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Multimodal Assessment of Protein Functional Deficiency Supports Pathogenicity of BRCA1 p.V1688del

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Abstract

Unequivocal discrimination between neutral variants and deleterious mutations is crucial for appropriate counseling of individuals with a *BRCA1* or *BRCA2* sequence change. An increasing number of variants of uncertain significance (VUSs) are being identified, whose unclassified biological effect poses clinical concerns. A multifactorial likelihood-based approach recently suggested disease causality for BRCA1 p.V1688del, a VUS recurrent in Italian breast/ovarian cancer families. Whether and how this single amino acid deletion in the BRCA1 BRCT domain affects the function of the mutant protein (Δ ValBRCA1) has not been elucidated. We undertook comprehensive functional characterization of Δ ValBRCA1, comprising comparative structural modeling, analysis of protein stability and associations, and analysis of DNA repair function. Our model predicted BRCT domain destabilization and folding disruption caused by BRCA1 p.V1688del. Consistently, the recombinant Δ ValBRCA1 was less stable than wtBRCA1 and, unlike the latter, failed to associate with BRIP1, CtIP, and Rap80, and to re-localize to sites of DNA damage. Yeast two-hybrid analysis revealed a compromised interaction with FHL2 and with KPNA2, which is likely responsible for improper subcellular localization of Δ ValBRCA1. In addition, we found four new breast/ovarian cancer families of Italian ancestry who carried this sequence alteration. These results provide the first evidence of the effect of BRCA1 p.V1688del on protein stability and function, supporting the view that it is a deleterious mutation. Multimodal analyses like ours could advance understanding of

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tumor suppression by BRCA1, and ultimately contribute to developing efficient strategies for screening and characterization of VUSs.

Keywords

BRCA1; variants of uncertain significance; hereditary breast/ovarian cancer; BRCT repeats; tumor suppressors

INTRODUCTION

The inheritance of a loss-of-function allele in either of the two tumor suppressor genes, *BRCA1* and *BRCA2*, confers a significantly increased lifetime risk to develop breast and/or ovarian cancer (1). Mutational screening of the *BRCA* genes is important in clinical practice and has become a valuable tool for breast/ovarian cancer risk estimation and reduction. To appraise the cancer proclivity of each detected sequence alteration can be challenging, leaving risk communication and management uncertain.

The full-length *BRCA1* gene product, a 220 kDa nuclear phosphoprotein, functions in multiple cellular processes, including homologous recombination (HR)-mediated DNA damage repair, cell cycle checkpoint control, transcriptional regulation, centrosome duplication, heterochromatin maintenance, and mitosis (2,3). The BRCA1 protein has a long, intrinsically disordered central region (4) bracketed by two evolutionarily conserved domains: an amino (N)-terminal RING finger domain and two tandem carboxyl (C)-terminal *BRCA1* C-terminus (BRCT) repeats (BRCT domain). The RING finger exhibits E3 ubiquitin ligase activity upon heterodimerization with the structurally-related partner protein, *BRCA1*-associated RING domain (BARD1) (5). The BRCTs are highly structured ~95 amino acid (aa) motifs, found in more than 50 proteins involved in DNA repair and cell cycle checkpoint regulation (6). They are characterized by a distinct cluster of hydrophobic amino acids, which constitute the core of the repeat fold (6), and contribute to the stability of BRCA1 (7). The two BRCT repeats behave as a single functional unit, which specifically binds phospho-serine (pSer)- or phospho-threonine (pThr)-containing proteins (8,9). Interactions with several such proteins, *e.g.* BRIP1 (*BRCA1* interacting protein 1), also known as BACH1 (*BRCA1*-associated C-terminal helicase 1) (10), and CtIP (*C*-terminal binding protein (CtBP)-interacting protein) (11) have been elucidated in detail, providing insights into ligand recognition (12–14).

Most functionally detrimental *BRCA1* mutations identified thus far are frame-shift and nonsense sequence changes that result in premature translational termination (15). Genomic rearrangements, missense mutations and splice site mutations account for the remainder of the *BRCA1* mutational spectrum (15). An increasing number of variants of uncertain significance (VUSs) are being identified and catalogued in the Breast Cancer Information Core (BIC) database¹. Their biological and clinical relevance still awaits elucidation, with consequent delays in decision-making. Up to 20% (this percentage being higher in non-white populations (16)) of all *BRCA1* sequence changes are currently categorized as VUSs (17).

Several reported methods aim to determine whether or not a VUS is cancer-predisposing. A recently developed (18), and subsequently expanded (17) or adapted (19,20), multifactorial-likelihood model, which integrates data from several sources, seems to represent the most comprehensive strategy to reliably state for or against causality. Studies providing functional support to the modeled predictions are always an invaluable and sought-after adjunct. *Ad*

¹<http://research.nhgri.nih.gov/bic/>

hoc functional assays are currently available only for sequence changes residing in the structurally and functionally well-characterized RING and BRCT domains.

The application of a multifactorial likelihood-based approach has recently suggested BRCA1 p.V1688del (c.5181_5183delGTT), a sequence variant recurrent amongst Italian families, as a likely pathogenic alteration (21). No studies have yet been carried out to ascertain whether and how this single-amino acid in-frame deletion in the BRCA1 C-terminus impacts the biological function of the mutant protein.

Here, we employed a multidimensional approach to investigate the functional repercussions of BRCA1 p.V1688del. Our results show that this sequence alteration profoundly destabilizes the BRCT hydrophobic core and compromises protein stability, thus implying its detrimental effect.

MATERIALS AND METHODS

Structural modeling

For comparative protein structural modeling we used the MODELLER program (22). The crystallographic structure of the human wild-type (wt) BRCA1 BRCT domain (wtBRCT) (PDB code: 1jnx) served as a template (7). The model was validated with the Verify3D Structure Evaluation Server², and by Ramachandran plot analysis. Figures 1A and B were created using PyMOL software³.

Constructs

The three nucleotide-deletion at the relevant site was generated using the Quick Change site-directed Mutagenesis kit (Stratagene). Wild-type templates were: pcDNA3βHA-BRCA1 (23) (kindly provided by R. Scully), pcDNA3.1Myc/3xHA-BRCA1 and pcDNA3.1Myc/3xHA-BRCA1 fragment #6 (aa: 1314–1863) (24) (kindly provided by S. Pathania). Constructs were fully sequenced before use. Protein and cDNA numbering are based on GenBank⁴ entries AAA73985 and U14680, respectively.

Cell culture and transfection

Both 293T and HeLa cells were maintained in Dulbecco's modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (Gemini) and antibiotics, at 37°C in a humidified incubator containing 10% (vol/vol) CO₂. Upon growth to 50% confluence, cells were transfected with 6 μg of relevant plasmid (and, when specified, with 0.5 μg of pEGFP-C2 plasmid (Clontech)) using Fugene 6 transfection reagent (Roche).

Immunoprecipitation, Western-blotting and protein stability assays

Immunoprecipitations, Western-blotting, and Cycloheximide-chase experiments were carried out as previously described (25).

Gamma irradiation and fluorescence microscopy

Forty hours after transfection, cells (seeded onto coverslips placed in 6 well plates) were either mock treated or exposed to 8 Gy of ionizing radiation (IR) using a Gammacell 40 Exactor (Nordion), and allowed to recover for up to 5 hours at 37°C. When necessary, soluble proteins were pre-extracted with Digitonin before fixation. Cells were, then, fixed in 3%

²<http://www.doe-mbi.ucla.edu/Services/Verify3D.html>

³<http://www.pymol.org/>

⁴<http://ncbi.nlm.nih.gov/GenBank/>

paraformaldehyde/2% sucrose, permeabilized in Triton X-100 0.5%, and incubated with relevant (primary or secondary) antibodies, for 1 hour at 37°C. Coverslips were mounted over Dapi-containing Vectashield mounting medium (H-1200, Vector Laboratories). Images were obtained using an Axioskop 2 (Zeiss) equipped with an AxioCam HRc (and with a Plan APOCHROMAT 63×/1.4 Oil DIC objective), and analyzed using the AxioVision software (Zeiss).

Antibodies

The polyclonal antibodies used were: anti-BARD1 (BL518) (Bethyl); affinity-purified anti-BRIP1 182 (25); anti-HA (ab9110) (Abcam); anti-gamma (γ) H2AX (Bethyl); anti-Rap80 (kindly provided by R. Greenberg and B. Sobhian). The monoclonal antibodies used were: anti-BRCA1 MS110 (23); anti-CtIP 14-1 (generous gift from R. Baer); anti-GFP, clone C163 (Zymed Laboratories Inc); anti-HA.11, clone 16B12 (Covance); anti-Myc, clones 9B11 (Cell Signaling Technology) and 4A6 (Upstate Biotechnology); anti-alpha tubulin, clone DM1A (Sigma). Immunodetection was carried out using: HRP-conjugated sheep anti-mouse IgG, HRP-conjugated donkey anti-rabbit Ig, or HRP-conjugated protein A (Amersham Biosciences). Fluorescein Isothiocyanate (FITC)- or Rhodamine Red-X-conjugated goat anti-mouse and anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) were used in fluorescence microscopy experiments.

Yeast two-hybrid analysis

Wild-type and ΔVal BRCA1 full-length ORFs were PCR-amplified with primers tailed with the AttB1 and AttB2 Gateway recombinational cloning sequences and subcloned via BP clonase reaction in pDONR223 vector. Entry clones were verified by sequencing, and transferred individually into both pDB (DNA Binding domain)-dest- and pAD (Activation Domain)-dest-CYH destination vectors by Gateway recombinational cloning using LR clonase (Invitrogen). Yeast two-hybrid tests for interactions were carried out as described (26) with DB-ORF- and AD-ORF-allele fusions transformed into *MATα* Y8930 or *MATα* Y8800 yeast strains, respectively. Since the DB-fusion wtBRCA1 protein displayed auto-activation of the reporters, as previously shown (27), the analysis was carried out in reverse orientation using AD-fusion wt (pAD-wtBRCA1) and ΔVal (pAD-ΔBRCA1) BRCA1 clones against DB-fusion clones of 35 known binary interacting partners present in an available ORFeome collection (28). Diploid yeast cells were tested for growth on SC-Leu-Trp-His+1mM 3AT (synthetic medium without leucine, tryptophan and histidine, containing 1 mM 3-amino-1,2,4-triazole) plates to confirm *GAL1::HIS3* transcriptional activity and/or on SC-Leu-Trp-Ade (synthetic medium without leucine, tryptophan and adenine) plates to confirm *GAL1::ADE2* transcriptional activity.

Pedigree collection

Information about additional families carrying BRCA1 p.V1688del was solicited from several cancer genetics programs in the United States and in Europe. The four families (DFCI#10145, UPH#934, PI#254 and PI#276) reported herein were identified by the Cancer Risk and Prevention Clinic at the Dana-Farber Cancer Institute, the Cancer Risk Evaluation Program at the University of Pennsylvania Health System Hospital, and the Genetic Counseling Service at the University of Pisa.

RESULTS

BRCA1 p.V1688del is predicted to partially disrupt BRCT folding

The BRCA1 p.V1688del variant lies within the first of two BRCT repeats that, connected by a conserved linker, pack head-to-tail at the BRCA1 C-terminus (7). To investigate the structural

consequences of this in-frame deletion, we used the available X-ray crystal structure of wtBRCT (7) as a template and modeled the mutated sequence onto it. Whether V1687 or V1688 is the real target of the deletion cannot be discriminated due to the presence of two tandem GTT codons (21). Both V1687 and V1688 reside in the third strand of a four-strand beta-sheet packed against two alpha-helices on one side and one alpha-helix on the other side. We adopted the name first given to the variant, which indicates the most 3' GTT codon (21), and we built a three-dimensional structural model taking V1688 as the deleted residue.

In the wtBRCT, the buried V1688 interacts with neighboring hydrophobic amino acids, creating a large, stabilizing hydrophobic core (Fig. 1A). As a result of the deletion, the bulging V1688 residue would no longer contribute to formation of the hydrophobic core, causing loss of stabilization energy with compensatory local structural rearrangements and partial but significant alteration of protein folding (Fig. 1B). Furthermore, our model suggests the possible rupture of a short, main-chain atom to main-chain atom hydrogen bond interaction ([Met1689 (N)---(O)Val1714: 2.64 Å] in the wt structure) with consequent further loss of stabilization energy (Fig. 1B). In the wtBRCT this hydrogen bond participates in holding one of the alpha-helices in place against the beta-strand.

Though not surface-exposed and therefore unlikely to be directly involved in protein-protein interactions, V1688 seems to play an important functional role by contributing to the overall stability of the BRCT domain. The deletion of V1688 might result in loss of structural stability and cause regional rearrangements, which could affect binding to partner proteins. Of note, L1657, one of the neighboring residues likely impacted by the V1688 deletion (Fig. 1), is reportedly involved in phospho-peptide recognition and binding (29).

Given the similar local environment of V1687 and V1688, which, pointing in opposite directions, interact with several hydrophobic amino acids and form stabilizing hydrophobic contacts, we believe that an analogous destabilization would occur on the other side of the beta-strand, should V1687 be the deleted residue.

BRCA1 p.V1688del compromises protein stability

To probe the steady-state expression levels of the mutated vs. wt BRCA1 protein, we generated constructs encoding a p.V1688del-carrying BRCA1 protein (Δ ValBRCA1) or BRCA1 C-terminal fragment (Δ ValCtermBRCA1) by site-directed mutagenesis of wtBRCA1 and wtCtermBRCA1-encoding templates, respectively. Protein expression was analyzed in transiently transfected 293T cells. Myc and/or HA tags allowed discernment of recombinant wt and mutant BRCA1 proteins from endogenous BRCA1. Δ ValBRCA1 was always less abundant than wtBRCA1 (Fig. 2A), despite equal amounts of constructs transfected and equivalent expression detected for both exogenous transcripts (Supplementary Fig. S1A). We obtained analogous results in HeLa cells (Supplementary Fig. S1B). The reduced abundance of Δ ValBRCA1 did not seem due to decreased protein solubility (data not shown). The effect of BRCA1 p.V1688del appeared more pronounced when wtCtermBRCA1 and Δ ValCtermBRCA1 fragments were expressed and compared (Supplementary Fig. S1B). To examine whether deletion of V1688 affects stability of BRCA1, we carried out a chase experiment in which *de novo* protein synthesis was prevented by cycloheximide treatment of cells transfected with the appropriate constructs. We observed an almost unvarying wtBRCA1 signal, as opposed to a decreasing intensity of Δ ValBRCA1 (Fig. 2B), indicating a shorter half-life for the latter (Supplementary Fig. 1C). Thus BRCA1 p.V1688del compromises protein stability. We next tested whether it also affects protein function.

ΔValBRCA1 displays a deficient DNA damage response function

Control of genome integrity via involvement in DNA double strand break (DSB) repair by HR is currently believed to be the major contribution of BRCA1 to tumor suppression (30). In cells exposed to IR, phosphorylated BRCA1 re-localizes to sites of DNA DSBs (31,32), where it participates in assembly of several DNA repair complexes. As integrity of the BRCT domain is critical for both BRCA1 DSB repair and HR, we examined the DNA repair-related function of ΔValBRCA1. Cells transfected with wt or ΔVal BRCA1-encoding plasmids were irradiated and subjected to fluorescence microscopy. Although the abundance of the exogenously expressed protein made detection of typical post-DNA damage staining pattern difficult, we could identify some wtBRCA1-transfected cells unambiguously displaying characteristic foci where HA and γH2AX (used as a marker of DNA DSBs) signals co-localized (Fig. 3). In contrast, punctate HA staining was not observed in cells expressing ΔValBRCA1 (Fig. 3). Hence, ΔValBRCA1 has impaired BRCT-mediated DNA damage response and repair functions.

ΔValBRCA1 fails to associate with known partner proteins

Association with partner proteins is essential for BRCA1 to properly execute its multiple functions. To learn whether the structural and folding anomalies of the BRCT domain in ΔValBRCA1 disrupt any such association(s), we carried out immunoprecipitations from cells expressing epitope-tagged wt and ΔVal BRCA1 proteins or C-terminal fragments, and characterized the corresponding immunocomplexes.

BRIP1, a member of the DEAH helicase family and a Fanconi Anemia gene (*FANCF*) (33), encodes a protein that binds with high affinity to the BRCA1 BRCT domain from S through M phase of the cell cycle (9,10). BRIP1-containing BRCA1 complexes have a role in post-DNA damage cell cycle checkpoints (31,34). We readily detected an interaction between HAwtBRCA1 and endogenous BRIP1, but not between BRIP1 and HAΔValBRCA1 (Fig. 4A). We obtained analogous results when wt and ΔVal CtermBRCA1 polypeptides were expressed (Supplementary Fig. S2A). We then tested whether associations with other known partners were compromised.

CtIP transiently interacts with the BRCA1 BRCT domain in the late S/G2 phase of the cell cycle (34) although with lower affinity compared to BRIP1 (14), and participates in BRCA1-dependent G2/M checkpoint response following DNA damage (31,34). CtIP was readily co-immunoprecipitated from wtBRCA1-transfected cells but not from ΔValBRCA1-transfected cells (Fig. 4B). Reciprocal immunoprecipitation experiments showed that HAwtBRCA1 but not HAΔValBRCA1 was detectable in CtIP-immunocomplexes (data not shown). Identical results were obtained with constructs encoding wt and ΔVal CtermBRCA1 fragments (Supplementary Fig. S2A). Consistent with the BRCT domain being dispensable for N-terminal RING finger-mediated associations (35), ΔValBRCA1 still bound BARD1 (Fig. 4B). In summary, BRCA1 p.V1688del disrupts the interaction with the known BRCT partners, BRIP1 and CtIP, while not affecting the RING finger domain-mediated binding to BARD1.

BRCA1 also associates with Rap80 (receptor-associated protein 80), a ubiquitin-interaction motif (UIM)- and zinc finger-containing protein (36). The Rap80-BRCA1 association, which seems to occur via bridging across coiled-coil domain containing protein 98 (CCDC98), known also as Abraxas (36), is likely involved in proper targeting of BRCA1 to DNA damage sites (36). We readily detected endogenous Rap80 associated with exogenous wtBRCA1 (or wtCtermBRCA1), but not with exogenous ΔValBRCA1 (or ΔValCtermBRCA1) (Fig. 4C and Supplementary Fig. S2B). As expected, Abraxas was detected in HAwtBRCA1 but not in HAΔValBRCA1 immunoprecipitates (Supplementary Fig. S2C). These results indicate a compromised association between ΔValBRCA1 and proteins known to be involved in the

recruitment of BRCA1 to sites of DNA damage. This defect might underlie the inefficient re-localization to IR-induced foci observed for Δ ValBRCA1.

Δ ValBRCA1 does not interact with a component of the nuclear transport signal receptor

We employed a yeast two-hybrid assay to carry out a wider search for disrupted or preserved interactions, and tested pAD-wtBRCA1 and pAD- Δ BRCA1 constructs against confirmed DB-fusion BRCA1 binding partners. We recapitulated the compromised interaction between Δ ValBRCA1 and CtIP (Fig. 4D). In addition, we observed abrogated interactions of Δ ValBRCA1 with FHL2 (four and a half LIM-only protein 2) and KPNA2 (Karyopherin subunit alpha-2) (Fig. 4D). BRCA1 binds FHL2 via the second BRCT repeat, and reportedly enhances FHL2 transcriptional activity (37). The BRCA1/FHL2 interaction is disrupted by clinically relevant *BRCA1* mutations (37). KPNA2 is a component of the nuclear transport signal receptor that participates in BRCA1 NLS (Nuclear Localization Signal) recognition and contributes to BRCA1 import into the nucleus (38). Of note, Δ ValBRCA1 displayed prevalent cytoplasmic localization, and did not localize to discrete nuclear foci in non-irradiated cells (Supplementary Fig. S3). These observations suggest that the destabilization of the BRCT domain caused by the V1688 deletion might also have repercussions on other regions of the BRCA1 protein.

BRCA1 p.V1688del is common in families of Italian origin

BRCA1 p.V1688del has been described in 12 Italian families, who share a common ancestor (21). We identified four additional families carrying this sequence alteration, all of Italian ancestry (Figure 5 and Supplementary Data). Pedigree analysis evidenced characteristics of a *BRCA1* gene defect (multiple breast cancer cases, bilateral breast cancer, co-occurrence of breast and ovarian cancer in the same individual, and early age of disease onset). When possible, we collected information regarding the breast cancer phenotype. Both tumors in the proband of family DFCI#10145 were classified as triple negative (*i.e.* negative for estrogen and progesterone receptors and lacking amplification of Her-2/neu) invasive ductal carcinomas, as is true for up to 80% of BRCA1-associated cancers (39). Breast tumors of the index individuals of families UPH#934 and PI#257 were reported as breast cancers of special type (a lobular carcinoma and a papillary carcinoma, respectively). These data buttress the notion that BRCA1 p.V1688del is common amongst Italian families.

DISCUSSION

Correct management of individuals who carry a *BRCA1* sequence variant relies upon accurate assessment of whether or not the alteration confers increased risk of breast/ovarian cancer. Using a multidimensional analysis, we identified several mechanisms by which the BRCA1 p.V1688del VUS, recurrent in families of Italian ancestry, compromises BRCA1 cellular functions. The disruptive effect on the BRCT domain and the significant functional impairment of the Δ ValBRCA1 protein, provide experimental evidence that BRCA1 p.V1688del is a damaging sequence alteration and support its ascribed pathogenicity (21).

Since the multifaceted nature of BRCA1 eludes ascertainment via a single assay, we combined several approaches to gain a broad view on the functional repercussions of BRCA1 p.V1688del. Comparative protein modeling suggested an architectural change in the hydrophobic core of the BRCT domain that could affect its folding and stability, as already described for cancer-associated missense BRCA1 BRCT mutations (7,40). This steric alteration likely causes a series of local structural rearrangements that would ultimately impact the ability of the mutant protein to interact with known binding partners. Consistently, our experiments confirmed decreased stability of Δ ValBRCA1 and its failure to localize to post-DNA damage foci and to associate with BRIP1, CtIP and Rap80. The latter proteins are components of biochemically

distinct BRCA1-containing complexes, which form after DNA damage and participate in the execution of different DNA repair and/or checkpoint control activities (31,36). Thus, BRCA1 p.V1688del has an adverse effect on the ability of the BRCT repeats to function as phospho-peptide binding modules involved in protein targeting during DNA damage response. Increasing evidence suggests that such defect would likely undermine BRCA1 tumor suppression, as clinically ascertained BRCA1 BRCT mutations abolish the association with BRIP1 (10), CtIP (11,14), Rap80 and Abraxas (36), and impair DNA DSB repair function (12,29,31). Our observations also revealed that inefficient nuclear transport might be an additional factor contributing to inability of Δ ValBRCA1 to re-localize to post-DNA damage foci. Consistent with the prevalent cytoplasmic localization of the exogenous Δ ValBRCA1, BRCA1 p.V1688del disrupts interaction with KPNA2, a component of the nuclear transport signal receptor (38). This result is congruent with previous observations suggesting that BRCA1 BRCT mutations that alter protein folding adversely affect BRCA1 nuclear import (hence localization), possibly by rendering the NLSs less accessible (41). BRCA1 nuclear trafficking reportedly relies on a NLS/importin α/β mechanism and on a RING/BARD1-dependent mechanism, which also prevents BRCA1 nuclear export (42). Given the preserved interaction between Δ ValBRCA1 and BARD1, the BARD1-dependent mechanism, likely saturated by the excessive amount of exogenous protein, might suffice for nuclear accumulation of Δ ValBRCA1 in an endogenous setting. Even so, Δ ValBRCA1 would still be unable to carry out its BRCT-mediated functions, as shown above.

In addition to its main role in phospho-peptide recognition, the BRCA1 C-terminus can activate transcription when fused to a heterologous DNA-binding domain (43,44). Although this property may not necessarily reflect the physiological function of BRCA1, as BRCA1 is unlikely a *bona fide* transcriptional activator, it appears to depend critically upon the integrity of the BRCT domain (40). Since certain cancer-predisposing BRCA1 BRCT mutations abolish this activity (43,44), transcription-based assays have been used to screen VUSs residing in the BRCT domain (45–47). One such screening found that BRCA1 p.V1688del compromises transactivation (46), reinforcing our results indicative of loss of function.

Currently, the integrated model for VUS classification relies substantively on genetic and family data (48), while information derived from functional assays serves only as supportive, qualitative evidence (47,49). To be used as risk assessment tools, functional assays are required to be systematically validated, and ultimately to have the weight of their contribution quantified (47,49). Thus far, only few assays, including the aforementioned transcriptional activation assay, meet the validation prerequisite (47). Lack of detailed knowledge on the mechanisms of BRCA1 tumor suppression, and scarce availability of genetic evidence (for cross-validation), hinder development and standardization of new functional tests, ultimately impacting VUS classification. In this view, analyses like ours, albeit too labor intensive to be performed routinely in clinical laboratories, could provide valuable information as well as insights into yet undefined aspects of BRCA1 pathobiology. For instance, in light of Δ ValBRCA1 mislocalization and presumably intact E3 ubiquitin ligase activity, due to preserved binding to BARD1, one wonders whether any gain of interaction(s) or spurious substrates could contribute to cancer predisposition. Deeper understanding of mechanisms underlying BRCA1 dysfunction would, in turn, ultimately help devise more effective strategies for rapid and successful functional screening of BRCA1 VUSs. In this regard, we have recently developed an experimental platform, which can test whether the phenotypic manifestations of a mutation arise from loss of one or a handful of all interactions with binding partners⁵. This strategy could be adapted to BRCA1 and, once standardized, ease analysis of sequence alterations, including those that disrupt binding surfaces while affecting folding insignificantly

⁵Zhong Q, Li Q-R, Charlotiaux B, Heuze F, Simonis N, Klitgord N, Tam S, Mou D, Dricot A, Milstein S, Szeto D, Venkatesan K, Brasseur R, Hill DE, Cusick ME, Vidal M. Interaction-specific network perturbations of human disease proteins. Submitted.

(12,13,50). Such variants, likely missed by a single-assay or domain-specific assessment, would require a combination of structure-based predictions and *ad hoc* analyses.

Given that many VUSs are described in single families (16), the availability of genetic and epidemiologic data for deliberation about clinical significance is often limited. It is likely that the contribution of functional assays to VUS classification will expand both as combinations of standardized tests that assess different functions or interactions and as more in-depth investigations aimed at elucidation of BRCA1 dysfunction. Those who tackle the issue of classifying sequence variants will confront the challenge of deciding whether and how to incorporate functional data already available or likely to emerge as new insights are gained into the multiple functions of this versatile protein. Still, as indirect (i.e. other than genetic) evidence is not considered sufficient to confidently classify a sequence variant as pathogenic (48), exclusive reliance on functional assays to guide clinical decisions would require caution (16,47).

Our study, though based on data from assays not yet systematically validated, supports existing genetic and epidemiologic data (21), and represents a further step toward definitive classification of BRCA1 p.V1688del. Hopefully, combination of multiple strands of available evidence will ultimately allow for appropriate clinical counseling of individuals carriers of this sequence alteration regarding options for surveillance, prevention and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparative structural modeling of Δ ValBRCT

A, cartoon representation of the wtBRCT domain crystal structure. The hydrophobic residues (yellow) surrounding V1688 (purple) form a hydrophobic core that helps stabilize the fold. **B**, cartoon of the same BRCA1 region in which the theoretical model of Δ ValBRCT (light blue) is superimposed to the wtBRCT crystal structure (green). The overlay shows that the V1688 bulge observed in the wtBRCT is evened out in the Δ ValBRCT model, and that the contribution of the V1688 side chain to the stabilization of the hydrophobic core is lost. The hydrogen bonding interaction (broken line) observed in the wtBRCT between the main chain O and N atoms of V1714 and M1689, respectively, might be also lost in the mutant.

Figure 2. BRCA1 p.V1688del affects protein stability

A, lysates from 293T cells transfected with GFP alone or together with either HAwt or HAΔVal BRCA1-encoding constructs were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with an anti-HA antibody. Note reduced abundance of HAΔValBRCA1 relative to the wt counterpart (lane 3 vs. 2). For loading and transfection efficiency control, blots were probed for alpha tubulin and GFP, respectively. Molecular mass, in kilodaltons, is indicated on the left of each blot. **B**, stability of wtBRCA1 and ΔValBRCA1 assessed by cycloheximide-chase analysis. 293T cells were transfected with HAwt or HAΔVal BRCA1-encoding constructs and, after 40 hrs, treated with 25 μg/ml cycloheximide. Lysates prepared at the indicated time points were analyzed by

Western-blotting. Decreasing intensity of Δ ValBRCA1 (compared to the steady level of wtBRCA1) indicates decreased stability. For more accurate comparison of wtBRCA1 and Δ ValBRCA1 expression, a longer exposure of the HA signal is also displayed for the latter (second lower panel).

Figure 3. Δ ValBRCA1 fails to localize to sites of DNA damage

Images of Myc/3xHAwt or Myc/3xHA Δ Val BRCA1-transfected 293T cells, irradiated and subjected to fluorescence microscopy with anti-HA and anti- γ H2AX antibodies. Digitonin pre-extraction removed excess of soluble over-expressed protein and maximized nuclear foci detection. Note the co-localization of HA and γ H2AX signals in typical post-DNA damage foci in wtBRCA1-transfected cells, versus the absence of HA foci in Δ ValBRCA1-transfected cells.

Figure 4. BRCA1 p.V1688del impairs association with known BRCA1 partner proteins

Whole cell extracts (WCEs) from 293T cells transfected with HAwt or HAΔVal BRCA1-encoding constructs were immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE and analyzed by Western-blotting. **A**, BRIP1 was found in HAwtBRCA1 complexes even when the immunoprecipitate (IP) was diluted up to 10-fold, whereas it was undetectable in undiluted HAΔValBRCA1 complexes (lanes 3–6 vs. 7). **B**, co-immunoprecipitated endogenous CtIP was detected in serial dilutions of the HA-IP from wtBRCA1-transfected cells but not in undiluted HA-IP from ΔValBRCA1-transfected cells (lanes 4–7 vs. 8). Note the retained interaction between ΔValBRCA1 and BARD1 (lane 8). **C**, endogenous Rap80 was detectable in a 1:2 dilution of HAwtBRCA1 immunocomplexes but not in undiluted HAΔValBRCA1 immunocomplexes (lane 1 vs. 2). **D**, yeast two-hybrid analysis of BRCA1 interactions. As DB-fusion CtIP (pDB-CtIP) exhibits self-activation on the less stringent SC-Leu-Trp-His+1mM 3AT growth medium, diploid yeast cells containing pAD-wtBRCA1 or pAD-ΔBRCA1 and pDB-CtIP were tested for growth on SC-Leu-Trp-Ade plates (left panel). Diploid yeast cells containing pAD-wtBRCA1 or pAD-ΔBRCA1 and DB-fusion FHL2 (pDB-FHL2) or DB-fusion KPNA2 (pDB-KPNA2) were tested for growth on SC-Leu-Trp-His+1mM 3AT plates (right panels). Interactions were observed only when the wtBRCA1 construct was used as bait. The pAD-dest-CYH vector (pAD-vector) served as control.

Figure 5. Four new families of Italian ancestry carrying the *BRCA1* p.V1688del variant
 Pedigrees of A, family DFCI#10145, B, family UPH#934, C, families PI#257 (left) and PI#276 (right). In each family, an arrowhead indicates the proband, an asterisk indicates individuals harboring the *BRCA1* p.V1688del variant. None of the individuals tested carried two wt *BRCA1* alleles. Site of tumor and age at diagnosis are given, when available. Patients with breast cancer are marked with a black upper left quadrant or, for bilateral disease, with a black left half. Patients with ovarian cancer are marked with a lower right gray quadrant. Other cancer types are marked as an upper right gray quadrant. BRAIN= brain tumor, BR= breast cancer, COL= colon cancer, LG= lung cancer, LK= leukemia, OV= ovarian cancer, PAN= pancreatic cancer, RCC= renal cell carcinoma, STO= gastric cancer, UNP= unknown primary tumor.