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

Georgia Chenevix-Trench,1 Sue Healey,1 Sunil Lakhani,1,2 Paul Waring,4 Margaret Cummings,2 Ross Brinkworth,3 Amie M. Deffenbaugh,7 Lynn Anne Burbidge,1 Dmitry Pruss,7 Thad Judkins,7 Tom Scholl,1 Anna Bekessy,1 Anna Marsh,1 Paul Lovelock,1,3 Ming Wong,6 Andrea Tesoriero,1 Helene Renard,9 Melissa Southey,5,9 John L. Hopper,6 Koulis Yannoukakos,10 Melissa Brown,3 kConFab Investigators,4 Douglas Easton,11 Sean V. Tavtigian,9 David Goldgar,8 and Amanda B. Spurdle1

1Queensland Institute of Medical Research; 2Department of Pathology and 3School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Australia; 4Peter MacCallum Cancer Centre; 5Department of Pathology and 5Centre for Genetic Epidemiology University of Melbourne, Melbourne, Australia; 6Myriad Genetic Laboratories, Inc.; 7Department of Medical Informatics, University of Utah, Salt Lake City, Utah; 8Molecular Diagnostics Lab., National Center for Scientific Research Demokritos, Athens, Greece; and 10Strangeways Laboratory, University of Cambridge, Cambridge, England

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 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 immunohistochemical 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 Cuningham 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

Note: J.L. Hopper and G. Chenevix-Trench are National Health and Medical Research Council Senior Principal and Principal Research Fellows.
Requests for reprints: Amanda B. Spurdle, Queensland Institute of Medical Research, 300 Herston Road, QLD 4029, Brisbane, Queensland 4006, Australia. Phone: 617-33-620-371; Fax: 617-33-620-105; E-mail: mandyS@qimr.edu.au.
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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 >1,000:1 were considered pathogenic, and those with odds of causality <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 immunohistochemical 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.aspx). The kConFab currently defines as pathogenic all missense variants that are well-characterized 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 5351 G > A (G1788R). 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 TCoffee and 3Doffee (28). The BRCA1 alignment contained 13 full-length sequences, the most divergent of which was from the tuna Cliona 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 ≥ 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 SipSiteFinder to determine whether any unclassified variant altered or created a consensus splice site (http://www.genet.sickkids.on.ca/~ali/spsitesefinder.html) and by ESEfinder (http://rulai.cshl.edu/tools/ESE3/) 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).
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 (ABCSF; 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. 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 to clarify genotypes where 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).

**Causality analysis.** Bayes factor analysis was done by computing the likelihood ratio of the pedigree and genotype data under causality and neutrality using the approach described in Thompson (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, 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

<table>
<thead>
<tr>
<th>Variant</th>
<th>Forward sequence (5' &gt; 3')</th>
<th>Reverse sequence (5' &gt; 3')</th>
<th>Melt temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPLC primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA1 544 C &gt; A (P142H)</td>
<td>GGGTTTCTCTGTTGCTTGTGA</td>
<td>CAATGTTTTCACCAGAGGGA</td>
<td>55</td>
</tr>
<tr>
<td>BRCA1 4719 G &gt; A (V1534M)</td>
<td>GCTGCCACAGTATGATT</td>
<td>TGGTCTCGTAAATCGTGTG</td>
<td>58 and 59</td>
</tr>
<tr>
<td>BRCA1 1225 del 3 (D369del)</td>
<td>GGACTCCCGACAGAAAGAA</td>
<td>TCCCCATCATCGGATCATC</td>
<td>57</td>
</tr>
<tr>
<td>BRCA1 1605 C &gt; T (R499C)</td>
<td>TGGGAAACTATGGGAAGAGAG</td>
<td>CGGTGTGTTAGTTCCCTGA</td>
<td>57</td>
</tr>
<tr>
<td>BRCA1 2531 G &gt; C (Q804H), BRCA1 2640 C &gt; T (R841W)</td>
<td>GGCACTGAGAAATGTACCTG</td>
<td>AATGACTGCGGTTTGAACAC</td>
<td>58</td>
</tr>
<tr>
<td>BRCA2 3582 G &gt; C (D1155H)</td>
<td>CCTGAAGATATGGTGAACATCC</td>
<td>GCTTTTGCAAAAACAGCAGA</td>
<td>56</td>
</tr>
<tr>
<td>BRCA2 5121 G &gt; A (R1699Q), BRCA2 5124 C &gt; T (A1708V)</td>
<td>CAGGCTCTTTGCTTTAGGAC</td>
<td>CCCAGCTACCAGCTTACCT</td>
<td>56</td>
</tr>
<tr>
<td>BRCA2 5331 G &gt; A (G1738R)</td>
<td>TGACGTTGCCTGCTCCATTC</td>
<td>CAGAGTCTGTTGATGAGGT</td>
<td>59</td>
</tr>
<tr>
<td>BRCA2 5242 C &gt; T (A1708V)</td>
<td>TCTTTAAGCTCTGTTGGCTC</td>
<td>TGGTTCTCTGTGATGAGGT</td>
<td>54</td>
</tr>
<tr>
<td>BRCA2 1379 C &gt; T (S384F)</td>
<td>AGCAAGCTGATGATGATG</td>
<td>CCATCTGGCTCTCATTAGA</td>
<td>57</td>
</tr>
<tr>
<td>BRCA2 2834 C &gt; T (S669L)</td>
<td>GCTGTGCTACCACTGAAAT</td>
<td>CTAAGCTGTTTTGATGAGT</td>
<td>54</td>
</tr>
<tr>
<td>BRCA2 4021 T &gt; A (C1265S)</td>
<td>TGGATGCTCAGAAAGCTG</td>
<td>TTTCTCTAACAAGATGTCGAC</td>
<td>54</td>
</tr>
<tr>
<td>BRCA2 6173 G &gt; C (S1982T)</td>
<td>TCACCTTGTGTTGTGTTTGA</td>
<td>TGGAAACTGTGCTTTCTCA</td>
<td>56</td>
</tr>
<tr>
<td>BRCA2 7049 G &gt; T (G2274V)</td>
<td>TGAGGATGATGGAGTACAGA</td>
<td>TTGTTTCAACAGAATGTC</td>
<td>56</td>
</tr>
<tr>
<td>BRCA2 7181 G &gt; A (R2318Q)</td>
<td>ATGAGGATGATGAGTACAGA</td>
<td>TWAACAGACACTTTTTCATG</td>
<td>54</td>
</tr>
<tr>
<td>BRCA2 8204G &gt; C (R2659T)</td>
<td>ATGGAAACTGGCAGCTATGG</td>
<td>CACAGACTGCTCTTGTCACC</td>
<td>57</td>
</tr>
<tr>
<td>BRCA2 8395 G &gt; C (D2723H), BRCA2 8234 C &gt; T (S869L)</td>
<td>TGGCACTCAGGAAAGTATCG</td>
<td>GCTTTTGACAAATGTTTA</td>
<td>54</td>
</tr>
<tr>
<td>BRCA2 8234 C &gt; T (S869L)</td>
<td>GGGTTTCTCTGCTCCTTTGC</td>
<td>CAGGACCTCAGGTTAGCCT</td>
<td>57</td>
</tr>
<tr>
<td>BRCA2 8536 G &gt; A (A2770T)</td>
<td>TGCAAAAACACTTGTTCTGTT</td>
<td>TGGTTTCAACAGAATGTC</td>
<td>56</td>
</tr>
<tr>
<td>BRCA2 8237 C &gt; T (S2670L)</td>
<td>ATGGAAACTGGCAGCTATGG</td>
<td>CACAGACTGCTCTTGTCACC</td>
<td>57</td>
</tr>
<tr>
<td>BRCA2 8237 C &gt; T (S2670L)</td>
<td>TGGCACTCAGGAAAGTATCG</td>
<td>GCTTTTGACAAATGTTTA</td>
<td>54</td>
</tr>
<tr>
<td>BRCA2 8237 C &gt; T (S2670L)</td>
<td>TGGCACTCAGGAAAGTATCG</td>
<td>GCTTTTGACAAATGTTTA</td>
<td>54</td>
</tr>
</tbody>
</table>

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, 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.

**Evaluation of Unclassified Variants of BRCA1 and BRCA2**
variant carrier LCLs was PCR-amplified using primers designed to span (41), cDNA synthesized from RNA extracted from wild-type control and negative controls. Briefly, following methodology detailed in Tesoriero et al. BRCA1 SpliceSiteFinder and ESEfinder to alter splicing, using out for BRCA1 causality. 1.2:1 in favor of causality, whereas scores of 1 to 2 gave odds of 2:1 against useful; tubule formation scores of 3 were associated only with odds of 

in situ cases of ductal carcinoma. 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, 3) were compared with the frequency distribution in 258 age-matched controls in each of the six possible combinations of ER status (+/−) and grade (1, 2, 3) were compared with the frequency distribution in 258 age-matched controls. The specific histopathologic features of grade and ER status 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, 3) were compared with the frequency distribution in 258 age-matched controls in each of the six possible combinations of ER status (+/−) and grade (1, 2, 3) were compared with the frequency distribution in 258 age-matched controls.

Table 2. Classification of variants in BRCA1

<table>
<thead>
<tr>
<th>BRCA1 variant</th>
<th>Occurrence in 180 ABCFS controls</th>
<th>Frequency in Myriad database (with a deleterious mutation)</th>
<th>Bayes factor (no. families)</th>
<th>Co-occurrence (no. families)</th>
<th>Sequence conservation</th>
<th>A-GVGD</th>
<th>LOH (no. tumors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>544 C &gt; A (P142H)</td>
<td>0</td>
<td>15 (1)</td>
<td>1.7 (2)</td>
<td>0.0043</td>
<td>0.003</td>
<td>Not valid</td>
<td>0.0055, no LOH (2), loss of UV (1)</td>
</tr>
<tr>
<td>1225 del5 (D369del)</td>
<td>0</td>
<td>10 (1)</td>
<td>0.0213 (1)</td>
<td>0.0034</td>
<td>0.003</td>
<td>Not valid</td>
<td>0.0045, loss of UV (2)</td>
</tr>
<tr>
<td>1605 C &gt; T (R96C)</td>
<td>0</td>
<td>52 (1)</td>
<td>1.95 (1)</td>
<td>0.0224</td>
<td>0.003</td>
<td>Not valid</td>
<td>1, NA</td>
</tr>
<tr>
<td>2531 G &gt; C (Q804H)</td>
<td>0</td>
<td>18 (0)</td>
<td>1.12 (0)</td>
<td>2.244</td>
<td>0.003</td>
<td>Valid, ED</td>
<td>0.001951, no LOH (5)</td>
</tr>
<tr>
<td>2640 C &gt; T (R841W)</td>
<td>1</td>
<td>207 (8)</td>
<td>0.59 (2)</td>
<td>&lt;0.0001</td>
<td>0.003</td>
<td>Not valid</td>
<td>0.2860, loss of UV (1)</td>
</tr>
<tr>
<td>3582 G &gt; C (D1155H)</td>
<td>0</td>
<td>0</td>
<td>0.0025 (1)</td>
<td>1.000</td>
<td>0.003</td>
<td>Valid, ED</td>
<td>0.0670, loss of UV (1)</td>
</tr>
<tr>
<td>4719 G &gt; A (V1534M)</td>
<td>0</td>
<td>32 (1)</td>
<td>0.0086 (1)</td>
<td>4.0220</td>
<td>0.001</td>
<td>Valid, ED</td>
<td>0.0055, no LOH (1)</td>
</tr>
<tr>
<td>5215 G &gt; A (R1699Q)</td>
<td>0</td>
<td>19 (0)</td>
<td>1.04 (1)</td>
<td>2.3470</td>
<td>58</td>
<td>Valid, ED</td>
<td>1.0000 (NA)</td>
</tr>
<tr>
<td>5242 C &gt; T (A1708V)</td>
<td>0</td>
<td>1 (0)</td>
<td>0.227 (1)</td>
<td>1.0457</td>
<td>58</td>
<td>Valid, ED</td>
<td>1</td>
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<tr>
<td>5331 G &gt; A (G1738R)</td>
<td>0</td>
<td>4 (0)</td>
<td>20.25 (3)</td>
<td>1.2</td>
<td>58</td>
<td>Valid, ED</td>
<td>27.7729, loss of WT (2)</td>
</tr>
</tbody>
</table>

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

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 G1738R.
Asp1840, the biological significance of which is not clear. For BRCA2 that is enriched for neutral substitutions. The A-GVGD analyses of R1699Q, A1708V, and G1738R fall into an A-GVGD set BRCA1 issue because the residue is on the outside of the protein, but the arginine side chain. For G1738R, there would probably be insufficient space for the long carbon and is thus likely to cause incompatibility with a bend structure in the helix. For BRCA1 acid with two methyl groups on the h, not BRCA1-like (1) 0.0069, not BRCA1-like (2) 0.0073 1.000 0.0001 0.001 0.001 Neutral G1738R variant were available for 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. 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...
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 × 5.27 = 27.81. 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 × 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 × 20.25 × 58 × 27.8 × 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

<table>
<thead>
<tr>
<th>BRCA2 unclassified variant</th>
<th>Occurrence in 180 ABCFS controls</th>
<th>Frequency in Myriad database (with a deleterious mutation)</th>
<th>Bayes factor (no. families)</th>
<th>Co-occurrence</th>
<th>Sequence conservation</th>
<th>A-GVGD</th>
<th>LOH (no. tumors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>333 A &gt; G (Y42C)</td>
<td>1</td>
<td>0.023 (2)</td>
<td>&lt;0.0001</td>
<td>0.190</td>
<td>Not valid</td>
<td>0.08180</td>
<td>no LOH (2)</td>
</tr>
<tr>
<td>1379 C &gt; T (S384F)</td>
<td>0</td>
<td>0.0987 (1)</td>
<td>0.2104</td>
<td>0.008</td>
<td>Not valid</td>
<td>0.06700</td>
<td>loss of UV (1)</td>
</tr>
<tr>
<td>2834 C &gt; T (S869L)</td>
<td>0</td>
<td>9.43 (1)</td>
<td>1.1560</td>
<td>0.005</td>
<td>Valid, EN</td>
<td>0.01916</td>
<td>no LOH (1)</td>
</tr>
<tr>
<td>4021 T &gt; A (C1265S)</td>
<td>0</td>
<td>0.042 (1)</td>
<td>1.0294</td>
<td>0.008</td>
<td>Valid, EN</td>
<td>0.28600</td>
<td>no LOH (1)</td>
</tr>
<tr>
<td>7049 G &gt; T (G2274V)</td>
<td>0</td>
<td>0.215 (1)</td>
<td>2.2490</td>
<td>0.008</td>
<td>Valid, EN</td>
<td>0.01916</td>
<td>no LOH (1)</td>
</tr>
<tr>
<td>7181 G &gt; A (R2318Q)</td>
<td>0</td>
<td>0.1264 (2)</td>
<td>1.0294</td>
<td>0.008</td>
<td>Valid, ED</td>
<td>0.01916</td>
<td>no LOH (1)</td>
</tr>
<tr>
<td>8204 G &gt; C (R2659T)</td>
<td>0</td>
<td>14.7 (1)</td>
<td>1.0000</td>
<td>5.000</td>
<td>Valid, ED</td>
<td>0.28600</td>
<td>no LOH (2)</td>
</tr>
<tr>
<td>8216 A &gt; T (E3663V)</td>
<td>0</td>
<td>1.0 (1)</td>
<td>1.2250</td>
<td>5.000</td>
<td>Valid, ED</td>
<td>0.06700</td>
<td>loss of UV (1)</td>
</tr>
<tr>
<td>8222 A &gt; G (D3665G)</td>
<td>1</td>
<td>0.0021 (1)</td>
<td>1.7330</td>
<td>5.000</td>
<td>Valid, ED</td>
<td>0.06700</td>
<td>loss of UV (1)</td>
</tr>
<tr>
<td>8237 C &gt; T (S2670L)</td>
<td>0</td>
<td>2.0 (1)</td>
<td>1.2250</td>
<td>0.190</td>
<td>Not valid</td>
<td>0.06700</td>
<td>loss of UV (1),</td>
</tr>
<tr>
<td>8395 G &gt; C (D2723H)</td>
<td>0</td>
<td>7.4 (2)</td>
<td>3.3720</td>
<td>5.000</td>
<td>Valid, ED</td>
<td>0.27000</td>
<td>loss of WT (1)</td>
</tr>
<tr>
<td>8536 G &gt; A (A2770T)</td>
<td>0</td>
<td>0.41 (1)</td>
<td>1.0000</td>
<td>5.000</td>
<td>Valid, ED</td>
<td>1.00000</td>
<td>NA</td>
</tr>
<tr>
<td>8714 A &gt; G (del exon 19)</td>
<td>0</td>
<td>39.20 (1)</td>
<td>1.0000</td>
<td>5.000</td>
<td>Valid, ED</td>
<td>0.28600</td>
<td>no LOH (1)</td>
</tr>
<tr>
<td>8795 A &gt; C (E2856A)</td>
<td>1</td>
<td>1.05 (2)</td>
<td>1.0640</td>
<td>0.190</td>
<td>Valid, EN</td>
<td>0.00449</td>
<td>loss of UV (2)</td>
</tr>
</tbody>
</table>

Abbreviations: UV, unclassified variant; WT, wild type; NA, not applicable; EN, enriched neutral; ED, enriched deleterious.
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 that the single tumor we examined showed loss of the variant allele and did not manifest a BRCA1-related histopathology.

BRCA2 Y42C is in a transactivation assay and is likely to lead to loss of function. Williams et al. (16) predicted that the BRCT region due to G1738R mutation has been predicted by 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. Mircovic et al. (12) predicted that this change at a flexible position. Moreover, the destabilization of 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 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. 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.

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 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.

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

<table>
<thead>
<tr>
<th>Histopathology (no. tumors)</th>
<th>Odds of causality, genetic data (frequency with a deleterious mutation and segregation data)</th>
<th>Odds of causality, sequence data (sequence conservation and A-GVGD)</th>
<th>Odds of causality, pathology data</th>
<th>Odds of causality, without pathology data (LOH and immunohistopathology)</th>
<th>Combined odds of causality (genetic, sequence, and pathology data)</th>
<th>Model classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.440 (2)</td>
<td>&lt;0.00001</td>
<td>1.000</td>
<td>0.11779</td>
<td>&lt;0.00001</td>
<td>&lt;0.00000</td>
<td>Neutral</td>
</tr>
<tr>
<td>1.000 (NA)</td>
<td>0.02077</td>
<td>1.000</td>
<td>0.06700</td>
<td>0.02077</td>
<td>0.00139</td>
<td>Neutral</td>
</tr>
<tr>
<td>0.520 (1)</td>
<td>10.90108</td>
<td>0.005</td>
<td>0.00996</td>
<td>0.05451</td>
<td>0.00054</td>
<td>Neutral</td>
</tr>
<tr>
<td>1.000 (NA)</td>
<td>0.04323</td>
<td>0.008</td>
<td>0.28600</td>
<td>0.00035</td>
<td>0.00010</td>
<td>Neutral</td>
</tr>
<tr>
<td>1.200 (1)</td>
<td>0.48354</td>
<td>0.008</td>
<td>0.02599</td>
<td>0.00387</td>
<td>0.00009</td>
<td>Neutral</td>
</tr>
<tr>
<td>1.000 (NA)</td>
<td>0.13012</td>
<td>5.000</td>
<td>0.01916</td>
<td>0.65058</td>
<td>0.01247</td>
<td>NA</td>
</tr>
<tr>
<td>1.200 (1)</td>
<td>14.70000</td>
<td>5.000</td>
<td>0.34320</td>
<td>73.50000</td>
<td>25.22520</td>
<td>NA</td>
</tr>
<tr>
<td>0.520 (1)</td>
<td>1.22500</td>
<td>5.000</td>
<td>0.03484</td>
<td>6.12500</td>
<td>0.21340</td>
<td>NA</td>
</tr>
<tr>
<td>1.200 (1)</td>
<td>0.00364</td>
<td>5.000</td>
<td>0.08040</td>
<td>0.01830</td>
<td>0.00146</td>
<td>Neutral</td>
</tr>
<tr>
<td>1.000 (NA)</td>
<td>2.45000</td>
<td>1.000</td>
<td>0.06700</td>
<td>2.45000</td>
<td>0.16415</td>
<td>NA</td>
</tr>
<tr>
<td>0.624 (2)</td>
<td>24.95280</td>
<td>5.000</td>
<td>3.28848</td>
<td>124.76400</td>
<td>410.28392</td>
<td>NA</td>
</tr>
<tr>
<td>1.200 (1)</td>
<td>0.41000</td>
<td>5.000</td>
<td>1.20000</td>
<td>2.05000</td>
<td>2.46000</td>
<td>NA</td>
</tr>
<tr>
<td>0.520 (1)</td>
<td>39.20000</td>
<td>5.000</td>
<td>0.14872</td>
<td>196.0</td>
<td>29.14912</td>
<td>NA</td>
</tr>
<tr>
<td>1.200 (1)</td>
<td>1.11720</td>
<td>0.190</td>
<td>0.00539</td>
<td>0.21227</td>
<td>0.00114</td>
<td>Neutral</td>
</tr>
</tbody>
</table>

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 transactivation domain function was disrupted, and because the concluded that 1738E was likely to be disease-related because its C\textsuperscript{1738R} 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 \textit{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. However, 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 ascertainmentcontrols so that the relevance of these control frequencies can be quantified.

Classification of variants in \textit{BRCA1} and \textit{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 \textit{BRCA1} and \textit{BRCA2} and thereby increase our understanding of the function of the proteins.

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\section*{References}


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

Georgia Chenevix-Trench, Sue Healey, Sunil Lakhani, et al.


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