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Functional categorization of *BRCA1* variants of uncertain clinical significance in homologous recombination repair complementation assays

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Statement of Translational Relevance

Germline loss of function (LoF) variants in *BRCA1* (MIM 113705) strongly predispose to hereditary breast and ovarian cancer (HBOC). Therefore, sequence analysis of *BRCA1* is routinely conducted in counselees from families with early-onset breast- and/or ovarian cancer. Besides variants that clearly abolish protein function and are known to increase cancer risk, a large number of variants of uncertain significance (VUS) have been identified for which the associated cancer risk is unknown. Information on functional consequences of these *BRCA1* variants is important, because it will (i) aid genetic counseling, as variants that lead to a functional defect are likely to confer high cancer risk, and (ii) guide the choice for optimal treatment strategies for cancer patients carrying LoF variants, because *BRCA1* deficiency renders tumors hypersensitive to platinum drugs and PARP inhibitors.

Abstract

Purpose: Because *BRCA1* is a high-risk breast/ovarian cancer susceptibility gene, *BRCA1* sequence variants of uncertain clinical significance (VUS) complicate genetic counseling. As most VUS are rare, reliable classification based on clinical and genetic data is often impossible. However, all pathogenic *BRCA1* variants analyzed result in defective homologous recombination DNA repair (HRR). Thus, *BRCA1* VUS may be categorized based on their functional impact on this pathway.

Experimental Design: 238 *BRCA1* VUS - comprising most *BRCA1* VUS known in the Netherlands and Belgium - were tested for their ability to complement *Brca1* deficient mouse ES cells in HRR, using cisplatin and olaparib sensitivity assays and a DR-GFP HRR assay. Assays were validated using 25 known benign and 25 known pathogenic *BRCA1* variants. For assessment of pathogenicity by a multifactorial likelihood analysis method, we collected clinical and genetic data for functionally deleterious VUS and VUS occurring in three or more families.

Results: All three assays showed 100% sensitivity and specificity (95% CI = 83%-100%). Out of 238 VUS, 45 showed functional defects, 26 of which were deleterious in all three assays. For 13 of these 26 variants we could calculate the probability of pathogenicity using clinical and genetic data, resulting in the identification of seven (likely) pathogenic variants.

Conclusions: We have functionally categorized 238 *BRCA1* VUS using three different HRR-related assays. Classification based on clinical and genetic data alone for a subset of these variants confirmed the high sensitivity and specificity of our functional assays.

Introduction

A large fraction of all hereditary breast and ovarian cancers (HBOC) is caused by heterozygous pathogenic *BRCA1* variants (1), which greatly increase the risk of women to develop breast cancer or ovarian cancer at any point during their lives (2,3). Consequently, patients with suspected HBOC are generally tested for the presence of germline *BRCA1* variants. Besides known pathogenic and benign variants, this yields many variants for which the associated cancer risk is unknown (Variants of uncertain clinical significance (VUS)). This uncertainty complicates clinical management of variant carriers. Despite considerable international efforts to determine pathogenicity for these VUS using a multifactorial likelihood model (MLM) that combines clinical and genetic information (4), for most VUS this is not successful (5). In the absence of sufficient genetic information, assessment of pathogenicity is depending on *in silico* analyses and assays that measure effects on gene functions (6). *In silico* analyses assume that changes in evolutionary conserved amino acids are most likely to be pathogenic, especially when they result in pronounced alterations in the chemical characteristics of these amino acids (7). Although this is true in general, prediction algorithms are inferior to well validated functional assays (8,9). Especially for *BRCA1* and *BRCA2* there are now several functional assays with high sensitivity and specificity that allow accurate assessment of the effects of VUS on gene function (10–13). Many of these assays measure effects of variants on homologous recombination repair (HRR) of DNA double strand breaks, which is thought to be the main tumor suppressor activity of *BRCA1* and *BRCA2*. In fact, all known pathogenic variants of *BRCA1* and *BRCA2* analyzed lead to HRR deficiency. However, when functional assays are well validated also less direct readouts such as effects on proliferation and transcription activation may be used (14,15).

We have previously developed an assay system for the functional categorization of *BRCA1* VUS (10). The assay system is based on complementation of *Brca1* deficient mouse embryonic stem (ES) cells by human *BRCA1* cDNA variants. Using this assay system, we have now functionally categorized 238 *BRCA1* VUS, including all 232 *BRCA1* missense and in-frame deletion VUS reported by the clinical

genetic laboratories in the Netherlands and Belgium up to 2015. Specifically, we measured the effects of *BRCA1* VUS and a validation set of 25 known benign and 25 known pathogenic *BRCA1* variants on HRR using the HRR-deficiency targeted drugs cisplatin and olaparib and the DR-GFP HRR reporter (16). To investigate if these assays are able to detect deleterious VUS outside the RING and BRCT domains, we included three variants in the coiled-coil domain that are known to disrupt the interaction with PALB2 and attenuate HRR. As functional data alone are currently considered to be insufficient for VUS classification, we have also collected and analyzed available genetic and clinical data for deleterious VUS or VUS occurring in three or more Dutch or Belgian families.

Materials and Methods

BRCA1 VUS selection

In 2015 we used the LOVD *BRCA1* database ((https://databases.lovd.nl/shared/genes/BRCA1) to select all 232 *BRCA1* missense and in-frame deletion VUS identified in counselees and reported by the clinical genetic laboratories in the Netherlands and Belgium for analysis in our functional assays. In addition, we selected VUS c.4198A>G, c.4220T>C, c.4232T>C, c.4952C>T, c.5359T>A and c.5363G>A. In silico splice site prediction analysis was performed for all variants using Alamut Interactive Biosoftware (https://www.interactive-biosoftware.com/alamut-visual/).

Generation of human *BRCA1* knockin embryonic stem cells

Using Quick-Change Lightning site-directed mutagenesis (Stratagene), tandem AarI restriction sites were introduced to allow excision and replacement of eight consecutive segments of the human *BRCA1* RMCE (Recombinase-Mediated Cassette Exchange) vector (10). Of note, the *BRCA1* cDNA sequence of this RMCE vector contains the SNPs c.2082C>T p.(Ser694Ser), c.3113A>G p.(Glu1038Gly), c.4308T>C p.(Ser1436Ser), and c.4837A>G p.(Ser1613Gly), which are known to be benign and which are frequently observed in the population (https://brcaexchange.org). Excised segments were replaced by synthetic gBlocks (IDT) containing *BRCA1* variants using Gibson Assembly (17). Mutations and assembly boundaries were sequence verified and introduction of human *BRCA1* cDNAs via RMCE was conducted by cotransfection of $R26^{CreERT2/RMCE}$;$Brca1^{SCo/\Delta}$;$Pim1^{DR\text{-}GFP/wt}$ ES cells with RMCE vectors and pCAGGs-Flpo-IRES-puro using Lipofectamine 2000 (Invitrogen) as described (10). Cells that had successfully undergone RMCE were selected for 11 days using 200–800 µg/mL G418 and cryopreserved. Correct RMCE was confirmed by quantitative PCR (see Supplementary Materials and Methods) and expression of human BRCA1 was analyzed by Western blotting using a polyclonal antibody against human BRCA1 (9010; Cell Signaling Technology) as described (10). The ES

cells used in this study tested negative for *Mycoplasma*, as determined by the MycoAlert PLUS Mycoplasma Detection Kit (Lonza).

Functional assays

For functional assays, cells were thawed and cultured in batches with 17-21 VUS pools, with three independently transfected human *BRCA1* wild-type and empty RMCE vector control pools per batch. After ten days, mouse *Brca1* was switched off using one day (at least 24 hours) 0.5 µM 4-OHT incubation. Subsequently, cells were cultured for three days in 50% CM/2i-medium (10,18) (see Supplementary Materials and Methods) and seeded for cytotoxicity or DR-GFP HRR assays. Three-day cisplatin and olaparib sensitivity assays were performed as described (10), with some modifications: cells were seeded in triplicate at 1500 cells per well in 96-well plates and cultured in 50% CM/2i-medium. For DR-GFP HRR assays, 500000 cells were seeded in 12-well plates for transfections with I-SceI-mCherry plasmid (10). Two days after transfection, mCherry/GFP double-positive cells were monitored by flow cytometry on a CyAn (Beckman Coulter) or LSRFortessa (BD Biosciences) cell analyzer. Data were analyzed using FlowJo software, including correction for flow aberrations using FlowAI (FlowJo LLC, BD Biosciences). Experiments were repeated twice.

Functional data analysis and statistics

For the analysis of cytotoxicity data, the raw fluorescence intensities of the cisplatin or olaparib treated wells were converted per plate to a cell viability value; with 0 being the mean of the empty wells and 1 being the mean of the no-drug wells. IC_{50} values were calculated by taking the tested drug concentrations around the IC_{50} and calculating the IC_{50} from these points in log-space, since the dose-response curve is estimated to be linear in log-space around the IC_{50}. Samples that had a non-

monotonous curve course were excluded. In the DR-GFP assays, HRR activity was measured as the percentage of GFP positive transfected cells.

For each series of experiments, data were normalized to three independently transfected wild-type human *BRCA1* (WT) and empty RMCE vector (EV) controls. All samples and controls were tested in biological triplicates. Gaussian distributions were estimated for both the EV and WT controls. These two distributions were used to calculate p, the posterior probability for a variant to be deleterious (19) based on its mean normalized IC_{50} or GFP percentage over the three biological replicates in the assay. For olaparib, the natural logarithm of the mean normalized IC_{50} + 0.05 was used to estimate the distributions and to calculate p values. The prior probability of being deleterious was assumed to be equal (uninformative prior). The VUS were categorized as follows: p>0.99: deleterious, 0.95<p<=0.99: likely deleterious, 0.05<p<=0.95: intermediate, 0.01<p<=0.05 = likely neutral, and p<=0.01 = neutral. Categorizations were considered uncertain in case the average values +/- the standard deviation over the biological repeats resulted in opposite outcomes (i.e.: (likely) deleterious vs (likely) benign). To establish the sensitivity and specificity of our assays, we included 25 known pathogenic and 25 known neutral variants (20).

Multifactorial likelihood analysis

To increase the chance of an informative variant classification within the timeframe of our study, we selected 43 VUS that had been observed in at least three families and/or shown to impair protein function in the functional assays. Clinical and genetic data was collected as described (5). Information for co-segregation analysis was provided in the form of a de-identified pedigree for families with known carrier status in more than one individual. Pedigree details included gender, breast/ovarian cancer status and age at cancer diagnosis, or age at interview if unaffected. Unaffected individuals known to have undergone prophylactic surgery (mastectomy or oophorectomy) were censored at age of earliest surgery. Breast tumor pathology information

collected for known variant carriers included hormone receptor status (estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2)), and/or tumor grade.

LR of pathogenicity were established on the basis of co-segregation of the variant with breast cancer in families of mutation carriers (21); and breast tumor data (age of diagnosis, grade, hormone receptor status) (22). Prior probabilities of pathogenicity were derived from Align GVGD (7) and MaxEntScan splicing predictions (23) and are available on http://priors.hci.utah.edu/PRIORS. To estimate prior probabilities for in-frame deletion variants, we took the highest prior of the deleted bases (5).

Table S4 summarizes the LRs assigned for each component for each variant with at least one data point. Prior probabilities and LRs were combined to calculate posterior probabilities: (Prior Probability x Combined LR)/(Prior Probability x [Combined LR + (1- Prior Probability)]). Where multiple data points were available for a single data type e.g. co-segregation, pathology, LRs were combined multiplicatively. VUS were classified based on the 5-tier classification system introduced by an International Agency for Research on Cancer (IARC) Working Group (24).

Web Resources

Align GVGD, http://priors.hci.utah.edu/PRIORS/

BRCA1 Circos, https://research.nhgri.nih.gov/bic/circos/

BRCA Exchange, https://brcaexchange.org

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

ENIGMA Consortium, https://enigmaconsortium.org

LOVD *BRCA1* database, https://databases.lovd.nl/shared/genes/BRCA1

Results

Generation of human *BRCA1* complemented mouse *Brca1*^{SCo} embryonic stem cells

As the role of BRCA1 in HRR appears to be crucial for its tumor suppressor activity (10,20,25–29), we set out to functionally classify all known *BRCA1* missense and in-frame deletion VUS reported in the Netherlands and Belgium based on their effect on this DNA repair pathway in *Brca1* deficient mouse ES cells. For this purpose, we used cisplatin and olaparib sensitivity assays, as well as the DR-GFP reporter assay to measure HRR by gene conversion (16). In addition to the Dutch/Belgian VUS, we (re)analyzed three VUS, c.4198A>G p.(Met1400Val), c.4220T>C p.(Leu1407Pro) and c.4232T>C p.(Met1411Thr), that are known to attenuate the interaction of BRCA1 with PALB2 (10,30). To validate our assays, we included 25 known pathogenic and 25 known benign variants for which the clinical significance has previously been established on the basis of clinical and genetic data (Table S1) (reference 20 and the NIH ClinVar database). Because variant c.5359T>A was always observed together with variant c.5363G>A (likely *in cis*) when it was classified as pathogenic based on genetic data (20,24,31), we included the c.[5359T>A; 5363G>A] double mutation as a known pathogenic control.

Our previously described assay system (10) made use of site-directed mutagenesis (SDM) to introduce VUS in the *BRCA1* cDNA sequence of an RMCE vector. Although this method worked sufficiently well before, the large size of the *BRCA1* RMCE vector makes it relatively inefficient. In addition, when SDM is used the complete *BRCA1* cDNA needs to be sequence verified for absence of unwanted additional mutations. Therefore, we created modified *BRCA1* RMCE vectors in which we could easily replace part of the *BRCA1* cDNA sequence by a synthetic gene block containing a specific variant (Figure 1). We generated a library of *BRCA1* VUS and controls using Gibson assembly (17). As our previous categorizations were based on the cisplatin sensitivity assay only, we also included previously generated and analyzed VUS and controls (10) (Table S1, S2). The inserted gene blocks of all *BRCA1* RMCE vectors were sequence verified before transfection into *Brca1*^{SCo} ES cells containing

the DR-GFP reporter (10). Before performing functional analyses, pools of transfected cells were assayed by qPCR for RMCE efficiency, human BRCA1 protein expression was confirmed by western blotting and the presence of the correct *BRCA1* variant was verified using Sanger sequencing (Figure 1, S1A-B, and data not shown). In addition, we assayed all VUS with Alamut Interactive Biosoftware for potential effects on mRNA splicing, which cannot be evaluated with our cDNA approach. Seven missense VUS are predicted to affect consensus splice sites, which may be deleterious (Table S2): c.441G>C, c.670G>C, c.4096G>A, 4675G>A, c.5072C>T, c.5074G>A, and c.5074G>C. For five of these variants, there is also experimental evidence that they result in reduced or even abrogated levels of the normal *BRCA1* transcript (Table S2) (14,32–37). In addition, c.4675G>A and c.5074G>C have recently been classified as pathogenic by expert panel review (https://www.ncbi.nlm.nih.gov/clinvar/).

Cisplatin sensitivity assay

Confirming its specificity, all 25 known benign variants were correctly categorized as functionally neutral in the cisplatin assay (Figure 2A, Table S1). In addition, all 25 known pathogenic variants – including 15 variants we had not analyzed before – were categorized as deleterious. The results from the cisplatin sensitivity assay (Figure 2A, S2A) confirmed the functional categorization of previously analyzed VUS (Table S2) (10). However, technical optimizations now allowed the functional categorization of several VUS and controls that had previously remained uncategorized due to inconsistent data or technological flaws, including the well-known pathogenic truncating founder mutation 5382insC (c.5266dup). Of note, similar to previously published findings (38), variant c.5359T>A p. by itself only had an intermediate effect on cisplatin sensitivity (Table S2). The c.[5359T>A; 5363G>A] double mutation however was clearly deleterious (Table S1), in line with the assumption that c.5359T>A is only pathogenic *in cis* with c.5363G>A. In total, 27 VUS were deleterious in the cisplatin assay (Table 1, S2). For two additional VUS – c.5207T>C p.(Val1736Ala)

and c.5348T>C p.(Met1783Thr) – the mean cisplatin response suggested functional defects, but these variants could not be categorized as neutral or deleterious because of experimental variation (Figure S3A-D). Both p.(Val1736Ala) (10,39) and p.(Met1783Thr) (11,29) have previously been shown to confer partial defects in BRCA1 function.

Olaparib sensitivity assay

As we observed before (10), the differences in IC_{50} for the PARP1-inhibitor olaparib between neutral and deleterious variants were much more pronounced than for cisplatin (Figure 2B, S2B,C, Table S2). As the invariably low olaparib IC_{50} values for deleterious variants are accompanied by a large variation between repeat experiments for (partially) *BRCA1*-proficient variants, we used log-transformed olaparib IC_{50} values for functional categorization. This led to the identification of 33 clearly deleterious variants including seven variants (c.5074G>C, c.5207T>C, c.5238C>G, c.5348T>C, c.5363G>A, c.5419A>T and c.5512G>A) that were not identified as deleterious by the cisplatin assay. Fifteen variants, including c.134A>C which was deleterious in the cisplatin assay, showed intermediate or variable defects precluding categorization using the olaparib sensitivity assay (Figure S3E-H; Table 1, S2).

Thus, the olaparib assay showed functional impairment for both hypomorphic variants that could not be categorized in the cisplatin assay (c.5207T>C and c.5348T>C). In addition, all deleterious variants identified by the olaparib assay are present in the functionally important RING or BRCT domains. Together this suggests that the greater sensitivity of the olaparib assay does not come at the cost of reduced specificity. This is confirmed by the correct categorization of all known benign variants. Of note, also the c.5363G>A control for the known pathogenic c.[5359T>A; 5363G>A] double mutant showed loss of function in the olaparib assay.

HRR assay

It is known that the sensitivity of *Brca1* deficient mouse ES cells to cisplatin or PARPi is alleviated by restoration of HRR (10,40). Conversely, it seems likely that variants failing to complement cisplatin or olaparib sensitivity share a negative impact on HRR. We used the well-established DR-GFP reporter (10,16) to directly measure the effect of *BRCA1* variants on HRR. This reporter expresses GFP upon HRR of a DNA double strand break generated by the meganuclease I-SceI. Analysis by flow cytometry indeed showed that all variants identified as deleterious in the cisplatin or olaparib assays resulted in defective HRR (Table 1, S2, Figure 2C, S2D). In addition, there were a number of variants which only scored as (likely) deleterious in the DR-GFP assay: c.41T>C, c.4220T>C, c.4232T>C, c.5002T>C, c.5062G>C, c.5071A>G, c.5074G>A, c.5128G>A, c.5225A>G, c.5300G>C and c.5435C>G. Also c.134A>C, which was deleterious in the cisplatin assay but could not be categorized in the olaparib assay, scored as deleterious. Thus, the DR-GFP assay confirmed the deleterious nature of all variants with intermediate or variable defects in both drug sensitivity assays. Only two variants could not be categorized due to variable results (Figure S3I).

Comparison of the results from the three functional assays

In total we analyzed 238 *BRCA1* VUS and identified functional defects in 45 of them, 43 VUS in the RING and BRCT domains and two in the coiled-coil domain (Table 1, S2). With all 25 known pathogenic variants and all 25 known benign variants correctly categorized in the cisplatin, olaparib and DR-GFP HRR assays, each assay has a sensitivity and specificity of 100% (95% CI = 83%-100%). Although the results of our assays were highly concordant (Figure 3), a subset of variants was only scored as deleterious in the olaparib and/or DR-GFP assays (Figure 4, Table 1, S2). In most cases, these variants appear to be hypomorphic, conferring partial defects which were only significant in the HRR assay (Table 1, S2, Figure S4A). Interestingly, and in line with our previous observations (10), the coiled-coil variants c.4220T>C p.(Leu1407Pro) and c.4232T>C p.(Met1411Thr) only affect HRR

and have no significant effect on the (short-term) drug sensitivities of our complemented ES cells (Table 1, S2, Figure S4B). In contrast to deleterious RING or BRCT variants, both deleterious coiled-coil variants are also able to support proliferation when mouse *Brca1* is switched off (10), (Figure S5A). These data indicate that the proliferation defect and drug sensitivity of BRCA1 deficient mouse ES cells is not caused by a defect in the completion of HRR per se.

Comparison with other functional assays

Our functional assays are well validated by the correct functional categorization of the 25 known pathogenic and 25 known benign variants and measure effects on the clinically relevant BRCA1 function in HRR. In addition, RMCE constructs as well as transfected ES cells were sequence verified and assayed for expression of human BRCA1 protein (Figure SI). Nevertheless, the possibility of miscategorization can never be completely excluded, also because of the intrinsically artificial nature of any functional assay system. It is therefore useful to compare the results of different well-validated functional assays. The recent publication of a saturation mutagenesis screen of BRCA1 RING and BRCT single-nucleotide variants in haploid *LIG4*-knockout HAP1 chronic myeloid leukemia cells (14) offers an excellent opportunity for such comparison. This high-throughput mutagenesis screen makes use of CRISPR/Cas9 assisted targeting of endogenous *BRCA1* and is based on the finding that HAP1 cells do not tolerate *BRCA1* loss (41). The results of the HAP1 proliferation assay are highly concordant with those of our assays, with all overlapping 16 known pathogenic variants and 6 known benign variants categorized correctly (Table S3). Six known pathogenic variants and three VUS in the RING and BRCT encoding regions were not present in the HAP1 dataset. Most of the 64 shared VUS in the RING and BRCT encoding regions are categorized the same in all three of our assays and in the HAP1 screen. Ten discrepant cases may largely be attributed to a partial or intermediate loss of function that is only detected in the olaparib and/or DR-GFP assay(s) (Figure S4C, Table S3). For two of these VUS, c.5074G>A and c.5074G>C, also defective mRNA splicing (Table

S2) may contribute to their deleterious nature in HAP1 cells. Two other variants show opposite categorization in all three our assays compared to the HAP1 screen. Variant c.19C>T p.(Arg7Cys) appeared to have no effect on HAP1 proliferation, but was defective in all three of our assays. For c.5258G>C p.(Arg1753Thr) we noticed the opposite. Also in these cases it seems likely that the variants have intermediate defects, that may vary depending on cell type or assay specific factors. Of note, upon transient transfection of these VUS in our ES cell system DR-GFP HRR activity was only mildly affected by c.19C>T, while c.5258G>C lead to a strong HRR defect (Figure S5B). Cleary there is a discrepancy between the results with stably integrated *BRCA1* c.5258G>C versus transient expression, but sequence and Western blot analyses revealed no straightforward explanation (data not shown).

Interestingly, none of the 11 variants that were only identified as deleterious in the DR-GFP assay were picked up by the HAP1 proliferation screen. Of these, coiled-coil variants c.4220T>C and c.4232T>C were not included in the analysis. However, both variants supported mouse ES cell proliferation, suggesting that they would not have been deleterious in the HAP1 assay. Despite the well-described impact of coil-coil domain inactivating variants on HRR (25,30), it remains to be determined if variants that are only identified in our DR-GFP assay affect tumor suppression.

As observed before (10), in general the results of our assay system also correlate well with those of various other assays in which *BRCA1* germline variants are analyzed individually for functional effects (25,42,43). However, multiplexing as used in the HAP1 assay clearly allows higher throughput and avoids batch or experimenter dependent differences (14,26). In principle, there are no barriers for parallel RMCE transfections and multiplexed analysis of *BRCA1* variants in our assay system. To illustrate this, we performed parallel RMCE for 30 variants in the BRCA1 RING encoding region. Using high-throughput sequencing, we measured the relative abundance of individual *BRCA1* variants before and after switching off endogenous mouse *Brca1* and with or without adding olaparib. The results are highly concordant with those from the one-by-one analyses (Figure S6). Of note, variant

c.134A>C showed some functional activity in one out of three individual olaparib assays but was clearly deleterious in the pooled proliferation and olaparib analyses (Figure 6A-C, S3E-H).

VUS classification based on genetic and clinical data

Although the functional assays show high sensitivity and specificity, clinical classification requires multiple independent data sources. We set out to collect genetic and clinical data for VUS shown to impair protein function in the functional assays and VUS that had been observed in at least three families (Table S4). In total, we collected data for 35 variants from 98 families. The recent publication of a large scale MLM analysis of *BRCA1* and *BRCA2* VUS by the ENIGMA consortium (5) allowed us to add information for 18 of these variants, and obtain results for an additional set of 51 variants (Table S4).

The multifactorial likelihood analysis method was used to calculate the posterior probability of pathogenicity associated with VUS (4,44). This method incorporates data from e.g. co-segregation analysis and breast tumor pathology with a prior probability based on evolutionary conservation and chemical properties of the altered amino acids (7) or predicted effects on mRNA splicing (23). For the 35 VUS for which we collected clinical and/or genetic data, a likelihood ratio (LR) for pathogenicity was calculated. A combined LR between 0.5 and 2.0, especially when derived from a limited number of data points, is assumed to provide insufficient data to perform reliable integrated analysis (23). Therefore, posterior probabilities of pathogenicity were not calculated for eight of the 35 variants. As also 17 of the 51 variants only analyzed by Parsons et al. (5) yielded unreliable combined LRs, posterior probabilities for pathogenicity could be calculated for a total of 61 variants (Figure 5, Table S4). Nine variants are classified as (likely) pathogenic and 40 as (likely) benign using the five-tier classification scheme described in Plon et al. (24). For the 12 variants that remained VUS, posterior probability ranged from 0.948 to 0.052 (Figure 5, Table S4).

Thirty-seven out of the 40 variants classified as (likely) benign were functionally neutral in all three of our assays. For three likely benign variants the functional categorization was not consistent for the three assays. For c.134A>C, the categorization was not clear in the olaparib assay whereas the variant was sensitive to cisplatin and defective in HRR (Table S2, Figure 4E-H), whereas variants c.5062G>C and c.5419A>T only showed defects in the olaparib and/or HRR assays. Of note, for 20 variants predicted (likely) benign – including c.134A>C, c.5062G>C and c.5419A>T – no co-segregation data were available and MLM classification was solely based on tumor pathology and prior probabilities (Table S4).

Seven out of the nine variants predicted to be (likely) pathogenic were deleterious in all three of our assays. Both discrepant variants, c.4675G>A and c.5074G>C, are reported to affect mRNA splicing (33–37) and can therefore not be evaluated well in our cDNA assay. However, the amino acid change alone is already deleterious for c.5074G>C in the olaparib and DR-GFP assays. It should be noted that also c.5258G>C, for which we only observed an HRR defect upon transient transfections, almost reached the threshold for classification as likely pathogenic. For six unambiguously functionally deleterious variants the clinical and genetic data was not sufficient to provide a clinical classification and those variants remained VUS (Figure 5, Table S4).

In total, the combined MLM approaches classified 40 VUS as (likely) benign and nine as (likely) pathogenic. These results correlated well with classifications based on our functional assays. Although concordance may be less for variants with functional defects in only the olaparib and/or DR-GFP assays, we did not analyze sufficient numbers of this category to allow firm conclusions.

Discussion

In this work we present the functional categorization of 238 *BRCA1* VUS using complementation assays for cisplatin and olaparib sensitivity and homologous recombination. All three assays allow the identification of defects in DNA repair via HRR, either directly by the DR-GFP reporter or indirectly by HRR-defect targeted drugs. Loss of function variants in *BRCA1* or *BRCA2* confer high risk to breast and/or ovarian cancer, and the essential role of BRCA1 and BRCA2 in HRR is thought to be at the heart of their tumor suppressor function. This is illustrated by the fact that thus far all known (likely) pathogenic variants affecting BRCA1, BRCA2 and their shared interaction partner PALB2 result in defective HRR (10,13,25–29,45–47). We further optimized and validated our previously published mouse ES cell-based assay system, thereby increasing its robustness. All three assays achieved 100% sensitivity and specificity (95% CI = 83%-100%) based on the validation sets of 25 known pathogenic and 25 known benign variants.

Of the 238 *BRCA1* VUS analyzed, 27 showed a clear functional defect in the cisplatin sensitivity assay we previously used for *BRCA1* variant categorization (10). Confirming the tight relation between the three assays, these 27 variants were also identified as deleterious in the olaparib sensitivity and/or DR-GFP HRR assays. However, a subset of functionally deleterious VUS were only identified in the olaparib and/or DR-GFP assays and not in the cisplatin assay. In most of these cases, the functional defects seemed to be less severe than for variants that were deleterious in all three the assays. Although there are indications that hypomorphic variants confer moderate risks of breast cancer (48), functional assays for *BRCA1* VUS are currently not validated for their identification. Thus far, only *BRCA1* c.5096G>A (p.Arg1699Gln) is known to confer intermediate loss of tumor suppression activity based on clinical and genetic data (49,50) and this variant was deleterious in all of our assays.

It is also possible that some functionally deleterious *BRCA1* variants are not identified in all three the assays because they confer a different type of defect. In contrast to the deleterious RING and BRCT

variants we tested, coiled-coil variants c.4220T>C p.(Leu1407Pro) and c.4232T>C p.(Met1411Thr) are defective in HRR but can still complement ES cells in proliferation and cisplatin or olaparib response. These coiled-coil variants lead to a specific type of HRR defect by disrupting the interaction with PALB2, which links BRCA1 and BRCA2 in the response to DNA damage (30). Apparently, this type of HRR defect has different consequences in different experimental settings, because p.(Leu1407Pro) and p.(Met1411Thr) do affect cisplatin and olaparib response of MDA-MB-436 breast cancer cells (25). At present, it is not clear if deleterious BRCA1 coiled-coil variants affect the tumor suppressive capacity of BRCA1 to the same extent as deleterious RING or BRCT variants. In summary, all three our functional assays are equally well validated. The cisplatin assay is most stringent, and it remains to be investigated if variants that are only deleterious in the olaparib and/or DR-GFP assays have milder effects on tumor predisposition.

Given the large number of *BRCA1* VUS (currently over 2000 listed in the NIH ClinVar database) and the current technical possibilities to modify endogenous genes using CRISPR/Cas9, it is clear that multiplexed saturated mutagenesis approaches as shown in HAP1 cells (14) hold great promise for future functional annotations. Although neither the HAP1 assay nor our mouse ES cell assays are likely to recapitulate all aspects of *BRCA1*-associated tumorigenesis, they show a near perfect overlap in functional categorizations. The few discrepancies may be attributed to partial loss of function variants, of which the consequences for tumor suppression are not yet known.

Nevertheless, because of their inherent limitations, at this moment functional assays alone are not considered sufficient for the classification of VUS. Therefore, we collected available clinical and genetic data for a subset of variants. Furthermore, we took advantage of a recently published dataset from the ENIGMA consortium (5). For 86 VUS we could calculate a combined LR for pathogenicity based on genetic data and tumor pathology, and for 61 of these VUS the magnitude of the combined LRs was sufficient to perform a valid integrated evaluation (23). With the MLM 49 VUS were classified as either (likely) pathogenic (9) or (likely) benign (40), and 12 variants remained of

uncertain significance. Our functional data correlated well with the MLM classifications, although three variants that may be hypomorphic variants (i.e. they were not categorized as deleterious in all assays) were predicted likely benign by the MLM approach. It should be noted that the multifactorial likelihood model is designed to distinguish high-risk variants from variants with no clinical significance and thus is less suitable to identify hypomorphic variants. Moreover, since there were no co-segregation data, MLM classification of these specific variants and 17 other predicted (likely) benign VUS depended heavily on prior probabilities derived from *in silico* analysis (7,23). Although these are predictive, co-segregation analysis would have provided more direct evidence for the probability of disease association. Unfortunately, for the majority of the variants, we could not collect sufficient genetic and clinical data to classify them using the MLM.

As our analyses show, international data sharing through networks such as the ENIGMA consortium is important for the classification of *BRCA1* VUS. In addition, the use of well validated functional assays is incorporated in the guidelines of the American College of Medical Genetics and Genomics (6) and expected to become essential in the classification of the majority of *BRCA1* VUS. We are convinced that in the near future the use of validated functional assays will allow the classification of the large majority of *BRCA1* VUS, and thereby aid clinical decision making. Besides facilitating genetic counseling, VUS classification may also guide personalized cancer therapy, as *BRCA1*-associated breast and ovarian tumors often show loss of the wild-type allele and consequent hypersensitivity to platinum drugs and PARP inhibitors.

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Table 1: *BRCA1* VUS with functional defects in the cisplatin, olaparib or DR-GFP HRR assay.

DNA Variant	Protein Change	Cisplatin Assay	Olaparib Assay	DR-GFP Assay
c.19C>T	p.(Arg7Cys)	Deleterious	Deleterious	Deleterious
c.35A>C	p.(Gln12Pro)	Deleterious	Deleterious	Deleterious
c.41T>C	p.(Val14Ala)	Likely Neutral	Not clear[a]	Deleterious
c.110C>G	p.(Thr37Arg)	Deleterious	Deleterious	Deleterious
c.116G>A	p.(Cys39Tyr)	Deleterious	Deleterious	Deleterious
c.134A>C	p.(Lys45Thr)	Deleterious	Not clear[a]	Deleterious
c.139T>C	p.(Cys47Arg)	Deleterious	Deleterious	Deleterious
c.290C>T	p.(Thr97Ile)	Deleterious	Deleterious	Deleterious
c.4220T>C	p.(Leu1407Pro)	Neutral	Neutral	Deleterious
c.4232T>C	p.(Met1411Thr)	Neutral	Neutral	Deleterious
c.4951T>C	p.(Ser1651Pro)	Deleterious	Deleterious	Deleterious
c.4964C>T	p.(Ser1655Phe)	Deleterious	Deleterious	Deleterious
c.5002T>C	p.(Phe1668Leu)	Neutral	Neutral	Deleterious
c.5057A>G	p.(His1686Arg)	Deleterious	Deleterious	Deleterious
c.5058T>A	p.(His1686Gln)	Deleterious	Deleterious	Deleterious
c.5062G>C	p.(Val1688Leu)	Neutral	Neutral	Deleterious
c.5066T>A	p.(Met1689Lys)	Deleterious	Deleterious	Deleterious
c.5071A>G	p.(Thr1691Ala)	Neutral	Intermediate	Deleterious
c.5072C>T[b]	p.(Thr1691Ile)	Deleterious	Deleterious	Deleterious
c.5074G>A[b]	p.(Asp1692Asn)	Neutral	Neutral	Deleterious
c.5074G>C[b, c]	p.(Asp1692His)	Neutral	Deleterious	Deleterious
c.5114T>C	p.(Leu1705Pro)	Deleterious	Deleterious	Deleterious
c.5128G>A	p.(Gly1710Arg)	Neutral	Neutral	Likely Deleterious
c.5154G>T	p.(Trp1718Cys)	Deleterious	Deleterious	Deleterious
c.5203G>A	p.(Glu1735Lys)	Deleterious	Deleterious	Deleterious
c.5207T>C	p.(Val1736Ala)	Not clear[a]	Deleterious	Deleterious
c.5216A>G	p.(Asp1739Gly)	Deleterious	Deleterious	Deleterious
c.5216A>T	p.(Asp1739Val)	Deleterious	Deleterious	Deleterious
c.5225A>G	p.(Asn1742Ser)	Neutral	Neutral	Deleterious
c.5228G>A	p.(Gly1743Glu)	Deleterious	Deleterious	Deleterious
c.5238C>G	p.(His1746Gln)	Neutral	Deleterious	Deleterious
c.5300G>C	p.(Cys1767Ser)	Neutral	Neutral	Deleterious
c.5339T>C	p.(Leu1780Pro)	Deleterious	Deleterious	Deleterious
c.5348T>C	p.(Met1783Thr)	Not clear[a]	Deleterious	Deleterious
c.5357T>C	p.(Leu1786Pro)	Deleterious	Deleterious	Deleterious
c.5363G>A	p.(Gly1788Asp)	Neutral	Deleterious	Deleterious
c.5365G>A	p.(Ala1789Thr)	Deleterious	Deleterious	Deleterious
c.5419A>T	p.(Ile1807Phe)	Neutral	Deleterious	Deleterious
c.5425G>T	p.(Val1809Phe)	Deleterious	Deleterious	Deleterious
c.5435C>G	p.(Pro1812Arg)	Neutral	Neutral	Deleterious
c.5495T>G	p.(Val1832Gly)	Deleterious	Deleterious	Deleterious
c.5497G>A	p.(Val1833Met)	Deleterious	Deleterious	Deleterious
c.5509T>C	p.(Trp1837Arg)	Deleterious	Deleterious	Deleterious
c.5512G>A	p.(Val1838Met)	Neutral	Deleterious	Deleterious
c.5513T>G	p.(Val1838Gly)	Deleterious	Deleterious	Deleterious

[a]: Not clear because of opposite categorization +/- the standard deviation of repeat experiments
[b]: Predicted/known to affect consensus mRNA splice site
[c]: Recently classified as pathogenic upon expert panel review

Figure legends

Figure 1: Workflow for the functional categorizations of *BRCA1* variants in mouse embryonic stem cells. Flp RMCE vectors containing a *BRCA1* cDNA segment deletion were complemented with synthetic *BRCA1* variant containing gene blocks by Gibson Assembly. Sequence verified vectors were transfected into *R26*$^{CreERT2/RMCE}$;*Brca1*$^{SCo/\Delta}$;*Pim1*$^{DR\text{-}GFP/wt}$ mouse ES cells and pools of cells that underwent correct RMCE were used in functional assays essentially as described (10).

Figure 2: Results of the functional analyses of *BRCA1* variants. (A) Normalized cisplatin IC_{50} values for *BRCA1* wild-type (WT) controls (green), known benign variants (light-green), VUS (blue), known pathogenic variants (pink) and empty RMCE vector (EV) controls (red). Averages and standard deviations of biological repeats are shown as dots and shades respectively. Horizontal solid and dotted grey lines mark (from top to bottom) 0.99 and 0.95 probability of being neutral and 0.95 and 0.99 probability of being deleterious. (B) Normalized olaparib $\log_e IC_{50}$ values, depicted as in A. (C) Normalized percentages GFP positive transfected cells as a measure of HRR, depicted as in A.

Figure 3. Comparison of the results of the three functional assays. The average normalized (n) outcome of the cisplatin (nIC_{50}), olaparib ($\log_e nIC_{50}$) and DR-GFP (%GFP+) assays for each *BRCA1* variant is plotted in a three-dimensional space. Data are shown for all VUS (blue) and known benign (green) and known pathogenic (red) variants analyzed.

Figure 4: Overlap between functional categorizations of *BRCA1* VUS. Venn diagram showing the number of functionally deleterious BRCA1 VUS for each assay and the overlap between them. In total, 27 VUS showed a functional defect in the cisplatin assay, the olaparib assays identified 7 additional deleterious VUS (adding up to 34 in total) and 11 more deleterious VUS were identified by the DR-GFP assay (adding up to 45 in total).

Figure 5: Classification of *BRCA1* VUS using multifactorial likelihood analysis. Posterior probability of pathogenicity was calculated based on MLM for variants functionally deleterious in all assays

(red), only deleterious in the olaparib and/or DR-GFP assays (grey) or neutral (green). For variants scoring above the dotted red line the posterior probability is larger than 0.95, indicating that these are (likely) pathogenic. Below the dotted green line variants are predicted to be (likely) benign. For most variants the posterior probability of pathogenicity is too low to be visible on the graph. *: affects mRNA splicing, †: c.134A>C could not be classified in the individual olaparib assay, but was deleterious in the pooled olaparib assay, ‡: c.5258G>C was HRR defective upon transient transfection, [1]: MLM results from this study, [2]: MLM results from this study combined with Parsons et al. (5), [3]: MLM results from Parsons et al. (5).

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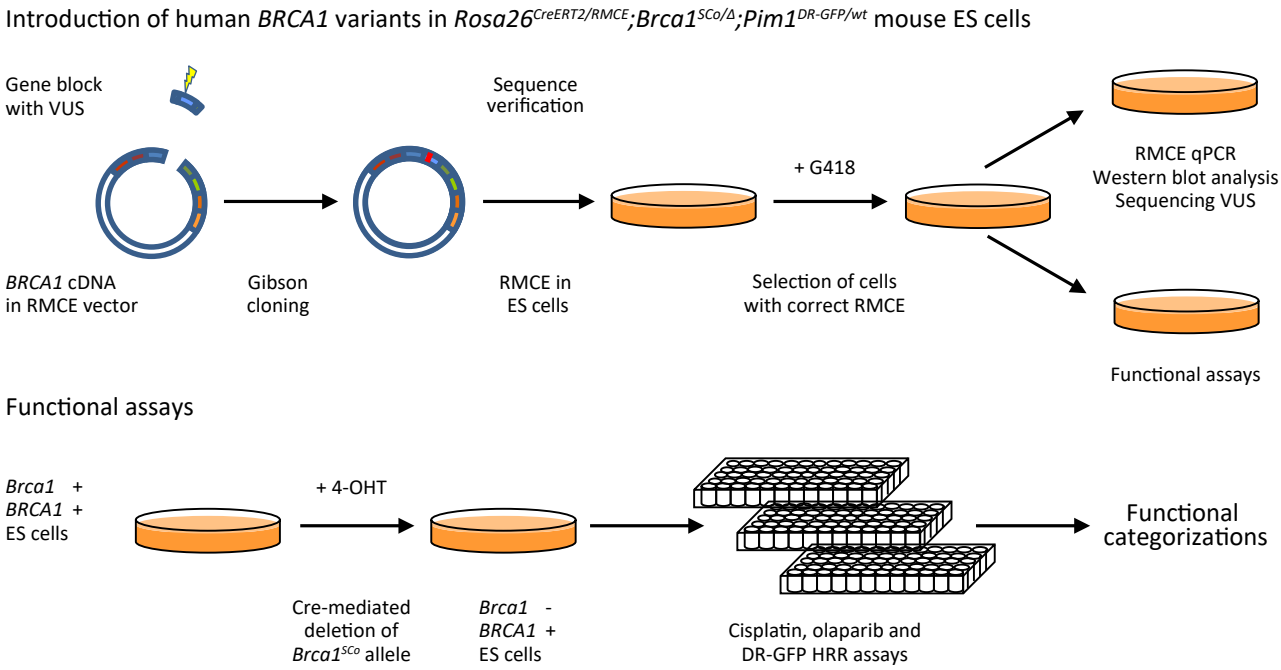
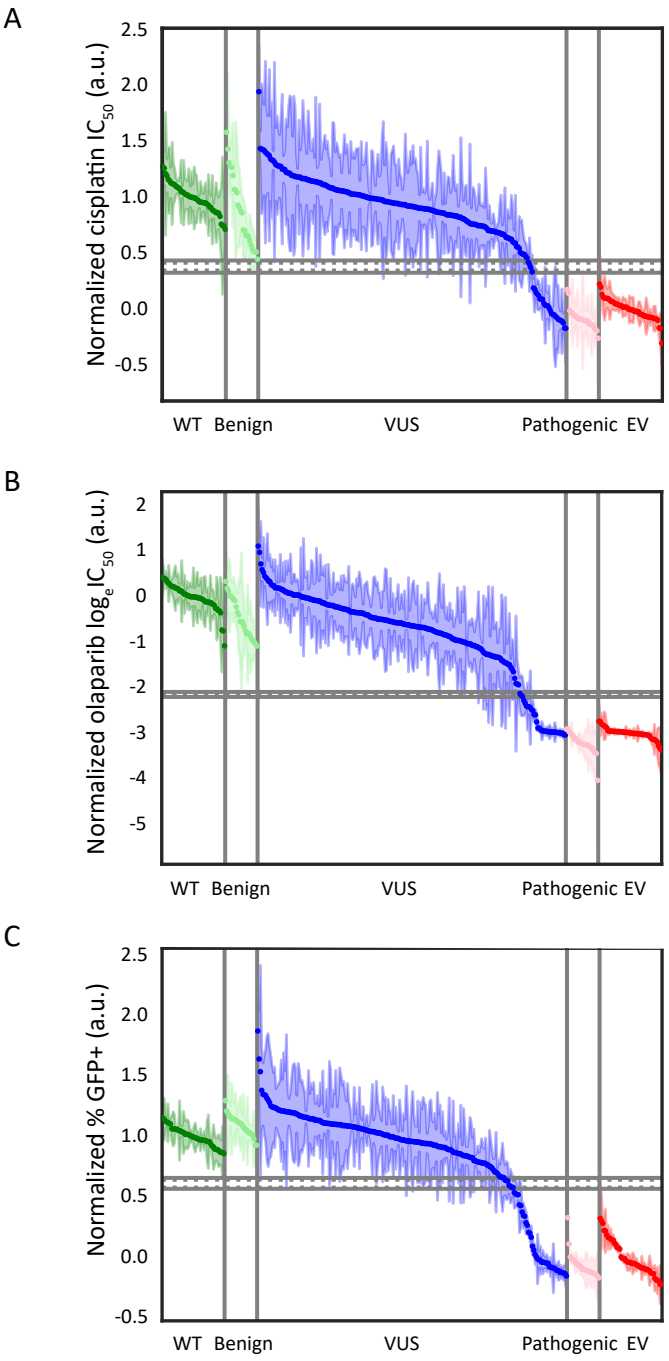
Figure 1



Author Manuscript Published OnlineFirst on June 16, 2020; DOI: 10.1158/1078-0432.CCR-20-0255
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 2



Author Manuscript Published OnlineFirst on June 16, 2020; DOI: 10.1158/1078-0432.CCR-20-0255
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 3

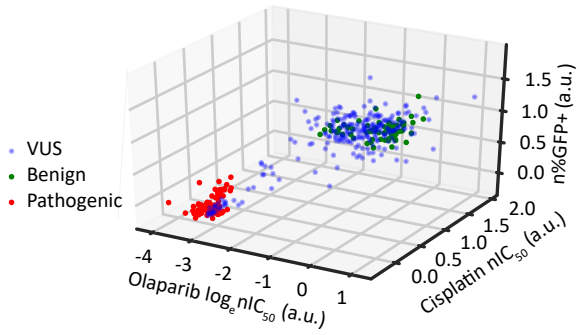


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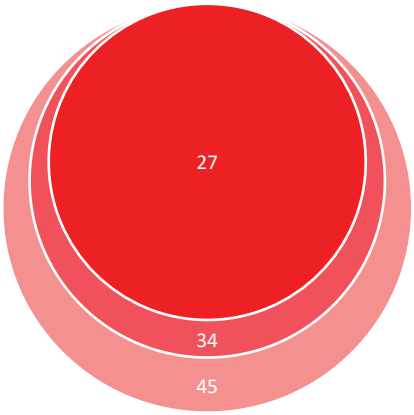
Figure 4



Cisplatin deleterious

c.19C>T	c.5057A>G	c.5228G>A
c.35A>C	c.5058T>A	c.5339T>C
c.110C>G	c.5066T>A	c.5357T>C
c.116G>A	c.5072C>T	c.5365G>A
c.134A>C	c.5114T>C	c.5425G>T
c.139T>C	c.5154G>T	c.5495T>G
c.290C>T	c.5203G>A	c.5497G>A
c.4951T>C	c.5216A>G	c.5509T>C
c.4964C>T	c.5216A>T	c.5513T>G

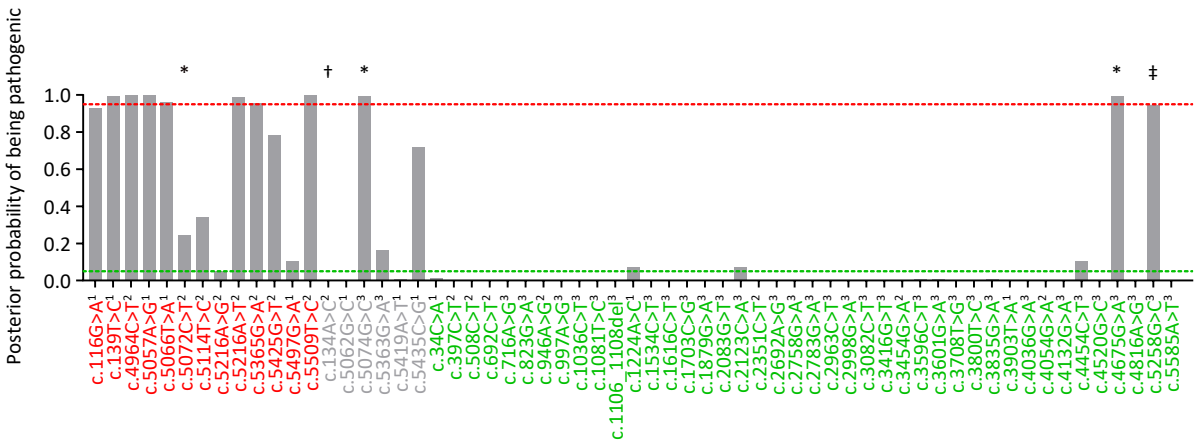
Additional olaparib deleterious

c.5074G>C	c.5348T>C	c.5419A>T
c.5207T>C	c.5363G>A	c.5512G>A
c.5238C>G		

Additional DR-GFP deleterious

c.41T>C	c.5062G>C	c.5225A>G
c.4220T>C	c.5071A>G	c.5300G>C
c.4232T>C	c.5074G>A	c.5435C>G
c.5002T>C	c.5128G>A	

Figure 5



AACR American Association for Cancer Research

Clinical Cancer Research

Functional categorization of BRCA1 variants of uncertain clinical significance in homologous recombination repair complementation assays

Peter Bouwman, Ingrid van der Heijden, Hanneke van der Gulden, et al.

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