Analysis of a set of missense, frameshift, and in-frame deletion variants of *BRCA1*

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ABSTRACT

Germline mutations that inactivate *BRCA1* are responsible for breast and ovarian cancer susceptibility. One possible outcome of genetic testing for *BRCA1* is the finding of a genetic variant of uncertain significance for which there is no information regarding its cancer association. This outcome leads to problems in risk assessment, counseling and preventive care. The purpose of the present study was to functionally evaluate seven unclassified variants of *BRCA1* including a genomic deletion that leads to the in-frame loss of exons 16/17 (Δ exons 16/17) in the mRNA, an insertion that leads to a frameshift and an extended carboxy-terminus (5673insC), and five missense variants (K1487R, S1613C, M1652I, Q1826H and V1833M). We analyzed the variants using a functional assay based on the transcription activation property of BRCA1 combined with supervised learning computational models. Functional analysis indicated that variants S1613C, Q1826H, and M1652I are likely to be neutral, whereas variants V1833M, Δ exons 16/17, and 5673insC are likely to represent deleterious variants. In agreement with the functional analysis, the results of the computational analysis also indicated that the latter three variants are likely to be deleterious. Taken together, a combined approach of functional and bioinformatics analysis, plus structural modeling, can be utilized to obtain valuable information pertaining to the effect of a rare variant on the structure and function of *BRCA1*. Such information can, in turn, aid in the classification of *BRCA1* variants for which there is a lack of genetic information needed to provide reliable risk assessment.

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1. Introduction

BRCA1 is a tumor suppressor gene and germline mutations which disrupt its biological activity contribute to breast and ovarian cancer susceptibility [1]. Carriers of these inactivating mutations are at increased risk of developing breast and ovarian cancer with an estimated cumulative risk of breast cancer ranging from 36% to 71% at age 70 and up to 90% at age 80 in some populations [2–5]. The magnitude of risk remains controversial and varies according with the population studied and with study design [3,6]. Neverthe-

less, it is significantly higher than the risk of breast cancer in the general population making genetic testing an important factor in the decision to undergo increased surveillance, chemoprevention, or prophylactic surgery [7]. The *BRCA1* gene codes for a nuclear protein of 1863 amino acids that has been found to play a role in many cellular processes including DNA-damage repair, transcriptional activation, cell cycle regulation, apoptosis, and genomic stability [8].

Truncations and missense substitutions are two of the predominant types of *BRCA1* mutations that have been identified (Breast Cancer Information Core – BIC – Database; http://research.nhgri.nih.gov/bic/). While most truncating mutations have been found to be cancer-associated, missense variants have proven more difficult to classify [9]. Over 300 different missense variants of *BRCA1* have been identified but presently their clinical significance is unknown despite intense efforts (BIC) [10,11].

In situations in which there is a lack of clinical and genetic data to classify these variants, functional studies which assess specific biochemical properties of the protein can contribute to the classification of the variant as either deleterious or neutral [12,13]. The BRCA1 transcription activation (TA) assay evaluates the ability of the COOH-terminus of the protein to function as a transactivation domain and has been used as a monitor of the structural integrity of the domain [12,14–16]. Cancer-associated missense variants of BRCA1 have been found to exhibit loss of function with respect to transcriptional activity while neutral variants display activity similar to the wild type protein [12,14]. Prediction of mutation impact on protein function by structure-based models has also been used in the classification of these rare variants [17–19].

Here, we examine functionally seven unclassified variants of BRCA1, three of which have not been reported previously. Those include a deletion of exons 16 and 17 (∆ exons 16/17), an insertion mutation (5673insC) leading to a frameshift that produces a protein which is 15 amino acids longer than the wild type, and missense variant Q1826H. Four missense variants (K1487R, S1613C, M1652I and V1833M) previously reported in the BIC database were also functionally evaluated. We provide a bioinformatics-based prediction of their impact, and for the variants that affect the BRCA1 COOH-terminal (BRCT) domains (∆ exons 16/17, 5673insC, M1652I, Q1826H and V1833M) we also provide a rationalization of their impact, based on structural modeling.

2. Materials and methods

2.1. Constructs

Constructs coding for exons 13–24 (amino acids 1396–1863) of wild type (wt) BRCA1, positive (S1613G; neutral), and negative (M1775R and Y1853X; deleterious) controls were previously described [14–16]. Mutations were generated by splicing by overlapping extension PCR [20] using p385-BRCA1 [14] (for ∆ exons 16/17, K1487R, S1613C, Q1826H, M1652I, and V1833M) or F3-BRCA1 [1] (for 5673insC) as template. PCR products were cloned into pLex9 or pGBT9 vectors. To obtain GAL4 DNA binding domain (DBD) fusions in a mammalian expression vector, GAL4 DBD fusion fragments were isolated from pGBT9 and subcloned into pCDNA3. Mutation nomenclature follows the nucleotide numbering found in the BIC database and uses GenBank U14680 as a reference for *BRCA1* cDNA with the corresponding protein accession number AAA73985.

2.2. Yeast assays

Saccharomyces cerevisiae strain EGY48 (*Matα, ura3, trp1, his3, 6 lexA operator-LEU2*) has an integrated *LEU2*-reporter gene under the control of 6 LexA ops [21] and when the gene is activated, cells grow in the absence of leucine. EGY48 transformants expressing either wt or mutant BRCA1 constructs in pLex9 were grown overnight and saturated cultures were then used to inoculate either synthetic drop-out (SD) medium lacking both leucine and tryptophan or only tryptophan and growth was measured by OD at 600 nm. EGY48 yeast cells were also co-transformed with the *lacZ* reporter plasmid pRB1840, which contains a *lacZ* reporter gene under the control of a single LexA operator, and a pLex9 plasmid coding for either the wt or mutant constructs [22]. β-galactosidase activity was determined using *o*-nitrophenyl-

β-D-galactopyranoside (ONPG) as the substrate for three independent clones each [23].

2.3. Mammalian assays

Human embryonic kidney (HEK) 293T cells were co-transfected with a reporter plasmid pG5Luc, which contains a firefly luciferase gene under the control of five GAL4 binding sites (5 × Gal4 bs), a pCDNA3 plasmid coding for either the wt or mutants fused to the GAL4 DBD, and an internal control, phRG-TK, which contains a *Renilla* luciferase gene under a constitutive thymidine kinase basal promoter. Transcriptional activity was determined using a dual luciferase substrate system (Promega).

2.4. Western blotting

Protein levels in yeast or in HEK-293T cells were determined by western blotting using α-LexA DBD (Upstate) or α-GAL4 DBD (Clontech) monoclonal antibodies [24]. Because of co-migration with nonspecific bands, protein levels in HEK-293T cells expressing ∆ exons 16/17 or 5673insC were determined by immunoprecipitation using α-GAL4 DBD monoclonal antibody followed by blotting with the same antibody (Santa Cruz).

2.5. Structural analysis

Prediction of the impact of amino acid changes in the BRCT domains was obtained by previously described bioinformatics supervised learning computation models [18,25–27]. To investigate the putative structural changes generated by the 5673c insertion and deletion of exons 16 and 17 (∆ exons 16/17), we built structural models with SAM-T06 and UNDERTAKER, an all-heavy-atom, fragment packing program for protein structure prediction [28].

2.6. Patients

BRCA1 mutations and sequence variants were either obtained from published data or public databases, or discovered in individuals attending oncogenetic counseling. All patients gave informed consent for the study. Besides the variants reported, no other mutation in *BRCA1* (or *BRCA2*) was detected unless otherwise noted.

2.7. Conceptual basis of the assay

The classification used in this manuscript is based on the ability of BRCA1 constructs fused to a heterologous DNA binding domain to activate transcription of a reporter gene [12]. Previous studies have shown that constructs containing variants that disrupt transcriptional activation are likely to be disease-associated, while variants that do not affect activity and behave similarly to the wt BRCA1 construct are not likely to be disease-associated [[14] and references therein]. In this heterologous context, the assay is not interpreted as a measure of a bona fide biochemical function of BRCA1 but rather as a monitor of protein integrity. Thus, reduction or abrogation of activity is not only considered to indicate whether certain variants affect BRCA1 tumor suppressive activities mediated by its role in transcription [29] but also to indicate whether a variant affects other functions required for tumor suppression mediated by the COOH-terminus of BRCA1, for example phosphopeptide binding [30]. At least three lines of evidence support this conclusion. First, variants that affect transcriptional activation have been shown to also affect the integrity of the BRCT domain as measured by protease sensitivity or direct crystal structure determination [19,31,32]. Second, a bioinformatics structure-based analysis designed to pinpoint surface residues of the BRCT domain that affect transcription activation identified a surface patch which was independently shown to correspond to the phosphopeptide binding pocket in the BRCT domain [33,34]. Finally, a recent study [14] showed that the transcriptional assay correctly classified all BRCA1 missense variants that have been classified by co-occurrence with other deleterious variants [35] or by integrated methods based on multifactorial likelihood models [9,11].

2.8. Term definitions for classification and limitations of the assay

Variants subjected to functional analysis are classified as "deleterious", "neutral", or left unclassified. "Deleterious" is a variant for which a significant negative impact on protein function has been verified when compared to the wt control. Because inactivation of BRCA1 function has been shown to underlie cancer predisposition, a compromised activity in the assay suggests that the variant is worthy of further investigation as causally-related to cancer predisposition. Conversely, "neutral" is a variant for which no negative impact on protein function has been verified. A series of internal controls is always included to assess reproducibility and to prevent misinterpretation due to unlikely but possible technical issues (*e.g.* plasmid mix-ups, yeast cross-contamination, degradation of luciferase reagents). These controls have been classified by genetic and/or integrative methods: (a) S1613G, a neutral polymorphic allele of BRCA1 [36,37] and (b) missense and nonsense deleterious variants M1775R and Y1853X, respectively [1,14,15,37,38].

To transform a quantitative measure, *i.e.* protein activity, into a qualitative classification we defined thresholds of activity (<45% of wild type activity: deleterious and >50% of wild type activity: neutral) considering the known deleterious variant that displays the highest activity and the known neutral variant displaying the lowest activity [14]. Variants whose range of percent activity (taking into account the standard deviation) falls in the intermediate range (between 45% and 50%) are left unclassified. Embedded in this classification is the simplifying notion that variants are either high or low (comparable to the risk in the general population) risk with no intermediate risk variants. For a more detailed discussion on intermediate risk see [9,14,39]. Lastly, the resulting classes derived from the functional assays in general (deleterious or neutral) have different degrees of certainty. For example, the disruption of function is due, in most cases, to a general effect on folding and it is likely to be deleterious to any other function dependent on that region. However, a cancer-causing surface change that does not affect folding and does not participate in the interaction being assessed in the assay may be incorrectly classified as neutral [9,13]. Although no such case has been documented it remains a possibility.

3. Results

3.1. Rationale for choice of variants

The transcriptional assay has been widely used to classify missense variants but more dramatic changes in protein structure have not been systematically analyzed. Most nonsense and frameshift mutations are expected to completely disrupt the BRCT domain or eliminate it altogether, in a way that we can infer that they constitute loss of function mutations [24]. However, this is not the case for frameshift mutations that occur relatively close to the COOH-terminus. These variant proteins may still retain function. Thus, we decided to analyze the 5673insC variant which causes a change near the end of BRCA1.

Similarly, in-frame deletions that cause loss of complete exons but retain the frame for the remaining ones may also have activity. Because in-frame deletions need to be confirmed using clinical samples (to verify that the protein product is indeed what was predicted from the DNA sequence) we chose variant Δ exons 16/17 for which these data were available.

We also analyzed five additional unclassified missense variants (Fig. 1A). Two of them (K1487R, S1613C) were chosen in order to increase coverage of the unstructured region of BRCA1 COOH-terminus and test the hypothesis that variants outside conserved domains are likely to be neutral. The remaining three (M1652I, Q1826H and V1833M) are missense variants in the BRCT domain and were chosen because they have been previously analyzed for binding to phosphopeptides (M1652I and V1833M) [31,33], or exposed at the surface of the domain (Q1826H) which represents a class of poorly studied variants [18].

3.2. Novel variants

3.2.1. Δ exons 16/17

This variant was identified in an Australian proband in which no other mutation in *BRCA1* or *BRCA2* was detected (Fig. 2A). This variant is a large deletion of the genomic region including exons 16 and 17, which produces stable mRNA lacking exons 16 and 17. The net effect of the mutation is the in-frame loss of amino acids 1559–1692 (Fig. 1B). Using an RNA-based test, we confirmed the presence of the mutant mRNA transcript, lacking exons 16 and 17 (Fig. 2B and C), but retaining the correct frame in the splicing of exon 15–18 (Fig. 2D).

3.2.2. 5673insC

Variant 5673insC codes for an insertion of a cytosine at nt5673 in exon 24, leading to a frameshift that changes the last 12 amino acids of the protein to a modified 27-amino acid segment (Fig. 1C). This variant was identified in a Swedish proband with ovarian cancer (at 59 y) and whose mother also had ovarian cancer (at 46 y) (Fig. 2A). The proband's grandmother died of rectal cancer at age 43. LOH
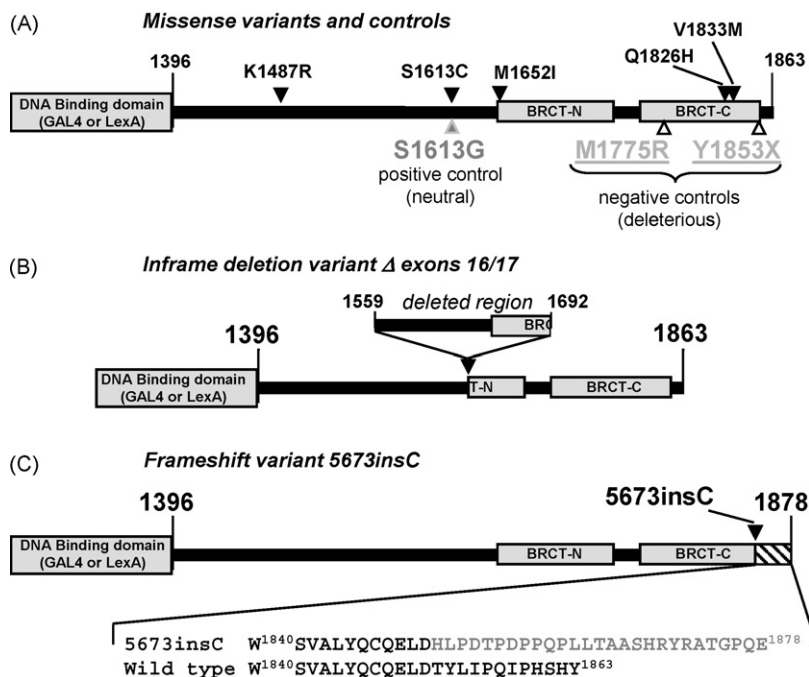

Fig. 1. Diagram of constructs containing BRCA1 variants analyzed in this study. (A) Location of missense variants (black arrowhead), positive control (grey arrowhead) and negative controls (open arrowheads). Grey boxes indicate the DNA binding domain (of GAL4 or LexA, depending on the construct) and the BRCT domains. (B) Construct containing in-frame deletion Δ exons 16/17. Diagram shows the region that is left out after splicing of exon 15 to exon 18, disrupting the BRCT-N. (C) Construct containing variant 5673insC with site of insertion indicated (black arrowhead). Bottom panel shows detail of wild type and mutant amino acid sequence.
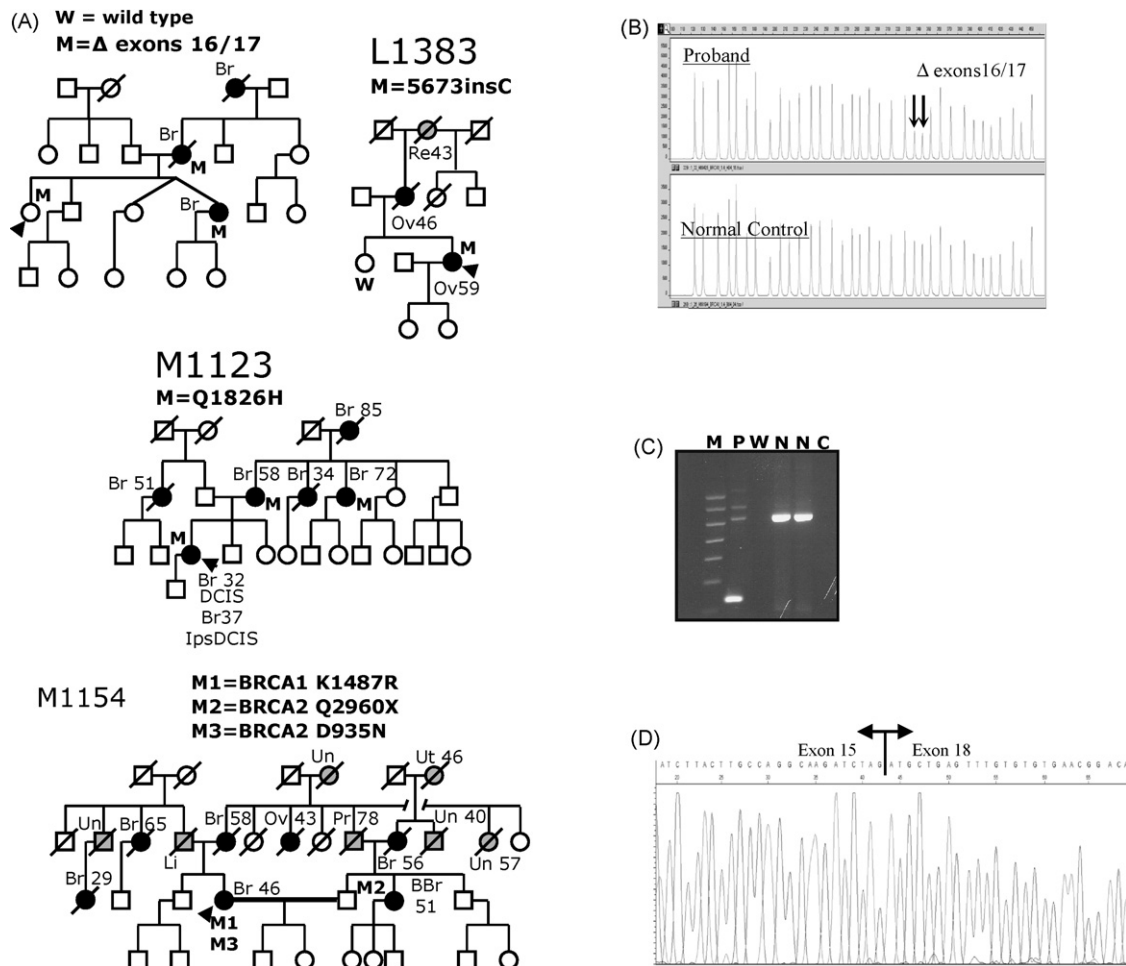
Fig. 2. Clinical data for BRCA1 variants. (A) Pedigrees of families carrying different variants. The presence (M) or absence (W) of the variants in the germ line of tested individuals is indicated. Proband is indicated by black arrowhead. Individuals affected by breast or ovarian cancer are denoted by a black circle or square and individuals affected by other cancers are denoted by a grey circle or square; site of tumor and age of diagnosis, when available is also indicated: Br, breast (individuals with two Br, reflect contralateral breast cancer); BBr, bilateral breast cancer; DCIS, ductal carcinoma in situ; Ips, ipsilateral; Li, liver; Ov, ovary; Pr, prostate; Re, rectal; Sk, skin; Un, unknown site and Ut, uterine. (B) Multiplex Ligation-dependent Probe Amplification (MLPA) analysis of genomic DNA showing *BRCA1* Δ exons 16/17. Note the halved intensity of the peaks indicated by arrows (corresponding to probes for exons 16 and 17, respectively) in the proband sample relative to the normal control specimen. It indicates genomic hemizygosity for *BRCA1* Δ exons 16/17. (C) Agarose gel of PCR products from cDNA with primers situated in exons 15 and 18 to amplify across the region containing the deletion shows *BRCA1* deletion of exons 16 and 17 in proband's RNA. M, molecular weight marker; P, Proband; W, water blank; N, normal control and C, RT blank. (D) Sequencing of PCR amplicon from cDNA showing *BRCA1* Δ exons 16/17 in proband's RNA. Note that splicing of exon 15 to exon 18 preserves the reading frame.

analysis of the proband's tumor revealed loss of the wild type BRCA1 allele (data not shown).

3.2.3. Q1826H

This variant was identified in family M1123 (Fig. 2A). This variant results from nucleotide change 5597G → T in *BRCA1*. The variant was predicted by two different computational prediction tools (ESEfinder, http://exon.cshl.edu/ESE/and RESCUE-ESE, http://genes.mit.edu/burgelab/rescue-ese/) to destroy a potential exonic splicing enhancer (ESE) sequence in exon 24. However, an RT-PCR analysis on a lymphoblastoid cell line derived from the proband did not detect any altered cDNA in the BRCA1 C-terminal region (data not shown). Moreover, sequencing of the amplified cDNA fragment suggested that the mutant allele was expressed at a level comparable to that of the wild type.

3.2.4. K1487R

This variant was identified in family M1154 (Fig. 2A). BRCA analysis revealed an additional missense variant in BRCA2 (D935N),

which has recently been classified in the BIC database as of no clinical importance.

3.2.5. Limitations of pedigree analysis

Because pedigree analysis was mostly uninformative and insufficient to classify the respective variants (Fig. 2A), we turned to structural-based predictions and functional analyses to characterize the *BRCA1* variants (see below).

3.3. Functional and computational analysis

3.3.1. Bioinformatics analysis

We applied our previously published bioinformatics method LS-SNP [18,25–27] to predict whether the BRCT missense variants M1652I, Q1826H, and V1833M have a deleterious or neutral effect on BRCA1. The method uses supervised learning algorithms that integrate a variety of bioinformatics-derived predictive features, including properties of amino acid sequence changes, evolutionary conservation, location in protein structure and predicted impact

on protein structure. These algorithms are "black boxes" and do not explain why a variant may be deleterious. It is possible that a variant BRCA1 protein is unable to fold into a stable structure, that it partially folds and is rendered more susceptible to proteolytic degradation than the wild type, or that binding interactions with ligands or protein partners are adversely affected.

We also assess the predictions of our method, when compared to available genetic or integrative classifications, and compare them to five other bioinformatics missense variant prediction methods: SIFT [40], PolyPhen [41], nsSNPanalyzer [42], PMUT [43] and Panther [44] (Tables 2 and 3). We used the web servers for these five methods and submitted 20 variants to each during the first week in August, 2008. Taking into account the genetic/integrative classifications, it appears that SIFT overpredicts Tolerated class, and that

PolyPhen, PMUT and Panther overpredict the Damaging, Pathological and Deleterious classes.

3.3.2. Functional analysis and structural rationalizations

In order to assess the functional impact of the changes in BRCA1 we determined the transcriptional activity of the C-terminal region of BRCA1 in which the variants were introduced. First, we assessed the activity of constructs containing in-frame deletion and frameshift variants (Fig. 1B and C) in yeast and mammalian cells. Yeast cells were co-transformed with a LexA-responsive β-galactosidase reporter gene (see Fig. 3) and a LexA DBD fusion to residues 1396–1863 of wild type BRCA1 (wt), or the same fragment carrying the Δ exons 16/17 or the 5673insC variants. We used the wt and the S1613G neutral polymorphism as positive con-
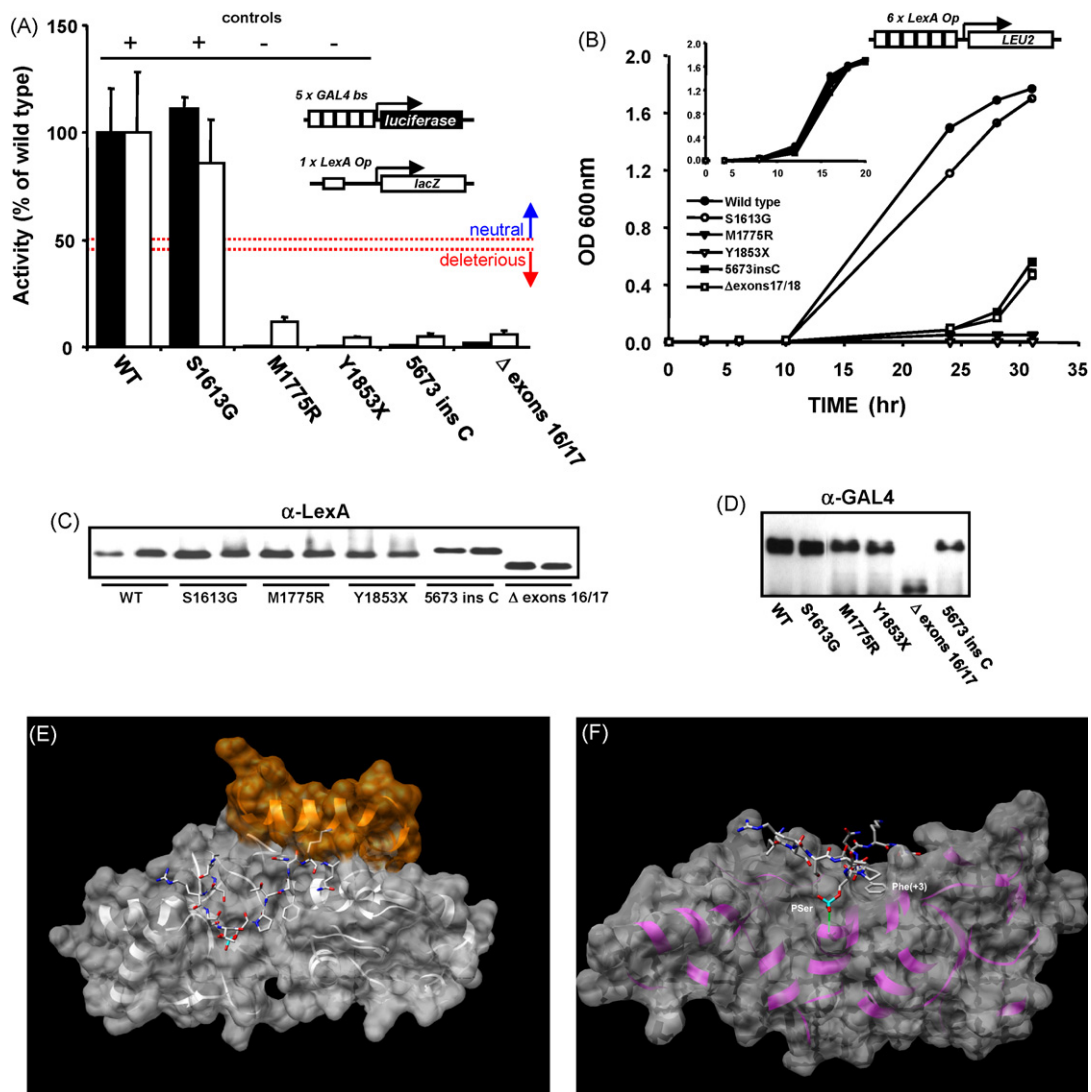

Fig. 3. Functional analysis and structural modeling of frameshift and splicing variants in *BRCA1*. (A) Quantitative transcriptional assay in yeast (white bars) and in mammalian cells (black bars). (B) Transcriptional assay in yeast using a less stringent reporter (diagram shown above the graph) driving *LEU2*. EGY48 cells expressing wt or mutant BRCA1 constructs were grown in the absence of tryptophan and leucine. Inset, control growth assay in the absence of tryptophan only. To control for possible variations in protein expression levels, samples were analyzed by western blot using α-LexA DBD polyclonal antibody in yeast extracts (C) or mouse α-GAL4 DBD monoclonal antibody in mammalian cell extracts (D). (E) Model of the structural changes induced by the 5673insC variant built with SAM-T06 and UNDERTAKER [25], an all-heavy-atom fragment packing program for protein structure prediction. The insertion may form a novel 13-residue helix (shown in gold). (F) Model of the structural changes induced by the Δ exons 16/17 variant built with SAM-T06 and UNDERTAKER. The image shows a transparent surface representation of the model (light gray) superimposed on a ribbon representation (purple) with BACH1 peptide (stick representation colored by element). The two key binding residues Phe(+3) and phosphoserine are labeled. The deletion of exons 16/17 does not impact the hydrophobic binding pocket of Phe(+3), but a hydrogen bond between the backbone nitrogen of GLY 1656 in BRCT-N and the phosphoserine O2P oxygen is disrupted as GLY 1656 is not present in the deletion mutant. Images generated with UCSF Chimera [25,54,55] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

trols (+). Deleterious mutations M1775R and Y1853X were used as negative controls (−). Three independent yeast clones were tested in triplicates. Mammalian cells were co-transfected with a GAL4-responsive firefly luciferase reporter gene (see Fig. 3), a *Renilla* luciferase driven by a constitutive promoter (internal control, not shown), and a GAL4 DNA binding domain (DBD) fusion to residues 1396–1863 of wild type BRCA1 (wt), or the same fragment carrying the Δ exons 16/17 or the 5673insC variants. Controls are the same as described above but fused to GAL4 DBD. Measurements were done in triplicates and normalized against the internal transfection controls. The activity of the wild type *BRCA1*:DBD fusion construct was then expressed as 100%, with the other results placed on this scale. The thresholds of activity (see Section 2) used for classification are indicated by the dotted lines in Fig. 3. Constructs containing the Δ exons 16/17 and 5673insC variants failed to activate transcription of the *lacZ* or the luciferase reporter genes in yeast as well as in mammalian cells, respectively (Fig. 3A).

In order to determine whether the constructs containing the variants had any residual activity, we also assessed their activity in a less stringent growth assay. Instead of using a reporter gene driven by a single binding site, as in the previous experiment, we used a reporter with six LexA binding sites which allows growth in the absence of leucine when the reporter is activated [21]. Yeast expressing Δ exons 16/17 or 5673insC exhibited a growth pattern similar to the negative controls up to 24 h, with modest growth at 28 h and 32 h (Fig. 3B). In non-selective medium their growth was similar to wt indicating that the expression of the variants did not result in impaired growth (Fig. 3B, inset). Both variant constructs are expressed at levels comparable to the wt in yeast cells (Fig. 3C) but display somewhat reduced levels in mammalian cells (Fig. 3D)

indicating that the mutations might confer a destabilizing effect in human cells.

In order to investigate the putative structural changes generated by the 5673insC insertion we built a structural model that suggests that the insertion could generate a novel 13-residue α-helix that might modify the binding of phosphopeptide to the BRCT binding pocket (Fig. 3E). We also built a model for the putative structure of the Δ exons 16/17 variant in which the β1, β2, β3 strands, and the α1 helix are missing (Fig. 3F). Of the two key binding residues in the BACH1 peptide (Phe + 3 and phosphoserine) [33], the Phe + 3 binding pocket is intact in the deletion model but a hydrogen bond between BRCT-N (backbone nitrogen of GLY 1656) and the phosphoserine O2P oxygen is disrupted because GLY 1656 is missing. We predict that these changes will result in weaker phosphopeptide binding. Taken together, the functional data and the structure prediction suggest that both variants constitute deleterious mutations.

The missense variant series (K1487R, S1613C, M1652I, Q1826H, and V1833M) was evaluated for transactivation activity exclusively in the context of stringent reporters (Fig. 4A) as previously defined for missense variants [12,14–16]. Variants S1613C, M1652I, and Q1826H exhibited activity significantly higher than 50% of the wt activity and are likely to constitute neutral variants (Fig. 4A). Variant V1833M showed reduced activity of ∼30% compared to the wild type in yeast and a markedly reduced activity in mammalian cells. Thus, it is likely to be a deleterious variant (Fig. 4A). Variant K1487R displayed ∼50% of the wt activity, which is considered to be the threshold for classification into deleterious or neutral [14] and therefore cannot be classified with the current data. Differences in activity could not be accounted for by differences in protein levels, as expression of fusion constructs did not necessarily correlate with activity (Fig. 4B).
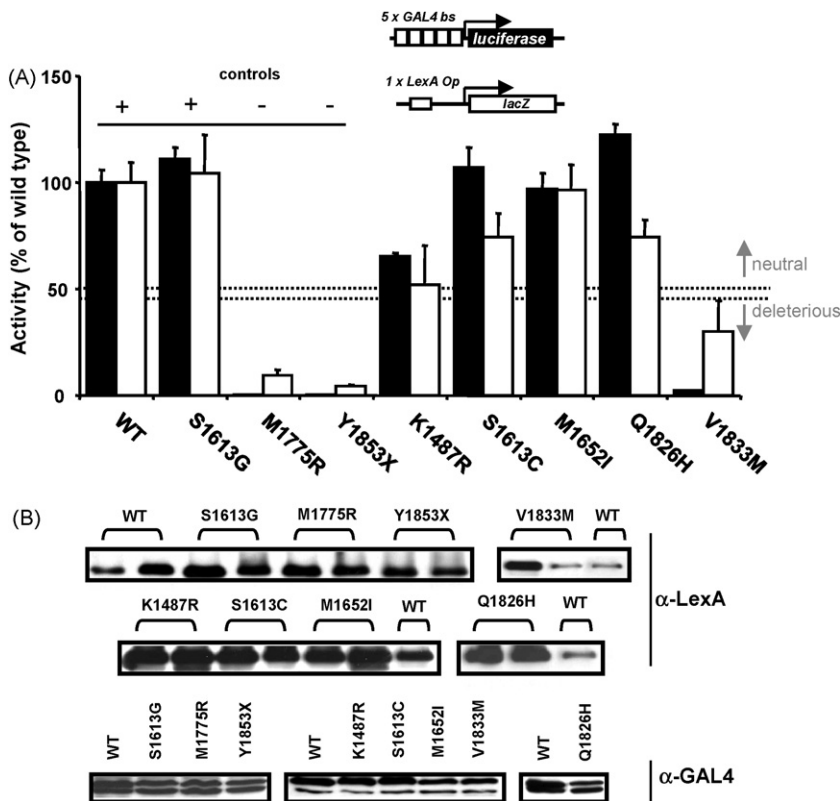

Fig. 4. Functional analysis of missense variants in BRCA1. (A) Quantitative assay in yeast (white bars) and mammalian cells (black bars) performed as described in Fig. 3. The thresholds of activity (see Section 2) used for classification are indicated by dotted lines. (B) Protein levels determined by western blot using α-LexA DBD polyclonal antibody for yeast extracts (top panels) or mouse α-GAL4 DBD monoclonal antibody in mammalian cell extracts (bottom panel).

Table 1
Structure-based analysis of BRCA1 variants in the BRCT domains.

Class	Variant	Notes
Hydrophobic core disruption	V1833M	V1833 in BRCT-C is completely inaccessible to solvent. It lies in the β4 strand, an edge strand of a four-stranded parallel β-sheet. The sidechain points inward towards the protein core. Valine is a β-branched residue that is more favored in β-strands than methionine and methionine is larger than valine, so this replacement likely disrupts the tight packing in the domain's hydrophobic core.
No Evidence for Functional Impact	Q1826H	Q1826 in BRCT-C is on the protein surface and its sidechain points outward to the solvent. There are no known or predicting binding interactions at this position.
	M1652I	M1652 in BRCT-N is completely inaccessible to solvent. It lies in the β1 strand, an interior strand of a four-stranded parallel β-sheet. Isoleucine is a hydrophobic, β-branched residue that is more favored in β-strands than methionine. We expect that this replacement is benign with respect to BRCT function and may even have a protective, stabilizing effect in the presence of other destabilizing mutations.

Table 1 summarizes the results obtained when the missense variants located at the BRCT region (M1652I, Q1826H, and V1833M) were rationalized based on our structural models. Variant V1833M is predicted to disrupt the hydrophobic core of the BRCT domain. V1833 in BRCT-C is completely inaccessible to solvent. It lies in the β4 strand, an edge strand of a four-stranded parallel β-sheet. The side chain points inward towards the protein core. Valine is a β-branched residue that is more favored in β-strands than methionine. In addition, methionine is larger than valine, so this replacement likely disrupts the tight packing in the hydrophobic core of the BRCT-C domain. On the other hand, we found no evidence for functional impact in variants M1652I and Q1826H. Residue Q1826 in BRCT-C is on the protein surface and its side chain points outward to the solvent. There are no known or predicted binding interactions at this position. M1652 is located in BRCT-N and is completely inaccessible to solvent. It lies in the β1 strand, an interior strand of a four-stranded parallel β-sheet. Isoleucine is a hydrophobic, β-branched residue that is more favored in β-strands than methionine. We expect that this replacement is benign with respect to BRCT function and may even have a protective, stabilizing effect in the presence of other destabilizing mutations.

3.4. Validation

Recently we evaluated the specificity and sensitivity of the transcriptional assay using a set of variants that had been classified by genetic and integrative methods [14]. Importantly, we only considered missense variants, and in that case the assay correctly classified all 24 variants tested [14]. This resulted in 100% sensitivity (95% CI 69% – no defined upper bound) and 100% specificity (95% CI 77% – no defined upper bound) [14]. Here, we update the validation for the transcription assay taking into consideration additional variants for which genetic or integrative data has emerged and classified variants with truncations, frameshift, or in-frame deletions, totaling 31 variants (Table 2). Sensitivity for the updated set is 100% (95% CI 79.4% – no defined upper bound). Specificity was 93% (95% CI 68.1–99.8%) with the assay correctly classifying 14/15 neutral variants. Variant V1736A was classified as deleterious in the tran-

Table 2
Cross validation of the transcription assay with genetic data.

Variants[a]	Genetic or integrative classification	Functional classification	References
H1402Y	Neutral	Neutral	Judkins et al. [35]; Phelan et al. [15]
R1443G	Neutral	Neutral	Overall odds against causality[b] >50:1; Carvalho et al. [12,14]
S1512I	Neutral	Neutral	Tavtigian et al. [36]; Phelan et al. [15]
V1534M	Neutral	Neutral	Chenevix-Trench et al. [56]; Easton et al. [11]; Carvalho et al. [12,14]
D1546N	Neutral	Neutral	Tavtigian et al. [36]; Carvalho et al. [12,14]
T1561I	Neutral	Neutral	Judkins et al. [35]; Hayes et al. [24]
L1564P	Neutral	Neutral	Judkins et al. [35]; Tavtigian et al. [36]; Carvalho et al. [12,14]
S1613G	Neutral	Neutral	Tavtigian et al. [36]; Phelan et al. [15]
P1614S	Neutral	Neutral	Judkins et al. [35]; Carvalho et al. [12,14]
M1628T	Neutral	Neutral	Judkins et al. [35]; Tavtigian et al. [36]; Phelan et al. [15]
M1652I	Neutral	Neutral	Tavtigian et al. [36]; this study
A1669S	Neutral	Neutral	Judkins et al. [35]; Vallon-Christersson et al. [16]
T1685I	Deleterious	Deleterious	Easton et al. [11]; Carvalho et al. [12,14]
V1688del	Deleterious	Deleterious	Easton et al. [11]; Vallon-Christersson et al. [16]; Malacrida et al. [57]
M1689R	Deleterious	Deleterious	Easton et al. [11]; Mirkovic et al. [17]
R1699W	Deleterious	Deleterious	Easton et al. [11]; Vallon-Christersson et al. [16]
R1699Q	Deleterious	Deleterious	Chenevix-Trench et al. [56]; Vallon-Christersson et al. [16]
G1706E	Deleterious	Deleterious	Easton et al. [11]; Carvalho et al. [12,14]
A1708E	Deleterious	Deleterious	Easton et al. [11]; Carvalho et al. [12,14]
S1715R	Deleterious	Deleterious	Easton et al. [11]; Vallon-Christersson et al. [16]
T1720A	Neutral	Neutral	Easton et al. [11]; Phelan et al. [15]
V1736A	Neutral	Deleterious	Easton et al. [11]; Carvalho et al. [12,14]
G1738R	Deleterious	Deleterious	Chenevix-Trench et al. [56]; Easton et al. [11]; Carvalho et al. [12,14]
5382insC	Deleterious	Deleterious	Miki et al. [1]; Monteiro et al. (1996)
L1764P	Deleterious	Deleterious	Easton et al. [11]; Carvalho et al. [12,14]
I1766S	Deleterious	Deleterious	Easton et al. [11]; Carvalho et al. [12,14]
M1775K	Deleterious	Deleterious	Tischkowitz et al. [32]
M1775R	Deleterious	Deleterious	Miki et al. [1]; Phelan et al. [15]
G1788V	Deleterious	Deleterious	Easton et al. [11]; Phelan et al. [15]
V1804D	Neutral	Neutral	Easton et al. [11]; Carvalho et al. [12,14]
Y1853X	Deleterious	Deleterious	Friedman et al. [37]; Phelan et al. [15]

[a] Variants in bold are nonsense, frameshift or deletions.
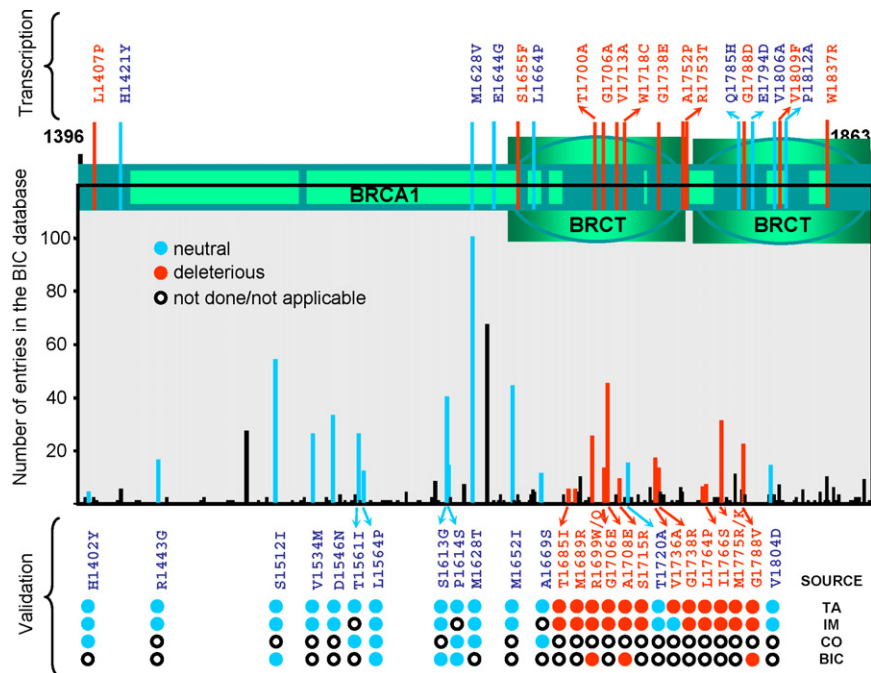[b] D. Goldgar, personal communication.

Fig. 5. Classification of missense variants in the carboxy-terminal region of BRCA1. _Top_, Diagram of human BRCA1 depicting the carboxy-terminal region including the BRCT domains. Regions showing conservation above 0.75 in a multiple sequence alignment (from human to puffer fish) are shown as dark green boxes. Variants that have been tested by the transcription assay are indicated by red (deleterious) or blue (lines) but have not yet been classified by other integrative methods. _Bottom_, graph aligned to the top diagram shows the number of entries in the BIC database for each missense variants recorded to date. Bars record entries for all variants for a particular codon (number of entries for specific variant may be lower than what is shown because different substitutions may have been recorded). The lower part of the graph shows cross-validation of the transcription assay (TA) with integrative methods (IM), the BIC classification (BIC), or co-occurrence data (CO). Variants are shown as black (unclassified), red (deleterious), and blue (neutral) bars. All deleterious and neutral variants as classified in the BIC database are shown. Note preferential localization of deleterious variants in regions of recognized domains (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

scriptional assay which was supported by structure-based analysis [14]. However, integrative analysis using family data resulted in odds of 2219:1 in favor of neutrality [11]. Interestingly, this variant is also classified as deleterious by all other prediction models except SIFT (Table 3). Myriad Genetics considers this variant unclassified (Michelle Martin, Myriad Genetics, personal communication). Despite the uncertainty about V1736A classification we decided to be consistent and keep it in the validation set. Fig. 5 shows a diagram of BRCA1 C-terminal region and the classification of all variants tested to date. For a complete list of variants in _BRCA1_ and their current classification the reader is referred to the Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/). In summary, the transcription assay is highly accurate and, despite the small number of variants studied to date, can reliably classify truncations, frameshift, and in-frame deletions.

4. Discussion

Due to the low frequency of most _BRCA1_ unclassified variants there is a shortage of clinical data to base a decision as to whether or not they will predispose to disease. Here we have combined functional analysis and bioinformatics-based prediction models to aid in the classification of in-frame deletion, frameshift, and missense variants of _BRCA1_. We have recently provided validation for our functional assays for missense variants and used the validated functional data to fine tune structure-based computation models [12,18]. Based on these methods we provide a tentative classification for the variants tested. Currently, there is no genetic or clinical data available for these variants that is conclusive and which could contribute to the classification. Although it is not possible to conclude with certainty, without additional genetic data, whether or not our classification reflects the in vivo biology, we believe

it provides an important addition to our knowledge about these variants.

The data obtained in vitro indicate that frameshift (5673insC) and in-frame deletion (Δ exons 16/17) novel variants display a loss-of-function phenotype and are likely to constitute cancer-predisposing variants of _BRCA1_ in vivo. Deletion analysis revealed that the integrity of the most BRCA1 C-terminal hydrophobic cluster (I1855, L1854, and Y1853) is necessary for transcriptional activity [24]. The Δ exons 16/17 variant, although still retaining an intact minimal transactivation domain (aa 1796–1863) [45,46], results in loss of function. These results are in line with the idea that the tandem BRCT repeats function as an integrated unit [17,47,48]. Unfortunately, there is no additional published data for these two variants.

Interestingly, modeling of the 5673insC suggested the existence of an α-helix that could disrupt binding to the BRCT phosphopeptide binding pocket (Fig. 3E). It is important to stress that structural models are inherently less reliable than experimental structures obtained through X-ray crystallography. In addition, although our functional assay has been validated ([12] and the present study) we have thus far only examined missense variants, and have not yet conducted a systematic analysis of the behavior of insertion or deletion variants. Therefore, caution should be used when considering structural implications of these variants.

Recently, using a large set of variants we have determined a tentative threshold of activity below which variants are likely to be cancer-predisposing [14]. Neutral and deleterious variants display >50% or <45% of wild type activity, respectively. In this context, variants S1613C, M1652I and Q1826H are likely to represent neutral variants. Variant M1652I is documented as a validated cSNP (rs1799967) in NCBI's SNP database (http://www.ncbi.nlm.nih.gov/SNP/) with 0.002 of heterozygosity.

Variant S1613C failed to activate transcription in yeast using a less sensitive qualitative assay, providing a cautionary note to the interpretation of functional assays [49]. Interestingly, variant S1613C is located at the same residue as one of the most common neutral polymorphisms in *BRCA1*, S1613G. S1613G does not contribute to risk for breast and ovarian cancer, and in functional assays its activity is comparable to the wild type BRCA1 [14]. For this reason, it is commonly used as a neutral control (see Figs. 3 and 4).

M1652I is one of the most common missense variants with a heterozygote frequency of 4.08% in a control population [50]. Taking into account co-occurrence with other known deleterious mutations and examination of the range of amino acid variation in other BRCA1 orthologs, Tavtigian et al. arrived at an integrated likelihood ratio of $<1.0 \times 10^{-10}$, and was considered neutral [36]. Moreover, this variant is comparable to the wt in the yeast growth assay and in its phosphopeptide binding properties [33,51]. Thus, taken together, the functional and computation prediction data presented here and the published data from multiple independent sources indicate that the M1652I constitutes a neutral variant. There is no additional available data for Q1826H.

The K1487R variant exhibits an amino acid change (Lys to Arg) with no alteration in charge and with large resemblance in structure in a position that allows Glycine in rodent species. In the present analysis, this variant displayed a decreased activity only slightly above 50% of the wild type, making it difficult to classify as either deleterious or neutral. To the present date, all tested variants located in this unstructured region of BRCA1, bordered by a coiled-coil region on its amino-terminus and the BRCT on its carboxy-terminus, have so far shown a pattern of activity compatible with a neutral classification [14]. Thus, although the conservative nature of the change and its location in a nonconserved and unstructured region may suggest neutrality, it is not possible to provide a classification for this variant without additional data.

On the other hand, variant V1833M presented activity comparable to that of the negative control variants (*i.e.*, significantly below 45% of the wild type) and is therefore classified as a deleterious allele. It has also been shown to destabilize the BRCT in vitro but its thermal unfolding seems to be only moderately affected and it remains able to bind phosphopeptides when measured by isothermal titration calorimetric experiments [31,52]. It should be noted that an early version of a computational prediction model suggested that M1652I and V1833M were likely to be neutral. While the results for the M1652I were a rationalization of a preliminary qualitative functional assay, the V1833M was a prediction by the model. The rule-based decision tree used by the model arrived at a neutral classification by considering solely that the valine to methionine substitution was a likely amino acid change. Indeed, the Grantham score [53] for Val/Met is very low, which is unusual for a deleterious missense mutation. However, the bioinformatics analysis and

Table 3
Bioinformatics predictions of the impact of BRCA1 variants in the BRCT domains.

Variant	LS-SNP	SIFT	PolyPhen	nsSNPanalyzer	PMUT	Panther
M1652I	Neutral	Tolerated	Benign	Disease	Pathological	Not deleterious
A1669S	Neutral	Tolerated	Benign	Neutral	Neutral	Not deleterious
T1685I	Deleterious	Not Tolerated	Probably Damaging	Disease	Pathological	Deleterious
M1689R	Deleterious	Tolerated	Probably Damaging	Disease	Pathological	Deleterious
R1699W	Deleterious	Not Tolerated	Probably Damaging	Disease	Pathological	Deleterious
R1699Q	Deleterious	Tolerated	Probably Damaging	Disease	Pathological	Deleterious
G1706E	Deleterious	Not Tolerated	Probably Damaging	Disease	Pathological	Deleterious
A1708E	Deleterious	Not Tolerated	Probably Damaging	Neutral	Pathological	Deleterious
S1715R	Deleterious	Not Tolerated	Probably Damaging	Disease	Pathological	Deleterious
T1720A	Neutral	Not Tolerated	Possibly Damaging	Neutral	Neutral	Not deleterious
V1736A	Deleterious	Tolerated	Possibly Damaging	Disease	Pathological	Deleterious
G1738R	Deleterious	Tolerated	Probably Damaging	Disease	Pathological	Deleterious
L1764P	Deleterious	Not Tolerated	Probably Damaging	Disease	Pathological	Deleterious
I1766S	Deleterious	Not Tolerated	Probably Damaging	Disease	Pathological	Deleterious
M1775K	Deleterious	Tolerated	Probably Damaging	Disease	Pathological	Deleterious
M1775R	Deleterious	Tolerated	Probably Damaging	Disease	Pathological	Deleterious
G1788V	Deleterious	Tolerated	Probably Damaging	Disease	Pathological	Deleterious
V1804D	Neutral	Not Tolerated	Benign	Neutral	Pathological	Not deleterious
Q1826H	Neutral	Tolerated	Probably Damaging	Neutral	Pathological	Deleterious
V1833M	Deleterious	Tolerated	Benign	Disease	Neutral	Deleterious

The three BRCT missense variants analyzed in this study are shaded in gray. We also show predictions of 17 additional BRCA1 variants for which there is a genetic or integrative classification available ("Validation set", Table 2). Red in the "Variant" column indicates that the genetic or integrative classification is deleterious; Blue that it is neutral. We compare the predictions of our LS-SNP method [18,25] with SIFT [40], PolyPhen [41], nsSNPanalyzer [42], PMUT [43] and Panther [44].

rationalization of structural impact performed here (Tables 1 and 3) suggest that the Val/Met change, in the context of the tight packing of the BRCT-C domain, may be more disruptive than previously believed.

In conclusion, we analyzed a series of missense, frameshift, and in-frame deletion variants of *BRCA1* for which clinical information was not available or not informative. Using a combination of functional analysis and structural predictions we tentatively classify variants V1833M, 5673insC and Δ exons 16/17 as likely to be deleterious and missense variants M1652I, S1613C, and Q1826H as likely to be neutral.

Competing interests

The authors declare that there are no competing interests.

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