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Functional assays for analysis of variants of uncertain significance in *BRCA2*

Lucia Guidugli^{1,*}, Aura Carreira^{2,3,*}, Sandrine M. Caputo^{4,*}, Asa Ehlen^{2,3}, Alvaro Galli⁵, Alvaro N.A. Monteiro⁶, Susan L. Neuhausen⁷, Thomas V.O. Hansen⁸, Fergus J. Couch^{1,#}, and Maaïke P.G. Vreeswijk^{9,#} on behalf of the ENIGMA consortium

¹Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA

²Institut Curie, Research Center, Centre Universitaire, Orsay, France ³CNRS UMR3348,

Genotoxic Stress and Cancer, Orsay, France ⁴Institut Curie, Service de Génétique, Paris, France

⁵Laboratorio di Terapia Genica e Molecolare Istituto di Fisiologia Clinica, CNR Area della Ricerca

CNR, Pisa, Italy ⁶Cancer Epidemiology Program, H. Lee Moffitt Cancer Center, Tampa, Florida,

USA ⁷Department of Population Sciences, Beckman Research Institute of City of Hope, Duarte,

CA, USA ⁸Center for Genomic Medicine, Rigshospitalet, University of Copenhagen, Copenhagen,

Denmark ⁹Department of Human Genetics, Leiden University Medical Center (LUMC), Leiden,

the Netherlands

Abstract

Missense variants in the *BRCA2* gene are routinely detected during clinical screening for pathogenic mutations in patients with a family history of breast and ovarian cancer. These subtle changes frequently remain of unknown clinical significance because of the lack of genetic information that may help establish a direct correlation with cancer predisposition. Therefore alternative ways of predicting the pathogenicity of these variants are urgently needed. Since *BRCA2* is a protein involved in important cellular mechanisms such as DNA repair, replication and cell cycle control, functional assays have been developed that exploit these cellular activities to explore the impact of the variants on protein function. In this review we summarize assays developed and currently utilized for studying missense variants in *BRCA2*. We specifically depict details of each assay, including VUS analyzed, and describe a validation set of (genetically) proven pathogenic and neutral missense variants to serve as a golden standard for the validation of each assay. Guidelines are proposed to enable implementation of laboratory-based methods to assess the impact of the variant on cancer risk.

Keywords

Variants of uncertain significance; VUS; *BRCA2*; Genetic testing; Functional analysis; Breast cancer; Ovarian cancer

Genetic epidemiology of *BRCA2*

Germline mutations in the *BRCA2* tumor suppressor gene (MIM# 600185) confer predisposition to breast and ovarian cancer and increase the risk for several other cancer types including male breast cancer, pancreatic cancer, and prostate cancer (van Asperen et al., 2005; Easton et al., 2007). In addition, hypomorphic mutations in *BRCA2* are involved in the cancer predisposition syndrome Fanconi Anemia (FA), leading to other types of tumors such as acute myeloid leukemia (Howlett et al., 2002).

BRCA2 mutations are rare in the population and account for less than 10% of familial breast cancer cases. Inactivating mutations in this gene confer an average cumulative breast cancer and ovarian cancer risk by age 80 of 51% and 12% respectively (Antoniou et al., 2008). Individuals carrying pathogenic *BRCA2* mutations can benefit from risk assessment and management strategies including enhanced cancer surveillance and/or prophylactic mastectomy and oophorectomy. In addition, mutation carriers diagnosed with breast or ovarian cancer may benefit from therapies such as platinum agents or PARP inhibitors that selectively target *BRCA2* related tumors (Evers et al., 2008; Fong et al., 2009; Audeh et al., 2010; Tutt et al., 2010; Gelmon et al., 2011; Sandhu et al., 2013).

While *BRCA2* mutations that result in truncation or inactivation of the protein are classified as pathogenic, genetic screening has also identified many variants of uncertain significance (VUS), i.e. missense and intronic variants or small insertions/ deletions, for which the impact on protein function and therefore the clinical significance is unknown. Results from the Breast Cancer Information Core Database (<http://research.nhgri.nih.gov/bic/>) (Szabo et al., 2000) and the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) (Spurdle et al., 2012), indicate that over 1,600 unique *BRCA2* missense VUS have been identified to date. The inclusion of intronic and small in-frame alterations places the number of unique variants at more than 2,000. Since it is unknown whether these variants are associated with an increased risk of cancer, the clinical management of carriers of VUS is complicated and predictive testing is not offered to family relatives.

Therefore, there is a strong demand for reliable tests to rapidly assess the clinical significance of VUS, providing carriers of these variants with the necessary information to make an informed clinical decision and refining cancer treatment by personalized therapy strategies.

Evaluation of the clinical relevance of VUS has been significantly improved by the development of a posterior probability model for *BRCA1* (MIM# 113705) and *BRCA2* variants introduced by an International Agency for Research on Cancer (IARC) Working Group (Plon et al., 2008). This method combines prior probabilities of causality derived from an evolutionary sequence conservation model (Align-GVGD) (Tavtigian et al., 2008) with likelihoods of causality derived from measures of association between the VUS and cancer (Goldgar et al., 2004); (Lindor et al., 2012). The latter, known as multifactorial likelihood approach, includes family-based evidence (personal and family history of cancer and co-segregation of variants with cancer in families) and individual evidence (histopathological tumor features and effects on RNA splicing). This method allows to

calculate the probability that a variant in *BRCA1* or *BRCA2* presents the features of known pathogenic mutations (Goldgar et al., 2008; Plon et al., 2008; Tavtigian et al., 2008; Lindor et al., 2012; Vallee et al., 2012). Accordingly to this model Class 1 (posterior probability < 0.001) and Class 2 (0.001 < posterior probability < 0.049) VUS are non-pathogenic and likely non-pathogenic VUS. Class 3 (0.05 < posterior probability < 0.949) VUS remain unclassified because of lack of sufficient family information for classification. Class 4 (0.95 < posterior probability < 0.99) and Class 5 (posterior probability > 0.99) VUS are likely pathogenic and pathogenic, respectively.

The multifactorial approach is most powerful when there are sufficient numbers of families with a given variant for analysis. The ENIGMA consortium was initiated in 2009 to facilitate pooling of genetic, clinical and histopathological information from a worldwide network of laboratories and hospitals (Spurdle et al., 2012). However, because the majority of unique VUS are very rare (1/10,000 to 1/100,000; <http://research.nhgri.nih.gov/bic/> (Szabo et al., 2000)), family-based analyses often lack statistical power to accurately predict contribution to cancer susceptibility. As a result, the use of *in vitro* assays that evaluate the consequences of a given variant on the structure and function of the protein is an essential alternative approach to predict VUS pathogenicity. Such assays have been developed for *BRCA1* and *BRCA2* and those available for *BRCA1* have recently been reviewed by Millot and authors (Millot et al., 2012). The goal of this review is primarily to describe and discuss the functional assays available for *BRCA2*. Furthermore, the manuscript discusses the promising steps that have been taken towards the translation of the functional outcome into a probability of pathogenicity associated with these variants.

BRCA2 architecture and interacting proteins

BRCA2 has 26 coding exons that encode a 3418 amino acids protein. The protein comprises a multi-domain structure (Figure 1) with limited overall sequence homology to any known protein (Lo et al., 2003). Sequencing studies identified full or partial homologous proteins to human *BRCA2* in various eukaryotic organisms, from plants to fungi, but none in yeast or bacteria (Lo et al., 2003). These *BRCA2*-like proteins differ greatly in size but contain at least a BRC repeat, a DNA binding domain (DBD) and a nuclear localization signal (NLS). Based on these features, human *BRCA2* is generally divided into three regions: the N-terminus; the BRC repeat region containing also one nuclear export signal (NES); and the C-terminal region containing the NLS, another NES and the DBD.

The N-terminus of *BRCA2* (residues 10–40) interacts with PALB2/FANCN (Partner and Localizer of *BRCA2*, Supp. Figure S1A) (MIM# 610355), that physically links *BRCA1* and *BRCA2* (Sy et al., 2009; Zhang et al., 2009a, 2009b) and is critical for maintenance of the DNA repair function of these proteins (Xia et al., 2006; Oliver et al., 2009; Tischkowitz and Xia, 2010; Bowman-Colin et al., 2013; Huo et al., 2013). The same region has also been implicated in binding to EMSY (MIM# 608574) and HP1 (MIM# 259900) proteins that are involved in chromatin remodeling (Hughes-Davies et al., 2003). In addition, the region encoded by exon 3 (residue 24–105) has been proposed to be implicated in transcriptional activation (Milner et al., 1997).

Residues 200–600 have been shown to interact with centrosome and midbody associated complexes CEP55-TSG101 and CEP55-ALIX that are required for completion of abscission and cytokinesis (Mondal et al., 2012).

The central region of BRCA2 (residues 900–2000) contains eight interspersed BRC repeats of about 35 residues (Bork et al., 1996), through which BRCA2 binds to RAD51 (MIM# 179617) (Supp. Figure S1B) (Chen et al., 1998), the recombinase that mediates strand invasion to process homologous recombination (HR) in humans. This series of repeats has been recently shown to work in two groups (BRC1-4 and BRC5-8) that display unique functional characteristics to ultimately facilitate loading of RAD51 onto single stranded DNA (ssDNA) at sites of DNA damage (Carreira et al., 2009; Carreira and Kowalczykowski, 2011). In the context of HR, the main function of BRCA2 is to mediate this loading (Yuan et al., 1999). Biochemical assays have shown that the purified full length BRCA2 protein binds about six molecules of RAD51, targets RAD51 loading onto ssDNA leading to displacement of RPA, and promotes the DNA repair activity of RAD51 (Jensen et al., 2010; Liu et al., 2010; Thorslund et al., 2010).

This region also harbors one NES (residues 1383–1393) necessary for centrosome localization of BRCA2 (Han et al., 2008). Downstream the BRC repeats there is a DMC1 (MIM# 602721) binding site implying an involvement of BRCA2 in meiotic recombination (Thorslund et al., 2007).

Apart from the PALB2/FANCN binding site in the N-terminus, BRCA2 has been shown to interact with two other FA proteins. FANCG (MIM# 602956) mapped in two regions: 499–994 and 2350–2545. FANCD2 (MIM# 613984) also binds this last region (Hussain et al., 2003; Hussain et al., 2004).

The C-terminal region (amino-acids 2459–3190, Supp. Figure S1C) contains a DBD. This domain is the most conserved portion of BRCA2 across metazoans, plants and fungal orthologs (Holloman, 2011). It is composed of a helical domain, three oligonucleotide binding (OB) folds that are ssDNA-binding modules and a tower domain (TD) that protrudes from OB2 and has been suggested to bind dsDNA (Supp. Figure S1C). The DBD also binds to DSS1 (MIM# 601285) (deleted in split-hand/split foot syndrome, Supp. Figure S1C), a protein involved in ubiquitin dependent protein turnover (Funakoshi et al., 2004; Sone et al., 2004) and possibly in stabilizing BRCA2 (Yang et al., 2002; Josse et al., 2006; Li et al., 2006). Recently it was shown that the nuclear localization of BRCA2 is controlled by DSS1 interaction with BRCA2 through a NES present at that position (Jeyasekharan et al., 2013). In addition, the C-terminus of BRCA2 contains another RAD51-binding site (residues 3265–3330), that is phosphorylated by cyclin dependent kinase 1 (CDK1 (MIM# 116940)). CDK1 regulates BRCA2 interaction with RAD51 filaments (Davies and Pellegrini, 2007; Esashi et al., 2007), and is important for the entry into mitosis (Ayoub et al., 2009). More recently, this phosphorylation site has been linked to inhibition of MRE11 (MIM# 600814) -dependent degradation of DNA strands at stalled DNA replication forks during S-phase (Schlacher et al., 2011). The C-terminus of BRCA2 also contains two functional NLS (residue 3263–3269 and 3381–3385) essential for the translocation of BRCA2 to the nucleus (Spain et al., 1999). Additional interacting partners of BRCA2 have

been identified, supporting the notion that BRCA2 is a multifunctional protein (Gudmundsdottir and Ashworth, 2006; Roy et al., 2012).

BRCA2 functions

A role for the BRCA2 protein in DNA repair and genome stability maintenance was first indicated by the hypersensitivity of *BRCA2*-deficient cells to DNA damaging agents (Sharan and Bradley, 1997; Patel et al., 1998). Specifically, these cells showed inability to repair DNA double-strand breaks (DSBs) by HR (Moynahan et al., 2001). The HR repair mechanism uses sister chromatids as templates for the accurate repair of replication-associated DSBs during S and G2 phases of the cell cycle. While other mechanisms of DSB repair, including non-homologous-end-joining (NHEJ), are error-prone and result in translocations and deletions, the HR pathway is error-free and contributes to the maintenance of genome stability.

BRCA2 inactivation leads to micronuclei and centrosome amplification in different cell lines (Tutt et al., 1999; Kraakman-van der Zwet et al., 2002), suggesting a role for BRCA2 in the control of centrosome duplication and/or spindle-pole function (Tutt et al., 1999). Moreover, the interaction of BRCA2 with BRAF35 (MIM# 164757) and chromatin during early phases of mitotic chromosome condensation suggests involvement of BRCA2 in regulation of cell cycle progression (Marmorstein et al., 2001). In addition, BRCA2 deficient MEFs exhibit delayed cytokinesis, an abnormal number of nuclei, and defective furrow formation suggesting a regulatory role for the protein in the cytokinesis process (Daniels et al., 2004). Indeed, *BRCA2* heterozygosity has been associated with delayed cytokinesis in primary human fibroblasts (Jonsdottir et al., 2009) and BRCA2 has been shown to be a component and regulator of midbody structure and function (Mondal et al., 2012).

The role of BRCA2 in the maintenance of genome integrity is also reflected by involvement in FA. FA is a rare disease characterized by the hypersensitivity to DNA cross-linking agents like Mitomycin C (MMC). Both FA and hereditary breast and/or ovarian cancer (HBOC) are inherited syndromes whose pathogenesis stem from defects in DNA repair. FA patients of the complementation group D1 carry biallelic mutations in *BRCA2* (Howlett et al., 2002). At the molecular level, these disorders are fundamentally related because products of the FA and BRCA genes operate in concert to repair specific types of DNA damage, in particular, inter-strand crosslinks. Furthermore, the interaction with FA proteins and RAD51 is necessary for BRCA2 role in protecting nascent DNA strands from degradation at stalled DNA replication forks (Schlacher et al., 2011; Schlacher et al., 2012). Interestingly, both the role of BRCA2 in the protection of replication forks and regulation of midbody structure in cytokinesis appear to be independent of BRCA2 HR function.

BRCA2 behaves as a tumor suppressor gene with functional inactivation of BRCA2 associated with tumorigenesis, often in combination with loss of other DNA damage response (DDR) mediators such as TP53 (MIM# 191170). However, the evidence in support of the loss of the wild-type allele in *BRCA2* related tumors is inconsistent (Clarke et al., 2006; Spearman et al., 2008; Walsh et al., 2008; Brozek et al., 2009).

BRCA2 deficient tumors show gross chromosomal rearrangements and display extensive chromosomal instability (van Beers et al., 2005; Roy et al., 2012). The absence of a functional HR pathway in these tumors has been exploited for chemotherapy treatment with platinum salts (carboplatin and cisplatin) that promote interstrand crosslinks and DNA breaks leading to lethal levels of genomic aberrations and chromosomal rearrangements. Similarly, PARP inhibitors, that induce single and double strand DNA breaks (Bryant et al., 2005; Farmer et al., 2005) and promote error prone repair of DNA breaks and crosslinks in the absence of efficient HR repair, are being evaluated as targeted therapies for BRCA1 and BRCA2 deficient tumors (Lord and Ashworth, 2012).

BRCA2 functional assays

Based on the presence of several protein domains associated with different functions of the BRCA2 protein, distinct *in-vitro* assays have been developed to analyze the effect of variants on BRCA2 function in a qualitative or quantitative way. As some functions attributed to BRCA2 may not necessarily influence cancer risk, the sensitivity (percentage pathogenic variants that is correctly classified) and specificity (percentage of non-pathogenic variants that is correctly classified) of each assay should be carefully evaluated against a series of pathogenic and non-pathogenic variants that have been classified using clinical genetic data (Table 1). Here we describe the characteristics of functional assays established for BRCA2.

Homology-directed repair (HDR) assay

This assay is based on gene conversion repair of an I-SceI induced DSB in a green fluorescent protein (DR-GFP) reporter construct in *Brca2* deficient cells (Moynahan et al., 2001). Complementation of the DSB repair deficiency in *Brca2* deficient cells by full-length human *BRCA2* cDNA expression constructs results in reconstitution of a functional GFP gene. Quantitation of the proportion of GFP-positive cells by flow cytometry serves as a measure of homology directed repair activity. By using the reporter assay in V-C8 *Brca2* deficient hamster lung fibroblast cells (*XRCC11*) it has been possible to measure and compare the HR activity of wild-type and mutant forms of BRCA2 (Wu et al., 2005; Farrugia et al., 2008; Guidugli et al., 2013) (Table 2). Most recently, the sensitivity and specificity of the assay for VUS in the *BRCA2* DBD has been evaluated using a set of 13 known pathogenic and 18 known non-pathogenic mutations (defined by family-based genetic data). Sensitivity has been estimated at 100% (95% CI: 75.3%–100%), and specificity at 100% (95% CI: 81.5%–100%) (Guidugli et al., 2013). Therefore a statistical classifier for estimating the probability of pathogenicity of VUS using the functional results was developed. Through this probability model 18 variants for which the clinical significance was unknown were predicted as pathogenic and eight as non-pathogenic (Guidugli et al., 2013) (Table 2).

Interestingly, several variants have displayed intermediate HR activity in this assay. It is not known whether intermediate function equates to intermediate risk or if the reduction in activity is sufficient to confer a high-risk of breast and ovarian cancer (see Discussion).

Homologous recombination assay in human cells

Recently, Balia and authors (Balia et al., 2011) developed an assay that determines the effect of the transient overexpression of BRCA2 VUS on restoration of a recombination reporter substrate. It utilizes a HeLa G1 cell line containing a recombination substrate with two differentially mutated hygromycin resistance (HygR) genes. An intra-chromosomal recombination event leads to the restoration of the wild-type form of HygR and cell survival in the presence of hygromycin. The frequency of intra-chromosomal recombination events is calculated as the total number of viable cells after selection with hygromycin. Wild-type BRCA2 overexpression only marginally enhances the number of viable cells after hygromycin selection. However, when a loss-of function BRCA2 variant is overexpressed, an increase in the number of viable cells is observed, indicating enhanced intra-chromosomal recombination events that result in HygR gene restoration and cell survival. Albeit the nature of the specific recombination event was not examined by the authors, Larminat et al. have previously reported that loss of *BRCA2* in V-C8 cells increases intra-chromosomal recombination most likely by SSA (Larminat et al., 2002). Similarly, in the HeLa G1 cells, the expression of a loss-of function BRCA2 variant may, in a dominant-negative manner, result in an increase of recombination, thereby restoring the HygR gene. The authors selected 15 *BRCA2* variants that localize in different regions of the BRCA2 protein (Table 2). The assay was calibrated using three neutral variants and one pathogenic mutation. The latter increased recombination by 3.5 fold relative to the wild-type. Eleven variants that had not previously been described or classified were also tested. Five variants did not enhance recombination and were classified as non-pathogenic. Six other variants increased recombination as much as the pathogenic mutation and were classified as “probably pathogenic”. However, the p.Gly2353Arg variant that had the strongest effect on recombination activity and was therefore classified as “probably pathogenic” has recently been shown to be a neutral variant (Class 1) (Lindor et al., 2012). This latter data suggest a reduced specificity of the assay. Importantly, this method does not directly measure a known BRCA2 function but it rather measures error-prone repair mechanisms such as SSA in the absence of functional BRCA2. Consequently, the application of this assay to evaluate the functional effect of VUS is a matter of debate.

Yeast recombination assay

This assay involves expression of human full-length BRCA2 wild-type protein in the diploid strain of *Saccharomyces cerevisiae*, RS112, which contains two substrates to measure HR (Spugnesi et al., 2013). The intra-chromosomal HR substrate consists of two *HIS3* alleles, one with a deletion at the 3' end and the other with a deletion at the 5' end, sharing 400 bp of homology. An intra-chromosomal recombination event leads to *HIS3* reversion. The diploid RS112 strain also contains the two alleles, *ade2-40* and *ade2-101*, located in two homologous chromosomes that allow the measurement of inter-chromosomal recombination events. Expression of wild-type BRCA2 increased both intra- and inter-chromosomal HR in yeast and enhanced resistance to methyl methanesulfonate without affecting the number of RAD51 nuclear foci, suggesting that BRCA2 interferes with yeast DNA repair. The influence of the expression of three neutral BRCA2 variants (i.e. p.Leu1019Val, p.Thr1915Met and p.Ala2951Thr) and the known pathogenic variant (p.Gly2748Asp) on HR

was evaluated. The neutral variants induced a significant increase in HR whereas p.Gly2748Asp did not. In addition, seven VUS have been tested with this assay. Five out of seven *BRCA2* variants induced at least one recombination event in yeast (Table 2). These results suggest that *BRCA2* missense variants that increase HR in the yeast assay are not pathogenic because they confer the same phenotype as the *BRCA2* wild-type. On the other hand, variants that do not stimulate HR may be associated with an increased risk of cancer.

Centrosome-amplification assay

Based on the observation that *BRCA2* inactivation leads to centrosome amplification, a cell-based functional assay that measures the influence of *BRCA2* variants on centrosome number has been developed (Wu et al., 2005; Farrugia et al., 2008). Briefly, centrioles and centrosomes were visualized by immunofluorescence and enumerated in 293T or HeLa cells 72 h after transfection with GFP-tagged full-length *BRCA2* cDNA (wild-type or mutant forms of *BRCA2*) (Wu et al., 2005). Wild-type *BRCA2* and neutral variants did not influence centrosome number, whereas the c.5946delT and p.Asp2723His pathogenic mutations induced a significantly increased number of centrosomes (Wu et al., 2005).

The influence of *BRCA2* on centrosome numbers was also assessed using a reconstitution approach in *BRCA2* deficient V-C8 cells (Wu et al., 2005). Wild-type *BRCA2* reduced the number of amplified centrosomes whereas the pathogenic p.Asp2723His mutation did not change the level of centrosome amplification (Wu et al., 2005). The sensitivity and specificity of the centrosome assay was assessed using eight classified pathogenic mutations and eight neutral variants (Farrugia et al., 2008). The assay showed a sensitivity of 85% (95% CI 47–100) since the pathogenic p.Arg3052Trp did not induce centrosome amplification. Specificity was 100% (95% CI, 63–100).

Mitomycin C (MMC) survival assay

BRCA2-null cells exhibit hypersensitivity to DNA damaging agents (Connor et al., 1997; Sharan et al., 1997; Morimatsu et al., 1998; Patel et al., 1998) and the re-introduction of a *BRCA2* functional gene significantly reduces this sensitivity (Kraakman-van der Zwet et al., 2002). Based on this observation, *BRCA2*-deficient V-C8 cells were complemented with a series of known neutral and pathogenic *BRCA2* missense changes and evaluated for survival by clonogenic survival assay following exposure to MMC (Wu et al., 2005). Wild-type *BRCA2* and five neutral variants displayed 5-fold enhanced survival and 12 to 14-fold improved viability relative to control V-C8 cells, while the mutations c.5946delT and the p.Asp2723His could not rescue the sensitivity to MMC (Table 2). The influence of other DNA damaging agents such as ionizing radiation, cisplatin or ultraviolet radiation (UV) can also be tested using this approach.

Embryonic stem cell (ESC)-based functional assay

A versatile system for assessing the clinical relevance of *BRCA2* VUS is represented by the mouse Embryonic Stem (ES) cell assay. The mouse ES cells contain one defective mouse *Brca2* allele (*mBrca2*) and one functional *mBrca2* allele that can be conditionally switched off. The assay is based on the observation that functional *BRCA2* is essential for ES cell viability. Transfection of a BAC clone containing a wild-type human *BRCA2* (*hBRCA2*)

allele will rescue mouse *Brca2* deficient ES cells (Sharan et al., 1997; Kuznetsov et al., 2008). The advantage of this system is that ES cells are not inherently prone to genomic instability and therefore represent a more stable system for studying BRCA2 function with respect to the majority of mammalian cancer cell lines. Furthermore, any type of mutation, including regulatory mutations and intronic alterations, can be introduced and subsequently assessed for influence on ES cell survival. Variants that fail to rescue the lethality of the *mBrca2* deficient ES are pathogenic, variants that (partially) rescue the lethality of the *mBrca2* deficient ES cells, are analyzed by a series of biological assays (e.g. drug sensitivity, HR, chromosomal instability). Briefly, assays that measure the sensitivity to chemical agents or radiation (e.g. UV and ionizing radiation) are performed using a cell viability assay such as the XTT-assay (Scudiero et al., 1988). Drug sensitivity is expressed as the percentage of surviving cells compared to untreated cells. An HR assay is performed using a DR-GFP reporter construct stably integrated in the ES cells as for the HDR assay described above, or using two gene-targeting vectors (Kuznetsov et al., 2008). In addition, the RAD51 foci assay is used to assess the ability of the cells to perform HR after induction of DSB by ionizing radiation (Kuznetsov et al., 2008). Chromosomal aberrations are scored by karyotype analysis of metaphase ES cells (Kuznetsov et al., 2008). Variants that are deficient in any of the functional assays are scored as pathogenic. The ES survival assay has been used to successfully discriminate between six known pathogenic and seven known neutral *BRCA2* missense changes (Table 2 and Figure 1). In addition, 14 missense variants that map to the N-terminal PALB2-binding and the C-terminal DBD have been analyzed using this approach (Table 2) (Kuznetsov et al., 2008; Biswas et al., 2011; Biswas et al., 2012), including variants that have been described to induce RNA splice aberrations in human (Claes et al., 2004; Thomassen et al., 2006; Farrugia et al., 2008). Using this assay, aberrant splice transcripts similar to those observed in carriers were identified, supporting the value of this approach for the analysis of variants that may affect RNA splicing.

Syngeneic human cancer BRCA2 knockout cell line model (SyVal model)

Targeted introduction of variants into the p53 deficient human epithelial colorectal cancer cell line DLD1 has been described as a method for characterizing *BRCA2* missense variants (Hucl et al., 2008). Targeted disruption of *BRCA2* exon 11 (*BRCA2* ex11/wt) yields a hemizygous human cancer cell that was further modified to contain nucleotide changes resulting in four unclassified missense variants and one synonymous change in exon 27 of *BRCA2* (Hucl et al., 2008). A clone bearing one functional copy of *BRCA2* (*BRCA2* wt/ex11) was established as a wild-type control and clones containing the pathogenic *BRCA2* p.Tyr3308X mutation and a biallelic disruption of the gene (*BRCA2* ex11/ex11) were established as negative controls. RAD51 foci formation and sensitivity to MMC or etoposide was evaluated in each clone (Table 2). The *BRCA2* wt/ex11 clone, the *BRCA2* synonymous mutant, and three clones with VUS, induced RAD51 foci and did not show hypersensitivity to MMC or etoposide indicating that they did not affect *BRCA2* function. The *BRCA2*-null clone, the pathogenic mutant and VUS p.Ser3291Glu showed no induction of RAD51 foci upon DNA damage and enhanced sensitivity to MMC or etoposide, indicating a severe defect in *BRCA2* function. This model provides the first human syngeneic *BRCA2* model and therefore seems promising for the characterization of *BRCA2* VUS.

Nuclear localization assay

The BRCA2 protein is predominantly localized within the nucleus (Spain et al., 1999) (Yano et al., 2000), but can relocalize to certain structures, such as centrosomes, during S and early M phases of the cell cycle (Nakanishi et al., 2007). To examine whether variants alter the subcellular localization of BRCA2, which might be associated with altered protein function, the influence of variants on localization of GFP-tagged BRCA2 was assessed (Wu et al., 2005). Studies in *BRCA2*-deficient (V-C8) and proficient cells (293T and HeLa) showed that BRCA2 protein encoded by non-pathogenic variants was localized in the nucleus. By contrast, BRCA2 protein encoded by pathogenic missense variants localized fully or partially in the cytoplasm (Wu et al., 2005). Variants in the N-terminal region of *BRCA2* that showed impaired HR activity (p.Gly25Arg, p.Trp31Arg, and p.Trp31Cys) displayed proficient nuclear localization (Xia et al., 2006; Biswas et al., 2012). The mechanism by which some variants are mislocalized remains to be elucidated. As these missense changes do not disrupt the known C-terminal NLS, the mutant proteins may be retained in the cytoplasm due to protein misfolding. The development of this method as a quantitative functional assay is complicated as the amount of protein overexpression, which is variable, would affect the protein mislocalization and misfolding.

BRCA2 protein-protein interaction-based assays

As described above, the functions of BRCA2 rely on interaction with other proteins. Differences in binding affinity due to the presence of a variant in one of the binding domains of BRCA2 can be tested directly by various biochemical methods such as pull downs, co-immunoprecipitation, or yeast two-hybrid strategies. In addition, these variants can be further analyzed by other assays to pinpoint the function affected. Several studies have assessed the effect of BRCA2 variants on the interaction with PALB2 (Xia et al., 2006; Biswas et al., 2011), APRIN (Brough et al., 2012), or with midbody components (Mondal et al., 2012). A similar rationale could be used to interrogate mutants located in the DBD of BRCA2 using EMSA (Electrophoretic Mobility Shift Assay) with the purified mutant protein as described for the wild type BRCA2 (Yang et al., 2002; Jensen et al., 2010; Biswas et al., 2012). While there is strong evidence that VUS influencing the interaction with PALB2 and ssDNA are cancer-associated *BRCA2* mutations (Xia et al., 2006; Biswas et al., 2012), the significance of disruption of other interactions remains to be established.

Analysis of variants that affect RNA splicing

There is strong evidence that a number of variants influence BRCA2 function and are associated with increased breast cancer risk due to induction of aberrant splicing of *BRCA2*. This subject is extensively addressed in a number of recent papers published by the ENIGMA splicing working group (Houdayer et al., 2012; Spurdle et al., 2012; Thomassen et al., 2012). Characterization of these variants relies on minigene-based splicing assays or analysis of RNA derived from blood or tissue samples from patients. Importantly, there are examples showing that RNA analysis is not sufficient to elucidate the clinical relevance of a subset of variants influencing consensus splice sites or forming *de novo* splice sites. In some cases, the absence of exonic SNPs in the patient will render RNA analysis uninformative since the allelic contribution of aberrant and normal transcripts cannot be distinguished. In

other cases, albeit the nature of the aberrant transcript might be elucidated, the effect on protein function might still be unclear (e.g. if there is a stable transcript with an in-frame deletion of a whole exon or an in-frame retention of a few intronic bases). Furthermore, some missense variants may cause incomplete aberrant splicing and also influence BRCA2 function associated with the non-spliced mutant transcript (Farrugia et al., 2008; Walker et al., 2010). In these cases, assays based on genomic DNA (such as the ES cell assay) are required to assess the functional effect of the aberrant RNA splicing (Kuznetsov et al., 2008; Biswas et al., 2011; Biswas et al., 2012).

Phenotype in heterozygous carriers

Several studies have described approaches to look for differences in the DNA damage response between cells from *BRCA2* heterozygous mutation carriers and healthy controls. These assays have been conducted using lymphoblastoid cells, non-immortalized lymphocytes, skin fibroblasts and breast tumor cells. Although these studies have yielded conflicting results and show large inter-individual differences, there are indications that cells derived from heterozygous *BRCA2* carriers might respond differently than controls at the gene expression level (Kote-Jarai et al., 2006; Waddell et al., 2008) or show different level of chromosomal damage following exposure to ionizing radiation (Buchholz et al., 2002; Baeyens et al., 2004; Ernestos et al., 2010; Becker et al., 2012). If robust assays can be developed with high specificity and sensitivity, this approach might also be applicable for interpreting VUS pathogenicity.

Discussion

An important obstacle to improved risk assessment for breast and ovarian cancers is due to the presence of a large number of *BRCA2* variants for which cancer association has not been determined. Here we provided detailed background about specific functional assays that have been developed to determine whether these variants may be associated with increased cancer risk. Although functional assays are very useful, they are technically demanding and their use is currently restricted to research laboratories. In addition it is important to highlight that there are two main caveats for the use of functional assays as a way of inferring the disease risk associated with *BRCA2* variants. The first caveat resides in the multifunctional role of BRCA2, its interaction with different proteins and the lack of a comprehensive understanding of every single biological function of BRCA2. A variant with no impact on one function may lead to severe impact on another function. Therefore, a combination of functional approaches for characterizing variants is necessary. The second caveat relates to the fact that distinct BRCA2 functions may contribute differently to the tumor suppressor activity of the protein and variants that disrupts specific BRCA2 functions may or may not be relevant in terms of cancer risk. For this reason it is essential to determine the sensitivity and specificity of each assay.

Validation panel

A functional assay should be validated against a panel of variants for which the clinical significance has been determined with the posterior probability approach using direct genetic evidence (e.g. co-segregation analysis, family history, co-occurrence). This

validation set, consisting of Class 4/5 (pathogenic) and Class 1 (neutral) variants will allow determination of the sensitivity and specificity of an assay (Table 1). A low-sensitivity test will identify a large number of false negatives (pathogenic variants misclassified as non-pathogenic) while a low specificity test will identify a large number of false positives (non-pathogenic variants misclassified as pathogenic). While validation using a panel of known pathogenic and neutral variants is essential, the nature of the validation panel will vary depending on whether the assay is being applied to a specific domain or to the entire protein. Assays for specific domains should be validated with known pathogenic and neutral variants in the same domain. In contrast, because the function of BRCA2 is still not completely elucidated, and depends on interactions interspersed throughout the sequence, any assay being applied to VUS throughout the protein should be validated using classified variants across the entire gene. However, the lack of known pathogenic mutations, defined by the multifactorial likelihood model, in the BRC repeats and the N-terminus of BRCA2 suggests that this approach may currently not be possible. Up to date, the HDR assay is the only functional approach that has been validated using a sufficient number of variants to establish the sensitivity and specificity of the assay. Other assays first have to be extensively validated before they can be used for clinical purpose.

Technical and practical considerations

A series of important considerations apply for almost all assays. Some of these issues were already addressed in a recent review that describes the functional assays available for variants in *BRCA1* (Milot et al., 2012) and are only briefly mentioned here.

- The use of proper controls in each experiment (Milot et al., 2012). At least one neutral variant (or polymorphism) and one pathogenic mutation should be included as controls in each independent experiment. These controls should map in the BRCA2 domain interrogated by the specific functional assay
- Replicates of each sample (i.e. construct carrying the variant) should be included within each independent experiment and each independent experiment should be repeated at least twice (Milot et al., 2012).
- Whenever assays are performed using transient transfection approaches, the transfection efficiency associated with each variant must be measured and used to normalize BRCA2 activity. In addition, since in transient transfection experiments protein levels are more variable compared to stable transfection experiments, it is essential to examine whether proteins are expressed at a similar level preferably by western blots. Ideally one should assess protein expression in the same sample being used for functional analysis (Milot et al., 2012).
- When performing transient transfection experiments, plasmids should be prepared and assayed in batches to control variability in the assay caused by differences in DNA quality (Milot et al., 2012).
- The use of partial BRCA2 proteins may lead to misinterpretation of the clinical relevance of certain *BRCA2* variants. For instance p.Tyr42Cys encoded by exon 3 was first shown to influence transcription (Milner et al., 1997) and a fragment

containing this variant has been shown to disrupt the BRCA2 interaction with RPA (Wong et al., 2003). However it was later clearly shown by genetic evidence (Goldgar et al., 2004) and the ES cell assay (Kuznetsov et al., 2008) that p.Tyr42Cys is a neutral allele with no influence on BRCA2 function. Therefore there is a clear preference for the use full-length BRCA2 proteins in assays, despite the associated problems with expression and detection of the 384 kDa protein.

- Assay results should be quantified and thresholds of activity that separate pathogenic and neutral variants should be established (Millot et al., 2012). Only the HDR assay has been developed in this manner (Farrugia et al., 2008; Guidugli et al., 2013). Highly quantitative assays will be necessary to discriminate between variants that totally inactivate or only partially inactivate protein function.
- A panel of different assays representative for different BRCA2 functions should be used to evaluate variants in order to minimize the risk that a specific functional effect of the protein will be overlooked. Conversely, *in-vitro* assays can suggest an impact of a variant on a certain function that does not necessarily translate into a defect *in vivo*, especially when the specific effect is compensated by other factors and/or functions.

Development and improvement of functional assays

While mouse ES cells and the *BRCA2*-deficient V-C8 hamster lung fibroblasts have been widely utilized for *BRCA2* functional assays, the lack of an easy to manipulate, stable *BRCA2* deficient human cell has hampered the development of assays in human cells. It is to be expected that improved methods for genetic manipulation of human cells, such as TAL endonucleases or RNA-guided engineering (Ding et al., 2013; Mali et al., 2013) will facilitate the study of VUS. In fact the introduction of variants into the endogenous gene as opposed to transient transfection will likely improve the reliability of functional assays. Similarly, inducible methods for expression of mutant forms of *BRCA2* in *BRCA2*-deficient cells may improve functional assays. Additional assays might be used in the future for the functional evaluation of variants in *BRCA2*. For example immortalized MEFs containing one allele with an exon 27 deletion (*lex1*) and one allele with a deletion of part of exon 26 and whole 27 (*lex2*) (*brca2^{lex1/lex2}*) have been developed (Donoho et al., 2003). *Brca2^{lex1/lex2}* MEFs showed mild sensitivity and hypersensitivity to MMC and γ -radiation, respectively. The influence of hypomorphic missense alterations on *BRCA2* function could also be analyzed by this approach. Also, the work described by Schlacher and colleagues (Schlacher et al., 2011), elucidating the role of *BRCA2* in the protection of DNA at stalled replication forks, could form the basis of an assay for evaluation of HR independent activity of *BRCA2*. The single-molecule fiber analysis method is in principle suitable for testing variants located at the C-terminal domain of *BRCA2*. Whether the method is applicable to variants in other domains of *BRCA2* remains to be determined.

A complementary approach to evaluate the role of variants in tumorigenesis is to study them in genetically engineered mouse models as has recently been described for *BRCA1* (Drost et

al., 2011; Shakya et al., 2011). These models are invaluable for studying the effect of different *BRCA2* variants on *BRCA2* function, impact on tumorigenesis and therapy response. Although conditional *Brca2/Trp53* mice are available (Jonkers et al., 2001; Evers and Jonkers, 2006), the effect of *Brca2* variants has only rarely been studied in mouse models (Xia et al., 2006; Kuznetsov et al., 2008; Biswas et al., 2012).

The challenge of risk prediction: Intermediate function, intermediate risk?

Functional analysis may also identify variants with intermediate function that may have intermediate or moderate effects on cancer risk (Monteiro and Couch, 2006; Lovelock et al., 2007; Kuznetsov et al., 2008; Guidugli et al., 2013). These variants do not fully complement the deficient phenotype or show discordant results using different functional assays. It is difficult to classify such missense changes using the standard multifactorial likelihood approach that is based on comparing data for a particular variant under the hypothesis that the variant is a fully penetrant pathogenic mutation against the hypothesis that the variant is neutral or of no clinical significance with respect to risk. As shown for the p.Arg1699Gln variant in *BRCA1*, the standard co-segregation analysis yielded odds of only 5:1 in favor of the variant being pathogenic compared to the >6000:1 odds when a lower penetrance was allowed (Spurdle et al., 2012). To determine whether intermediate function translates to intermediate cancer risk, family history and clinical information of families carrying intermediate function variants need to be collected to estimate age related and/or cumulative risk of breast/ovarian cancer associated with these missense changes.

Translating functional effects to cancer risk; the use of functional test results in the multifactorial likelihood model

Genetic studies based on family history, co-segregation or co-occurrence, have established a clear impact of certain variants on cancer risk. Nevertheless, functional assays to interpret the VUS pathogenicity are essential since many individual variants are unique in the population and there is not enough genetic information for classification purposes. However, impairment of a specific function does not translate directly to cancer predisposition. While a variant that completely knocks out gene function in an assay could be regarded as a “pathogenic mutation”, a genetic counselor would like to see additional evidence of pathogenicity from the clinical presentation of the patient and from family history before taking a definitive decision.

The use of quantitative measurements for assays that have been extensively validated is the first step towards developing a model for predicting the VUS pathogenicity (Guidugli et al., 2013). The model developed by Guidugli and authors for *BRCA2* VUS analyzed with the HDR assay derives a probability of pathogenicity for each variant using estimates of the mean and the variances of the distribution of the HDR results for the known pathogenic and the non-pathogenic mutations. Such probabilities can be converted into likelihood ratios (LR) for inclusion in a Bayesian integrative analysis, in which data from other sources (e.g. family history, co-segregation, *in silico* protein prediction analysis) can be combined with functional data to generate an overall likelihood that a given variant is pathogenic or neutral (Iversen et al., 2011).

In time, functional assay data will form part of a multivariate model for the evaluation of VUS carriers and in this way will be a valuable and indispensable tool for the assessment of the clinical relevance of variants of uncertain significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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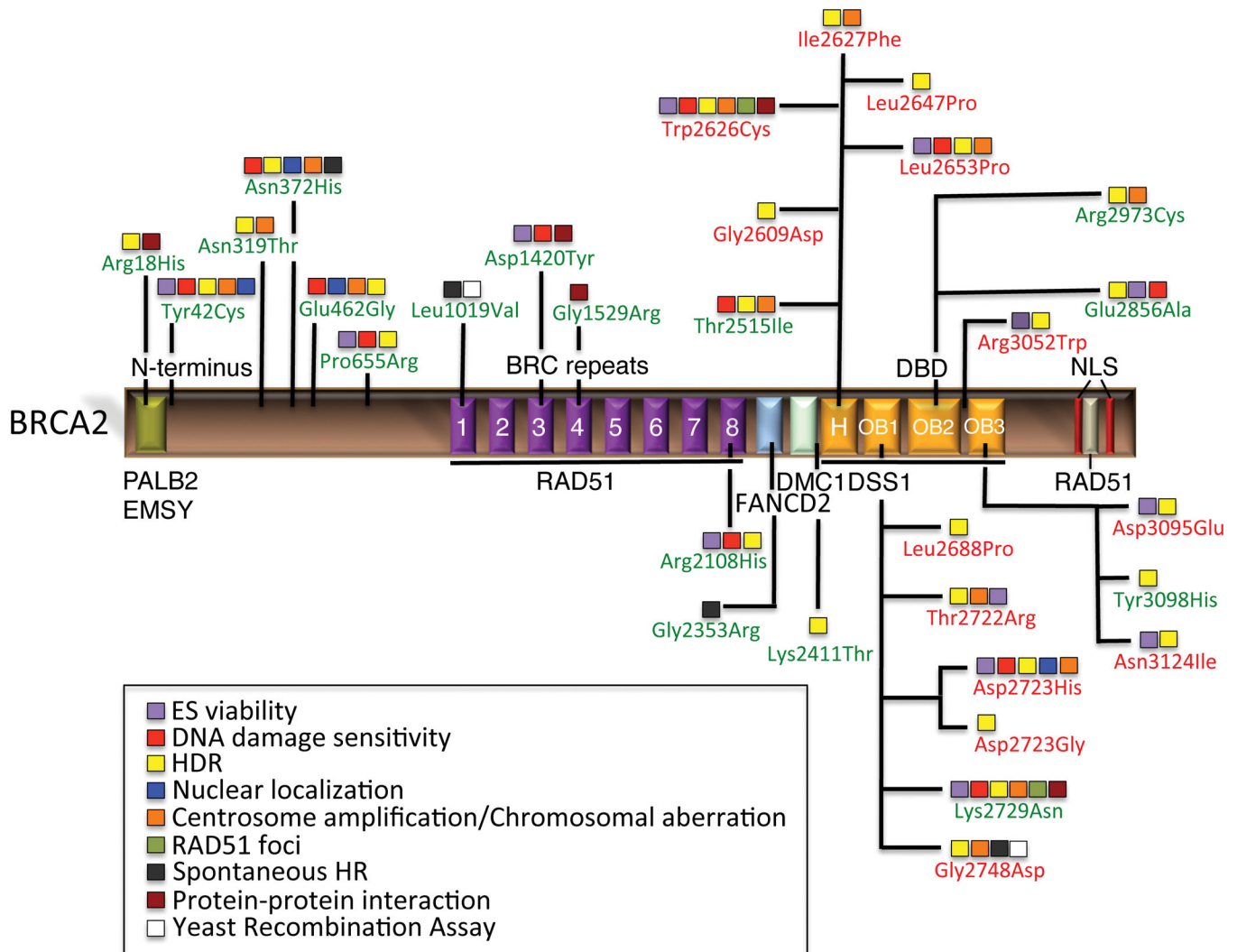


Figure 1.

Landscape of classified *BRCA2* variants evaluated by functional assays. Schematic representation of *BRCA2* protein and functional domains showing the approximate location of Class 1, 4 and 5 missense variants that have been analyzed in one or more functional assays and classified by the posterior probability model (Plon et al., 2008; Lindor et al., 2012). Class 1 or neutral variants are shown in green; Class 4/5 or pathogenic and likely pathogenic mutations are shown in red. H represents the helical domain; OB represents the oligonucleotide/oligosaccharide-binding fold; NLS depicts the nuclear localization signals.

Table 1

Validation panel

HGVs: protein level	HGVs: DNA level [#]	BIC: DNA level [#]	Functional domain/interacting proteins [‡]	IARC Class [*]	References
p.Arg18His	c.53G>A	281G>A	PALB2 interaction	1	Spurdle et al., 2008
p.Tyr42Cys	c.125A>G	353A>G	PALB2 interaction	1	<i>b</i> Turner et al., 1999; Spitzer et al., 2000; <i>b</i> Edwards SM et al., 2003; <i>b</i> Bergthorsson et al., 2001; Goldgar et al., 2004; Wu et al., 2005; Salazar et al., 2006; <i>‡</i> Chenevix-Trench et al., 2006; Farrugia et al., 2008; Gomez-Garcia et al., 2009; <i>‡</i> Spearman et al., 2008; Lindor et al., 2012
p.Asn56Thr	c.167A>C	395A>C	/	1	Easton et al., 2007; Lindor et al., 2012
p.Ala75Pro	c.223G>C	451G>C	/	1	Diez et al., 2003; Easton et al., 2007; Lindor et al., 2012
p.Pro168Thr	c.502C>A	730C>A	/	1	Easton et al., 2007; Lindor et al., 2012
p.Asn319Thr	c.956A>C	1184A>C	CEP55-TSG101 and CEP55-ALIX interaction	1	Farrugia et al., 2008; Lindor et al., 2012
p.Ser326Arg	c.978C>A	1206C>A	CEP55-TSG101 and CEP55-ALIX interaction	1	<i>‡</i> Spearman et al., 2008;; Edwards SM et al., 2003; Lindor et al., 2012
p.Asn372His	c.1114C>A	1342C>A	CEP55-TSG101 and CEP55-ALIX interaction	1	<i>b</i> Healey et al., 2000; <i>b</i> Edwards SM et al., 2003; <i>b</i> Hadjisavvas A et al., 2003; Diez et al., 2003; Wu et al., 2005; Johnson et al., 2007; Farrugia et al., 2008; Lindor et al., 2012
p.Pro375Ser	c.1123C>T	1351C>T	CEP55-TSG101 and CEP55-ALIX interaction	1	Easton et al., 2007; Lindor et al., 2012
p.Ser384Phe	c.1151C>T	1379C>T	CEP55-TSG101 and CEP55-ALIX interaction	1	Diez et al., 2003; Wappenschmidt et al., 2005; <i>‡</i> Chenevix-Trench et al., 2006; Salazar et al., 2006; <i>‡</i> Spearman et al., 2008; Lindor et al., 2012
p.Glu462Gly	c.1385A>G	1613A>G	CEP55-TSG101 and CEP55-ALIX interaction	1	Wu et al 2005; Easton et al., 2007; Farrugia et al., 2008; Mohammadi et al., 2009; Gomez-Garcia et al., 2009; Lindor et al., 2012
p.Lys513Arg	c.1538A>G	1766A>G	CEP55-TSG101 and CEP55-ALIX interaction	1	Easton et al., 2007; Spurdle et al., 2008; Lindor et al., 2012
p.Cys554Trp	c.1662T>G	1890T>G	CEP55-TSG101 and CEP55-ALIX interaction	1	Easton et al., 2007; Lindor et al., 2012
p.Thr582Pro	c.1744A>C	1972A>C	CEP55-TSG101 and CEP55-ALIX interaction	1	Wagner et al., 1999; Easton et al., 2007; Lindor et al., 2012
p.Gly602Arg	c.1804G>A	2032G>A	/	1	Easton et al., (2007); Lindor et al., 2012
p.Thr630Ile	c.1889C>T	2117C>T	/	1	Easton et al., (2007); Lindor et al., 2012
p.Pro655Arg	c.1964C>G	2192C>G	/	1	Goldgar et al., 2004; <i>‡</i> Spearman et al., 2008; Lindor et al., 2012
p.Asp806His	c.2416G>C	2644G>C	/	1	Easton et al., 2007; Lindor et al., 2012
p.Val894Ile	c.2680G>A	2908G>A	/	1	Easton et al., 2007; Lindor et al., 2012
p.Leu929Ser	c.2786T>C	3014T>C	/	1	<i>‡</i> Spearman et al., 2008; Lindor et al., 2012

HGVs: protein level	HGVs: DNA level#	BIC: DNA level#	Functional domain/interacting proteins#	IARC Class*	References
p.Asp935Asn	c.2803G>A	3031G>A	/	1	Wagner et al., 1999; Edwards et al., 2003; Salazar et al., 2006; Spurdle et al., 2008; Lindor et al., 2012
p.Asn987Ile	c.2960A>T	3188A>T	/	1	‡Spearman et al., 2008; Lindor et al., 2012
p.Leu1019Val	c.3055C>G	3283C>G	BRC1	1	Edwards et al., 2003; Easton et al., 2007; †Balia et al., 2011; Lindor et al., 2012
p.Asn1102Tyr	c.3304A>T	3532A>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Ser1172Leu	c.3515C>T	3743C>T	/	1	Easton et al., 2007; Spurdle et al., 2008; ‡Spearman et al., 2008; Lindor et al., 2012
p.Arg1190Trp	c.3568C>T	3796C>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Gly1194Asp	c.3581G>A	3809G>A	/	1	Easton et al., 2007; Lindor et al., 2012
p.Asn1228Asp	c.3682A>G	3910A>G	BRC2	1	Easton et al., 2007; Lindor et al., 2012
p.Cys1265Ser	c.3793T>A	4021T>A	/	1	‡Chenevix-Trench et al., 2006; Lindor et al., 2012
p.Asp1280Val	c.3839A>T	4067A>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Val1306Ile	c.3916G>A	4144G>A	/	1	Easton et al., 2007; Lindor et al., 2012
p.Ile1349Thr	c.4046T>C	4274T>C	/	1	Easton et al., 2007; Lindor et al., 2012
p.Thr1354Met	c.4061C>T	4289C>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Cys1365Tyr	c.4094G>A	4322G>A	/	1	Easton et al., 2007; Lindor et al., 2012
p.Gln1396Arg	c.4187A>G	4415A>G	/	1	Easton et al., 2007; Lindor et al., 2012
p.Asp1420Tyr	c.4258G>T	4486G>T	BRC3	1	Wagner et al., 1999; Spitzer et al., 2000; Edwards SM et al., 2003; †Scott et al., 2003; Deffenbaugh et al., 2004; ‡Spearman et al., 2008; Lindor et al., 2012
p.Phe1524Val	c.4570T>G	4798T>G	BRC4	1	Easton et al., 2007; Lindor et al., 2012
p.Gly1529Arg	c.4585G>A	4813G>A	BRC4	1	Easton et al., 2007; Lindor et al., 2012
p.Lys1690Asn	c.5070A>C	5298A>C	BRC5	1	Easton et al., 2007; Lindor et al., 2012
p.Ser1733Phe	c.5198C>T	5426C>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Gly1771Asp	c.5312G>A	5540G>A	/	1	Wagner et al., 1999; Edwards SM et al., 2003; Easton et al., 2007; Lindor et al., 2012
p.Pro1819Ser	c.5455C>T	5683C>T	/	1	Easton et al., 2007; ‡Spearman et al., 2008; Lindor et al., 2012
p.Leu1904Val	c.5710C>G	5938C>G	/	1	Easton et al., 2007; ‡Spearman et al., 2008; Lindor et al., 2012
p.Thr1915Met	c.5744C>T	5972C>T	/	NA	Wagner et al. (1999); Spitzer et al. (2000); Diez et al. (2003); Johnson et al. (2007)

HGVS: protein level	HGVS: DNA level#	BIC: DNA level#	Functional domain/interacting proteins#	IARC Class*	References
p.His1918Tyr	c.5752C>T	5980C>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Leu1929Val	c.5785A>G	6013A>G	/	1	Easton et al., 2007; Lindor et al., 2012
p.Arg2034Cys	c.6100C>T	6328C>T	/	1	Edwards SM et al., 2003; Diez et al., 2003; Deffenbaugh et al., 2004; Spearman et al., 2008; Caputo et al., 2011; Salazar et al., 2006; Johnson et al., 2007; Lindor et al., 2012
p.Asn2048Ile	c.6143A>T	6371A>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.His2074Asn	c.6220C>A	6448C>A	BRC8	1	Wagner et al., 1999; Easton et al., 2007; Lindor et al., 2012
p.Arg2108His	c.6323G>A	6551G>A	/	1	Diez et al., 2003; Easton et al., 2007; Lindor et al., 2012
p.Asn2113Ser	c.6338A>G	6566A>G	/	1	Easton et al., 2007; Lindor et al., 2012
p.Thr2250Ala	c.6748A>G	6976A>G	/	1	Easton et al., 2007; Lindor et al., 2012
p.Ile2285Val	c.6853A>G	7081A>G	/	1	Easton et al., 2007; Spearman et al., 2008; Lindor et al., 2012
p.Asp2312Val	c.6935A>T	7163A>T	/	1	Easton et al., 2007; Lindor et al., 2012
p.Gly2353Arg	c.7057G>C	7285G>C	FANCD2 interaction	1	Ballia et al., 2011; Lindor et al., 2012
p.Gln2384Lys	c.7150C>A	7378C>A	FANCD2 interaction	1	Easton et al., 2007; Lindor et al., 2012
p.Leu2396Phe	c.7188G>T	7416G>T	FANCD2 interaction	1	Easton et al., 2007; Lindor et al., 2012
p.Lys2411Thr	c.7232A>C	7460A>C	FANCD2 interaction	1	Easton et al., 2007; Lindor et al., 2012
p.Asn2436Ile	c.7307A>T	7535A>T	FANCD2 interaction	1	Easton et al., 2007; Lindor et al., 2012
p.Ala2466Val	c.7397C>T	7625C>T	FANCD2 interaction	NA	Wagner et al., 1999; Freedman et al., 2004
p.Lys2472Thr	c.7415A>C	7643A>C	HD/FANCD2 interaction	1	Spurdle et al., 2008; Lindor et al., 2012
p.Thr2515Ile	c.7544C>T	7772C>T	HD	1	Wagner et al., 1999; Diez et al., 2003; Campos et al., 2003; Wu et al 2005; Spurdle et al., 2008; Lindor et al., 2012
p.Gly2609Asp	c.7826G>A	8054G>A	HD	4	Lindor et al., 2012; Guidugli et al., 2013
p.Trp2626Cys	c.7878G>C	8106G>C	HD	5	Easton et al., 2007; Lindor et al., 2012
p.Ile2627Phe	c.7879A>T	8107A>T	HD	5	Spitzer et al., 2000; Easton et al., 2007; Farrugia et al 2008 Lindor et al., 2012
p.Leu2647Pro	c.7940T>C	8168T>C	HD	4	Farrugia et al., 2008; Lindor et al., 2012
p.Leu2653Pro	c.7958T>C	8186T>C	HD	5	Easton et al., 2007; Farrugia et al., 2008; Lindor et al., 2012
p.Leu2688Pro	c.8063T>C	8291T>C	OBI	4	Guidugli et al., 2013; Lindor et al., 2012
p.Ala2717Ser	c.8149G>T	8377G>T	OBI	1	Edwards SM et al., 2003; Diez et al., 2003; Salazar et al., 2006; Spurdle et al., 2008; Lindor et al., 2012

HGVS: protein level	HGVS: DNA level [#]	BIC: DNA level [#]	Functional domain/interacting proteins [‡]	IARC Class [*]	References
p.Thr2722Arg	c.8165C>G	8393C>G	OBI	5	Easton et al., 2007; Farrugia et al 2008; Lindor et al., 2012
p.Asp2723His	c.8167G>C	8395G>C	OBI	5	Goldgar et al. 2004; Wu et al 2005; [‡] Chenevix-Trench et al, 2006; Farrugia et al., 2008; Lindor et al., 2012
^a p.Asp2723G	c.8168A>G	8396A>G	OBI	5	Easton et al., 2007; Farrugia et al 2008; Walker et al., 2010; Lindor et al., 2012
p.Lys2729Asn	c.8187G>T	8415G>T	OBI	1	Easton et al., 2007; Farrugia et al 2008; Lindor et al., 2012
p.Gly2748Asp	c.8243G>A	8471G>A	OBI	5	Easton et al., 2007; Farrugia et al 2008; Lindor et al., 2012
p.Arg2842His	c.8525G>A	8753G>A	Tα	1	Easton et al., 2007; Lindor et al., 2012
p.Glu2856Ala	c.8567A>C	8795A>C	Tα	1	Spitzer et al., 2000; Edwards SM et al., 2003; [‡] Chenevix-Trench et al., 2006; Johnson et al., 2007; [‡] Spearman et al., 2008; Lindor et al., 2012
p.Arg2888Cys	c.8662C>T	8890C>T	Tα	1	Easton et al., 2007; Gomez-Garcia et al., 2009; Lindor et al., 2012
p.Ala2951Thr	c.8851G>A	9079G>A	Tα	NA	Wagner et al., 1999; Spitzer et al., 2000; Diez et al., 2003; Deffenbaugh et al., 2004; Bergthorsson et al. 2001; ^b Hammet et al., 2007
p.Val2969Met	c.8905G>A	9133G>A	OB2	1	Wagner et al., 1999; Easton et al., 2007; Lindor et al., 2012
p.Arg2973Cys	c.8917C>T	9145C>T	OB2	1	Easton et al., 2007; Farrugia et al 2008; [‡] Spearman et al., 2008; Lindor et al., 2012
p.Arg3052Trp	c.9154C>T	9382C>T	OB2	5	Farrugia et al 2008; Gomez-Garcia et al., 2009; Mohammadi et al., 2009; Walker et al., 2010; Lindor et al., 2012
p.Val3079Ile	c.9235G>A	9463G>A	OB3	1	Easton et al., 2007; Lindor et al., 2012
p.Asp3095Glu	c.9285C>G	9513C>G	OB3	4	Easton et al., 2007; Farrugia et al 2008; Lindor et al., 2012
p.Tyr3098His	c.9292T>C	9520T>C	OB3	1	Edwards SM et al., 2003; Easton et al., 2007; Lindor et al., 2012
p.Asn3124Ile	c.9371A>T	9599A>T	OB3	4	Lindor et al., 2012; Guidugli et al., 2013
p.Asp3170Gly	c.9509A>G	9737A>G	OB3	1	Easton et al., 2007; Lindor et al., 2012
p.Cys3198Arg	c.9592T>C	9820T>C	/	1	Easton et al., 2007; Lindor et al., 2012
p.Thr3349Ala	c.10045A>G	10273A>G	/	1	Diez et al., 2003; Easton et al., 2007; Lindor et al., 2012

GenBank Reference *BRCA2* NM_000059.3

NA not applicable because the variant is a well-defined polymorphism* 1=Class 1 Not Pathogenic or “of No Clinical Significance (posterior probability<0.001); 2=Class 2 Likely Not Pathogenic or “of Little Clinical Significance” (0.001<posterior probability<0.049).4=Class 4 Likely Pathogenic (0.95<posterior probability<0.99); 5=Class 5 Definitely Pathogenic (posterior probability >0.99).

^a Asn2723Gly variant was shown to cause aberrant splicing (deletion of 163 bp from exon 18) of a small proportion of the mutant allele (Walker LC et al., 2010)

^b Report suggesting a classification of variants that is divergent from the classification based on the multifactorial approach.

Bold=classified Class 4/5 mutations.

/ no functional domains identified

✗ LOH is improperly utilized as a measure of association of the variant with the disease.

Nucleotide numbering in “HGVS: DNA level” reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, (<http://www.hgvs.org/mutomen>).

Nucleotide numbering in “BJC: DNA level” refers to the original nomenclature for *BRCA1* and *BRCA2* before adoption of HGVS standards where *BRCA1* +119 and *BRCA2* +228 correspond to the A of the ATG translation codon in the reference sequences.

List of publications that have defined the pathological importance of the variants through the multifactorial and/or the posterior probability methods or through other genetic approaches including the frequency in cases and controls.

✗ **BRC**=interspersed BRC repeats of about 35 residues in the central region of BRCA2; **HD**=helical domain within the DNA binding domain in the C-terminal region of BRCA2 (DBD); **OB**= oligonucleotide-binding folds within the DBD; **Tα**= tower domain which is an insertion within OB2.

Table 2

Analysis of VUS; Results from functional assays

HDR assay [‡]		Embryonic stem cell (ESC)-based survival assay [#]							Centrosome-amplification assay [*]		Spontaneous HR assay ^{**}		Yeast recombination assay ^{***}		MMC survival assay [±]		SyVal model ^{††}		
		Viability	DNA-damaging agents	HDR	RAD51 foci	Chromosomal aberration	+	-											
+	p.Pro9Leu	+	p.Lys2729Asn	+	p.Gly25Arg	+/-	/	+	p.Tyr42Cys	+	p.Gly173Val	+	p.Ser286Pro	+	p.Tyr42Cys	+	p.Tyr3308Tyr	+	+
+	p.Phe12Val	-	p.Gly2748Asp	-	p.Trp31Arg [#]	NA	NA	NA	p.Asn319Thr	+	p.Asp191Val	-	p.Met927Val	+	p.Asn372His	+	p.Pro3292Leu	+	+
+	p.Arg18His	-	p.Arg2784Gln	-	p.Trp31Cys	NA	NA	NA	p.Asn372His	+	p.Ser286Pro	+	p.Thr1011Arg	-	p.Glu462Gly	+	p.Pro3280His	+	+
-	p.Gly25Arg	-	p.Arg2784Trp	-	p.Tyr42Cys	/	/	/	p.Glu462Gly	+	p.Asn372His	+	p.Leu1019Val	+	p.Thr1302del	-	p.Ser3291Glu	-	-
+	p.Ile27Val	+	p.Arg2787His	+	p.Pro655Arg	+	/	/	p.Thr1302del	-	p.Met927Val	+	p.Asn1878Lys	+	p.Glu1382del	-	p.Ser3291Ala	+	+
-	p.Trp31Arg	-	p.Leu2792Pro	-	p.Asn991Asp	/	/	/	p.Glu1382del	+	p.Thr1011Arg	+	p.Thr1915Met	+	p.Thr2515Ile	-			
-	p.Trp31Cys	-	p.Gly2793Glu	-	p.Asp1420Tyr	/	/	/	p.Thr2515Ile	+/-	p.Leu1019Val	+	p.Ser2006Arg	-	p.Asp2723His	-			
+	p.Tyr42Cys	+	p.Gly2793Arg	-	p.Arg2108His	+	/	/	p.Arg2520Gln	+	p.Asn1878Lys	-	p.Arg2108Cys	+	p.Val2908Gly	+			
+	p.Asn319Thr	+	p.Pro2800Ser	+/-	p.Phe2406Leu	+	/	/	p.Ile2627Phe	-	p.Met1915Thr	+	p.Val3091Ile	+					
+	p.Asn372His	+	p.Pro2800Arg	+/-	p.Ile2490Thr	+	+	+	p.Ala2643Gly	+	p.Ser2006Arg	-	p.Gly2748Asp	-					
+	p.Glu462Gly	+	p.Ser2807Leu	+	p.Leu2510Pro	+/-	-	-	p.Thr2722Arg	-	p.Arg2108Cys	-	p.Ala2951Thr	+					
+	p.Thr1302del	+	p.Gly2812Glu	+/-	p.Trp2626Cys	+/-	-	-	p.Lys2729Asn	+	p.Gly2353Arg	-							
-	p.Glu1382del	-	p.Gly2813Glu	+	p.Leu2653Pro	+/-	/	/	p.Gly2748Asp	-	p.Gly2748Asp	-							
+	p.Lys2411Thr	+	p.Arg2842Leu	+/-	p.Ser2695Leu	+	/	/	p.Arg2784Trp	+	p.Ala2951Thr	+							
+	p.Ser2414Leu	+	p.Arg2842Cys	-	p.Thr2722Arg	-	NA	NA	p.Leu2865Val	+	p.Val3091Ile	-							
-	p.Leu2510Pro	-	p.Glu2856Ala	+	p.Asp2723His	-	NA	NA	p.Val2908Gly	+									
+/-	p.Thr2515Ile	+/-	p.Leu2865Val	+	p.Lys2729Asn	+	+	+	p.Arg2973Cys	+									
+	p.Arg2520Gln	+	p.Gly2901Asp	+	p.Val2728Ile	+	/	/											
-	p.Gly2585Arg	-	p.Val2908Gly	+	p.Glu2856Ala	+	/	/											
+	p.Pro2589His	+	p.Ala2951Thr	+	p.Gly2901Asp	+	/	/											
-	p.Gly2609Asp	-	p.Asp2965His	+	p.Ile2944Phe	+	+	/											
-	p.Trp2626Cys	-	p.Arg2973Cys	+	p.Ser2988Gly	+	/	/											
-	p.Ile2627Phe	-	p.Glu3002Lys	-	p.Glu3002Lys	-	NA	NA											
+	p.Ala2643Gly	+	p.Glu3002Asp	+/-	p.Arg3052Trp	-	NA	NA											

HDR assay [‡]	Embryonic stem cell (ESC)-based survival assay [#]					Centrosome-amplification assay [*]	Spontaneous HR assay ^{***}	Yeast recombination assay ^{****}	MMC survival assay [±]	SyVal model [†]		
	Viability	DNA-damaging agents	HDR	RAD51 foci	Chromosomal aberration					RAD51 foci	DNA-damaging agents	
p-Leu2647Pro	-	p-Thr3013Ile	+	p-Arg3052Gln	+/–	/	/	/				
p-Leu2653Pro	-	p-Ser2030Cys	+	p-Asp3095Glu	NA	NA	NA	NA				
p-Leu2654Pro	-	p-Thr3033Ile	+/-	p-Asn3124Ile	NA	NA	NA	NA				
p-Arg2659Gly	-	p-Arg3052Trp	-									
p-Tyr2660Asp	-	p-Pro3063Ser	+									
p-Asp2665Gly	+	p-Asp3073Gly	-									
p-Met2676Thr	+	p-Gly3076Val	-									
p-Asp2679Gly	+	p-Gly3076Glu	-									
p-Asp2679Tyr	+	p-Tyr3092Cys	+									
p-Leu2688Pro	-	p-Tyr3092Ser	+/-									
p-Thr2722Arg	-	p-Asp3095Glu	-									
p-Asp2723Ala	-	p-Tyr3098His	+									
p-Asp2723Gly	-	p-Asn3124Ile	-									
p-Asp2723His	-											
p-Tyr2726Cys	-											

[‡] Wu K. et al., 2005; Xia et al., 2006; Farrugia DJ. et al., 2008; Guidugli L. et al., 2013.

^{*} Wu K. et al., 2005; Farrugia DJ. et al., 2008

[#] Kuznetsov et al., 2008; Biswas et al., 2011; Biswas et al. et al., 2012 (this assay is also applicable for the analysis of intronic variants but these are not listed here).

^{**} Balia et al., 2011

^{***} Spugnese et al 2013

[±] Wu K. et al., 2005

[†] This missense change causes skipping of the exon 3 of BRCA2 that in turn generates an in-frame deletion of 83 amino acids essential for BRCA2 interaction with PALB2 (Biswas et al., 2012; Xia et al., 2006). Loss of exon 3 has been associated with increased breast/ovarian cancer (Nordling et al., 1998).

[‡] Hucl T. et al., 2008

NA=not applicable; / =not done; (+) denotes the missense variant has no effect on BRCA2 function; (-) denotes the missense variant disrupts BRCA2 function.

Italics=variants classified as Class 1 with the posterior probability method (Plon et al., 2008) or well-defined polymorphisms

Bold=variants classified as Class 4/5 with the posterior probability method (Plon et al., 2008)