A high-throughput functional complementation assay for classification of BRCA1 missense variants

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Running title: Functional analysis of BRCA1 sequence variants

Keywords: BRCA1, HBOC, VUS, functional classification

Financial support: This work was supported by the Dutch Cancer Society (NKI 2008-4116 to J.J. and P.B), the TI Center for Translational Molecular Medicine (CTMM) BreastCare project, and the Cancer Genomics Centre Netherlands.

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The authors declare no conflict of interests.

Word count: 5268

Number of figures: 6
Number of tables: 1

Abbreviations: 4OHT, BAC, BIC, DSB, ES cells, HBOC, HR, IARC, IC₅₀, ICL, NHEJ, RMCE, RSE, SCo, SDM, VUS
Abstract

Mutations in BRCA1 and BRCA2 account for the majority of hereditary breast and ovarian cancers, and therefore sequence analysis of both genes is routinely performed in patients with early-onset breast cancer. Besides mutations that clearly abolish protein function or are known to increase cancer risk, a large number of sequence variants of uncertain significance (VUS) have been identified. Although several functional assays for BRCA1 VUS have been described, thus far it has not been possible to perform a high-throughput analysis in the context of the full-length protein. We have developed a relatively fast and easy cDNA-based functional assay to classify BRCA1 VUS based on their ability to functionally complement BRCA1-deficient mouse embryonic stem cells. Using this assay we have analyzed 74 unclassified BRCA1 missense mutants, of which all predicted pathogenic variants are confined to the BRCA1 RING and BRCT domains.

Significance

This manuscript describes the generation, validation and application of a reliable high-throughput assay for the functional classification of BRCA1 sequence variants of uncertain significance. BRCA1 VUS are frequently found in patients with hereditary breast or ovarian cancer and present a serious problem for clinical geneticists.
Introduction

Germline loss-of-function mutations in BRCA1 and BRCA2 are known to result in an approximately 10-fold increased lifetime risk of developing breast or ovarian cancer. Thus far, no other genes have been identified with such a strong link to hereditary breast and ovarian cancer (HBOC) and during the past decades many women have been screened for germline mutations in BRCA1 or BRCA2. This has resulted in the identification of numerous pathogenic mutations as well as a large number of sequence variants of which the clinical relevance is not clear. In the most recent publication of the ENIGMA group, an international consortium for the evaluation of BRCA1 or BRCA2 sequence variants, a total number of 1273 unique BRCA1 variants is mentioned (1) but this number may even increase further because of the implementation of high-throughput sequencing methods. Of the BRCA1 VUS, 920 are non-truncating exonic mutations that may affect protein function or mRNA splicing, but for which there is not enough linkage information to indicate whether they are pathogenic. To aid genetic counseling of individuals with BRCA1/2 VUS, both genetic and functional classification methods have been developed. Genetic analysis of BRCA1 or BRCA2 VUS relies on co-segregation with disease, co-occurrence with known pathogenic mutations, and family history of cancer. These data have been integrated into computational models to calculate the likelihood that a VUS is disease-causing (2). Additional in silico analysis of the evolutionary conservation of the amino acids affected by the mutation and the predicted impact of the mutant amino acids on protein folding are also implemented in such models. Functional assays do not rely on pre-existing data, but directly test the effect of BRCA1/2 VUS on known functions of the encoded proteins (3). Although this may seem relatively straightforward, it can be difficult to extrapolate data from functional assays into cancer risks for patients. For instance, in most
assays only part of the BRCA1 protein is analyzed. In addition, some of the more elegant assays are technically demanding and not suitable to analyze large numbers of mutations. We reasoned that a good functional assay should fulfill three basic requirements: [1] it should investigate the biological effects of a BRCA1 VUS in the context of the full-length protein; [2] it should be performed under normal physiological conditions in a non-cancerous cell type; and [3] it should be based on a highly standardized and reproducible protocol.

These considerations led us to develop a functional assay based on physiological expression of full-length human BRCA1 cDNA in mouse embryonic stem (ES) cells that are genetically engineered to allow conditional deletion of endogenous Brca1. Mutant BRCA1 cDNAs are generated using site-directed mutagenesis (SDM) and introduced in a defined genomic locus of mouse ES cells by recombinase-mediated cassette exchange (RMCE). In this way, we have analyzed 86 BRCA1 variants for their effects on cell proliferation and drug sensitivity, including 74 clinically relevant VUS.
Results

Generation of mouse embryonic stem cells expressing BRCA1 sequence variants

Although BRCA1-deficient tumor cells proliferate rapidly in situ, loss of BRCA1 in normal cells leads to a severe proliferation defect (4). We decided to make use of this phenotype for the functional analysis of BRCA1 variants in Brca1 selectable conditional knockout (SCo) mouse ES cells (5). These cells carry one Brca1-null allele and a selectable conditional Brca1SCo allele, which contains, in addition to loxP sites around exons 5-6, a split puromycin resistance marker that is activated upon Cre-mediated deletion of exons 5-6. They also contain a CreERT2 allele in the Rosa26 locus, which allows for controlled activation of Cre via administration of 4-hydroxy tamoxifen (4OHT). Thus, BRCA1-deficient ES cells can be easily obtained via 4OHT-induced inactivation of the Brca1SCo allele and subsequent selection for puromycin resistance (Fig. 1) (5). To allow efficient integration of human BRCA1 variants in one specific genomic locus, we supplied the other Rosa26 allele with F3 and Frt recombination sites for RMCE by the site-specific recombinase Flp (Fig. 1 and Supplementary Fig. S1) (6). Cells that have undergone successful RMCE can be selected because they express a truncated neomycin selection marker under control of the endogenous Rosa26 promoter, which further increases targeting efficiency. Flp-mediated recombination ensures single-copy integration of BRCA1 expression constructs at the same Rosa26 locus, thus avoiding position-effect variegation and copy number-dependent differences in expression.

RMCE vectors were equipped with a human BRCA1 cDNA expression construct, which was modified using SDM to introduce defined mutations in BRCA1. The focus of our analysis was on Dutch and Belgian VUS that were found in families with HBOC. We also included a number of variants that were previously classified using functional assays (7,8)
or a multifactorial likelihood model (9), as well as M1400V, L1407P and M1411T, which have been reported to attenuate the interaction between BRCA1 and PALB2 (10). To allow validation of our functional complementation assay, we included a series of 8 BRCA1 variants, which are known to be deleterious or neutral according to the Breast Cancer Information Core database (BIC; http://research.nhgri.nih.gov/bic/; Supplementary Table S1). These controls include the well-known pathogenic BRCA1 founder mutations 185delAG and 5382insC and the neutral polymorphisms Y105C, R866C and E1250K. The BIC designation is supported by the classification according to the International Agency for Research on Cancer (IARC; http://iarc.fr/), which includes the Align GVGD score that indicates biophysical and evolutionary alterations (http://agvgd.iarc.fr/; Supplementary Table S1) (11,12). Align GVGD scores vary between C0 (likely neutral) and C65 (likely deleterious). Sequence verified constructs that contained the intended VUS, but no other BRCA1 mutations, were transfected into R26CreERT2/RMCE;Brca1Sc/A ES cells to undergo RMCE (Fig. 2). Neomycin-resistant clones were pooled and RMCE was confirmed by PCR analysis. Subsequently, protein expression of the human BRCA1 variants was analyzed by western blot with a human BRCA1-specific antibody (Fig. 2 and Supplementary Fig. S2). Comparison with BRCA1 protein levels in ES cells expressing human BRCA1 from a bacterial artificial chromosome (BAC), which is known to rescue embryonic lethality of Brca1-null mice (7,13), showed that transcription of the human BRCA1 cDNA from the EF1A gene promoter results in physiological levels of BRCA1 protein (Supplementary Fig. S1). Most BRCA1 mutants were expressed at equal levels, allowing comparison of their functional activities. A number of C-terminal BRCA1 mutants showed low levels of protein expression (Supplementary Table S2, Supplementary Fig. S2). RT-PCR analysis showed that decreased protein expression of these mutants was not caused by decreased mRNA expression (Supplementary Fig. S3), suggesting that the
low abundance of BRCA1 protein results from post-translational events. In fact, for most of these variants mutation-associated protein instability was already documented in previous publications (8,14,15) (Supplementary Table S2).

Functional complementation assay of BRCA1 sequence variants in mouse Brca1-null ES cells

As a first functional test we assayed the ability of BRCA1 mutants to restore the proliferation defect of switched R26CreERT2/RMCE;Brca1SCo/g507 ES cells (Fig. 2). Expression of endogenous mouse Brca1 was shut-off through overnight induction of cre activity by 4OHT and 7 days after switching cells were plated in 96-well plates for proliferation assays, which were analyzed using Sulphorhodamine B staining. For each group of mutants tested, we included positive and negative controls consisting of ES cells containing respectively wild-type hBRCA1 cDNA and an empty RMCE vector. BRCA1 variants were evaluated on their ability to support growth compared to these controls (Supplementary Tables S3 and S4). Although we tested 28 mutations in the central domain encoded by exon 11 (aa 224-1366), BRCA1 variants that were unable to rescue the proliferation defect of Brca1-null mouse ES cells to BRCA1 wild-type levels were confined to the conserved N- and C-terminal domains of BRCA1.

Cisplatin sensitivity assay for classification of BRCA1 variants

While the ability of BRCA1 variants to support proliferation appears to be indicative of VUS function, results were not always clear-cut (Supplementary Tables S3 and S4). BRCA1 is known to be important for DNA interstrand crosslink (ICL) repair through mechanisms that are both dependent (5) and independent (16) of its function in homologous recombination (HR). The role of BRCA1 in ICL repair is stressed by the occurrence of genetic reversion
mutations restoring BRCA1 protein expression in platinum-resistant ovarian tumors in 

BRCA1-mutation carriers (17,18). We therefore decided to perform a 96-well based 
cisplatin sensitivity assay to allow a more stringent evaluation of BRCA1 VUS functionality. 
Half-maximal inhibitory concentrations (IC50) of cisplatin were determined using a 
resazurin cell viability assay and BRCA1 variants were again classified in comparison to 
wild-type BRCA1 and an empty RMCE vector. To obtain corrected cisplatin IC50 values, 
we fitted a log-logistic curve constrained at 1 and 0. We excluded a fit if the residual 
squared error (RSE) exceeded 0.1. We then applied a Bayesian predictor to classify 
BRCA1 VUS as pathogenic or benign. Most variants that showed a less than wild-type 
activity in the proliferation assay also scored as functionally impaired in the cisplatin 
sensitivity assay and were classified as deleterious (Table 1, Fig. 3). Variants showing 
increased cisplatin sensitivity were tested at least twice before they were classified. The 
positive and negative controls classified as expected, although the known pathogenic 
truncation mutation 5382insC scored as neutral in one out of three assays, stressing the 
need for repeat experiments. Also BRCA1 variants that were previously tested in other 
assays performed as expected. It should be noted that the artificial variants S308A and 
S1497A rescued proliferation and cisplatin response of mouse Brca1-deficient ES cells in 
BAC complementation assays, but are predicted to be deleterious based on their effects 
on ES cell differentiation and the response to γ-irradiation respectively (7). The V1804D 
mutation scored as a neutral variant in our assay, which is in line with most published data 
(8,9), except for the results of an ES cell-based BAC complementation assay (7). Again, all 
BRCA1 mutations that were classified as deleterious were confined to regions encoding 
the conserved N- and C-terminal domains (Fig. 4), despite the observation that deletion of 
the central region encoded by exon 11 leads to genetic instability in mice (12). 
Remarkably, the three mutations that diminish the interaction between BRCA1 and PALB2
(10) - just C-terminal of the region encoded by exon 11 - had no effect on cisplatin sensitivity in this assay. Of note, there was in general good correlation between our classification and the Align GVGD score (Supplementary Table S4). Notable exceptions are the neutral control variant R866C, which validates our assay but scores as likely deleterious (C65) using Align GVGD, and G1770V, which is scored as likely neutral (C0) by Align GVGD but classified as deleterious in our assay. Our assay also classifies some variants for which the Align GVGD scores are less clear, emphasizing the usefulness of functional assays to complement in silico analysis.

We also analyzed the possible effects of all BRCA1 variants on mRNA splicing, which may have deleterious consequences but cannot be assessed in our cDNA based assay. Although the predictive value for exonic variants outside the consensus splice sites is questionable (19,20), 4 missense mutations are present in existing splice sites (Supplementary Table S4). While c.5154G>T (encoding W1718C) is deleterious, three of these variants are classified as neutral in our assay. One of them, c.441G>C (encoding L147F) has an increased probability to damage the splice donor site of exon 7. Another variant, c.5072C>T (encoding T1691I) might also affect splicing but no defect was measured in blood samples of mutation carriers (19). The third exonic splice site mutation that was classified as neutral in our cisplatin sensitivity assay, c.133A>C (K45Q), is not predicted to lead to aberrant splicing.

Results from proliferation and cisplatin sensitivity assays correlate with HR activity of BRCA1 variants

BRCA1 and BRCA2 are involved in DNA repair via HR (12,21). Together with non-homologous end joining (NHEJ), HR forms the cellular defense against DNA double-strand breaks (DSBs), a severe type of DNA damage that is lethal if unrepaired. While HR is
essentially error-free, NHEJ is error-prone and therefore defects in HR are known to lead
to genomic instability. Although it is not clear whether other functions of BRCA1 are also
important for tumor suppression (22,16,23–26), its role in HR is likely to be relevant.
Therefore we used the DR-GFP assay (27) to measure the effects on HR for a subset of
our BRCA1 VUS. A number of predicted pathogenic variants and controls were shuttled
into R26CreERT2/RMCE;Brca1SCo/g507;Pim1DR-GFP/wt ES cells carrying the DR-GFP reporter in the
Pim1 locus. Expression of endogenous mouse Brca1 was switched off and cells were
transfected with a plasmid encoding the I-SceI meganuclease as well as an mCherry
fluorescent marker to control for transfection efficiency. Repair of I-SceI-induced DNA
DSBs in DR-GFP via HR leads to expression of GFP, which can be monitored by flow
cytometry. All but one predicted pathogenic variants tested resulted in defective HR,
thereby confirming our functional classification (Fig. 5). The only predicted deleterious
variant that does not significantly differ from wild-type BRCA1, R1699Q, appears to
support intermediate levels of HR activity.

PARP inhibitor sensitivity assay for classification of BRCA1 variants with intermediate
activity
Deleterious effects of variants with intermediate or partial activity may escape detection in
certain functional assays. Although cisplatin sensitivity assays allow robust and
reproducible classification of several functionally impaired BRCA1 variants, assays using
other compounds may have additional value. It is known that BRCA1 and BRCA2 deficient
cells are extremely sensitive to PARP1 inhibition (28,29), leading to a larger dynamic
range between BRCA2-deficient cells and isogenic BRCA2-proficient controls than for
cisplatin (30). We therefore tested complementation of PARP inhibitor sensitivity for a
number of BRCA1 mutants and the BRCA1 wild-type control. Given the unexpected
neutral effects of the M1400V, L1407P, and M1411T mutations in the PALB2 interaction
domain, we decided to include these variants in this series, as well as the R1699Q and
V1736A variants that have recently been shown to confer a partial defect (31,32). To allow
direct comparison of results from different assays, we repeated the cisplatin sensitivity and
proliferation assays in parallel to the olaparib sensitivity assay. Our results indicate that
BRCA1 wild-type and empty vector controls indeed show a larger difference in sensitivity
for olaparib than for cisplatin (Fig. 6). However, this increase in dynamic range is
accompanied by an increased variation between repeat experiments for BRCA1-proficient
samples. Nevertheless, the functional defect of the R1699Q and V1736A mutations
becomes more evident, and there also appears to be a less than wild-type response for
the PALB2 interaction mutant L1407P. In the concurrent proliferation analysis, R1699Q
and V1736A both show an intermediate functional defect (Supplementary Fig. S4). Also
the M1411T mutation seems to affect the response to PARP inhibition but the difference
with BRCA1 wild-type is not significant. Interestingly, the L1407P and M1411T variants
have previously been shown to be more defective than M1400V in a gene conversion
assay (10). It should be noted that, in contrast to the large-scale classification
experiments, also the cisplatin sensitivity assay performed in parallel to the PARP inhibitor
assay identified significant functional defects for L1407P and V1736A.
Discussion

Over the past few years several functional assays for classification of BRCA1 VUS have been developed. Several of these assays are restricted to functions of the BRCA1 protein that reside in the evolutionary conserved RING or BRCT domains. Examples include in vitro transactivation assays for BRCT peptides (8) and measurement of ubiquitin ligase activity for protein fragments encompassing the N-terminal RING domain (33). Other assays were designed to evaluate the functions of full-length mutant BRCA1 protein, either by monitoring general effects on proliferation or response to DNA damage (7) or by directly focusing on the role of BRCA1 in DNA repair via HR (34).

Since BRCA1 VUS are not restricted to the N- or C-terminal domains - and given the observation that interaction between these domains is required for recruitment of BRCA1 to damaged DNA (35) - functional assays for the full-length protein would be most ideal. In principle such assays can be performed in cell lines derived from BRCA1-mutated tumors, but there are indications that the outcome of assays for BRCA1 function depends on the cellular context. Since absence of BRCA1 leads to loss of cellular viability, it is thought that additional mutations are required for BRCA1-associated tumorigenesis. For example, loss of p53 alleviates the consequences of BRCA1 deficiency both in vitro and in vivo (4) and is common in BRCA1-deficient tumors (36,37). Also depletion of 53BP1 is known to suppress the defects caused by BRCA1 deficiency (5,38,39). Therefore, aberrations in BRCA1-deficient tumor cells may mask functional defects of BRCA1 VUS. We reasoned that assays in normal cells that can be depleted from endogenous BRCA1 expression are most likely to reveal functional defects of BRCA1 VUS. The usefulness of this approach has been previously demonstrated by BAC complementation assays in mouse ES cells (7,40). However, mutagenesis of large BAC clones by recombineering and functional
complementation of cells with these mutant constructs is time-consuming and technically demanding, and can therefore not be performed in a high-throughput setting. We therefore set out to develop a cDNA-based functional complementation assay in mouse ES cells that is easier to control and scale-up and more suitable for routine functional classification of \textit{BRCA1} sequence variants.

One advantage of BAC transgenics in complementation assays is that genes are expressed at physiologically relevant levels. This is the result of low copy number integrations and the presence of natural regulatory elements required for proper gene expression. We decided to use RMCE to allow single-copy integration of \textit{BRCA1} cDNAs at one specific genomic locus. The use of RMCE effectively prevents multiple or partial integrations, concatemers and position-effect variegation. As a result, all variants are expressed at equal levels. Moreover, transcription of \textit{BRCA1} cDNAs from the \textit{EF1A} gene promoter results in physiological levels of \textit{BRCA1} protein, comparable to those observed in \textit{Brca1}-null ES cells complemented with a BAC containing the human \textit{BRCA1} locus. Indeed, the wild-type human \textit{BRCA1} cDNA was able to complement \textit{Brca1}-null ES cells in cellular proliferation, drug sensitivity and HR assays. Our RMCE strategy employs \textit{BRCA1} cDNA constructs in which mutations can be swiftly introduced using SDM, enabling a higher throughput than introduction of mutations via BAC recombineering. In addition, the introduction of \textit{BRCA1} cDNAs via RMCE obviates the need to analyze multiple ES cell clones for correct integration and expression.

As a proof of principle we used our functional complementation assay to analyze exonic \textit{BRCA1} VUS that were identified in families with HBOC in the Netherlands and Belgium, as well as a set of previously analyzed \textit{BRCA1} variants. A number of mutations resulted in
reduced BRCA1 protein levels, most likely because of structural destabilization. In all cases this led to diminished capacity for functional complementation. Also several variants that gave rise to normal BRCA1 protein levels were unable to rescue the proliferation defect and cisplatin sensitivity of Brca1-null ES cells. Since BRCA1 loss-of-function is associated with increased cancer risk, variants that score as functionally impaired in our ES cell assay system may be causally involved in tumor formation. This notion is supported by the fact that 7 out of 8 known pathogenic or neutral control variants in our validation series were correctly classified by the cisplatin sensitivity assay. The pathogenic 5382insC truncation mutation could not be classified because scored as neutral in 1 out of 3 transfection series. This was probably due to technical reasons, as the 5382insC mutation did not restore HR activity in Brca1-deficient ES cells, in contrast to R1699Q, which was recently shown to confer intermediate risk of HBOC (32).

Our assay system yielded ambivalent results for 9 other variants: S4F, S308A, S1651P, S1651F, T1691I, V1736A, E1735K, H1746Q and R1753T. T1691I and E1735K were classified as functionally impaired in only one cisplatin sensitivity test, while values from repeat experiments could not be taken into account because of RSE values above 0.1. S4F, S1651P, S1651F, V1736A, H1746Q, and R1753T were differently classified in repeat experiments, which may reflect technical flaws or intermediate activity of these variants. Of note, the V1736A mutation was recently identified as a pathogenic variant with hypomorphic activity in DNA repair (31). While we did not measure significant HR activity of V1736A in a DR-GFP gene conversion assay, intermediate activity of this variant is supported by the results of the proliferation assays and additional cisplatin sensitivity assays. S1651F showed HR activity similar to wild-type BRCA1, whereas R1753T was HR-deficient. S308A is an artificial mutation of a BRCA1 phosphorylation site that was able
to support proliferation and resistance to DNA damage in an ES cell-based BAC complementation assay. However, S308A complemented ES cells did show increased apoptosis when cultured in embryoid bodies (7), indicating a partial defect that might explain the ambivalent results for this mutation in our assays.

Our results show that BRCA1 variants should ideally be assayed in triplicate to avoid misclassification. This also applies to BRCA1 VUS that we classified as neutral, most of which were tested only once because in our current study we focused on variants that showed functional impairment. The striking restriction of unambiguously predicted pathogenic mutants to the terminal RING and BRCT domains suggest that some plasticity is allowed for the central domain of BRCA1. However, our dataset is still limited and more experiments are required to gain insight in the function of this domain.

Our cDNA-based system allows for several additional functional assays that have been described previously for BAC transgenic ES cells (7). These include assays for defects during in vitro and in vivo ES cell differentiation but also treatments with other cytotoxic agents. As a proof of principle, we investigated the activity of a number of mutants in the response to the PARP inhibitor olaparib. These included M1400V, L1407 and M1411T, which were previously shown to impair PALB2 binding and have a negative effect on BRCA1 function (10). While the differences in sensitivity of BRCA1-deficient versus BRCA1-proficient ES cells are larger for olaparib than for cisplatin, increased variation between repeat experiments only allowed us to identify functional defects for L1407 and not for the other two variants in the PALB2 interaction domain, M1400V and M1411T. However, the R1699Q and V1736A variants, which are known to have hypomorphic activity, clearly showed a defect in the response to olaparib. Interestingly, both mutations
are in the BRCT domain and it has recently been shown that especially mutation of this
domain confers sensitivity to PARP inhibition (41). Together, our results show that PARP
inhibitor sensitivity assays may have added value, especially for the classification of
BRCA1 VUS with intermediate phenotypes.

Platinum drugs and PARP inhibitors are selectively toxic to BRCA1-deficient cells because
they target HR deficiency. Although the role of BRCA1 in HR is thought to be essential for
maintaining genomic integrity and preventing accumulation of (oncogenic) mutations, other
activities may also contribute to its tumor suppression function. These activities may
include the HR independent role for BRCA1 in ICL repair, which has been attributed to the
facilitation of FANCD2 accumulation at cross-linked DNA (16). Nevertheless, we observed
a good correlation between the results of the cisplatin sensitivity assay and the results of
the DR-GFP HR assay. All BRCA1 variants that failed to restore the cisplatin response in
Brca1-null ES cells were also defective in catalyzing gene conversion, thereby confirming
our functional classification. It will be interesting to see if this holds true for all BRCA1
variants or whether there are also pathogenic mutations that have no effect on HR.

As with any other in vitro approach, our functional complementation assay system might
still fail to identify all pathogenic variants because it does not necessarily recapitulate all
aspects of BRCA1 function in vivo. A limitation of our cDNA-based assay is that it cannot
be used to investigate effects on mRNA splicing. Although algorithms have been designed
to predict possible splice defects, especially the consequences of mutations outside of the
consensus splice sites require functional validation experiments. For this purpose, BAC
complementation assays (7), minigene-based splicing assays or BRCA1 transcript
analysis of patient blood samples (19) may be instrumental. However, also trans-acting
factors affect splicing and these may be tissue specific (42). A possible solution would be
to determine the presence of BRCA1 splice variants in tumor tissue from BRCA1 VUS
carriers and use this information to generate a cDNA construct for analysis of the
functional consequences. To evaluate BRCA1 VUS, there remains a need for multifactorial
models that combine results from functional assays and in silico analyses with genetic
evidence and other information from mutation carriers. This also includes DNA copy
number data from tumors from BRCA1 VUS carriers, since it is known that BRCA1-
associated breast tumors show distinct genomic aberrations (43,44). Our functional assay
system does however provide a robust and easily implementable tool for the functional
characterization of large numbers of BRCA1 VUS within the context of the full-length
protein. It is our hope that our assay system will find its way to clinical genetics
laboratories where it can be used to aid genetic counseling. Ideally, these tests should be
coordinated on an international level and in close collaboration with the ENIGMA
consortium.
Materials and Methods

Generation of RMCE vectors containing human BRCA1 sequence variants

Human BRCA1 cDNA from a pCDNA3-BRCA1 expression construct (45) was subcloned into the pRNA 251-MCS RMCE exchange vector under control of the EF1A gene promoter. BRCA1 mutations were introduced by site-directed mutagenesis using the QuickChange Lightning protocol (Stratagene) and constructs were verified by sequencing the entire human BRCA1 cDNA (see Supplementary Methods).

Generation of human BRCA1 transgenic ES cells

R26CreERT2/wt;Brca1SCoΔ ES cells were generated by gene targeting in 129/Ola E14 IB10 ES cells (46). The presence of correctly targeted alleles was verified using Southern blotting, western blotting and PCR analysis (5,47). The wild-type Rosa26 allele of R26CreERT2/wt;Brca1SCoΔ ES cells was equipped with Frt and F3 sites for Flp RMCE as described (6), see Supplementary Figure S1. Introduction of human BRCA1 cDNAs via RMCE was performed by co-transfection of R26CreERT2/RMCE;Brca1SCoΔ ES cells with RMCE vectors and pFlpe (48) using Lipofectamine 2000 (Invitrogen). Cells that had successfully undergone RMCE were selected using 200-400 μg/ml G418. Correct RMCE was confirmed by PCR and expression of human BRCA1 was analyzed by western blotting using a polyclonal antibody against human BRCA1 (9010; Cell Signaling) or the monoclonal antibody MS110 (Ab-1, OP92; Calbiochem). The generation of R26CreERT2;Brca1SCoΔ ES cells expressing human BRCA1 from the BAC clone RP11-812O5 was performed essentially as described (49).
Cytotoxicity and proliferation assays

Cre-mediated inactivation of the endogenous mouse Brca1Sc0 allele was achieved by overnight incubation of cells with 0.5 μM 4OHT (Sigma). One week after switching, cells were seeded in triplicate at 1000 cells/well in 96-well plates for cisplatin or olaparib (AZD2281) sensitivity assays essentially as described (5). In addition, cells were seeded in triplicate at 500 cells/well on 96-well plates to monitor proliferation.

HR reporter assays

For DR-GFP assays we used a modified version of the p59X DR-GFP construct (12) (kindly provided by T. Ludwig), in which the puromycin resistance marker was inactivated by inversion of an internal SalI fragment. To allow selection of targeted integration of this construct to the Pim1 locus, we equipped the wild-type Rosa26 allele of R26CreERT2/wt;Brca1Sc0/ ES cells with Frt and F3 sites for RMCE using a targeting vector in which zsgreen, the hygromycin resistance marker and Flpe were replaced by a puromycin resistance marker. Subsequently, the DR-GFP construct was targeted to the Pim1 locus, BRCA1 variants were introduced using RMCE as described, and cells were subcloned to allow HR reporter assays. HR reporter assays were performed by Lipofectamine 2000 transfections of an I-SceI-mCherry plasmid, which was generated by cloning CMV-mCherry (Clontech) into the cBas I-SceI expression plasmid. Three days after transfection mCherry/GFP double positive cells were monitored by flow cytometry on a FACS CyAn (Beckman Coulter) using Summit software (Beckman Coulter).

Statistics

We calculated the cisplatin IC50 values from the 96-well plate-based cisplatin sensitivity assays for VUS classification by fitting a log-logistic curve, normalized to the no-drug
control, constrained between 1 and 0 using the drc package in the R programming language. We discarded fits that exceeded a 0.1 RSE. To allow comparison between plates we normalized using a linear model based on the positive and negative controls, resulting in corrected IC$_{50}$ scores. We estimated normal distributions of the corrected IC$_{50}$ score for both the pooled positive and negative controls across plates. We used these estimated normals to calculate the probability of pathogenicity for each VUS.

Statistical significance for the HR and cytotoxicity assays on selected groups of variants was calculated by two-tailed Student’s t test using Prism 6 Software. Significant differences are indicated by *p<0.05, **p<0.01, and *** p<0.001, ****P<0.0001. NS stands for non-significant (p>0.05).

Computational analysis

Alamut software was used to obtain genomic annotations and Align GVGD scores (Human to Sea urchin) for all variants.

See Supplementary Methods for a detailed protocol for the classification of BRCA1 sequence variants.
Acknowledgements

We thank Sandra Niehaves (TaconicArtemis GmbH, Cologne, Germany) for technical assistance; D.P. Silver (Dana-Farber Cancer Institute, Boston, MA) for the human BRCA1 cDNA expression construct; L. van der Weyden (Wellcome Trust Sanger Institute, Hinxton, UK) for the pFlpe expression construct; F. Stewart (BioInnovations Zentrum, Dresden, Germany) for the pFlpo expression construct; and M. Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY) and T. Ludwig (Colombia University, New York, NY) for the I-SceI expression plasmid and the DR-GFP reporter plasmid. We thank Jo Morris (University of Birmingham, UK), Annegien Broeks and Petra Nederlof for helpful comments on the manuscript. The Dutch/Belgium VUS workgroup is gratefully acknowledged for providing data on the occurrence of BRCA1 VUS in the Netherlands and Belgium. Workgroup members are: Frans B.L. Hogervorst (Family Cancer Clinic, Netherlands Cancer Institute, Amsterdam), Johannes J.P. Gille (Department of Clinical Genetics, VU Medical Center, Amsterdam), Juul T. Wijnen and Maaike Vreeswijk (Department of Human Genetics & Department of Clinical Genetics, Leiden University Medical Center, Leiden), Rob van de Luijt (Department of Medical Genetics, University Medical Center Utrecht, Utrecht), Marinus J. Blok (Department of Clinical Genetics, University Hospital Maastricht, Maastricht), Ans van den Ouweland (Department of Clinical Genetics, Family Cancer Clinic, Erasmus University Medical Center, Rotterdam), Danielle Bodmer and Arjen Mensenkamp (Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen), Annemiek van der Hout (Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen). Belgian Collaborators: Katrien Storm (Department of Medical Genetics, University and University Hospital Antwerp, Antwerp), Kathleen Claes (Center for Medical Genetics, Ghent University Hospital, Ghent),
Grant Support

This work was supported by grants from the Dutch Cancer Society (NKI 2008-4116 to J.J. and P.B), the Cancer Genomics Centre Netherlands, and the TI Center for Translational Molecular Medicine (CTMM) BreastCare project.
References


**Table 1: Functional classification of BRCA1 VUS based on cisplatin response**

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a Type of mutation indicates if variants are a VUS according to the BIC database (http://research.nhgri.nih.gov/bic/).

b Functionally impaired in the DR-GFP and/or combined PARP inhibitor/cisplatin sensitivity assay.
Figure Legends

Figure 1

Schematic overview of the RMCE procedure in R26\textsuperscript{CreERT2/RMCE};Brca1\textsuperscript{SCo/Δ} ES cells

Before introduction of a human BRCA1 cDNA, R26\textsuperscript{CreERT2/RMCE};Brca1\textsuperscript{SCo/Δ} ES cells are mouse BRCA1-proficient and sensitive to both neomycin and puromycin. Targeting of single-copy human BRCA1 cDNA variants to the Rosa26 locus by Flp recombinase-mediated cassette exchange (RMCE) results in expression of human BRCA1 and neomycin resistance. Addition of 4OHT leads to CreERT2-mediated deletion of mouse Brca1 exons 5 and 6, resulting in loss of mouse BRCA1 protein and concomitant expression of puromycin from the PGK promoter. This enables selection of mouse BRCA1-deficient, human BRCA1 expressing R26\textsuperscript{CreERT2/hBRCA1};Brca1\textsuperscript{Δ/Δ} ES cells that can be used in functional complementation assays.

Figure 2

Workflow for the functional classification of BRCA1 sequence variants in Brca1-null ES cells

Outline of the generation of mouse Brca1-deficient ES cells expressing human BRCA1 variants and functional complementation assays. Indicated are the experimental steps and the time it takes one person to analyze 20 mutants.
Figure 3

Waterfall chart of cisplatin IC\textsubscript{50} values normalized for wild-type human BRCA1 and empty RMCE vector controls

Corrected cisplatin IC\textsubscript{50} values for all tested human BRCA1 variants and controls, including repeat experiments. BRCA1 variants are classified as functionally impaired or neutral when the corrected IC\textsubscript{50} values are similar to either the empty RMCE vector or the wild-type human BRCA1 controls (Pval<0.05).

Figure 4

Predicted pathogenic BRCA1 amino acid substitutions are confined to the evolutionary conserved N- and C-terminal domains

Schematic representation of the BRCA1 protein with the positions of variants classified as neutral (green) or deleterious (red) indicated. Positive and negative controls are depicted by open pinheads and the known deleterious truncation mutations 185delAG (N-terminal) and 5382insC (C-terminal) are marked in blue. The amino acid sequences of the evolutionary conserved RING (N-terminal) and BRCT (C-terminal) domains are specified to show the exact positions of deleterious (red) and neutral (green) VUS mutants. The zinc finger region of the RING domain is underlined and the amino acid substitutions or nonsense (X) mutations of positive and negative controls are encircled.

Figure 5

BRCA1 sequence variants classified as pathogenic do not restore HR

\textit{R26}^{\text{CreERT2/\textit{hBRCA1};\textit{Brca1}^{\text{Sco/\Delta};\text{Pim1}^{\text{DR-GFP/wt}}}}\text{ES cells carrying the DR-GFP reporter gene in the Pim1 locus and BRCA1 VUS mutants or controls in the Rosa26 locus were switched using 4OHT and transfected with a vector expressing I-SceI and mCherry. Transfected}
cells were analyzed for GFP expression as a measure of HR activity. Expression of 
*BRCA1* wild-type (WT) cDNA resulted in increased HR compared to the empty RMCE 
vector (Vector) control. Error bars indicate the standard deviation between the results of 
three independent I-SceI transfections. Significantly decreased HR activity compared to 
the wild-type control (green line) is indicated.

**Figure 6**

**PARP inhibitor sensitivity assay of BRCA1 sequence variants**

*R26CreERT2/hBRCA1;Brca1Sco/Δ* or *R26CreERT2/RMCE;Brca1Sco/Δ* ES cells carrying *BRCA1* VUS 
mutants or *BRCA1* wild-type (WT) or empty RMCE vector (Vector) controls in the Rosa26 
locus were switched using 4OHT and assayed for sensitivity to cisplatin or the PARP 
inhibitor olaparib. The cytotoxicity assays were performed in parallel and data were 
normalized to the average of the wild-type controls. Error bars indicate the standard 
deviation between the results of biological triplicates for which the cells were 
independently switched. Significant deviation from the average IC₅₀ values of the wild-type 
control (green line) is indicated.
Figure 1

**R26CreERT2/RMCE Brca1SCo/Δ**

- **Brca1<sup>SCo-6</sup>**
  - Puro
  - PGK

- **Brca1<sup>Δ5-13</sup>**
  - 3
  - 14

- **R26CreERT2**
  - SA
  - CreERT2

- **R26RMCE**
  - SA
  - FLP
  - Hyg
  - zsgreen

- **Neo<sup>R</sup> selection**

- **Introduction of human BRCA1 by RMCE**

- **hBRCA1<sup>+</sup>, mBrca1<sup>+</sup>, neo<sup>S</sup>, puro<sup>S</sup>**

---

**Brca1<sup>Δ5-13</sup>**

- 3
  - 14

- **R26CreERT2**
  - SA
  - CreERT2

- **R26RMCE**
  - SA
  - zsgreen
  - Hyg
  - FLP

- **Neo<sup>R</sup> selection**

---

**Cre-mediated deletion of mouse Brca1 by 4OHT addition**

- **Puro<sup>R</sup> selection**

- **hBRCA1<sup>+</sup>, mBrca1<sup>-</sup>, neo<sup>R</sup>, puro<sup>S</sup>**
Introduction of *BRCA1* variants in *R26^{creERT2/RMCE}, Brca1^{Sco/Δ}* ES cells

**Functional assays**

- **SDM and sequencing**
- **RMCE in ES cells**
- **Selection of clones with correct RMCE**
- **hBRCA1 expression analysis**
- **Functional assays**

**Timecourse for the analysis of 20 *BRCA1* variants**

SDM and RMCE → Functional assays

0 1 2 3 4 5 6 7 8 weeks
Figure 3

The graph shows the corrected cisplatin IC50 (a.u.) for various conditions. The conditions include:

- Mutant hBRCA1
- Wild-type hBRCA1
- Positive control
- Empty RMCE vector
- Negative control

The y-axis represents the corrected cisplatin IC50 (a.u.), ranging from -3.0 to 4.0. The x-axis represents the concentration of cisplatin, with values ranging from -3.0 to 3.0.
Figure 4

RING domain

BRCT domains

- Deleterious truncation mutant
- Neutral VUS
- Deleterious VUS
- Neutral control variant
- Deleterious control variant
- Phe(+3)-binding pocket
- Phospherine-binding pocket
- Conserved zinc-binding residues

1 MDLSALRVEEVQNVINAMQKILEYECLEL

31 IKEPVSTKCDHIFFKFLMLKLLNQKKGPSQ

61 QPLKNDTQRSLQESTFSQVLVEELLKII

91 CAFQLDTGLEYANSYNAK 109

1646 VNKRMSMVFGLTEEFMLVYKFARKHHITNLITEEHTVVMMKTDAEFVCTELTLYFL

1706 GIAGGKWVSYFWVTQSIEKRMLENHDFEVRTGGDVNVGNHRQQPKRARESQDRKIFRGE

1766 ICYGPFTNMPTDQLEWMVQLCAGSVKELSSLTGLTVHPVQPDANEDNGFAIG

1826 QMCEAPVVTRWEVLSDVALYQCQLDLTYLIPQPHSHY 1863
Figure 6

IC_{50} relative to WT controls (%)