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Functional assays for BRCA1 and BRCA2

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Abstract

Genetic testing for the two major breast cancer susceptibility genes has now been available for several years with more than 70,000 people tested in the USA alone. While the current genetic testing identifies many sequence alterations there are problems with both sensitivity and specificity of the assay. In particular, the genetic testing is limited in its ability to determine which of the many missense mutations identified in *BRCA1* and *BRCA2* actually predispose to cancer and which are simply neutral alterations. Here we will focus on the limitations in test result interpretation and we will explore how biochemistry and cell biology can help to clarify these issues. Although we limit our discussion to genetic testing of *BRCA1* and *BRCA2*, the problem is common to an expanding group of genes, including *ATM* and *MSH2*, in which germ-line missense mutations may also confer increased risk of cancer. Here we advocate the use of functional assays to complement genetic data in the analysis of unclassified missense mutations and propose a set of standards to conduct and interpret these assays.

Keywords

BRCA1; BRCA2; functional assays; cancer risk; unclassified variants

INTRODUCTION

Every year approximately 200,000 women are diagnosed with breast cancer and 25,000 are diagnosed with ovarian cancer in the USA (Ries et al., 2005). While the majority of cases are sporadic, 5 to 10% of breast cancer cases can be classified as hereditary (Newman, Austin, Lee, & King, 1988). Linkage studies conducted with families displaying high risk of breast cancer have identified two major susceptibility genes: *BRCA1* and *BRCA2* (Fig. 1). Individuals with an inherited inactivating mutation in the *BRCA1* or *BRCA2* genes have an increased risk of developing early onset breast and ovarian cancer, and often have multiple close family members with the disease (Tavtigian et al., 1996; Ford et al., 1998; Miki et al., 1994; Wooster et al., 1995).

Identification of tumor suppressor genes associated with specific hereditary cancer syndromes constitutes an important step because it allows individuals at high-risk to be identified early

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(Marx, 1991). This is particularly important to promote changes in life style, foster increased surveillance and to allow informed decisions about preventive surgery or hormonal therapy which are now becoming available (Narod & Offit, 2005). Often overlooked is the benefit to individuals in high-risk families with mutations in *BRCA1* or *BRCA2* who do not themselves inherit the pre-disposing allele. In the absence of testing, these individuals might decide to undergo preventive surgery although they do not carry the predisposing allele. Clearly, informative genetic testing is of considerable benefit to individuals with a family history of breast and/or ovarian cancer.

Unfortunately, the limitations of genetic testing are seldom appreciated. Technical limitations of tests often result in limited sensitivity or an inability to detect all mutations in the gene of interest. Similarly, issues with specificity or the inability to discriminate inactivating mutations from neutral sequence changes often arise. Here we consider the limitations in specificity inherent in *BRCA1* and *BRCA2* testing. We focus on the current difficulties with interpretation of test results and we outline how biochemistry and cell biology can help to illuminate these issues. In the U.S., testing for *BRCA1* and *BRCA2* mutations is performed by Myriad Genetics (Salt Lake City, Utah) using direct sequencing of coding regions (Frank et al., 2002; Frank et al., 1998). Briefly, three main outcomes of testing are possible: a) Positive for a deleterious mutation indicates that the individual is at increased risk of developing cancer. It is important to stress that because mutations in *BRCA1* or *BRCA2* do not present complete penetrance, the chance of these individuals of developing cancer, albeit high, is not 100%; b) No mutation detected in *BRCA1* or *BRCA2* coding regions. This result is highly informative if a specific mutation has been found in other family members but not in the patient being tested, indicating the proband did not inherit the susceptibility allele responsible for cancer in the family. The risk therefore is low or comparable to the risk in the general population. However, the absence of mutations combined with no information for the family may reflect different scenarios. First, an inactivating mutation in *BRCA1* or *BRCA2* may be located in non-coding regulatory regions of the genes. Second, inactivation of the gene may be caused by large rearrangements, usually deletions, insertions, or duplications that are not detected by PCR-based tests such as direct sequencing (Agata et al., 2005; Unger et al., 2000; Puget et al., 1999). Finally, it may reflect the fact that the susceptibility in the family is due to the inactivation of an unknown susceptibility gene. Thus, a negative result may not necessarily rule out an increased risk of cancer; c) The third outcome, the focus of this review is the finding of an alteration in *BRCA1* or *BRCA2* for which the cancer risk has not been determined. These alterations have been termed unclassified variants (UCV) or variants of uncertain significance (VUS). The reasons for our inability to classify these alterations stem from the lack of genetic information that allows determination of cancer association.

BRCA1 AND BRCA2 GENES

Shortly after *BRCA1* was positionally cloned in 1994, *BRCA2* was identified (Tavtigian et al., 1996; Miki et al., 1994; Wooster et al., 1995). Almost a dozen years later, while we have important clues about the function of *BRCA1* and *BRCA2* determining their exact biochemical role has been a more challenging task. *BRCA1* and *BRCA2* encode large nuclear proteins widely expressed in different tissues, markedly during S and G2 phases (Chen et al., 1996; Scully & Livingston, 2000; Thomas, Smith, Tonkinson, Rubinfeld, & Polakis, 1997)(Fig. 1). They bear little resemblance to one another or to other proteins of known function (Venkitaraman, 2002). Both protein products have been consistently linked to various processes involved in the DNA damage response, acting as tumor suppressors. These include the repair of double-strand breaks (DSBs) by homologous recombination (HR), the repair of oxidative damage by transcription-coupled repair, and a possible role in nonhomologous end joining (Moynahan, Chiu, Koller, & Jasin, 1999; Moynahan, Pierce, & Jasin, 2001; Abbott et al., 1999; Xia et al., 2001; Zhong, Chen, Chen, & Lee, 2002; Zhong, Boyer, Chen, & Lee, 2002). *BRCA1* and

BRCA2 are also implicated in the maintenance of chromosome stability, possibly through their function in recombination (Venkitaraman, 2002; Narod & Foulkes, 2004; Scully, Xie, & Nagaraju, 2004).

BRCA1 is likely to participate as a sensor or transducer rather than directly as a repair factor (effector) (Zhou & Elledge, 2000) and it has been suggested that BRCA1 functions as a scaffold or platform to coordinate different activities needed for repair (Bochar et al., 2000; Cantor et al., 2001). However, the exact molecular functions of BRCA1 in the DNA damage response have remained elusive, although several biochemical activities have been proposed. They include transcriptional regulation (Monteiro, August, & Hanafusa, 1996; Scully et al., 1997a), mRNA polyadenylation (Kleiman & Manley, 1999), chromatin remodeling (Bochar et al., 2000; Hu, Hao, & Li, 1999; Neish, Anderson, Schlegel, Wei, & Parvin, 1998), and ubiquitination (Baer & Ludwig, 2002).

The molecular role of BRCA2 is somewhat better understood. BRCA2 interacts with and regulates the function of RAD51, the mammalian homolog of *Escherichia coli* RecA that has a catalytic activity central to HR (Galkin et al., 2005; Davies et al., 2001). The interaction involves a substantial proportion of the total cellular pool of each protein, suggesting that BRCA2 works directly to regulate the availability and activity of RAD51 in this key reaction (Chen et al., 1998). Taken together these observations suggest pleiotropic roles for BRCA1 and BRCA2 in the cellular response to DNA damage.

One defective copy of *BRCA1* or *BRCA2* in the germline is sufficient for cancer predisposition, but the loss of the second allele is required for cancer development (Miki et al., 1994; Friedman et al., 1994). Little is known about the mechanisms by which the wild-type allele is lost. Surprisingly, despite the association with inherited predisposition, somatic mutations in *BRCA1* and *BRCA2* are rare in sporadic breast and ovarian cancers (Futreal et al., 1994; Lancaster et al., 1996). Nevertheless, a role for *BRCA1* in sporadic tumors has been proposed based on the epigenetic silencing of *BRCA1* in high grade tumors (Wilson et al., 1999).

THE UNCLASSIFIED VARIANTS

Both genes are highly polymorphic with approximately 70% of reported variants leading to the absence of full-length BRCA1, through loss of expression or protein truncation. Several lines of evidence derived from population-based analysis and functional studies indicate that most mutations leading to premature termination of BRCA1 are associated with increased cancer susceptibility (Friedman et al., 1994; Hayes, Cayan, Barilla, & Monteiro, 2000; Hogervorst et al., 1995; Couch & Weber, 1996; Struwing et al., 1995; Szabo, Worley, & Monteiro, 2004). Inactivating germ-line mutations in the *BRCA2* gene are associated with 60 to 85% lifetime risk of breast cancer and 15 to 30% of ovarian cancer (Wooster & Weber, 2003). The majority of mutations in *BRCA2* that are classified as cancer predisposing variants truncate the protein prior to the carboxy-terminal nuclear localization signals, resulting in mislocalization to a different cell compartment and an inability to perform its nuclear functions (Bertwistle et al., 1997; Spain, Larson, Shihabuddin, Gage, & Verma, 1999). The few truncating mutations located beyond the nuclear localization sites appear to disrupt the ability of BRCA2 to interact with Rad51, thereby inactivating DNA DSB repair. However, any truncating mutation located in the last 100 amino acids beyond the polymorphic K3326X mutation is thought to be neutral due to their limited effect on function (Martin et al., 2005).

BRCA1 and *BRCA2* missense mutations, as well as in-frame deletions and insertions have emerged as the most problematic mutations for individual risk assessment because the effect of these mutations on protein function is unclear (Szabo et al., 2004). It is not surprising that the majority of missense mutations reported do not have a clear designation as either deleterious (cancer-associated) or neutral, but remain as unclassified variants (UCVs). UCVs account for

over 35% of all reported mutations in *BRCA1* and approximately 50% of *BRCA2* (Breast Cancer Information Core Database: <http://research.nhgri.nih.gov/bic/>). The inability to determine which mutations are disease causing generates significant problems in risk evaluation, counseling and preventive care. In order to determine the clinical relevance of UCVs in *BRCA1* and *BRCA2*, several functional assays have been or are being developed. These assays are intended to serve as independent classifiers of UCVs through assessment of the influence of the UCVs on protein function and also to provide additional data that can be combined with available genetic and epidemiological data in a likelihood model to predict cancer causality of UCVs.

WHAT DO WE CALL “FUNCTIONAL ASSAY”?

On a first look it seems paradoxical to develop a functional assay for a protein for which the exact biochemical function has not been elucidated. However, there are at least two ways to circumvent this problem. One is to generate assays that evaluate global biological functions for which the requirement for *BRCA1* or *BRCA2* has been demonstrated. The other is to use genetic information to identify domains in the protein that are essential for its function and then develop ways to monitor the integrity of these domains.

In order to facilitate analysis we make an arbitrary distinction between *functional data* and *functional assays*. Functional data would include all kinds of published data (biochemical or genetic) that generate information on a phenotype caused by a particular variant, such as sets of experiments in which a germ-line mutation is shown to abolish a defined protein-protein interaction or a function normally performed by the wild-type protein. Examples are the disruption of *BRCA1/BARD1* binding by introduction of the C61G mutation (Wu et al., 1996; Brzovic, Meza, King, & Klevit, 1998); the controversial role of the phosphorylated S664 and S745 *BRCA1* residues in the disruption of the *BRCA1* interaction with CtIP after irradiation (Li et al., 2000; Wu-Baer & Baer, 2001); or the questionable role of the Y42C variant in *BRCA2* in transcriptional activation (Goldgar et al., 2004; Milner, Ponder, Hughes-Davies, Seltnmann, & Kouzarides, 1997). These controversies exemplify the problems involved in using functional *data* in trying to infer causality.

Functional *assays* should represent experiments in which a series of variants have been systematically analyzed and compared to well-defined positive and negative controls (Phelan et al., 2005; Vallon-Christersson et al., 2001; Wu et al., 2005). We suggest that negative controls should be at least two highly penetrant mutations as defined by clinical data. Ideally these mutations should have 1,000:1 overall odds for causality as calculated by the recently proposed integrated likelihood method (Goldgar et al., 2004). Taking *BRCA1* as an example, the C61G mutation for the amino-terminal region and M1775R, A1708E and Y1853X for the carboxy-terminal region constitute appropriate negative controls. Positive controls should be the wild-type *BRCA1* sequence and at least one well-defined benign polymorphisms (e.g. S1613G) with 100:1 overall odds against causality (Goldgar et al., 2004).

For *BRCA1*, not only is there a wealth of functional data but also two tests meet the definition of a functional assay: the transcription activation assay (Monteiro & Humphrey, 1998; Phelan et al., 2005; Vallon-Christersson et al., 2001) and the small colony phenotype (Monteiro et al., 1998; Humphrey et al., 1997; Coyne, McDonald, Edgemon, & Brody, 2004). Unfortunately, these two assays are domain-specific and have only been explored for the carboxy terminus of *BRCA1*. In addition, two other experimental models have generated promising results: the rescue of radiation sensitivity assay (Scully et al., 1999) the ubiquitin ligase activity (Morris et al., 2006).

Similarly, much is known about the role of *BRCA2* in DNA repair and cell cycle regulation. Based on this knowledge a series of *in vitro* assays that measure (a) MMC sensitivity, (b)

homologous recombination DNA repair and (c) centrosome amplification have been proposed as possible functional assays for evaluation of BRCA2 mutations (Wu et al., 2005).

BRCA1 TRANSCRIPTION ACTIVATION (TA) ASSAY

The TA assay was derived from observations indicating that the carboxy-terminal region of BRCA1 had the ability to function as a transactivation domain when expressed as a fusion to a heterologous DNA binding domain (DBD) (Monteiro et al., 1996; Monteiro, 2000). In the most frequently used setup a fusion of GAL4 or LexA DBD to the carboxy-terminal region of BRCA1 (aa 1396–1863) containing the variant to be tested is expressed in yeast or mammalian cells (Fig. 2A). These cells have reporter genes integrated in the host genome or in episomal form that are driven by promoters containing GAL4 or LexA binding sites (Mirkovic, Marti-Renom, Weber, Sali, & Monteiro, 2004; Phelan et al., 2005; Vallon-Christersson et al., 2001; Monteiro et al., 1996). Quantitation of the reporter gene product or its activity permits an indirect assessment of the transcriptional activity mediated by the BRCA1 fusion protein. The degree of functional inactivation associated with a given sequence alteration can then be assessed (Phelan et al., 2005; Vallon-Christersson et al., 2001).

The TA assay can be performed using a LexA DBD:BRCA1 fusion in the yeast *Saccharomyces cerevisiae* strain EGY48 using the *LacZ* gene as a reporter. That permits colonies to be directly screened on X-gal containing plates for blue/white color without the need of replica plating (Carvalho et al., 2002). The β -galactosidase activity can also be quantitated in liquid cultures using ONPG as its substrate (Phelan et al., 2005; Vallon-Christersson et al., 2001). This setup has the additional advantage of a series of well characterized reporters with different stringencies (Estojak, Brent, & Golemis, 1995). Alternatively, it can be performed using a GAL4 DBD:BRCA1 fusion in strains HF7C or SFY546 (Monteiro et al., 1996).

A similar approach is used for human cells (e.g. HEK 293T or HeLa) using the GAL4DBD fused to BRCA1 C-terminal region and a luciferase gene as a reporter (Phelan et al., 2005; Vallon-Christersson et al., 2001). The assay is conducted in a quantitative manner by measuring firefly luciferase activity. To equalize fluctuations in cell viability and transfection efficiency, an internal control with a *Renilla* luciferase gene under a constitutive promoter is used (Phelan et al., 2005; Vallon-Christersson et al., 2001). Of more than 40 assays conducted to date in only one case the results between yeast and human cells were not concordant. In this case, variant R1699W was shown to be a temperature-sensitive mutant of BRCA1 (Worley, Vallon-Christersson, Billack, Borg, & Monteiro, 2002). However, it is important to perform parallel yeast assays because several transcripts show decreased stability in mammalian cells leaving open the question of whether the variant would be considered active if the results were normalized by expression levels. We found that these transcripts are consistently more stable in yeast allowing us to rule out that possibility. Caution needs to be used when interpreting these results as it is possible the instability in human cells is itself reflective of the role of the mutation in cancer predisposition.

Although several studies have provided solid support for a potential role for BRCA1 in different aspects of transcription, it is unlikely that BRCA1 represents a *bona fide* transcriptional activator (Monteiro, 2000; Lane, 2004). Classic transcription factors are defined as those that recognize and directly bind specific DNA sequences in promoter regions and recruit the RNA polymerase II machinery. To date BRCA1 has been shown to bind indirectly to several DNA binding sites probably acting as a co-activator or co-repressor of transcription (Monteiro, 2000; Lane, 2004). Importantly, the activity on the TA assay is a reliable monitor for the integrity of the C-terminal domain of BRCA1, a domain that has been shown to be required for most, if not all functions of BRCA1. Germ-line frameshift, nonsense or missense cancer-predisposing mutations in this region have been found to abolish transcription activation, both

in yeast and mammalian cells (Monteiro, August, & Hanafusa, 1997b; Monteiro et al., 1996; Phelan et al., 2005; Vallon-Christersson et al., 2001). Conversely, benign polymorphisms or neutral variants do not alter wild-type transcriptional activity (Monteiro, August, & Hanafusa, 1997a; Phelan et al., 2005). The TA assay has not yet been formally validated but it has shown a strong correlation between its results and cancer predisposition. Importantly, this assay is presently limited to the C-terminus of BRCA1 (aa 1396–1863) and it is not known whether other regions of the protein can be interrogated using this assay.

SMALL COLONY PHENOTYPE (SCP) ASSAY

Expression of constructs containing the C-terminus of BRCA1 inhibits the growth of yeast and results in formation of colonies much smaller than controls, generating the so-called small colony phenotype which can be assessed qualitatively (Fig. 2B) or quantitatively by counting the number of cells present in each colony (Humphrey et al., 1997; Coyne et al., 2004; Monteiro et al., 1998). The growth suppression by wild-type and neutral but not deleterious variants suggested that the C-terminal region of BRCA1 inhibits growth in yeast by a mechanism analogous to BRCA1 tumor suppression in humans (Humphrey et al., 1997). Alternatively, the SCP may be due to a generic transcriptional squelching (Gill, Sadowski, & Ptashne, 1990). The fact that cancer-predisposing mutations that abolish transcription also abolish the small colony phenotype and that the phenotype is enhanced by fusion of the BRCT domains to the transcription activation domain of yeast GAL4 is consistent with squelching (Hayes et al., 2000; Phelan et al., 2005; Vallon-Christersson et al., 2001; Monteiro et al., 1998; Monteiro et al., 1996; Coyne et al., 2004; Humphrey et al., 1997).

More recently, the SCP assay was better characterized and its potential in BRCA1 sequence variant classification was evaluated (Coyne et al., 2004). The structural integrity of the BRCT region (approximately the most C-terminal 213 amino acid residues) of BRCA1 is the most critical determinant in yeast SCP assay. To date, approximately 30 variants have been tested using this assay (Humphrey et al., 1997; Coyne et al., 2004). Importantly, variants tested upstream of the BRCT domains showed no detectable effect on growth indicating that this assay may be limited to the BRCT domains.

RESCUE OF RADIATION RESISTANCE (RRR)

BRCA1 plays a role in maintaining genome integrity in response to DNA DSBs arising during DNA replication or after exogenous insults to DNA. In response to DNA-damaging agents BRCA1 is required for efficient cell cycle checkpoint activation (Cortez, Wang, Qin, & Elledge, 1999; Zhang & Powell, 2005; Scully et al., 1997b; Foray et al., 1999; Moynahan, Cui, & Jasin, 2001). These findings suggest that efficient DSB repair is linked, albeit possibly in an indirect fashion to BRCA1 tumor suppressor function. Consistent with this notion, BRCA1-deficient cells show sensitivity to ionizing radiation and to drugs that produce DSBs (Shen et al., 1998).

The test, called here RRR (Fig. 2C), is based on the ability of neutral but not deleterious variants to rescue γ -radiation resistance to the human breast cancer cell line HCC1937 which is functionally null for BRCA1 (Scully et al., 1999). The RRR uses a retroviral infection approach leading to co-expression of *BRCA1* and GFP. To evaluate γ -radiation sensitivity, mixed cultures of GFP-negative (non-expressing cells) and GFP-positive (co-expressing wild-type or variant BRCA1) HCC1937 cells are treated with ionizing radiation and assayed for growth advantage relative to parental cells by FACS analysis 3 to 24 days later (Fig. 2C). Unfortunately, this assay is very labor intensive and fewer than 10 variants have been tested. The clear advantage of this assay is that it uses the full length protein to assess a global biological function of BRCA1. However, given the small number of variants tested so far it is not clear whether this indirect measure reflect the tumor suppressive activities of BRCA1. For

example it could be that a certain variant is able to rescue radiation resistance but disables another unknown activity of BRCA1 needed for its tumor suppression function *in vivo*.

UBIQUITIN LIGASE ACTIVITY

BRCA1 displays E3 ubiquitin ligase activity mediated by its RING-finger domain at the N-terminal region and this activity is potentiated by the interaction with BARD1 (Baer et al., 2002; Brzovic et al., 2003; Chen, Kleiman, Manley, Ouchi, & Pan, 2002; Hashizume et al., 2001; Lorick et al., 1999; Ruffner, Joazeiro, Hemmati, Hunter, & Verma, 2001; Starita et al., 2004; Starita et al., 2005; Wu-Baer, Lagazon, Yuan, & Baer, 2003; Xia, Pao, Chen, Verma, & Hunter, 2003). Variants that have been unambiguously classified as cancer-predisposing (*e.g.* C61G) within the RING finger lead to inactivation of ubiquitin ligase activity linking the loss of this activity to increased cancer risk (Ruffner et al., 2001). In a recent report, Morris et al. used the interaction of BRCA1 to BARD1 and of BRCA1 to UbcH5a (E2 ubiquitin conjugating enzyme) to systematically classify variants in the first 100 residues of BRCA1 (Morris et al., 2006)(Fig. 2D). Their approach combined parallel yeast 2-hybrid (Y2H) screenings of mutagenized BRCA1 N-terminal region using BARD1 or UbcH5a as baits and isolated changes that resulted in loss of interaction. These changes identified substitutions in residues found as germline changes in patients, again strengthening the correlation between loss of ubiquitin ligase activity to increased cancer risk. Next, using site-directed mutagenesis they introduced all the variants documented in the first 100 amino acids of BRCA1 and directly tested the interaction to BARD1 or UbcH5a via the Y2H method. Importantly, variants previously classified or expected to be deleterious were shown to disrupt the interaction to BARD1 and UbcH5a. Moreover, variants that lead to loss of interaction with UbcH5a correlate with loss of ubiquitin ligase activity (Morris et al., 2006). Similarly to the TA and SCP assay, the ubiquitin ligase assay is domain-specific and probably will not be used to interrogate variants in other regions of BRCA1.

BRCA2 FUNCTIONAL ASSAYS

Functional assays for BRCA2 have been developed to account for the known homologous recombination and cell cycle regulatory activities of this protein (Fig. 3). These cell based *in vitro* assays depend upon transient expression of the complete 380 kDa wild type or mutant BRCA2 protein. Transient expression studies have been utilized because it has proven difficult to generate BRCA2 inducible or stably expressing cell lines. Expression of full-length wild type or mutant protein has been used in order to better account for the influence of the mutation on the overall activity of this large multi-domain protein in cells. Studies of single domains such as the putative N-terminal transcriptional activation domain of BRCA2 have been performed (Milner et al., 1997), but this study has yielded results that are incompatible with genetic and epidemiological data and results of functional studies using full-length BRCA2 protein (Goldgar et al., 2004). This suggests that evaluation of BRCA2 UCVs using partial BRCA2 proteins is error-prone and that assays should focus on the use of full-length proteins whenever possible.

HOMOLOGOUS RECOMBINATION

BRCA2 associates with Rad51 and is thought to mediate HR repair of DNA DSBs. This has been confirmed by recent studies in BRCA2 mutant CAPAN-1 cells and mouse embryo fibroblasts using a GFP-dependent homology directed repair assay (Moynahan et al., 2001). In this system, a Direct Repeat (DR)-GFP reporter construct containing an I-SceI site is stably integrated in the genome of recipient cells. In this reporter, two GFP genes are placed as direct repeats, each containing different inactivating mutations, making both repeats inactive. One is inactivated by the introduction of an I-SceI recognition site, while the adjacent GFP gene is differentially mutated. I-SceI sites are not found in the human genome and therefore induction

of I-SceI enzyme will create a single site-specific DSB (at the DR-GFP reporter). Expression of an active GFP depends on the repair of the mutated GFP by homologous recombination using the downstream GFP repeat as a template after the induction of a DSB. Because both repeats are mutated in different places in the coding region, one repeat can use the other as a template to correct its mutation. Thus, a GFP positive cell indicates that, after the introduction of a DNA DSB at the I-SceI site, the GFP gene was reconstituted by HR.

This homologous recombination assay has now been established in VC-8 *BRCA2*-deficient cells (Wu et al., 2005), one of the few cell types that are *BRCA2* deficient or mutant and have the ability to ectopically express full length *BRCA2* protein. These cells containing the DR-GFP reporter can be transiently transfected with either wild type or mutant full-length *BRCA2* constructs in conjunction with a construct encoding the I-SceI enzyme. I-SceI induces a unique DSB in the VC-8 cell DNA (located at the DR-GFP reporter construct that has been previously integrated in the VC-8 genome). Upon repair by wild type *BRCA2*-dependent homology directed repair processes, cells express GFP which can be enumerated by FACS.

Nine *BRCA2* UCVs have been evaluated using this approach (Wu et al., 2005) and we report on another four UCVs in Figure 3A. VC-8 cells were co-transfected with *BRCA2* constructs and the I-SceI construct and the percentage of GFP expressing cells was measured after 72h. Positive controls were wild type and known polymorphic forms of *BRCA2*. The 6174delT truncating mutation served as a negative control because no other known deleterious variants were known at the time of the study (Wu et al., 2005). Three to four-fold differences in GFP expressing cells between wild type and mutant forms of *BRCA2* were identified. The results also consistently match with data from genetic studies, suggesting that the HR assay displays good specificity. These findings suggest that this assay is useful for discriminating between inactivating and neutral alterations in the *BRCA2* gene. However, additional studies are needed to more accurately determine the sensitivity and specificity of this functional assay.

CROSSLINK DEPENDENT SURVIVAL

It has been demonstrated that *BRCA2*-deficient cells are exquisitely sensitive to agents such as mitomycin C (MMC) that induce inter-strand crosslinks and DBS (Kraakman-van der et al., 2002). This observation suggested that measurement of cellular survival in response to treatment with these agents might directly reflect the contribution of mutant forms of *BRCA2* to cancer development. Thus, a functional assay that uses clonogenic survival assays to measure the influence of *BRCA2* UCVs on *BRCA2* function has been developed (Wu et al., 2005).

BRCA2-deficient VC8 cells are transiently transfected with GFP-tagged wild type or mutant full-length *BRCA2*, and GFP positive cells are selected by flow sorting. The enriched *BRCA2* expressing cell population is exposed to MMC for 72h and colonies are enumerated after five days. Wild type *BRCA2*, the N372H and K3326X polymorphisms were used as positive controls and the 6174delT truncation was used as the negative control (Wu et al., 2005). Importantly, the clonogenic survival assays can be replaced by trypan blue exclusion cell survival assays that directly measure numbers of surviving cells rather than colony formation. In a direct comparison, the results from the assays were essentially identical suggesting that the assays are measuring similar functions of *BRCA2* and that they display excellent specificity. However, as for the assays described above, greater numbers of positive and negative controls must be evaluated to establish the sensitivity and specificity of the assays.

CENTROSOME AMPLIFICATION

BRCA2-deficient cancer cell lines and mouse embryo fibroblasts grown in culture have significant aneuploidy and centrosome amplification, and all *BRCA2* mutant tumors in both

humans and mice have substantial centrosome amplification (Tutt et al., 1999; Yu et al., 2000). Centrosome amplification is known to result from direct effects on the centrosome duplication process in early S phase, uncoupling of DNA replication from centrosome duplication, and aborted cell division in the form of a failure of cytokinesis. As BRCA2 has been implicated in regulation of cytokinesis (Daniels, Wang, Lee, & Venkitaraman, 2004), centrosome duplication (unpublished data), and DNA replication it appears likely that measurement of the ability of BRCA2 variants to induce centrosome amplification is a useful method for identifying UCVs that disrupt non-DNA damage associated functions of BRCA2.

To measure the influence of BRCA2 on centrosome number, GFP-tagged full-length wt or mutant forms of BRCA2 are ectopically expressed in cells and after 72 hrs the cells are subjected to direct immunofluorescence using α -centrin-2 antibodies to detect centrioles and centrosomes (Fig. 3C). The numbers of centrosomes in each of 200 GFP positive BRCA2 expressing cells are subsequently enumerated using confocal microscopy. Recent studies have demonstrated that wild type, positive controls, and the E462G and Y42C UCVs display low levels of centrosome amplification in 293T cells. In contrast the D2723H and 6174delT mutations display 18% centrosome amplification. These results suggest that expression of mutant BRCA2 constructs can result in centrosome amplification in a dominant negative manner. In addition, wt BRCA2 and positive controls but not the D2723H or 6174delT mutants of BRCA2 can also partially rescue the centrosome amplification that is intrinsic to *BRCA2*-deficient VC8 cells. As the results from these two methods for quantitating centrosome effects of *BRCA2* mutations are consistent and in keeping with results from homologous recombination and cell survival assays, it appears that this assay may represent a useful functional assay for BRCA2. However further sensitivity and specificity studies are needed before the assay can be used for clinical purposes.

Importantly, a number of variants that alter centrosome number but have no effect on survival or homologous recombination have been identified (data not shown), suggesting that it is necessary to perform each of the assays to ensure that as many functions of BRCA2 as possible are evaluated. This finding also indicates that a failure to alter BRCA2 activity in these three assays does not exclude a UCV as a cancer predisposing mutation because the UCV might only influence another as yet undefined or unmeasured function of the protein.

CONCLUSION

Here we discussed a number of assays that have been developed to determine whether unclassified variants of BRCA1 and BRCA2 are associated with increased cancer risk. Although the assays discussed here have shown the ability to discriminate known variants and show great promise of clinical applicability, the most important limitation for these assays to reach the clinics resides in their lack of proper validation (sensitivity and specificity). Importantly, this problem is not inherent to these assays but rather to the problem in question in which genetic data (the gold standard for validation) is not readily available for most of these rare variants.

In addition, although a number of approaches have been developed to classify missense variants there are no standardized test to evaluate variants that may affect splicing or transcript instability. These assays will be an important addition to the clinical geneticist's armamentarium as they will allow a comprehensive view of all variants in BRCA1 and BRCA2 that have clinical relevance. It is tempting to speculate that results derived from functional assays besides revealing important aspects about the biology of BRCA1 and BRCA2 may also for the basis for prediction methods that incorporate structure information (Monteiro & Couch, 2006).

Another area that needs to be fully explored if we want to provide reliable risk assessment to individuals at high risk is the development of integrated methods to combine results from functional assays and other sources of information in validated likelihood models (Goldgar et al., 2004). We also expect that several lessons learned with BRCA1 and BRCA2 will help us devise better ways to classify alleles of several disease genes in the human genome even in the absence of genetic data.

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Abbreviations

BRCT	BRCA1 C-terminal domain
DBD	DNA binding domain
DR	direct repeat
DSB	double strand break
FACS	flow activated cell sorting
GFP	green fluorescent protein
HR	

	homologous recombination
MMC	mitomycin C
ONPG	2-Nitrophenyl- β -D-galactopyranoside
RRR	rescue of radiation resistance
SCP	small colony phenotype
TA	transcription activation
UCV	unclassified variants
VUS	variant of uncertain significance
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
Y2H	yeast two-hybrid

Figure 1. Features of BRCA proteins

Domains associated with specific assays or activities are depicted. BRCA1 contains the N-terminal RING domain, nuclear localization signal (NLS), and two C-terminal BRCT domains. BRCA2 contains eight repeats of the BRC motif and the Rad51 binding regions are depicted.

OUTLINE OF BRCA1 FUNCTIONAL ASSAYS

A. Transcription Activation

Introduce variant in yeast and mammalian expression vectors as a fusion to a DNA binding domain

Transfer constructs to yeast and mammalian cells harboring reporter genes

Determine the transactivation activity of constructs containing the variants in comparison with controls

B. Small Colony Phenotype

Introduce variant in yeast expression vectors to express a fusion of BRCA1 last 305 residues to GAL4 transactivation domain

Transform yeast cells and plate

Determine colony size (or cell number in each colony)

C. Rescue of Radiation Resistance

Introduce variant in full length BRCA1 construct in a bicistronic retroviral vector containing GFP

Infect HCC1937 cells expressing the murine ecotropic receptor by co-culture with packaging cell line producing the retrovirus

Sort GFP+ HCC1937 cells and mix them (1:8) with parental (GFP-) HCC1937 cells. Irradiate the co-culture, incubate for 18 days and measure the relative enrichment of GFP+ cells

D. Ubiquitin Ligase Activity Assay

Introduce variant in BRCA1 construct (aa 2-101) as a fusion to VP16 and LacZ genes.

Test for the interaction in a Y2H system against fusions of BARD1 (aa 27-146) or full length UbCH5a.

Heterodimers of BARD1 (aa 26-142) and BRCA1 (aa 1-147) carrying the variants are expressed in bacteria and used for an in vitro ubiquitination assay.

Figure 2. Outline of BRCA1 functional assays

A. Transcription activation (adapted from Vallon-Christersson et al. 2001). Activity in a heterologous transcription assay is plotted in comparison with wild type (wt) or deleterious (M1775R and Y1853X) variants. **B.** Small colony phenotype. Yeast cells expressing wild type (A) or deleterious variants (B) generate small and large size colonies, respectively. **C.** Rescue of radiation resistance (adapted from Scully et al. 1999). Relative enrichment of GFP-positive cells is plotted in comparison to wild type (wt) or deleterious (M1775R) controls. **D.** Ubiquitin ligase activity assay. Variants are subjected to an in vitro ubiquitination reaction in which deleterious variants fail to generate polyubiquitin chain structures (adapted from Morris et al. 2006).

Figure 3. Outline of BRCA2 functional assays

A. Homologous recombination assay. Fold increase in GFP-positive cells is plotted in comparison to wild type and deleterious controls. **B.** Crosslink-dependent survival. **C.** Centrosome amplification. Wild type or neutral variants display normal number of centrosomes, while deleterious variants generate multiple centrosomes as indicated by staining against centrin (circle). Note also that BRCA2 expression (green) is both nuclear and cytoplasmic while the neutral or wild type BRCA2 is mainly nuclear (adapted from Wu et al. 2005).