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A Guide for Functional Analysis of *BRCA1* Variants of Uncertain Significance (VUS)

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Abstract

Germline mutations in the tumor suppressor gene *BRCA1* confer an estimated lifetime risk of 56–80% for breast cancer and 15–60% for ovarian cancer. Since the mid 1990's when *BRCA1* was identified, genetic testing has revealed over 3,000 unique germline variants. However, for a significant number of these variants, the effect on protein function is unknown making it difficult to infer the consequences on risks of breast and ovarian cancers. Thus, many individuals undergoing genetic testing for *BRCA1* mutations receive test results reporting a variant of uncertain clinical significance (VUS), leading to issues in risk assessment, counseling, and preventive care. Here we describe functional assays for *BRCA1* to directly or indirectly assess the

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ONLINE SOURCES OF INFORMATION ON *BRCA1* VARIANTS

Breast Cancer Information Core Database (<http://research.nhgri.nih.gov/bic/>): Information on documented *BRCA1* and *BRCA2* variants. It is the locus-specific database for all *BRCA1* and *BRCA2* variants. (Couch and Weber, 1996; Friend, et al., 1995)

Breast Cancer Genes IARC Database (<http://brca.iarc.fr/>): Prediction of functional impact based on evolutionary conservation and on splicing prediction algorithms.

Leiden Open Variation Database (<http://brca.iarc.fr/LOVD/home.php>): Extensively annotated database with bibliographic references for individual variants.

Ex-VUS Database (<http://brca.iarc.fr/LOVD/home.php>) Information of former VUS that have been recently classified (Vallee, et al., 2011).

UMD-*BRCA1* mutations database (<http://www.umd.be/BRCA1/>): published and unpublished information about the *BRCA1/BRCA2* mutations reported in French diagnostic laboratories (Caputo, et al., 2012).

impact of a variant on protein conformation or function and how these results can be used to complement genetic data to classify a VUS as to its clinical significance. Importantly, these methods may provide a framework for genome-wide pathogenicity assignment.

Keywords

genetic testing; functional analysis; *BRCA1*; breast; ovarian; cancer; VUS

INTRODUCTION

Breast cancer (BC) is the most common cancer in women and the most common cause of cancer death globally. It is estimated that 1.38 million people (10.9% of the total number of cancer cases) were diagnosed with BC in 2008, second only to lung cancer (<http://globocan.iarc.fr/>). Hereditary BC accounts for 5–10% of all BC cases and refers to families in which multiple individuals from different generations are affected with cancer at a relatively young age and in which a mutation within a cancer susceptibility gene is present in the germline (Claus, et al., 1991; Easton, et al., 1993). Individuals with an inherited inactivating mutation in *BRCA1* (MIM# 113705) or *BRCA2* (MIM# 600185) have an increased risk of developing early-onset breast and ovarian cancers (Ford, et al., 1998; Miki, et al., 1994; Tavtigian, et al., 1996; Wooster, et al., 1995). Inherited mutations in *BRCA1* account for 40–45% of all hereditary BC cases but approximately 80% of cases in families with multiple cases of breast and ovarian cancers (Easton, et al., 1993).

Accurate risk assessment of developing cancer is an important component of cancer prevention and proper medical management for the proband and her family. The carrier of a pathogenic mutation can benefit from increased surveillance and a more informed decision about preventive surgery or hormonal therapy. Often ignored is the benefit to non-carrier family members in high-risk families, because in the absence of appropriate testing, these individuals might decide to undergo preventive surgery based on family history alone.

BRCA1 screening is largely based on direct sequencing of coding regions (Frank, et al., 2002). There are four possible findings: 1) no sequence alteration; 2) pathogenic variant; 3) polymorphism or neutral variant; and 4) variant of uncertain clinical significance (VUS). Outcome 1 for individuals with no family history of cancer means cancer risk is no greater than in the general population. In addition, outcome 1 is particularly informative if a specific pathogenic variant has been found in other affected family members but not in the individual tested, indicating no inheritance of the known susceptibility allele. In outcome 2 the individual is predisposed to hereditary breast and ovarian cancer syndrome (HBOC) with associated elevated risk of various cancers, primarily breast and ovarian cancers (Miki, et al., 1994). These individuals benefit from options available for early cancer detection and prevention that can reduce the overall risk. In outcome 3, the individual has a similar cancer risk as individuals (with no family history of cancer) in outcome 1. In outcome 4, the focus of this manuscript, the individual carries a genetic variant in *BRCA1* for which the clinical significance is unclear. The reason for the inability to classify variants comes from the lack of genetic information to determine cancer association. Most of these VUS are in-frame deletion/insertions, missense or silent mutations, or alterations in intronic and regulatory regions. The impact of these alterations generally cannot be inferred from previous knowledge and must be verified experimentally (Carvalho, et al., 2007a).

It has previously been estimated that ~10% of Caucasians undergoing testing receive a VUS report (Frank, et al., 2002), with a much higher proportion in Hispanics and African Americans (Kurian, 2010; Nanda, et al., 2005; Weitzel, et al., 2005). Although the

percentage of individuals receiving VUS reports has diminished due to recent improvements in classification it remains an important problem. The finding of a VUS generates uncertainty and anxiety when women are already facing several life-changing events and need to make informed decisions.

Initial attempts to evaluate the clinical significance of VUS in *BRCA1* were based on family data including family history and co-segregation with the disease in families; tumor data including histopathological features and loss of heterozygosity (LOH); and VUS features including the frequency in cases and controls, co-occurrence with a deleterious mutation in the same gene, the severity of the nucleotide or amino acid change and its conservation across species, and its effect on RNA splicing. A major advance has been the development of multifactorial classification models for *BRCA1*, which combines a number of independent features to establish the likelihood that the VUS has the characteristics of known pathogenic mutations (Easton, et al., 2007; Goldgar, et al., 2004).

For variants identified in multiple families, co-segregation, personal and family history as well as pathological characteristics of breast tumors can provide sufficient evidence of association with cancer risk. However, the majority of the VUS are rare, and therefore these family-based clinical analyses often lack statistical power. Thus, the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) Consortium (<http://enigmaconsortium.org/>) was created to assess pathogenicity of mutations in genes involved in cancer predisposition (Spurdle, et al., 2011). While genetic and epidemiological data can be used to help classify variants, in this manuscript we provide a critical evaluation of functional assays for *BRCA1* variants.

The BRCA1 protein functions in a number of different processes, including DNA damage response, control of cell cycle progression and centrosome number, and transcriptional regulation (Narod and Foulkes, 2004; Yun and Hiom, 2009). It is a 220KDa nuclear phosphoprotein with multiple functional domains and motifs (Fig. 1) many of which are highly conserved in orthologs (Narod and Foulkes, 2004; Yun and Hiom, 2009). Cancer-causing mutations in a tumor suppressor gene, such as *BRCA1*, are expected to impair the protein's biological activity. The pleiotropic nature of BRCA1 complicates the interpretation of functional data because it is not established which of its functions are important for tumor suppression.

BRCA1 is a highly polymorphic gene with more than 1600 unique documented variants (Breast Cancer Information Core BIC; <http://research.nhgri.nih.gov/bic/>). Population-based analysis and functional studies indicate that most variants leading to premature termination of the BRCA1 protein are associated with increased risk of cancer (Szabo, et al., 2004). In-frame deletions and insertions, as well as silent and missense variants pose a significant problem for individual risk assessment because their consequences on protein function are largely unknown (Szabo, et al., 2004). There are currently over 500 documented missense VUS in *BRCA1* (BIC).

FUNCTIONAL ASSAYS

The purpose of functional assays is to serve as independent classifiers of VUS by assessing, directly or indirectly, their influence on protein conformation or function and generating additional information that can be integrated with available genetic and epidemiological data into multifactorial likelihood models (Easton, et al., 2007; Goldgar, et al., 2004). For a detailed explanation of VUS classification multifactorial likelihood models, see (Lindor, et al., 2012).

In 2008, the unclassified genetic variant working group of the International Agency for Research on Cancer (IARC) proposed a 5-class system based on degree of likelihood of pathogenicity derived from multifactorial models. Class 1 (not pathogenic or of no clinical significance), Class 2 (likely not pathogenic or of little clinical significance), Class 3 (uncertain), Class 4 (likely pathogenic), and Class 5 (definitely pathogenic) have probabilities of being pathogenic of <0.001, 0.001–0.049, 0.05–0.949, 0.95–0.99, and >0.99, respectively (Plon, et al., 2008). Until recently, functional data were not incorporated into multifactorial models and functional information served as supplementary information (Easton, et al., 2007; Goldgar, et al., 2004). Development of a computational model to transform functional assay data into likelihood ratios now allows functional data to be taken into account to improve the performance of these predictive models (Iversen, et al., 2011). We use this 5-class system as a reference in the following sections to assess the sensitivity and specificity for some of the assays that functionally evaluate BRCA1 VUS.

Ubiquitin ligase activity and protein interaction

The amino-terminus of BRCA1 contains a zinc-binding RING domain (aa 8–96) that mediates binding to the RING domain of BARD1 (BRCA1 associated RING domain 1) and to UbCH5c (Brzovic, et al., 2003; Brzovic, et al., 2001)(Fig. 2A). The BRCA1/BARD1 complex displays E3 ubiquitin ligase activity promoting the formation of K-6 ubiquitin chains instead of the more common K-48 or K-63 chains (Baer and Ludwig, 2002; Wu-Baer, et al., 2003). It can ubiquitylate the variant histone H2AX *in vitro* and also γ -tubulin, RNA polymerase II, and estrogen receptor alpha (Chen, et al., 2002; Dizin and Irminger-Finger, 2010; Eakin, et al., 2007; Starita, et al., 2005; Starita, et al., 2004).

An approach combining experiments evaluating the ubiquitin ligase activity and parallel yeast two-hybrid assays to assess the impact of variants on the interaction between a BRCA1 N-terminus VUS and BARD1 or UBCH5a has been developed (Morris, et al., 2006). Interestingly, loss of E2 (UBCH5a) binding but not loss of BARD1 binding was correlated with reduced or abrogated ubiquitin ligase activity. Variants p.Met18Thr, p.Cys39Arg and p.Cys61Gly, classified as pathogenic by genetic studies (Class 4, 5 and 5 respectively)(Lindor, et al., 2012; Vallée, et al., 2011), displayed loss of E2 enzyme interaction and ubiquitin ligase activity while p.Asp67Tyr classified as not pathogenic by genetic studies (Class 1)(Lindor, et al., 2012; Vallée, et al., 2011) did not (Morris, et al., 2006). It will be important to test a larger number of known variants to estimate the specificity and sensitivity of the assay. A small number of variants in the BRCA1/BARD1 interaction surface has also been tested using a qualitative fluorescent complementation assay in bacteria but further investigation is still warranted to assess its accuracy (Sarkar and Magliery, 2008).

Intriguingly, the enzymatic activity of BRCA1 does not seem to be required for cell viability, homology-directed repair of double stranded breaks, chromosomal stability, senescence induction, centrosome number, spindle formation or resistance to genotoxic stress in mouse embryonic stem (ES) cells (Reid, et al., 2008) and tumor suppression in a mouse model (Shakya, et al., 2011). Shakya et al. showed that synthetic p.Ile26Ala variant, which abrogates ubiquitin ligase activity without altering binding to Bard1, prevents tumor formation to the same degree as does wt Brca1 (Shakya, et al., 2011). Drost et al. added yet another layer of complexity by showing that while the p.Cys61Gly variant (defective for both ubiquitin ligase activity and Bard1 binding) fails to prevent tumor formation as a pathogenic variant would, tumors associated with the p.Cys61Gly variant respond poorly to PARP inhibition and to platinum-based therapy (Drost, et al., 2011). These data suggest that the p.Cys61Gly variant still retains a residual activity.

In contrast, in a different mouse model, ubiquitylation was shown to be required for heterochromatin silencing and tumor suppression (Zhu, et al., 2011). While some of these discrepancies can be explained by different experimental models, taken together they suggest that there is no unifying mechanism of breast carcinogenesis linked to BRCA1 loss of function. Rather, BRCA1-mediated tumor suppression has contributions from different functions and distinct mutations will result in variations in risk and/or in the phenotype of the disease. This example illustrates the biological complexity of BRCA1 functions and serves as a cautionary note for the interpretation of laboratory functional assays.

Transcription activation (TA) assay

The carboxy-terminal region of BRCA1 is highly acidic and encompasses two tandem BRCT (BRCA1 Carboxy Terminal; PFAM: PF00533) repeats (aa 1646–1736 and aa 1760–1855)(Fig. 2B–C). BRCA1 was shown to interact with RNA polymerase II holoenzyme and a number of transcription factors, co-repressors, chromatin remodeling enzymes, and RNA processing factors; supporting its role in transcription regulation (Dapic and Monteiro, 2006). Consistent with this notion, the C-terminal region of the protein can transactivate a reporter gene when fused to a heterologous DNA binding domain (DBD)(Chapman and Verma, 1996; Monteiro, et al., 1996) and this activity forms the basis of the transcription activation (TA) assay (Vallon-Christersson, et al., 2001). The TA assay monitors the integrity of the C-terminus of BRCA1 (aa 1396–1863), which includes the tandem BRCT domains (Phelan, et al., 2005)(Fig. 1). The assay consists of a fusion of the viral repressor Lex A or the yeast GAL4 DBD to the C-terminus of BRCA1 (wild type or variant) coexpressed in yeast or mammalian cells with reporter genes driven by promoters containing Lex A or GAL4 binding sites (Carvalho, et al., 2007b; Monteiro, et al., 1996; Phelan, et al., 2005; Vallon-Christersson, et al., 2001).

The use of a series of well characterized reporter systems with different stringencies (Estojak, et al., 1995) allows the TA to quantitatively assess the impact of variants. In yeast, the assay is most frequently conducted with *Saccharomyces cerevisiae* strain EGY48 using Lex A DBD:BRCA1 fusion protein and the *LacZ* gene under control of Lex A operators as a reporter. This approach allows the use of different substrates for the assay such as X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and ONPG (2-nitrophenyl- β -D-galactopyranoside). The use of X-Gal allows the investigators to assay β -galactosidase activity qualitatively in a white/blue colony color screening manner (Carvalho, et al., 2002). The assay can also be performed using a GAL4 DBD:BRCA1 fusion protein in *S. cerevisiae* strains HF7C or SFY546 (Monteiro, et al., 1996). In mammalian cells, the assay is quantitative using a GAL4 DBD:BRCA1 fusion protein and the firefly luciferase gene under the control of a GAL4 responsive promoter as a reporter. To control for variation in transfection efficiency and cell viability an internal control with the *Renilla sp.* luciferase under control of a constitutive promoter is used (Carvalho, et al., 2007a; Carvalho, et al., 2007b; Phelan, et al., 2005).

Protease sensitivity assay

The protease sensitivity assay (Fig. 1) derives from an observation that BRCT domains containing a deleterious mutation were more susceptible to protease (elastase, trypsin, or chymotrypsin) digestion than the wild type (wt) counterpart (Williams, et al., 2003; Williams and Glover, 2003; Williams, et al., 2001). This assay can identify variants that cause defects in proper protein folding but may not detect defects caused by variants located on the protein surface that do not affect the folding. For this reason, its specificity is lower than the TA and phosphopeptide binding assays (Lee, et al., 2010).

Phosphopeptide binding assays

The BRCT domains in BRCA1 interact with phosphorylated protein targets containing the sequence pSer-x-x-Phe (Botuyan, et al., 2004; Clapperton, et al., 2004; Rodriguez, et al., 2003; Shiozaki, et al., 2004; Williams, et al., 2004; Yu, et al., 2003)(Fig. 2B). In this assay (Fig. 1), the BRCT domain including the VUS is transcribed and translated *in vitro*, pulled down with a biotinylated pSer-x-x-Phe-containing peptide coupled to streptavidin agarose beads. VUS are then tested for their binding activity (Lee, et al., 2010; Williams, et al., 2004). An unphosphorylated peptide version is used as a control and two different analyses can be performed. "Binding activity" corresponds to the pSer-x-x-Phe binding activity of the variant compared to the wt whereas "binding specificity" compares the binding of the variant to the phosphorylated peptide versus binding to an unphosphorylated peptide (Lee, et al., 2010).

The TA, protease sensitivity, and phosphopeptide binding assays have been extensively used for VUS analysis, and sensitivity and specificity measures are available for all assays. There were strong correlations between these assays and cancer predisposition indicating that they can be incorporated into multifactorial models (Iversen, et al., 2011; Lee, et al., 2010).

Small colony phenotype (SCP) assay

The SCP assay is derived from the observation that BRCA1 expression inhibits yeast growth leading to the formation of small size colonies (Coyne, et al., 2004; Humphrey, et al., 1997; Monteiro and Humphrey, 1998). The phenotype can be assessed qualitatively by looking at the size of the colonies or quantitatively by determining the number of cells in each colony. There is growth suppression with expression of wt or variants with no clinical significance but not with truncations or missense mutations known to be pathogenic (Humphrey, et al., 1997; Millot, et al., 2011). The integrity of BRCT domains is a critical determinant in the SPC assay and correlates well with results from the TA assay (Coyne, et al., 2004). Although the sensitivity and specificity were high for variants in the BRCT domain, 100% and 80% respectively, confidence intervals are still very broad (95%CI between 15% and 100%; and 28% and 99% respectively) and a more extensive validation needs to be performed. This assay seemed limited to interrogating variants located in the BRCT domains (aa 1646–1855) but its utility to interrogate the RING domain is being explored. The biochemical basis of the SCP is not yet understood. It has been proposed to be associated with cleavage of the RNA Polymerase II carboxy-terminal domain (Bennett, et al., 2008) and with the aggregation of BRCA1 in the yeast nucleus (Millot, et al., 2011).

Yeast localization phenotype (YLP) assay

In the YLP assay, the BRCA1 wt protein fused to the mCherry red fluorescent protein accumulates in a single inclusion body in the yeast nucleus providing qualitative information concerning the cellular localization of BRCA1 (Millot, et al., 2011). This event can be assessed quantitatively by determining the proportion of cells showing the nuclear aggregate in fluorescent microscopy. The BRCA1 wt fusion accumulates in approximately one third of all cells and this fraction is reduced when cells express a fusion containing a pathogenic variant. Truncations or pathogenic missense variants induce mislocalization of the protein to the cytoplasm similarly to what was found in mammalian cells (Rodriguez et al., 2004a). Interestingly, results from the YLP and SCP assays are highly correlated suggesting that the growth defect could be due to BRCA1 nuclear aggregation (Coyne, et al., 2004; Humphrey, et al., 1997; Millot, et al., 2011; Monteiro and Humphrey, 1998). The YLP assay can assess variants mapping to the RING and BRCT domains (Fig. 1) but extensive validation needs to be performed.

Embryonic stem cell (ESC)-based functional assay

Unlike the methods discussed above which are mainly domain-specific, the ESC assay (Chang, et al., 2009) can, in principle, interrogate variants irrespective of their location in the *BRCA1* sequence (Fig. 1). The approach is an extension of a strategy originally developed for the study of *BRCA2* variants (Kuznetsov, et al., 2008). *BRCA1* variants in mouse ESC are expressed in levels comparable to human cancer cell lines. This allows examination of VUS that affect expression due to impact on regulatory sites. *Hprt*-deficient mouse ESC were used to develop a conditional *Brca1* expressing cell line in which one *Brca1* allele was disrupted by deletion of exons 3–7. The second allele was modified by flanking the locus with two halves of the human *HPRT1* minigene along with two *loxP* sites to allow, simultaneously, *Brca1* silencing and selection of clones that undergo *Cre*-mediated recombination (Chang, et al., 2009). Because *BRCA1* is essential for the maintenance and survival of ESC (Gowen, et al., 1996; Hakem, et al., 1996), loss of the conditional allele is expected to produce no viable hypoxanthine, aminopterin and thymidine (HAT)-resistant cells. Expression of a full-length wt *BRCA1* transgene introduced as a bacterial artificial chromosome (BAC) clone rescues the lethality of *Brca1*-deficient mouse ESC. Based on the ability of wt human *BRCA1* to rescue these cells from lethality, *BRCA1* variants were quantitatively assayed to validate the ability of ESC-based system to classify these variants (Chang, et al., 2009). The assay was able to correctly discriminate between known pathogenic variants (p.Cys61Gly and p.Ala1708Glu) and a known non-pathogenic variant (p.Met1652Ile).

Interestingly, this model is also able to identify hypomorphic (mild to moderate effects) variants such as the p.Ser1497Ala that can rescue ES cell viability but exhibited hypersensitivity to IR and genomic instability (Chang, et al., 2009). It was also used to identify a hypomorphic effect of the p.Arg1699Gln variant and to uncover a novel mechanism of *BRCA1*-linked tumorigenesis by controlling the expression of the oncogenic miRNA 155 (Chang, et al., 2011).

Restoration of radiation resistance

BRCA1 plays a key role in maintaining genome integrity in response to DNA double-stranded breaks (DSBs) arising during DNA replication or exogenous insults (Dapic and Monteiro, 2006; Narod and Foulkes, 2004; Scully and Livingston, 2000). Ionizing radiation (IR) hypersensitivity is characteristic of several *BRCA1* mutant cell lines, consistent with the idea that this property is linked to the absence of a normal *BRCA1* function (Abbott, et al., 1999; Cortez, et al., 1999; Shen, et al., 1998). Thus, reconstituting the *BRCA1*-deficient breast cancer cell line HCC1937 (Tomlinson, et al., 1998) with wt or VUS can be used to assess the ability to restore radiation resistance (Scully, et al., 1999).

Retrovirus infected cells co-expressing green fluorescent protein (GFP) and *BRCA1* (at levels comparable to human breast cancer cell line MCF-7) and GFP-negative (non-infected control HCC1937) cells were mixed and treated with IR. After 3–24 days, cells were assayed for growth advantage compared to parental cells by fluorescence-activated cell sorting (FACS) analysis. The enrichment of GFP positive cells indicates a restoration of radiation resistance, discriminating the biological behavior of wt *BRCA1* alleles from functionally impaired ones. Four variants have been assessed this way (Scully, et al., 1999). A similar strategy has been employed by Ruffner and colleagues to test variants in the *BRCA1* RING finger (Ruffner, et al., 2001). These studies need to be extended to allow evaluation of its predictive value.

Homology-directed recombination (HDR) assay

Ransburgh et al. have developed an assay to assess the ability of a VUS to promote homology-directed recombination (Ransburgh, et al., 2010)(Fig. 1). It relies on two inactive alleles encoding GFP in a single locus in the genome of HeLa cells. A DSB can be induced in the first allele by the expression of the I-SceI endonuclease. If this break is repaired by homologous recombination (HR) using the second allele as the template, then the repaired copy becomes functional and cells express GFP. This effect is strongly compromised when BRCA1 is inactivated by a siRNA targeted to its 3' untranslated region (UTR). Ectopic expression of wild type BRCA1 from a transfected cDNA (which does not contain the 3'UTR and is therefore not a target of the siRNA) can restore GFP expression. Replacement of the full-length wt BRCA1 cDNA by a cDNA containing a VUS enables the determination of the effect of the VUS on HR. Sixteen variants located in the RING domain of BRCA1 were tested and 10 of them were found to affect the function of BRCA1 in HR but the assay has not been fully validated yet.

Centrosome amplification

This functional assay relies on the observation that depletion or down regulation of BRCA1 leads to centrosome amplification (Kais, et al., 2011; Starita, et al., 2004). The percentage of cells with centrosome amplification was determined for fourteen variants located in the RING domain. Variants were co-transfected with GFP-centrin 2 in Hs578T cell line, in which the endogenous BRCA1 was silenced using RNA interference against the BRCA1 3' untranslated region (UTR). Expression of all variants located at residues that coordinate zinc binding resulted in centrosome amplification. This analysis identified a variant (p.Leu52Phe) showing a defect in centrosome duplication but not in centrosome pairing, uncovering a potential novel function of BRCA1 in centrosome pairing. Interestingly, several of the BRCA1 variants tested in this assay have been found to have intermediate phenotype (*i.e.* percentage of cells with centrosome amplification intermediate between wt, ~4%, and pathogenic variants, >12%). Importantly, the authors analyzed the same set of BRCA1 variants tested in the HDR assay (Ransburgh, et al., 2010). Several variants showed different outcomes in HDR and in centrosome amplification assay suggesting that different regions in the RING domain have different contributions to distinct BRCA1 functions but further validation is required.

Yeast recombination assay

As BRCA1 functions in DNA repair and recombination, the yeast recombination assay evaluates the effect of the expression of BRCA1 missense variants located throughout the entire coding region on yeast homologous recombination (HR) using intrachromosomal and interchromosomal reporter assays in a diploid yeast strain (Caligo, et al., 2009)(Fig. 1). While the expression of full length wt *BRCA1* did not affect recombination, expression of three Class 5 variants induced a significant increase in intrachromosomal and interchromosomal HR. Conversely, expression of three Class 1 variants did not affect recombination in either assay. However, two Class 1 variants also showed effects on recombination. At this point it is not known whether this reflects a problem with the specificity of the test. Alternatively, it is also possible that the assay is highly sensitive to variants with low or moderate effects. There is additional functional evidence suggesting that one Class 1 variant (p.Tyr179Cys) may have some functional impact on the protein (Velkova, et al., 2010). A similar increase in recombination has been reported for BRCT mutations in human cells (Dever, et al., 2011). In order to evaluate its accuracy a systematic validation is still needed.

Subcellular localization assay

BRCA1 is a predominantly nuclear localized protein (Chen, et al., 1996; Coene, et al., 2005; Monteiro and Birge, 2000; Scully, et al., 1996) that has also been detected in the cytoplasm (Au and Henderson, 2005; Feng, et al., 2004; Rodriguez and Henderson, 2000; Rodriguez, et al., 2004b; Wang, et al., 2010). BRCA1 contains nuclear localization sequences (aa 503–508 and 606–65) and nuclear export sequences (aa 22–30 and 81–99) which control the subcellular localization and nucleo/cytoplasmic shuttling of BRCA1 (Rodriguez and Henderson, 2000). In the nucleus, BRCA1 is localized in S-phase-associated foci (Jin, et al., 1997; Scully, et al., 1997a) together with BARD1 and other DNA repair factors, but redistributes into DNA repair-associated nuclear foci following DNA damage (Scully, et al., 1997b). To examine whether a variant changes the subcellular location of BRCA1, the localization of GFP-tagged variant BRCA1 in transiently transfected cells is examined by confocal laser scanning microscopy. The cells are then scored as nuclear, cytosolic or nuclear/cytosolic in mock or IR-treated cells (Au and Henderson, 2005; Feng, et al., 2004; Rodriguez, et al., 2004a; Wang, et al., 2010). The assay examined two truncating mutations (p.Tyr1853X and c.5266dup aka 5382insC) and three Class 5 variants (Au and Henderson, 2005; Rodriguez, et al., 2004a). All except one (p.Cys61Gly) displayed increased cytoplasmic localization of BRCA1 and prevented the formation of BRCA1 foci either before or after DNA damage. Interestingly, tumor cells carrying the p.Cys61Gly generated from a mouse model (conditional deletion in mammary gland epithelial cells; *Brca1^{del/C61G}; p53^{del/del}*) showed RAD51 IR-induced nuclear foci, suggesting that the p.Cys61Gly variant has residual activity (Drost et al 2011).

Assessment of variants mapping to BRCA1 regulatory regions

BRCA1 is regulated at the transcriptional and post-transcriptional level (Saunus, et al., 2007; Saunus, et al., 2008; Signori, et al., 2001; Xu, et al., 1997b). Its transcription is driven by a bidirectional promoter (Xu, et al., 1997a) and regulated by several transcription factors including E2F1 and p53 (Arizti, et al., 2000; Bindra and Glazer, 2006). *Cis* regulatory elements have also been identified in introns 1 (repressor) and 2 (enhancer), and located at ~85kb at the 3' end of the gene (Suen and Goss, 2001; Tan-Wong, et al., 2008; Wardrop and Brown, 2005) but additional elements are likely to exist. Large deletions removing the *BRCA1* promoter, and regulatory elements in introns 1 and 2 have been described in familial breast cancer patients and shown to abolish *BRCA1* transcription (Brown, et al., 2002). Although mutation analysis of *BRCA1* has been, for the most part, restricted to coding regions and intron-exon boundaries (Frank, et al., 2002) pathogenic variants have been identified in the 5' UTR (Signori, et al., 2001) and *in vitro* and *in silico* analyses indicate that they may also occur in the 3' UTR (Brewster et al., personal communication) and deep within introns (Brandao, et al., 2011; Fackenthal, et al., 2002; Tesoriero, et al., 2005).

Recent approaches based on massively parallel sequencing have allowed for the analysis of the entire locus including introns, 3' and 5' UTRs, and upstream and downstream regulatory regions (Walsh, et al., 2010). The proportion of variants detected in non-coding regions will dramatically increase and systematic functional assays to determine their significance need to be developed. A hierarchical experimental approach, in which tests that are easier to set up, execute, and interpreted are followed by more in-depth studies only for those variants that show evidence of functional impact, seems appropriate to prioritize analysis. This approach will avoid conducting complex experiments on single nucleotide variants with a low likelihood of having a functional effect strong enough to lead to high risk of cancer in the carrier.

Several bioinformatics tools are available to predict the functional consequences in regulatory elements (Table 1). Evolutionary conservation is accepted as a good indicator of DNA and RNA sequence significance and can be determined with NCBI (<http://www.ncbi.nlm.nih.gov/>) or UCSC (<http://genome.ucsc.edu/>) resources. These tools have variable sensitivity and specificity but are useful to predict and prioritize variants for further analysis.

While functional impact on regulatory regions can be assessed by *in vitro* heterologous models (e.g. *in vitro* transcription and translation), particular attention should be given to assays that use adequate human cell hosts as several changes may have cell-type specific effects. It is still unclear which levels of transcript should be considered as a threshold of separation to determine functional impact but analyses of increasing number of variants in these regions may improve our ability to correctly distinguish variants with and without functional impact.

Candidate variants in putative promoter, enhancer or repressor sites (identified in the bioinformatics analysis) can be assessed by their ability to modulate transcription of a reporter gene (e.g. luciferase) in transient transfections (Wardrop and Brown, 2005). Reporter assays in which the wt or variant containing UTR is cloned upstream or downstream of the reporter gene driven by a viral promoter can be used to examine the effects of variants on UTR activity. These assays can be conducted in a high-throughput fashion for hundreds of variants but they also have limitations due to the use of artificial arrangements of DNA sequence elements or heterologous cell line models. Detection of long-range physical interactions between enhancers or repressors with target promoters by chromosome conformation capture techniques can further support the biological relevance of a regulatory site (Tan-Wong, et al., 2008). The ability of transcription factors to bind to elements containing sequence variants can be measured by electrophoretic mobility shift assay (EMSA)(Wardrop and Brown, 2005).

Effects of variants on post-transcriptional processes such as RNA stability and splicing of endogenous transcripts can be assayed using lymphocytes, lymphoblastoid cell lines, or fibroblasts provided that variant and wt alleles can be distinguished (Tesorio, et al., 2005; Walker, et al., 2010b; Whiley, et al., 2011). Further details about splicing variant analysis can be found elsewhere (Brandao, et al., 2011; Thomassen, et al., 2011). The use of transcription inhibitors, such as Actinomycin D enables analysis of effects on RNA stability (Saunus, et al., 2007). In addition, RNA protein interactions can be measured using RNA-based EMSA assays, IP-RT-PCR, and ectopic expression of the RNA binding protein in UTR reporter containing cell lines (Saunus, et al., 2008). As miRNA targets have been reported in the *BRCA1* 3'UTR, the role of miRNAs can also be analysed by cotransfecting miRNA expression constructs or synthetic miRs into reporter containing cell lines (Chen, et al., 2010; Garcia, et al., 2011).

Agnostic approaches

Microarray-based gene expression profiling is an indirect method with potential to distinguish pathogenic variants. The premise is that expression of certain genes and/or modulation of pathways may be different in the presence of a defective *BRCA1* protein compared to a wt protein and that a mutation will have enough impact to allow distinguishing heterozygous cells from homozygous wt cells (Baldeyron, et al., 2002). The use of microarrays has the advantage of testing the expression changes in several pathways in an agnostic manner. Kote-Jarai et al. have used short-term breast fibroblast cultures from healthy persons or from *BRCA1* mutation carriers subjected to DNA damage (Kote-Jarai, et al., 2004). However, the signature obtained was only able to distinguish *BRCA1*-mutation carriers from controls with a probability of 85%. Later, by using a microarray with a larger

number of genes, the accuracy improved to 95% (Kote-Jarai, et al., 2006). Using irradiated lymphoblastoid cell lines, Waddell et al. have reported 71% and 83% accuracy of prediction of *BRCA1* truncating and missense mutations, respectively (Waddell, et al., 2008). Walker et al. have shown that mitomycin C treatment could be more effective than IR to determine *BRCA1* and *BRCA2* status in lymphoblastoid cells, which was accomplished with 83% accuracy using a 9-gene signature (Walker, et al., 2010a). In summary, gene expression profile is a promising avenue but further studies and validation are needed.

DISCUSSION

Risk assessment is a key tool in cancer screening and prevention, particularly in the context of familial cancer. An important obstacle to improve risk assessment for breast and ovarian cancers is the large number of variants for which cancer association has not been determined. Here we describe and discuss several strategies of functional analysis, their advantages, and limitations. Depending on the location of the mutation, different assays are required. We also review results obtained so far and their implication for future research. Below we discuss several issues that are critical for the correct interpretation of functional results. The functional assays described above have inherent differences and one is strongly advised to read the original publications and to contact the authors to obtain step-by-step protocols. Nevertheless, there are several practices that should be followed for every procedure and they are summarized below:

Controls

It is recommended that every batch of tests contains at least one positive (wt, RefSeq) and one negative (a variant classified by genetic or multifactorial methods as pathogenic; Classes 4 or 5) control. It is also good practice, if possible, to include a variant classified as non-pathogenic (Classes 1 or 2). Importantly, the control variants should be located in the region that is being interrogated in the assay. For assays that rely on transfection, an internal control should be used to normalize results according to transfection efficiency and cell viability.

Replicates

For quantitative tests a minimum of triplicate samples should be run and the assays should be performed independently at least twice.

Expression

Most assays described here rely on the ectopic expression of *BRCA1* variants that may have widely different expression levels and stability. Thus, it is important to assess the level of expression (ideally by western blots, or alternatively by quantitative polymerase chain reaction) to differentiate between a loss of function phenotype due to a direct effect on the function or due to decreased stability of the variant protein. It is good practice, when possible to assess expression levels in the same sample being examined for the assay readout. If it is not possible, then a parallel set of transfections should be conducted.

Plasmid preparation

At least in the context of the TA assay, the most important source of variation between experiments is plasmid preparation. For example, preparation of separate stocks of control and test plasmids yields highly variable results. Thus, we recommend that one single plasmid preparation batch be used for all plasmids being tested simultaneously.

Controlled vocabulary

To avoid confusion, reports should use a controlled vocabulary. The current literature uses several terms to describe the same entity. Results from functional assays should classify a variant as “functional impact” (or “damaging protein conformation or function”) or “no functional impact” (or “not damaging protein conformation or function”). Intermediate phenotypes should be scored as “intermediate impact” or “mildly damaging protein conformation or function”. For a more detailed discussion on controlled vocabulary see (Lindor, et al., 2012).

Scoring phenotypes

Independent of whether the reported result is categorical (*e.g.* *Yes* × *No*; *Positive* × *Negative*) or quantitative (*e.g.* arbitrary luciferase units; densitometry measures), cutoff values that separate functional impact and no functional impact need to be clearly defined and their rationale clearly stated. Raw data should be retained to allow reanalysis of the data with different cutoff values. Quantitative assessments should be favored because they can more accurately assess intermediate effects. Significantly, current computational models can accommodate categorical and quantitative results (Iversen, et al., 2011).

Benchmarks and validation

The assays described herein include those that indirectly assess conformational changes (*e.g.* protease sensitivity), those that measure a specific biochemical activity (*e.g.* ubiquitylation), those that measure broad biological phenotypes such as viability or response to DNA damage, and those for which the biological function interrogated is unknown to date (*e.g.* SCP assay). The biological validity of a test should be assessed at two levels. First, by determining to what extent the assay relies on a natural function of the protein, but also by determining how this particular function contributes to tumor suppression. While we have strong evidence that many functions interrogated in the assays described here are *bona fide* functions of BRCA1, evidence that a certain function is required or even contributory to its tumor suppression activity has been difficult to demonstrate (Chang, et al., 2011; Shakya, et al., 2011; Tavtigian, et al., 2008; Velkova and Monteiro, 2011; Zhu, et al., 2011). In order for the results generated to be confidently interpreted or incorporated into a multifactorial model, the functional assay should be validated by assessing specificity and sensitivity (and confidence intervals) using a reference panel of variants classified by genetic or multifactorial methods. In Table 2, we provide an expanded reference panel of variants. Sensitivity is the proportion of true pathogenic variants in a sample which are correctly identified. A low-sensitivity test will identify a large number of false negatives (pathogenic variants misclassified as non-pathogenic). Specificity is the proportion of true non-pathogenic variants correctly identified. A low specificity test will identify a large number of false positives (non-pathogenic variants misclassified as pathogenic). For example, three functional tests (TA, protease sensitivity, and phosphopeptide binding), have been benchmarked using 14 pathogenic and 10 not pathogenic variants (Lee, et al., 2010).

The assays described here assess changes in protein structure and activity in a limited number of potential functions of BRCA1. Thus, caution is warranted when inferring the effect of the changes in cancer risk. Moreover, environment or genetic background interactions can have a strong influence on the phenotype (Burga, et al., 2011), particularly for variants that cause subtle changes, but are not usually taken into account in functional assays. Generation of mouse models carrying the variants in question (Shakya, et al., 2011) and the use of a multifactorial classification model (Easton, et al., 2007) should improve our ability to assess cancer risk from functional assay data.

Conclusions and future directions

Although the approach taken during the development of functional assays relies on testing whether a variant affects a particular function, it is also the case that large-scale variant testing can inform mechanistic studies. The identification of variants which have moderate effects on function (hypomorphic mutations) may reveal moderate risk variants, and recent evidence suggests that this might be the case, at least for some variants (Lovelock, et al., 2007). Additionally, variants can uncouple different molecular functions which will provide tools to dissect the mechanisms of tumor suppression (Chang, et al., 2011; Shakya, et al., 2011; Velkova and Monteiro, 2011).

As can be seen in Table 2 there is a dearth of information for variants located in the central region of BRCA1. Progress in the functional analysis of this region has not been as fast as in other regions because of the lack of a recognized biochemical function or a protein domain that can guide the development of functional tests. In addition, judging by the relative low similarity with other BRCA1 orthologs the expectation is that many variants in these regions are likely to have no functional impact, making it less attractive to conduct more difficult global functional analysis using the full length protein. Thus, one of the challenges for the future will be to obtain better coverage of these less well studied regions.

Finally, data from genome-wide genotyping and massively parallel sequencing projects of germline and somatic DNA are revealing large numbers of sequence variants whose impact on function (and ultimately its pathogenicity) cannot be easily inferred. The framework described here for *BRCA1* can be used to guide functional analysis of any gene in the genome and is going to be instrumental for individual risk assessment and personalized medicine.

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Figure 1. The landscape of BRCA1 as assessed by functional assays

A. Diagram of BRCA1 protein. RING domain (aa 8–96), NES, nuclear export signal (aa 22–30 and aa 81–99), NLS, nuclear localization signal (aa 503–508 and 606–615); AAR, auxiliary activation region (aa 1396–1559); TAD, transcriptional activation domain (aa 1560–1863), BRCT, BRCA1 C-terminal domains (aa 1646–1736 and 1760–1855). **B.** Sequences used by the different functional assays are indicated in grey. Limits of the aa sequence interrogated by the assays are indicated on the right. CA, centrosome amplification; ESC, embryonic stem cell; FL, full length; HDR, homology-directed recombination; RRR, restoration of radiation resistance; SCP, small colony phenotype; TA, transcription activation; YLP, yeast localization phenotype. Black box, full length sequence is used but assessment restricted to this region only.

Figure 2. Structural domains in BRCA1

A. Ring domain of BRCA1. Ribbon representation of the BRCA1-BARD1 heterodimer complex (pdb: 1jm7). BARD1 RING finger is colored in light grey. Black and dark grey indicates BRCA1 residues involved in the interaction to BARD1 or to UbH5c, respectively. **B.** BRCT domains. Ribbon representation of the BRCA1 BRCT domains (pdb: 1jnx) and the interaction with phosphopeptides from BACH1 (pdb: 1t15), CtIP (pdb: 1y98), and ACC1 (pdb: 3coj). Residues common to the binding to all three phosphorylated peptides are shown in black (Ser1655, Gly1656, Arg1699, Leu1701, Lys1702, Phe1704, Asn1774, Met1775, and Leu1839). Variants Arg1699Trp, Arg1699Gln, Met1775Arg and Met1775Lys have been classified as IARC Class 5 (Lindor et al. 2012). **C.** Ribbon representation of the BRCA1 BRCT domains. BRCT residues corresponding to variants that have already been classified listed in Table 2 are shown in black.

TABLE 1

Bioinformatics tools

Tool	Predictions	URL	References
<i>Transcription regulation</i>			
CoreBoost	start site	http://rulai.cshl.edu/tools/CoreBoost/	(Wang, et al., 2009)
7×regulatory potential	regulatory element	http://hgdownload.cse.ucsc.edu/goldenPath/hg18/regPotential7X/	(King, et al., 2005)
TFSearch	transcription factor binding sites	http://www.cbrc.jp/research/db/TFSEARCH.html	
oPOSSUM	transcription factor binding sites	http://www.cisreg.ca/oPOSSUM/	(Ho Sui, et al., 2005)
<i>Posttranscriptional regulation</i>			
UTResource	3'UTR motif		
Transterm	3'UTR motif	http://uther.otago.ac.nz/	(Jacobs, et al., 2009)
M-fold	RNA secondary structure	http://mfold.rna.albany.edu/?q=mfold	(Zuker, 2003)
Vienna RNAfold	RNA secondary structure	http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi	(Hofacker, 2003)
miRbase	miRNA binding sites	http://www.mirbase.org/	(Kozomara and Griffiths-Jones, 2011)
miRanda	miRNA binding sites	http://www.microrna.org/microrna/home.do	(Betel, et al., 2008)
Human Splicing Finder	splice site finder	http://www.umd.be/HSF/	(Desmet, et al., 2009)
Splice Site Prediction by Neural Network	splice site finder	http://www.fruitfly.org/seq_tools/splice.html	(Reese, et al., 1997)
MaxEntScan	splice site finder	http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html	(Yeo and Burge, 2004)
Gene Splicer	splice site finder	http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml	(Pertea, et al., 2001)

Table 2

Validation Panel (26 pathogenic; 97 not pathogenic)

Variant	Class ^a	Variant	Class ^a	Variant	Class ^a	Variant	Class ^a	Variant	Class ^a
Met11Ile	Clinically important ^b	Gln356Arg	1	Gln842Gly	1	Glu1250Lys	1	Thr1685Ile	5
Met18Thr	4	Asp369del	1	Tyr856His	1	Ser1266Thr	1	Val1688del	5
Leu22Ser	5	Asp369Asn	1	Lys862Glu	1	Ile1275Val	1	Met1689Arg	4
Thr37Lys	5	Ile379Met	1	Arg866Cys	1	Arg1347Gly	1	Arg1699Gln	5
Cys39Arg	5	Asp420Tyr	1	Pro871Leu	1	Thr1349Met	1	Arg1699Trp	5
Cys44Ser	5	Asn473Ser	1	Gly890Val	1	Met1361Leu	1	Gly1706Ala	1
Cys44Tyr	5	Phe486Leu	1	Val920Ala	2	His1402Tyr	1	Gly1706Glu	5
Lys45Gln	1	Arg496His	1	Ile925Leu	1	Glu1419Gln	1	Ala1708Glu	5
Cys61Gly	5	Arg496Cys	1	Met1008Ile	1	Arg1443Gly	1	Ser1715Arg	5
Cys64Tyr	Clinically important ^b	Arg504His	1	Met1008Val	1	Asn1468His	1	Thr1720Ala	1
Asp67Tyr	1	Asn550His	1	Arg1028His	1	Arg1495Met	1	Gly1738Arg	5
Tyr105Cys	1	Glu597Lys	1	Glu1038Gly	1	Ser1512Ile	1	Arg1751Gln	1
Ile124Val	1	Ala622Val	1	Ser1040Asn	1	Val1534Met	1	Leu1764Pro	5
Asn132Lys	1	Asp642His	1	Ile1044Val	1	Asp1546Asn	1	Ile1766Ser	5
Pro142His	1	Lys654Gln	2	Pro1099Leu	1	Asp1546Tyr	1	Met1775Lys	5
Glu143Lys	1	Leu668Phe	1	Ser1101Asn	1	Leu1564Pro	1	Met1775Arg	5
Gln155Glu	1	Asp693Asn	1	Lys1109Asn	1	Ser1613Gly	1	Pro1776His	2
Tyr179Cys	1	Asn723Asp	1	Ser1140Gly	1	Pro1614Ser	1	Cys1787Ser	5
Ser186Tyr	1	Glu736Ala	2	Asp1155His	1	Met1628Thr	1	Gly1788Val	5
Val191Ile	1	Val772Ala	1	Lys1183Arg	1	Pro1637Leu	1	Val1804Asp	1
Leu246Val	1	Gln804His	1	Gln1200His	2	Met1652Ile	1	Val1838Glu	5
Ala280Gly	1	Asn810Tyr	1	Arg1203Gln	1	Met1652Thr	1	Ile1858Leu	1
Met297Ile	1	Lys820Glu	1	Glu1214Lys	1	Phe1662Ser	1	Pro1859Arg	1
Pro334His	2	Thr826Lys	1	Pro1238Leu	1	Gly1682Lys	1		
Pro334Leu	1	Arg841Trp	1	Val1247Ile	1	Thr1685Ala	5		

^a Variants showing a calculated IARC class (Pilon, et al., 2008) are from (Lindor, et al., 2012).

^b Additional variants were included only if they have been classified at BIC either as clinically important or not and have associated information supporting the classification (additional information column in BIC Database). No missense variant classified as likely pathogenic or definitely pathogenic has been reported in the central part of BRCA1 to date (between p.Asp67Tyr and p.Glu1682Lys).