > T (S384F)	AGCAAACGCTGATGAATGTG	CCATCTGGGCTCCATTAGA	57
BRCA2 2834 C > T (S869L)	GCTGTGCCCACCTGAAAAAT	TCAAGTCTGTTTCATGAAGTTCC	54
BRCA2 4021 T > A (C1265S)	TGAAGCTCTGCAAAAAGCTG	TTTCTTCAACAAAAGTGCCAGT	54
BRCA2 6173 G > C (S1982T)	TCACCTTGATGTTAGTTTGGGA	TGGAACCTTGCTTTCCACTTG	56
BRCA2 7049 G > T (G2274V)	TGGAAGATGATGAACTGACAGA	TTGTTAGCATACCAAGTCTACTGAA	56
BRCA2 7181G > A (R2318Q)	CCGTACATTTCACTGAAAATTG	TAAAAACGAGACTTTTCTCATACTGT	54
BRCA2 8204G > C (R2659T)	ATGGAAACTGGCAGCTATGG	CAACTGGCTTGTGCAACATT	57
BRCA2 8216 A > T (E2663V),	TCCTAGCTACAAAAATTTTAATTCTCA	TTTGCCTCAATGAAATTATGT	55
BRCA2 8222 A > G (D2665G),			
BRCA2 8237 C > T (S2670L)			
BRCA2 8395 G > C (D2723H),	TGCAAAAACACTTGTCTCTGTG	CAGTACATCTAAGAAATTGAGCATCC	58
BRCA2 8536 G > A (A2770T)			
BRCA2 8714 A > G (del exon 19)	TCCTGACCCTAGACCTTTTCC	GCAAGAGACCGAAACTCCAT	56
BRCA2 8795 A > C (E2856A)	TGCCCTGGCCTGATACAATTA	TGTCCTTGTGTCTATTCTTTG	57
Sequencing primers			
BRCA1 5215 G > A (R1699Q),	GAGGCTCTTTAGCTTCTTAGGAC	AAACGTTAGGTGTGTAATAATGCAA	
BRCA1 5242 C > T (A1708V)			
BRCA2 8395 G > C (D2723H),	CATTGAGCGCAATATATCTGAAA	CAGGAGAGCCCACCAGTTC	
BRCA2 8536 G > A (A2770T)			

Table 2. Classification of variants in *BRCA1*

<i>BRCA1</i> unclassified variant	Occurrence in 180 ABCFS controls	Frequency in Myriad database (with a deleterious mutation)	Bayes factor (no. families)	Co-occurrence	Sequence conservation	A-GVGD	LOH (no. tumors)
544 C > A (P142H)	0	15 (1)	1.7 (2)	0.0043	0.003	Not valid	0.0055, no LOH (2), loss of UV (1)
1225 del3 (D369del)	0	10 (1)	0.0213 (1)	0.0034	0.003	Not valid	0.0045, loss of UV (2)
1605 C > T (R496C)	0	52 (1)	1.95 (1)	0.0224	0.003	Not valid	1, NA
2531 G > C (Q804H)	0	18 (0)	1.12 (2)	2.244	0.003	Valid, EN	0.001951, no LOH (5)
2640 C > T (R841W)	1	207 (8)	0.59 (2)	<0.0001	0.003	Not valid	0.2860, loss of UV (1)
3582 G > C (D1155H)	0	0	0.0025 (1)	1.0000	0.003	Valid, EN	0.0670, loss of UV (1)
4719 G > A (V1534M)	0	32 (1)	0.0086 (1)	4.0220	0.001	Valid, EN	0.2860, no LOH (1)
5215 G > A (R1699Q)	0	19 (0)	1.04 (1)	2.3470	58	Valid, ED	1.0000 (NA)
5242 C > T (A1708V)	0	1 (0)	0.227 (1)	1.0457	58	Valid, ED	1
5331 G > A (G1738R)	0	4 (0)	20.25 (3)	1.2	58	Valid, ED	27.7729, loss of WT (2)

Abbreviations: UV, unclassified variant; WT, wild type; NA, not applicable; EN, enriched neutral; ED, enriched deleterious.

blinded to mutation status. All the tumors were invasive, except for two cases of ductal carcinoma *in situ*. Sections were scored for variables recognized to be associated with *BRCA1* tumors (23), according to the kConFab criteria developed for the purpose of research. “*BRCA1*-like” phenotype was designated as “medullary” or “atypical medullary carcinoma,” or “ductal/no special type” histologic subtype, with high grade, a high mitotic count (>16 mitotic figures/10 high power fields), and one or more of the following features: >25% pushing margin, confluent necrosis, prominent lymphocytic infiltrate. Estrogen and progesterone receptor (ER and PR) results were available from clinical records for a subset of tumors, and for others, we carried out the ER immunohistochemistry ourselves (24). The specific histopathologic features of grade and ER status (*BRCA1*) and tubule formation (*BRCA2*) were used to estimate likelihood ratios, based on estimates of association with *BRCA1* and *BRCA2* mutation status from Lakhani et al. (25). Specifically, the frequencies of 600 tumors from women carrying a known *BRCA1* mutation diagnosed below age 60 in each of the six possible combinations of ER status (+/–) and grade (1, 2, and 3) were compared with the frequency distribution in 258 age-matched tumors from women with no known *BRCA1* or *BRCA2* mutation. For example, an ER[–] grade 3 tumor from a woman with an unclassified variant provides evidence of ~3:1 in favor of causality for that variant, whereas an ER⁺ grade 1 tumor would provide evidence of nearly 20:1 against causality because *BRCA1* mutation associated tumors are rare in this category compared with control tumors. For tumors where either grade or ER status was missing, the marginal frequencies were used. Unfortunately, *BRCA2* tumors are much more similar to their sporadic counterparts, and neither ER status nor grade offers much predictive power for a *BRCA2* unclassified variant. Only the tubule formation criterion within grade determination was significantly different in *BRCA2* tumors. However, this was only marginally useful; tubule formation scores of 3 were associated only with odds of 1.2:1 in favor of causality, whereas scores of 1 to 2 gave odds of 2:1 against causality.

Splicing analysis. Analysis of potential splicing aberrations were carried out for *BRCA1* 5215 G > A (R1699Q), which was predicted by SpliceSiteFinder to alter splicing, and *BRCA2* 8714 A > G, which was predicted by SpliceSiteFinder and ESEfinder to alter splicing, using *BRCA1* 5242 C > A (A1708V), *BRCA1* 5331 G > A (G1738R), and *BRCA2* 8237 C > T (S2670L) as negative controls. Briefly, following methodology detailed in Tesoriero et al. (41), cDNA synthesized from RNA extracted from wild-type control and variant carrier LCLs was PCR-amplified using primers designed to span

potential aberrations, and products were separated by high-resolution gel electrophoresis, extracted from the gel, cloned into pGEM-T Easy Vector and 24 to 50 clones of the wild-type and any variant band were analyzed by sequencing.

Results

Sixteen kConFab pedigrees were identified with a total of 10 unclassified variants in *BRCA1* (Table 2). Six of the *BRCA1* variants segregated in two pedigrees each. Of these 12 pedigrees, eight were found by the kConFab Central Registry to merge into four fused pedigrees, with some individuals registered in two pedigrees originally. Twenty-one kConFab pedigrees were identified with a total of 15 unclassified variants in *BRCA2* (Table 3). Five of the *BRCA2* variants segregated in two or more pedigrees each, but none of these pedigrees could be merged. Two families (0005.01.003 and 0010.00.006) were segregating two unclassified variants. All the index cases from these pedigrees underwent *BRCA1* and *BRCA2* mutation analysis by sequencing, DHPLC, or chemical cleavage of mismatch, and for two families, this revealed clearly pathogenic *BRCA1* variants in carriers of *BRCA2* unclassified variants, whereas another family had a pathogenic *BRCA2* mutation and an unclassified variant in *BRCA1*. Individuals with both pathogenic *BRCA1* and *BRCA2* mutations have been previously reported (42, 43); thus, these findings do not imply neutrality of the *BRCA2* unclassified variants.

None of the unclassified variants in this study resulted in loss or gain of a consensus splice site, with the exception of the *BRCA2* 8714 A > G variant, which resulted in the deletion of exon 19 (data not shown). The loss of an acceptor site caused by this substitution was also predicted as loss of an SF2/ASF motif by ESEfinder. No abnormal splicing was observed for *BRCA1* 5215 G > A (R1699Q) for which an alternative acceptor site was predicted.

Protein modeling predictions were possible for the three *BRCA1* variants mapping to regions of the protein for which the structure is known (i.e., R1699Q, A1708V, and G1738R). Analyses suggested that the probability of a mutational effect was “conditional” for *BRCA1* R1699Q and “high” for both *BRCA1* A1708V and *BRCA1*

Table 2. Classification of variants in *BRCA1* (Cont'd)

Histopathology (no. tumors)	Odds of causality, genetic data (frequency with a deleterious mutation and segregation data)	Odds of causality, sequence data (sequence conservation and A-GVGD)	Odds of causality, pathology data (LOH and immunohistopathology)	Odds of causality, without pathology data	Combined odds of causality (genetic, sequence, and pathology data)	Model classification
0.0069, not <i>BRCA1</i> -like (2)	0.0073	1.000	<0.0001	0.0073	<0.0001	Neutral
8.7025, not <i>BRCA1</i> -like (1)	<0.0001	1.000	0.0391	<0.0001	<0.0001	Neutral
1, NA	0.04368	1.000	1	0.0437	0.0437	NA
0.0087082, not <i>BRCA1</i> -like (5)	2.51328	0.003	<0.0001	0.00754	<0.0001	Neutral
0.4190, not <i>BRCA1</i> -like (1)	<0.0001	1.000	0.1198	<0.0001	<0.0001	Neutral
0.1390, not <i>BRCA1</i> -like (1)	0.0025	0.003	0.0093	<0.0001	<0.0001	Neutral
0.1390	0.0346	0.001	0.0397	<0.0001	<0.0001	Neutral
1.0000 (NA)	2.4409	58	1.0000	141.571	141.5710	NA
2.9500, <i>BRCA1</i> -like (1)	0.2374	58	2.95	13.7677	40.61467	NA
0.15, <i>BRCA1</i> -like (1), not <i>BRCA1</i> -like (1)	24.30	58	4.1659	1409.4	5871.468	Pathogenic

G1738R. For *BRCA1* A1708V, the valine is a β -substituted amino acid with two methyl groups on the β carbon and is thus likely to cause incompatibility with a bend structure in the helix. For *BRCA1* G1738R, there would probably be insufficient space for the long arginine side chain. For *BRCA1* R1699Q, there would be no space issue because the residue is on the outside of the protein, but the main effect would be to destroy the salt bridge between Arg¹⁶⁹⁹ and Asp¹⁸⁴⁰, the biological significance of which is not clear.

Alignment and Grantham analysis (A-GVGD) indicated that *BRCA1* R1699Q, A1708V, and G1738R fall into an A-GVGD set that is enriched for deleterious substitutions, and that three other substitutions (Q804H, D1155H, and V1534M) fall into a A-GVGD set that is enriched for neutral substitutions. The A-GVGD analyses of P142H, D369del, R496C, and R841W were inconclusive (Table 2). For *BRCA2*, A-GVGD suggested that seven of the variants were in the enriched deleterious group, four were likely to be neutral and three were uninformative.

Most of the unclassified variants seen in our families have been identified at least once previously by the Myriad Genetic Laboratories, but in most cases, not with a pathogenic mutation in the same gene (Tables 2 and 3). However, *BRCA2* S1982T has been found seven times by the Myriad Genetic Laboratories with the same pathogenic mutation in *BRCA2*, with which it is therefore assumed to be *in cis*, and the same pathogenic mutation of *BRCA2* was found in the kConFab carrier of this variant. We therefore did not attempt to classify the *BRCA2* S1982T variant. There were six unclassified variants [*BRCA1* 544 C > A (P142H), *BRCA1* 1225 del3 (D369del), *BRCA1* 1605 C > T (R496C), *BRCA1* 2640 C > T (R841W), *BRCA2* 353 A > G (Y42C), and *BRCA2* 1379 C > T (S384F)], which have been identified by the Myriad Genetic Laboratories *in trans* with a pathogenic mutation in the same gene and are therefore likely to be neutral. Furthermore, a homozygote with *BRCA2* 8795 A > C (E2856A) has been identified, further suggesting that this variant is likely to be neutral.

We screened 180 ABCFS controls for all the unclassified variants and found that four (*BRCA1* R841W, *BRCA2* Y42C, *BRCA2* D2665G, and *BRCA2* E2856A) occurred in 1 of 180 controls. Given a frequency of *BRCA1* and *BRCA2* mutations in the general Caucasian population of <0.1% (44–46), and the large number of different

pathogenic mutations that have been reported in both of these genes, the likelihood of even 1 of 180 controls carrying this variant, if it is pathogenic, is very small. However, in the absence of data on the frequency of the unclassified variant in cases from the same ethnic background and evaluated by the same methodology, it is not possible to integrate these data into the causality estimates. Nevertheless, even in the absence of precise estimates of causality, it would be reasonable to predict that these five unclassified variants (*BRCA1* R841W, *BRCA1* V1534M, *BRCA2* Y42C, *BRCA2* D2665G, and *BRCA2* E2856A) are likely to be neutral based on the control frequencies alone.

From the 33 kConFab pedigrees identified with one or more unclassified variants, 421 family members' germ line DNA samples were available. These were all genotyped for the relevant variants by DHPLC, and those ($n = 69$) occurring in amplicons with known SNPs were confirmed by direct sequencing. In addition, two Greek pedigrees carrying the *BRCA1* G1738R variant were available for analysis with two affected relatives available for genotyping. Bayes factor analysis was used to estimate the likelihood of causality by maximizing the evidence in favor of causality over the hazard ratio (ref. 4; Tables 2 and 3).

We examined 34 tumors from 24 families carrying 23 unclassified variants for loss of the variant or wild-type allele at the relevant (*BRCA1* or *BRCA2*) locus (Tables 2 and 3). Loss of the wild-type allele was seen in three tumors, from carriers of *BRCA1* G1738R and *BRCA2* D2723H. In 12 tumors, the allele carrying the unclassified variant (*BRCA1* P142H, *BRCA1* D369del, *BRCA1* R841W, *BRCA1* D1155H, *BRCA2* S384F, *BRCA2* S869L, *BRCA2* G2274V, *BRCA2* E2663V, *BRCA2* D2665G, *BRCA2* S2670L, and *BRCA2* E2856A) was lost, suggesting that these variants are neutral. In some cases, tumors from women carrying these same variants showed no LOH, either because of stromal contamination, or more likely because the rate of LOH in non-*BRCA* tumors is only around 30%. There were also three tumors in which the wild-type allele was lost, but this could be a reflection of background LOH (36) and a random loss of the wild-type allele and not necessarily infer that the variant allele is pathogenic. A caveat needs to be applied to the rationale for using LOH data in these analyses; that is, that most of the allelic loss data in the literature is derived from families with

Table 3. Classification of variants in *BRCA2*

<i>BRCA2</i> unclassified variant	Occurrence in 180 ABCFS controls	Frequency in Myriad database (with a deleterious mutation)	Bayes factor (no. families)	Co-occurrence	Sequence conservation	A-GVGD	LOH (no. tumors)
353 A > G (Y42C)	1	255 (11)	0.023 (2)	<0.0001	0.190	Not valid	0.08180, no LOH (2)
1379 C > T (S384F)	0	182 (4)	0.0987 (1)	0.2104	0.008	Not valid	0.06700, loss of UV (1)
2834 C > T (S869L)	0	1 (0)	9.43 (1)	1.1560	0.005	Valid, EN	0.01916, no LOH (1), loss of UV (1)
4021 T > A (C1265S)	0	0	0.042 (1)	1.0294	0.008	Valid, EN	0.28600, no LOH (1)
7049 G > T (G2274V)	0	19 (0)	0.215 (1)	2.2490	0.008	Valid, EN	0.01916, loss of UV (1), no LOH (1)
7181 G > A (R2318Q)	0	5 (0)	0.1264 (2)	1.0294	5.000	Valid, ED	0.01916, no LOH (1), loss of UV (1)
8204 G > C (R2659T)	0	1 (0)	14.7 (1)	1.0000	5.000	Valid, ED	0.28600, no LOH (2)
8216 A > T (E2663V)	0	9 (0)	1.0 (1)	1.2250	5.000	Valid, ED	0.06700, loss of UV (1)
8222 A > G (D2665G)	1	29 (1)	0.0021 (1)	1.7330	5.000	Valid, ED	0.06700, loss of UV (1)
8237 C > T (S2670L)	0	8 (1)	2.0 (1)	1.2250	0.190	Not valid	0.06700, loss of UV (1), no LOH (1)
8395 G > C (D2723H)	0	42 (0)	7.4 (2)	3.3720	5.000	Valid, ED	5.27000, loss of WT (1)
8536 G > A (A2770T)	0	0	0.41 (1)	1.0000	5.000	Valid, ED	1.00000 (NA)
8714 A > G (del exon 19)	0	0	39.20 (1)	1.0000	5.000	Valid, ED	0.28600, no LOH (1)
8795 A > C (E2856A)	1	239 (3)	1.05 (2)	1.0640	0.190	Valid, EN	0.00449, loss of UV (2)

Abbreviations: UV, unclassified variant; WT, wild type; NA, not applicable; EN, enriched neutral; ED, enriched deleterious.

truncating mutations of *BRCA1* or *BRCA2*, and it is possible that some pathogenic missense variants act as dominant-negative mutations, so that loss of the wild-type allele is less common in this situation (although loss of the mutant allele would still be unexpected).

We reviewed the histopathology of 15 tumors from carriers of *BRCA1* unclassified variants, as well as eight tumors from carriers of *BRCA2* unclassified variants as controls (Tables 2 and 3). Two tumors from *BRCA1* carriers (*BRCA1* G1738R and *BRCA1* A1708V) showed a typical *BRCA1*-like appearance, suggesting that these may be the only *BRCA1* unclassified variants we studied that are pathogenic. None of the six tumors from carriers of *BRCA2* unclassified variants that were reviewed for histopathologic features showed a typical *BRCA1*-like appearance.

To derive a combined odds of causality for each variant, we extended our previous model (22) with the addition of the tumor characteristics of histopathologic and immunohistochemical data and LOH. Because these are independent of each other, and the other data sources included in the model, it is straightforward to include the likelihood ratios from LOH and pathology as simple products with the other sources. For example, the *BRCA1* variant G1738R has been observed four times by the Myriad Genetics Laboratories, never with a deleterious mutation. This only provides minimal evidence in favor of causality of 1.2:1. Three pedigrees with multiple individuals typed for the variant were available; each showed quite good cosegregation with disease and under the assumed penetrance model resulting in odds in favor of causality of 20.25:1. Our *BRCA1* sequence alignments reveal that position G1738 is invariant through Ciona. Under the model of Abkevich et al. (9), this yields odds of 58:1 that the position is slowly substituting and therefore under strong functional constrain as

opposed to fast substituting and under little selective pressure. As the substitution, G1738R is outside of the observed range of variation; it falls in the A-GVGD "enriched deleterious" set, and we regard the sequence conservation likelihood ratio as valid. Two tumors were available for LOH analysis, and both exhibited loss of the wild-type allele. Under the assumed model, each tumor contributes $0.79/0.15 = 5.27:1$, and because the two tumors are independent, the combined evidence from LOH is $5.27 \times 5.27 = 27.8:1$. One of the two tumors examined by histopathology was grade 3 and ER⁻, which is typical of *BRCA1* tumors and provides odds of 2.95:1 in favor of causality, whereas the second tumor was quite atypical grade 1 ER⁺ and under the model is unlikely to be a *BRCA1* tumor with odds in favor of 0.05:1 (or 20:1 against). Thus, the histopathology component provides combined odds of $0.05 \times 2.95 = 0.15:1$. Because these sources are independent, we can simply multiply the individual likelihood ratios together to achieve the final odds shown in Table 2 of $1.2 \times 20.25 \times 58 \times 27.8 \times 0.15 = 5,871:1$, in favor of this variant being a pathogenic *BRCA1* mutation.

Discussion

We have extended the multifactorial likelihood model of Goldgar et al. (22) for classification of unclassified variants in *BRCA1* and *BRCA2* to include the joint effects of histopathology and immunohistochemistry and allelic loss. Using this extended model, we were able to classify 6 of 10 *BRCA1* (P142H, D369del, Q804H, R841W, D1155H, and V1534M) and 7 of 14 *BRCA2* (Y42C, S384F, S869L, C1265S, G2274V, D2665G, and E2856A) unclassified variants as neutral with odds of causality of <0.01. Three of the variants we evaluated were also analyzed by Goldgar et al. (22) who found convincing evidence that *BRCA1* R841W was neutral by Bayes

Table 3. Classification of variants in *BRCA2* (Cont'd)

Histopathology (no. tumors)	Odds of causality, genetic data (frequency with a deleterious mutation and segregation data)	Odds of causality, sequence data (sequence conservation and A-GVGD)	Odds of causality, pathology data	Odds of causality, without pathology data (LOH and immunohistopathology)	Combined odds of causality (genetic, sequence, and pathology data)	Model classification
1.440 (2)	<0.00001	1.000	0.11779	<0.00001	<0.00000	Neutral
1.000 (NA)	0.02077	1.000	0.06700	0.02077	0.00139	Neutral
0.520 (1)	10.90108	0.005	0.00996	0.05451	0.00054	Neutral
1.000 (NA)	0.04323	0.008	0.28600	0.00035	0.00010	Neutral
1.200 (1)	0.48354	0.008	0.02299	0.00387	0.00009	Neutral
1.000 (NA)	0.13012	5.000	0.01916	0.65058	0.01247	NA
1.200 (1)	14.70000	5.000	0.34320	73.50000	25.22520	NA
0.520 (1)	1.22500	5.000	0.03484	6.12500	0.21340	NA
1.200 (1)	0.00364	5.000	0.08040	0.01820	0.00146	Neutral
1.000 (NA)	2.45000	1.000	0.06700	2.45000	0.16415	NA
0.624 (2)	24.95280	5.000	3.28848	124.76400	410.28392	NA
1.200 (1)	0.41000	5.000	1.20000	2.05000	2.46000	NA
0.520 (1)	39.20000	5.000	0.14872	196.0	29.14912	NA
1.200 (1)	1.11720	0.190	0.00539	0.21227	0.00114	Neutral

factor analysis of six pedigrees. Such classification of R841W as neutral is consistent with our finding that the single tumor we examined showed loss of the variant allele and did not manifest a *BRCA1*-related histopathology. Goldgar et al. (22) also evaluated *BRCA2* Y42C in 17 pedigrees and concluded that the odds for causality were <0.000001. This conclusion is supported by our finding of the variant in 1 of 180 controls, and the odds of causality that we calculated of <0.000001, although it should be noted that our results are based on some of the same co-occurrence data in Goldgar et al. (20) and thus are not completely independent. Insufficient information was available for *BRCA1* R1699Q for classification, but Goldgar et al. found odds of 4:1 in favor of causality (22). We were not able to collect any tumor blocks for the family that carried this variant, and our study was also unable to classify it as deleterious or neutral, with odds of only 67:1 in favor of causality. Wappenschmidt et al. (47) have recently classified *BRCA2* S384F as neutral on the basis of co-occurrence, cosegregation, and LOH.

In addition, we have classified *BRCA1* 5331G > A (G1738R) as pathogenic with odds of 5871:1 in favor of causality and found evidence that *BRCA2* 8395G > C (D2723H) might be pathogenic, with odds of 410:1 in favor of causality. The odds for *BRCA2* D2723H from our data do not meet the recommendation of 1,000:1 for classification as a pathogenic mutation, but our results are consistent with the findings of Goldgar et al. (22) who reported odds of ~ 57,000:1 in favor of causality for this variant. G1738R is in the transactivation domain of *BRCA1*, as are three other variants we analyzed. Of these other three variants, we classified 4719 G > A (V1534M) as neutral with odds of <0.0001 in favor of causality, whereas 5215 G > A (R1699Q) and 5242 C > T (A1708V) had odds of 67:1 and 41:1, respectively, in favor of causality, which would be consistent with pathogenicity.

Including histopathology, immunohistochemistry, and allelic loss in the multifactorial likelihood model did not result in the

classification of any *BRCA1* variants that would have remained unclassified under the original model (22). However, it did result in the classification of four *BRCA2* variants (S384F, S869L, D2665G, and E2856A) as neutral, which would otherwise have remained unclassified. The apparent lack of value of including histopathology and immunohistochemistry to classify *BRCA1* variants seems surprising because of the distinctive phenotype of *BRCA1* tumors, but on examination, it is clear that whereas including the histopathology, immunohistochemistry, and allelic loss data did greatly change the odds of causality for all *BRCA1* variants, the inclusion of genetic and sequence conservation analysis alone was actually sufficient to show odds of <0.01:1 in favor of causality (Table 2). By contrast, although the histopathology of the *BRCA2* tumors did not greatly alter the odds of causality, the allelic loss data from this set of families was critical in the classification of these four *BRCA2* variants (Table 3).

Although there are currently no functional assays that are considered robust enough to include in the multifactorial likelihood model, it is interesting to examine the available data to determine whether they are consistent with the odds of causality that we have calculated. Wu et al. (19) predicted that *BRCA2* D2723H is disease causing based on multiple assays of cellular survival, homologous recombination repair, and genome instability, and this is consistent with our finding of 410:1 in favor of causality. Mircovic et al. (12) predicted that *BRCA1* G1738R might be deleterious because it would cause a very large amino acid volume change at a flexible position. Moreover, the destabilization of the BRCT region due to G1738R mutation has been predicted by Williams et al. (16). Destabilization of BRCT to partially unfolded intermediates (48, 49) will severely affect the interaction with the BACH1 phosphopeptide (50) and is likely to lead to loss of function. Although there are no reports of *in vitro* assays of the function of *BRCA1* G1738R, Hayes et al. (51) did evaluate another substitution at this site, *BRCA1* G1738E, in a transactivation assay and

concluded that 1738E was likely to be disease-related because its transactivation domain function was disrupted, and because the variant occurred in a conserved amino acid. This classification of 1738E as pathogenic would be consistent with our finding that both of the two tumors we evaluated from carriers of the *BRCA1* G1738R variant showed loss of the wild-type allele. However, only one of these two tumors had a typical *BRCA1*-like phenotype. Clapperton et al. (52) recently reported that *BRCA1* R1699Q loses phosphospecificity in its binding to BACH1, and reduced activity in mammalian transactivation assays has been reported for *BRCA1* R1699Q and another substitution at the same site, R1699W (15). Williams et al. (16) found that R1699W was much more protease sensitive than R1699Q. However, functional data alone are insufficient to classify either *BRCA1* R1699Q or R1699W as deleterious.

We used many different approaches to attempt to classify variants in *BRCA1* and *BRCA2*. Most of these were useful, although to a variable degree depending largely on the nature of the biospecimens available to us. The kConFab is an excellent resource for these types of studies because of the large number of bloods that have been collected per family and the availability of many archival tumor blocks. If the appropriate functional assays are ever developed, the ability to generate lymphoblastoid cell lines from every participant will also be an advantage. The only analysis that we carried out which was not fruitful was the investigation of splicing in lymphoblastoid cell lines from carriers of unclassified variants that were predicted to affect ESE. Through a series of *in silico* approaches, we have estimated that only a very small proportion of ESEs predicted by ESEfinder in *BRCA1* are likely to be functional (53); thus, our failure to find splicing aberrations in these carriers is not unexpected.

The extended multifactorial likelihood model for classification of unclassified variants in *BRCA1* and *BRCA2* could be modified for analysis of variants in other genetic diseases, particularly hereditary cancer syndromes, such as hereditary nonpolyposis colon cancer, where tumors have distinctive immunohistochemical profiles and inactivation of the wild-type allele is common. How-

ever, before the extended model can be more generally applied, it will be important to validate the LOH and histopathology results on larger data sets (e.g., from Myriad Genetic Laboratories, as they become available), and care must be taken that consistent scoring is applied to that used in the development of the likelihood ratio estimates. Interestingly, we found that all the unclassified variants we identified in 1 of 180 controls were subsequently classified as neutral. We therefore plan further developments of the model to include the frequency of the relevant unclassified variant in appropriately ascertained controls so that the relevance of these control frequencies can be quantified.

Classification of variants in *BRCA1* and *BRCA2* has obvious benefits for genetic counseling. Women found to carry a pathogenic variant can be advised to undergo regular screening by mammography or magnetic resonance imaging and to consider risk-reducing surgery (54, 55). In addition, their relatives who do not carry the pathogenic variant can be reassured that there are not at high risk. Furthermore, classification of these variants will help to define the functional domains of *BRCA1* and *BRCA2* and thereby increase our understanding of the function of the proteins.

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