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The functional impact of variants of uncertain significance in *BRCA2*

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Purpose: Genetic testing has uncovered large numbers of variants in the *BRCA2* gene for which the clinical significance is unclear. Cancer risk prediction of these variants of uncertain significance (VUS) can be improved by reliable assessment of the extent of impairment of the tumor suppressor function(s) of BRCA2.

Methods: Here, we evaluated the performance of the mouse embryonic stem cell (mESC)-based functional assay on an extensive set of *BRCA2* missense variants.

Results: Whereas all 20 nonpathogenic (class 1/2) variants were able to complement the cell lethal phenotype induced by loss of endogenous mouse *Brca2*, only 1 out of 15 pathogenic (class 4/5) variants (p.Gly2609Asp) was able to do so. However, in this variant the major tumor suppressive activity of BRCA2, i.e., homology directed repair (HDR), was severely abrogated. Among 43 evaluated VUS (class 3), 7 were unable to complement the lethal phenotype of mouse Brca2 loss while 7 other variants displayed a more severe reduction of HDR activity than observed for class 1/ 2 variants.

Conclusion: The mESC-based BRCA2 functional assay can reliably determine the functional impact of VUS, distinguish between pathogenic and nonpathogenic variants, and may contribute to improved cancer risk estimation for *BRCA2* VUS carriers.

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INTRODUCTION

Inherited pathogenic variants in *BRCA2* confer an average cumulative risk by age 70 years of about 60% to develop breast cancer in combination with an increased ovarian cancer risk up to 18%.[1,2] In addition, they may increase the risk to develop other cancer types including pancreatic and prostate cancer.[3,4] Clinical management of carriers of pathogenic variants consists of intensified screening programs, the option to undergo prophylactic surgery, and presymptomatic genetic testing of family members.[5] Furthermore, targeted treatment of BRCA1/2-associated tumors has recently become available following the registration of poly ADP ribose polymerase (PARP) inhibitors.[6]

Variants associated with high cancer risk typically disrupt BRCA2 function, but for many variants identified by genetic testing the functional impact cannot be inferred from sequence information alone. Those variants are therefore defined as variants of uncertain significance (VUS)[7] and they represent a major challenge for genetic counselling and clinical management of the families in which they are identified. Depending on the inclusion criteria used for genetic testing these may comprise up to 20% of all identified variants.[7–9] Worldwide, more than 2000 unique *BRCA2* VUS have been identified, including missense and silent substitutions, small in-frame insertions and deletions, and intronic variants.[10,11]

To classify *BRCA2* variants, a prior likelihood of pathogenicity, based on in silico analysis of the sequence alteration, can be combined with the available genetic and epidemiological data (such as family history of cancer, tumor histopathology, cosegregation with disease, and cooccurrence) to calculate the posterior probability that a variant is pathogenic, in a so-called multifactorial likelihood model.[9] Based on these posterior probabilities a 5-tier classification system has been introduced in which each class is associated with specific recommendations for clinical management.[12] Class 1/2 variants have very low posterior probabilities (<0.05) for pathogenicity and are probably benign, whereas class 4/5 variants have very high posterior probabilities (>0.95) for being associated with cancer risk equivalent to classical pathogenic variants that encode a truncated BRCA2 protein. By definition, a VUS falls in class 3. Unfortunately, as the occurrence of a VUS is usually rare, there are often insufficient clinical data to make clinically meaningful

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Table 1 Validation set of *BRCA2* missense variants evaluated in this study

Exon	Nucleotide[a]	Amino acid	Complementation[b]	HDR capacity (%)[c]	Cisplatin sensitivity (%)[d]
Class 1/2 *BRCA2* missense variants					
2	c.53G>A	p.Arg18His	Yes	95	68
3	c.125A>G	p.Tyr42Cys	Yes	72	59
6	c.502C>A	p.Pro168Thr	Yes	115	108
10	c.1114A>C	p.Asn372His	Yes	79	150
12	c.6853A>G	p.Ile2285Val	Yes	60	122
14	c.7057G>C	p.Gly2353Arg	Yes	82	108
15	c.7544C>T	p.Thr2515Ile	Yes	78	70
16	c.7766C>A	p.Pro2589His	Yes	52	127
18	c.8149G>T	p.Ala2717Ser	Yes	85	57
18	c.8187G>T	p.Lys2729Asn	Yes	70	53
19	c.8360G>A	p.Arg2787His	Yes	94	114
20	c.8567A>C	p.Glu2856Ala	Yes	82	63
21	c.8662C>T	p.Arg2888Cys	Yes	52	92
22	c.8851G>A	p.Ala2951Thr	Yes	72	80
22	c.8917C>T	p.Arg2973Cys	Yes	69	51
24	c.9155G>A	p.Arg3052Gln	Yes	50	105
24	c.9235G>A	p.Val3079Ile	Yes	97	48
25	c.9292T>C	p.Tyr3098His	Yes	86	54
26	c.9509A>G	p.Asp3170Gly	Yes	71	105
27	c.10045A>G	p.Thr3349Ala	Yes	102	65
Class 4/5 *BRCA2* missense variants					
2	c.3G>A	p.Met1Ile	No	NA	NA
17	c.7826G>A	p.Gly2609Asp	Yes	28	35
17	c.7878G>C	p.Trp2626Cys	No	NA	NA
17	c.7879A>T	p.Ile2627Phe	No	NA	NA
17	c.7940T>C	p.Leu2647Pro	No	NA	NA
17	c.7958T>C	p.Leu2653Pro	No	NA	NA
18	c.8057T>C	p.Leu2686Pro	No	NA	NA
18	c.8063T>C	p.Leu2688Pro	No	NA	NA
18	c.8165C>G	p.Thr2722Arg	No	NA	NA
18	c.8167G>C	p.Asp2723His	No	NA	NA
18	c.8168A>G	p.Asp2723Gly	No	NA	NA
18	c.8243G>A	p.Gly2748Asp	No	NA	NA
24	c.9154C>T	p.Arg3052Trp	No	NA	NA
25	c.9285C>G	p.Asp3095Glu	No	NA	NA
25	c.9371A>T	p.Asn3124Ile	No	NA	NA

[a]Nucleotide numbering reflects Human Genome Variation Society (HGVS) nomenclature where complementary DNA (cDNA) numbering +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (BRCA2 NM_000059.3). The initiation codon is codon 1

[b]Complementation by *BRCA2* variants of the cell lethal phenotype imposed by Cre-mediated loss of *Brca2* was visualized by methylene blue staining of arising HAT-resistant clones (Fig. 2)

[c]HDR capacity, as measured by the repair of an I-Sce1 induced double strand break (DSB) in the direct repeat green fluorescent protein (DR-GFP) reporter, relative to the HDR levels observed in wild type (WT) BRCA2-expressing cells

[d]Cisplatin sensitivity of BRCA2 variants normalized to the average sensitivity of WT BRCA2-expressing cells (IC_{50} values relative to the IC_{50} of WT controls presented in percentages)

NA not applicable since no clones were formed after loss of the conditional *Brca2* allele, *HDR* homology directed repair

inferences about their associated cancer risks and therefore it has been difficult to move a VUS into either class 1/2 or class 4/5 on the basis of clinical data. Information on protein conformation or function can serve as independent classifiers of VUS.[13–17] Quantitative functional data transformed into likelihood ratios might improve the performance of predictive models when used in combination with available genetic and epidemiological data. BRCA2 plays a major role in the DNA damage response (DDR), the network of interacting pathways that together execute the response upon DNA damage. Disruption of BRCA2 leads to a deficiency in homologous recombination and consequently sensitivity to DNA damaging agents that induce replication fork stalling and DNA double strand breaks (DSBs).[18] Various assays have been

Table 2 Selection of *BRCA2* VUS evaluated in this study

Exon	Nucleotide[a]	Amino acid	Complementation[b]	HDR capacity (%)[c]	Cisplatin sensitivity (%)[d]
3	c.73G>A	p.Gly25Arg	Yes	65	66
3	c.93G>T	p.Trp31Cys	Poor	24	39
3	c.165_167del	p.Asn56del	Yes	70	84
7	c.599C>T	p.Thr200Ile	Yes	98	150
10	c.1769T>G	p.Phe590Cys	Yes	79	106
11	c.4301A>T	p.Lys1434Ile	Yes	83	124
11	c.5733_5735del	p.Asp1911del	Yes	85	110
12	c.6867A>T	p.Leu2289Phe	Yes	125	123
12	c.6935A>T	p.Asp2312Val	Yes	78	46
15	c.7484T>C	p.Ile2495Thr	Yes	58	84
15	c.7522G>A	p.Gly2508Ser	Yes	50	37
15	c.7547C>G	p.Ser2516Cys	Yes	87	113
16	c.7751G>A	p.Gly2584Asp	Yes	70	65
16	c.7753G>A	p.Gly2585Arg	No	NA	NA
17	c.7875A>T	p.Arg2625Ser	No	NA	NA
17	c.7928C>G	p.Ala2643Gly	Yes	121	102
17	c.7954G>A	p.Val2652Met	Poor	45	39
18	c.7978T>G	p.Tyr2660Asp	No	NA	NA
18	c.8162T>A	p.Leu2721His	No	NA	NA
18	c.8111C>T	p.Ser2704Phe	Yes	116	45
18	c.8249_8251del	p.Lys2750del	No	NA	NA
19	c.8350C>T	p.Arg2784Trp	Poor	15	51
19	c.8351G>A	p.Arg2784Gln	Poor	15	36
19	c.8435G>A	p.Gly2812Glu	Yes	50	61
20	c.8524C>T	p.Arg2842Cys	Yes	31	107
20	c.8525G>T	p.Arg2842Leu	Yes	53	43
20	c.8552C>T	p.Ala2852Val	Yes	65	86
21	c.8738A>G	p.Asp2913Gly	Yes	46	94
21	c.8739C>G	p.Asp2913Glu	Yes	80	80
22	c.8897T>C	p.Val2966Ala	Yes	62	65
23	c.9004G>A	p.Glu3002Lys	No	NA	NA
23	c.9104A>C	p.Tyr3035Ser	Yes	50	52
23	c.9116C>G	p.Pro3039Arg	Yes	73	43
24	c.9218A>G	p.Asp3073Gly	No	NA	NA
25	c.9275A>C	p.Tyr3092Ser	Yes	39	36
27	c.9838C>T	p.Pro3280Ser	Yes	125	65
27	c.9839C>G	p.Pro3280Arg	Yes	86	70
27	c.9871T>G	p.Ser3291Ala	Yes	66	88
27	c.9872C>G	p.Ser3291Cys	Yes	81	90
27	c.9925G>T	p.Glu3309Ter	Yes	75	145
27	c.9945del	p.Glu3316Asn-fs*2	Yes	63	61
27	c.9976A>T	p.Lys3326Ter	Yes	104	44
27	c.10184del	p.Glu3395Gly-fs*32	Yes	67	64

[a]Nucleotide numbering reflects Human Genome Variation Society (HGVS) nomenclature where complementary DNA (cDNA) numbering +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (BRCA2 NM_000059.3). The initiation codon is codon 1

[b]Complementation by *BRCA2* variants of the cell lethal phenotype imposed by Cre-mediated loss of *Brca2* was visualized by methylene blue staining of arising HAT-resistant clones (Fig. 2)

[c]HDR capacity, as measured by the repair of I-Sce1 induced double strand breaks (DSBs) in the DR-GFP reporter, relative to the HDR levels observed in wild type (WT) BRCA2-expressing cells

[d]Cisplatin sensitivity of *BRCA2* variants normalized to the average sensitivity of WT BRCA2-expressing cells (IC_{50} values relative to the IC50 of WT controls presented in percentages)

NA not applicable because no clones were formed after loss of the conditional *Brca2* allele, *fs* frameshift, *HDR* homology directed repair, *VUS* variant of uncertain significance

developed to assess the functional impact of variants in *BRCA2*[19] which are able to accurately discriminate between nonpathogenic (class 1/2) and pathogenic variants (class 4/5).

A mouse embryonic stem cell (mESC)-based assay was developed for the functional assessment of human *BRCA2* variants.[20,21] This mESC-based system involves the introduction of human *BRCA2* into a hemizygous mouse *Brca2* mESC line and allows evaluation of the functional consequences of any *BRCA2* variant, including those located in either exonic or intronic sequences that may affect RNA splicing. Variants are being assessed for their ability to complement the loss of cell viability following Cre-mediated deletion of a conditional *Brca2* allele. Nonfunctional protein variants are unable to overcome *Brca2* loss while variants resulting in (partially) functional BRCA2 protein are able to complement cell lethality and can be assessed for their capacity to perform BRCA2 functions.[20]

An extensive validation was performed to determine the sensitivity and specificity of the assay. To this end, we evaluated its ability to correctly discriminate between pathogenic (class 4/5 [$n = 15$]) and nonpathogenic (class 1/2 [$n = 20$]) variants that had previously been classified on the basis of genetic and clinical data. In addition, we assessed its performance on variants characterized in previously published functional studies [14,17,20,22] ($n = 20$) and determined the functional impact of 23 VUS identified in the clinic.

MATERIALS AND METHODS

Selection of classified missense variants in *BRCA2*

To establish the sensitivity and specificity of the BRCA2 mESC-based assay, we selected all known pathogenic missense variants (class 4/5 [$n = 15$]) and a similarly sized set of nonpathogenic missense variants (class 1/2 [$n = 20$]) in *BRCA2* for which the clinical significance has been established on the basis of clinical and genetic data (Table 1).[19]

Selection of VUS in *BRCA2*

Functional analysis was performed for a set of VUS ($n = 43$) consisting of 3 variants that previously have been tested in a mESC-based system[20,22] and 17 variants characterized by a complementary DNA (cDNA)-based functional assay in V-C8 Chinese hamster cells.[14,17]

In addition, we included 23 variants that have been identified in counselees visiting one of the Dutch Clinical Genetic Centers and who were eligible for genetic testing according to the Dutch guidelines (Table 2). The majority of the variants were missense or single amino acid deletion variants. In silico splice site prediction analysis was performed for all variants using five different algorithms.[23] With the exception of c.6935A>T (p.Asp2312Val), which is predicted to lead to reduced donor site recognition of exon 12 by two programs, an effect on RNA splicing for the other variants is unlikely.

Generation of variants in the human *BRCA2* gene

A library of pUC19 plasmids each containing a single *BRCA2* exon and about 100 nucleotides of intron sequence upstream and downstream of the exon was generated to enable introduction of variants (QuickChange site-directed mutagenesis from Stratagene). Following sequence confirmation, homology arms were added to the exon containing the variant by polymerase chain reaction (PCR). Subsequently, Red/ET recombineering was used in combination with a positive/negative selection procedure to introduce the variant into a bacterial artificial chromosome (BAC) (clone RP11-777I19, BACPAC) containing the full length human *BRCA2* gene as described previously.[21]

Cell culture

mESC culture was performed as described previously.[21] Prior to transfections, protein/RNA isolations, and biological assays, cells were cultured on gelatin-coated plates using buffalo rat liver cell (BRL)-conditioned mESC medium.

Insertion of the DR-GFP construct in mouse embryonic stem cells

Insertion of the pX59 direct repeat green fluorescent protein (DR-GFP) construct at the *Pim1* locus in the *Brca2* $^{-/loxP}$ mESC line[21] was performed as described.[24] Integration of the construct was confirmed by Southern blot analysis and via PCR using the following primers:

Pim1Ex1F: 5′-AAGATCAACTCCCTG GCCCACCTGCG-3′, Pim1Ex4R 5′-TGTTCTCGTCCTTGATGTCG-3, and Hyg3A 5′-CCGCTCGTCTGGCTA AGAT-3′ (Figure S1).

Generation of mouse embryonic stem cells expressing *BRCA2* variants

BACs carrying *BRCA2* variants were transfected into the Pl2F7 conditional *Brca2* knockout mESC line containing the DR-GFP construct (*Brca2*$^{-/loxP}$; *Pim1*$^{DR\text{-}GFP/WT}$). This mESC line contains one conditional endogenous *Brca2* allele with a *loxP* site on either side of the complete *Brca2* locus and one disrupted endogenous *Brca2* allele in which exon 11 has been disrupted (Figure S2). Per variant 1.5×10^6 cells were transfected in suspension with 4 µg BAC DNA using Lipofectamine 2000 (Invitrogen). Transfected cells were subsequently split over two 60-mm cell culture dishes and cultured in the presence of G418 (200 µg/ml), starting 24 h post transfection. Ten days post transfection G418 resistant clones of each 60-mm dish were pooled (at least 50 clones) providing two independent polyclonal cell populations (PCPs).

RT-PCR and western blot

BRCA2 expression was determined in two independent PCP per variant by reverse transcription PCR (RT-PCR) and western blot analysis (Figure S3).[21] Western blot analysis was performed using NuPAGE™ Novex™ 3–8% Tris-Acetate Protein Gels (ThermoFisher Scientific). BRCA2 protein was detected with the rabbit polyclonal antibody (BETHYL, A303–434A-T-1) recognizing the region between amino acids 450–500 of human BRCA2. Protein signal was detected by electrochemiluminescence (Amersham ECL

RPN2235 Biocompare) and quantified using ImageQuant TL software.

Functional complementation of mouse embryonic stem cells expressing *BRCA2* variants

The conditional *Brca2* allele was removed following transient transfection with a Cre-expressing plasmid (pCAG-Cre:GFP from Addgene). Recombination between the loxP sites restores the *HPRT1* minigene and allows cells to grow in the presence of hypoxanthine–aminopterin–thymidine (HAT).[20] For each variant two independent PCPs of BAC clones were used for transfection. Per PCP 1.2×10^6 cells were transfected with 4 µg plasmid DNA in suspension using Lipofectamine 2000 (Invitrogen) and subsequently seeded in triplo on 90-mm cell culture dishes. The transfection efficiency was determined by monitoring GFP expression 16 h after transfection. Cells were cultured for 6 days in the presence of HAT and subsequently 5 days in the presence of hypoxanthine-thymidine (HT). Thirteen days after transfection one culture dish was used for visualization of clones by methylene blue staining. Clones arising on the remaining two culture dishes were pooled. This procedure allows downstream functional analysis of two independent PCPs per variant (Figure S4).

Cell cycle analysis

Per variant, two PCPs of HAT-resistant clones were subjected to functional analysis in the HDR assay and survival assays. In parallel 15×10^3 cells were seeded per well in 96-well plates to detect potential effects of the variant on cell cycle progression. Two days after seeding cells were fixed in ice cold ethanol (70%) and subsequently DNA was stained with propidium iodide (50 µg/ml) in the presence of RNaseA (0.1 mg/ml) and Triton X-100 (0.05%). The fluorescence intensity was measured by flow cytometry (Guava, Millipore) and used to determine cell cycle phase distribution (Flowing software 2). Cell cycle analysis was performed in duplo for each PCP (50,000 cells), resulting in four cell cycle measurements per variant.

HDR assay

BRCA2-expressing cells were seeded at a density of 15×10^3 cells per well on gelatin-coated 96-well plates in BRL-conditioned mES cell culture medium. On the next day, cells were transfected with an I-Sce1-mCherry plasmid[26] using Lipofectamine 2000. Three days after transfection the fraction of mCherry/GFP double-positive cells was determined by flow cytometry (Guava, Millipore). For each variant the proportion of GFP-positive cells was calculated relative to wild type (WT) *BRCA2*. HDR measurements were performed in triplo for each independent PCP resulting in six data points per variant.

Sensitivity assays with cisplatin and PARP inhibitor treatment

BRCA2-expressing cells were seeded at a density of 30×10^3 cells per well on gelatin-coated 96-well plates to measure the sensitivity of *BRCA2* variants to the DNA crosslinking agent cisplatin (Accord) and the PARP inhibitor KU-0058948 (Axon Medchem). Cells were treated in triplo with the following concentrations of cisplatin: 0 µM (1% phosphate-buffered saline [PBS]), 0.16 µM, 0.31 µM, 0.63 µM, 1.25 µM, and 2.5 µM. Applied concentrations of PARP inhibitor were 0 nM (1% dimethyl sulfoxide [DMSO]), 8 nM, 16 nM, 31 nM, 62.5 nM, and 125 nM. Cell counts were determined after 48 h of continuous exposure by flow cytometry (Guava, Millipore). Survival was calculated by dividing the number of surviving cells in treated samples by the number of untreated cells of the same variant. The IC50 values plotted for the cisplatin survival assays were calculated as previously described using the *drc* package in the R programming language.[26,27]

RESULTS

The widespread use of *BRCA1/2* pathogenic variant testing in individuals with a family history of breast or ovarian cancer has uncovered large numbers of VUS. Classification of a VUS as either class 1/2 or class 4/5 may be improved when the impact of a VUS on BRCA2 protein function can be taken into consideration. To this end, a semihigh-throughput assay with robust quantitative read-outs is needed. We therefore further optimized the mESC-based BRCA2 functional assay[20,21] (Fig. S2). Here, we validated its performance on a large series of pathogenic and nonpathogenic missense variants. The specificity and sensitivity of the optimized mESC system was determined by assessing the functional impact of a panel of class 1/2 and class 4/5 missense variants associated with respectively a low and high posterior probability of pathogenicity (Table 1, Fig. 1). In addition, we included an extensive series of *BRCA2* VUS to evaluate their effect on protein function (Table 2, Fig. 1).

Cell cycle analysis

HDR is selectively used for the repair of DSBs in the S and G2 phase of the cell cycle when a sister chromatid is available to serve as donor for homologous DNA sequences.[28,29] To exclude the possibility that a reduction in HDR activity observed for a *BRCA2* variant is the consequence of a smaller fraction of cycling cells, we performed cell cycle distribution analysis of all *BRCA2* variants by propidium iodide staining (Fig. S5), and observed no significant effect on cell cycle phase distribution among the nondeleterious *BRCA2* variants.

Survival and HDR activity of class 1/2 and class 4/5 *BRCA2* variants

Functional BRCA2 is essential for cell survival and normal embryonic development in mice.[30,31] We first tested the ability of 15 class 4/5 variants to rescue the lethal cell phenotype of loss of Brca2 function after Cre-mediated removal of the conditional *Brca2* allele. Fourteen of these variants were unable to form HAT-resistant clones in the absence of functional Brca2 (Table 1, Fig. 2), indicating that BRCA2 function was severely affected. Only the class 4 variant p.Gly2609Asp allowed outgrowth of a reduced

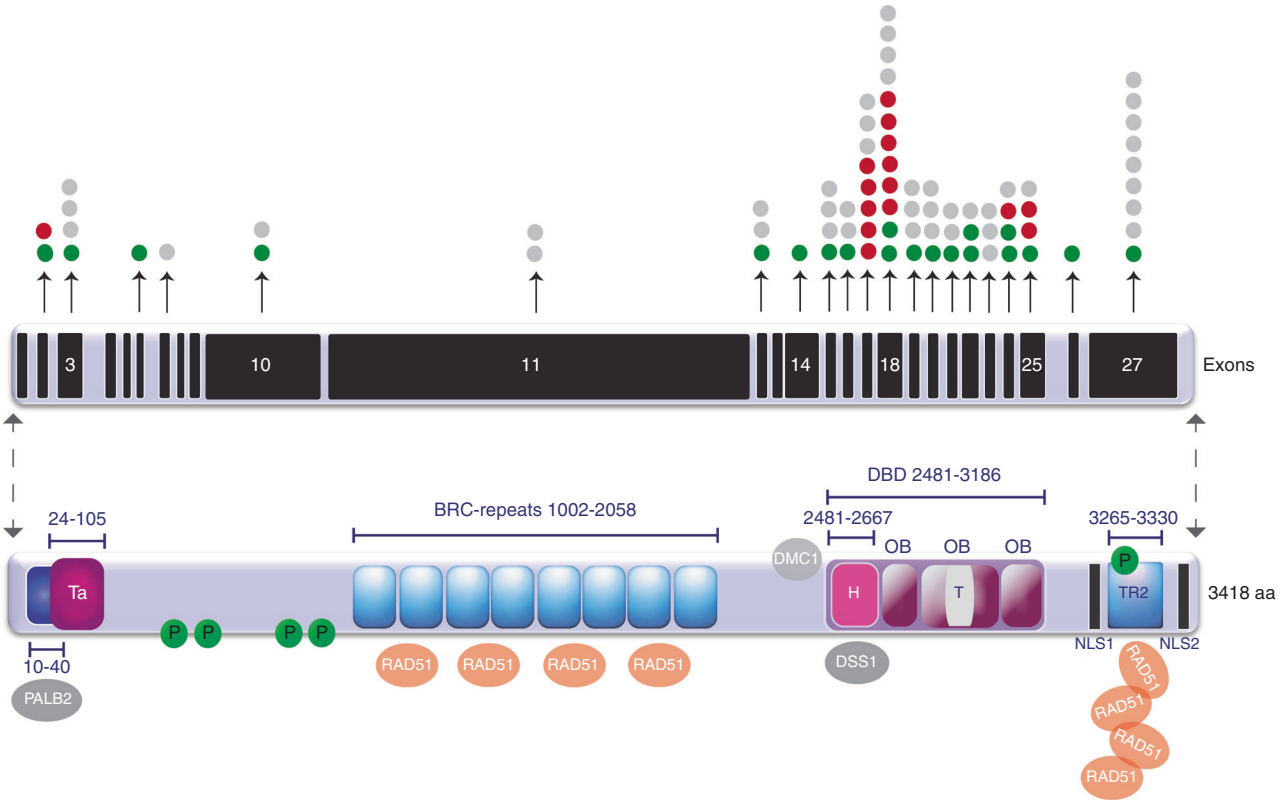

Fig. 1 Position of *BRCA2* variants. Schematic representation of the position of class 1/2 variants (green dots), class 4/5 variants (red dots), and *BRCA2* variants of uncertain significance (VUS) (gray dots) in the *BRCA2* gene. *Ta* transcriptional activation domain, *P* phosphorylation site, *H* helical domain, *DBD* DNA binding domain, *OB* oligonucleotide binding fold, *T* Tower domain, *TR2* C-terminal RAD51 binding site, *NLS* nuclear localization signal, *aa* amino acids[19,39,40]

number of HAT-resistant clones compared with WT BRCA2-expressing cells (Fig. 2). In contrast, all 20 class 1/2 variants were able to fully complement loss of endogenous *Brca2* (Table 1, Fig. 2).

Next, we assessed the capacity of surviving variants (i.e., all class 1/2 variants and class 4 variant p.Gly2609Asp) to use HDR for the repair of an I-Sce1-induced DSB in a DR-GFP reporter construct integrated at the *Pim1* locus.[25] HDR activity of class 1/2 variants relative to WT BRCA2 ranged between 50 and 120% (Fig. 3a), with the strongest reduction observed for variants p.Pro2589His, p.Arg2888Cys, and p.Arg3052Gln. In contrast, the class 4 variant p.Gly2609Asp displayed a more severe functional impairment of BRCA2 function with an HDR reduction of 72%. Based on the results obtained with the validation set, variants with an HDR level higher than 50% can be classified as class 1/2, whereas class 4/5 variants either are unable to rescue the lethal cell phenotype of *Brca2* loss or display an HDR level below 30% of WT *BRCA2*. Accordingly, both the sensitivity and specificity of the assay were estimated at 100% (95% confidence interval 78–100 and 83–100% respectively).

Survival and HDR activity of *BRCA2* VUS

Given the limited number of class 4/5 missense variants, we expanded our analysis with VUS that had previously displayed an impairment of BRCA2 function in other studies ($n = 20$) (refs. [17,19,22]) including 2 VUS significantly associated with an intermediate risk of breast cancer (Fig. S8).[32] In addition, we evaluated the functional consequences of 23 VUS (with focus on missense and single amino acid deletion variants) that had been identified by genetic screening (Table 2). The complementation phenotype of 7 of the 43 VUS tested, i.e., variants p.Gly2585Arg, p.Arg2625Ser, p.Tyr2660Asp, p.Leu2721His, p.Lys2750del, p.Glu3002Lys, and p.Asp3073Gly, resembled that of class 4/5 variants with respect to their inability to rescue the cell lethality imposed by Cre-mediated loss of *Brca2* (Fig. 2, Table 2). In the case of variants p.Trp31Cys, p.Val2652Met, p.Arg2784Trp, and p.Arg2784Gln, only small numbers of HAT-resistant clones arose after removal of the conditional *Brca2* allele indicating that BRCA2 function is compromised in these variants resulting in incomplete complementation (Fig. 2, Table 2).

The ability to perform HDR was evaluated for the 36 *BRCA2* VUS that were able to complement loss of the conditional *Brca2* allele (Fig. 3b). HDR levels varied widely between *BRCA2* VUS and a subset of variants revealed a more severe reduction in HDR activity than observed among the class 1/2 variants. Variants p.Val2652Met, p.Arg2842Cys, p.Asp2913Gly, and p.Tyr3092Ser showed a more than 50% reduction in HDR capacity compared with WT BRCA2-

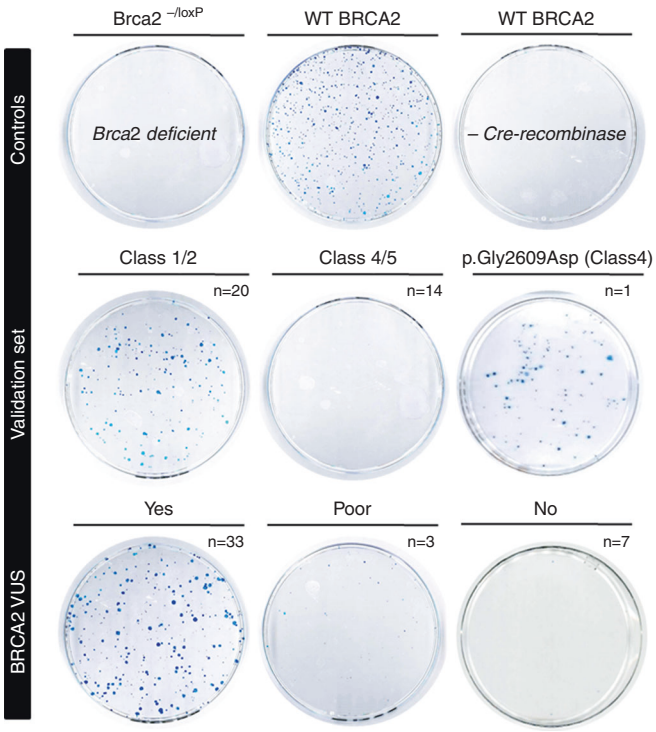

Fig. 2 Representative images of complementation phenotypes and controls. *Brca2*$^{-/loxP}$*Pim1*$^{DR\text{-}GFP/WT}$ cells expressing WT BRCA2 or BRCA2 variants were transfected with a Cre-GFP expression plasmid to induce loss of the conditional *Brca2* allele and restore the *Hprt* gene. Upon Cre-recombinase expression cells become *Brca2* deficient, which is lethal unless complemented by the expression of a (partially) functional BRCA2 variant. Untransfected cells that still contain the conditional *Brca2* allele lack *Hprt* expression and will subsequently not survive HAT selection as shown in the –Cre-recombinase control. Thirteen days post Cre-GFP transfection culture dishes were stained with methylene blue

expressing cells, while a 70–80% reduction was detected for variants p.Trp31Cys, p.Arg2784Trp, and p.Arg2784Gln.

We wondered to what extent our HDR measurements were similar to those previously obtained with a cDNA-based assay in *Brca2*- deficient V-C8 cells (Fig. S6).[14,17] Linear regression analysis on HDR data of 24 viable variants analyzed with both methods (11 class 1/2 variants, 1 class 4/5 variant, and 12 VUS) indicated a high level of concordance ($R^2 = 0.71$). Interestingly, using the linear equation from the regression analysis, extrapolation of the HDR activity for 19 BRCA2 variants (14 class 4/5 and 5 VUS) that were unable to complement loss of *Brca2* showed it to range between 7 and 26% (Fig. S6b). Thus, a residual HDR activity of at least 25% of WT appears to be required to allow variants to overcome *Brca*2 loss-induced cell lethality in mESC.

Sensitivity to cisplatin and PARP inhibitor

Due to the pivotal role of BRCA2 in the repair of DSBs, the absence of functional BRCA2 protein will render cells vulnerable to compounds that introduce toxic DNA lesions that impede cellular processes such as transcription and replication.[33] The crosslinking agent cisplatin, a clinically relevant chemotherapeutic for BRCA1/2-related tumors, was used for cell survival measurements in all variants. Analogous to the wide range of HDR activity detected, half-maximal inhibitory concentrations (IC50) of cisplatin varied considerably among class 1/2 variants (Fig. S7a, Table 1). Although IC50 values in eight class1/2 variants were lower than in WT BRCA2-expressing cells, class 4 variant p.Gly2609Asp displayed the largest increase in cisplatin sensitivity with a more than twofold reduced IC50 value. The low IC50 values calculated for variants p.Trp31Cys, p.Gly2508Ser, p.Val2652Met, p.Ser2704Phe, p.Arg2784Gln, p.Arg2842Leu, p.Pro3039Arg, p.Tyr3092Ser, and p.Lys3326Ter indicate hypersensitivity toward cisplatin treatment (Fig. S7b, Table 2).

The therapeutic strategy of PARP inhibition is a relatively novel approach to exploit the absence of efficient HDR. Clinical trials have revealed promising results for the use of PARP inhibitors in BRCA-associated breast and ovarian cancers.[34–36] It is, however, unclear to what extent partial impairment of HDR activity sensitizes cells to inhibition of PARP. We treated cells expressing *BRCA2* variants with different concentrations of a PARP inhibitor (KU-0058948) for 48 h and measured cell survival by flow cytometry. Neither the class 1/2 variants nor the class 4 variant p.Gly2609Asp displayed increased sensitivity towards PARP inhibitor treatment (Fig. S9). Apart from p.Trp31Cys and p.Arg2784Gln, none of the VUS, including variants with severely compromised HDR activity (>55% reduction), displayed increased sensitivity towards PARP inhibitor treatment (Fig. S9).

DISCUSSION

In this study, we determined the sensitivity and specificity of the BRCA2 mESC-based assay using a validation panel of classified *BRCA2* missense variants. The functional impact of variants associated with a high posterior probability (class 4/5) was distinctly different from that of variants with a low posterior probability (class 1/2) of pathogenicity. All class 1/2 variants ($n = 20$) complemented the lethal phenotype of *Brca2* deficient mESC and displayed capacities to repair an I-Sce1 induced DSB via HDR between 50 and 120% of WT BRCA2 activity. In contrast, 14 of 15 class 4/5 variants were unable to rescue *Brca2* loss-induced cell lethality. Although class 4 variant p.Gly2609Asp was able to complement loss of *Brca2* this variant displayed a severe reduction in HDR activity (28% of WT). Variant classification on the basis of complementation phenotype and level of HDR activity allows a clear separation of class 1/2 (HDR >50%) and class 4/5 (no complementation or HDR <30%) variants with 100% sensitivity and specificity.

Comparison of our HDR data with HDR measurements performed in *Brca2*- deficient V-C8 cells indicated them to be highly correlated ($R^2 = 0.71$).[14,17] In these studies an HDR cut-off of >95% probability of pathogenicity has been established at a level that is equivalent to a residual HDR activity of 30% in our measurements (Fig. S6b), in concordance with the HDR level (28%) that we obtained for class 4 variant p.Gly2609Asp.

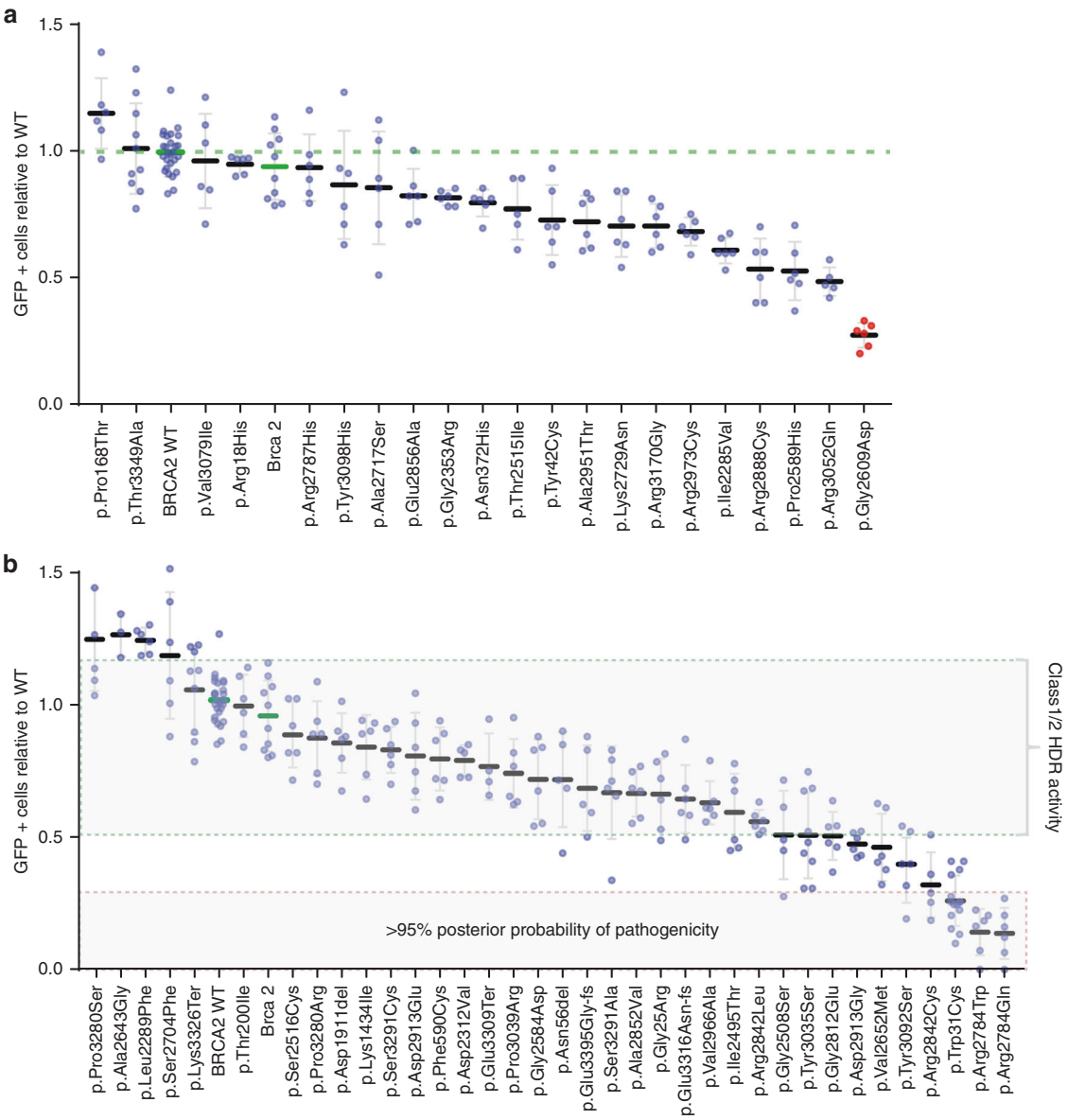

Fig. 3 Homology directed repair (HDR) activity of BRCA2 variants relative to wild type (WT) BRCA2 activity. GFP signal was measured in I-Sce1 expressing cells 2 days post transfection by flow cytometry for (**a**) classified *BRCA2* missense variants and (**b**) *BRCA2* variants of uncertain significance (VUS). Relative HDR activity is expressed as the ratio between the percentage of GFP-positive cells observed in *BRCA2* variant expressing cells and the percentage of GFP-positive cells in WT BRCA2-expressing cells (green line). Brca2 (green bar) represents the conditional *Brca2*$^{-/loxP}$*Pim1*$^{DR\text{-}GFP/WT}$ cell line expressing endogenous Brca2. The upper gray box represents the HDR range of class 1/2 *BRCA2* variants. The lower gray box represents the HDR range associated with >95% probability of pathogenicity as reported by Guidugli et al.[17] (see also Fig. S6b). The black bars correspond to the mean HDR activity and error bars indicate the SD of at least six independent GFP measurements per variant as represented by the dots (purple = class 1/2, red = class 4/5)

Among the 23 VUS identified by genetic screening in the Netherlands, two variants (p.Arg2625Ser and p.Lys2750del,) did not rescue loss-of-*Brca2*-mediated cell lethality, strongly indicative for their pathogenicity. However, clinical and genetic information is required to confirm their association with high cancer risk. Based on the level of HDR activity, variant p.Asp2913Gly is characterized as a hypomorphic variant (HDR between 30 and 50%), while the other VUS displayed HDR activities (>50%) similar to nonpathogenic class 1/2 variants. The question remains whether hypomorphic variants (in this study defined by HDR activity between 30 and 50% of WT) are associated with a moderate risk to develop breast cancer. A recent case-control study[32] showed an inverse correlation between breast cancer risk and HDR activity for four *BRCA2* missense variants (Fig. S8).

The amino acid changes that either lead to the inability to complement *Brca2* loss or that severely impair HDR activity cluster almost exclusively in the DNA binding domain of the protein (residues 2481–3186) (Fig. S10). The only exception is

variant p.Trp31Cys, located in exon 3 encoding the PALB2 interaction domain, in agreement with previous studies.[22,37]

Defective HDR has been shown to sensitize cells to treatment with agents that induce DNA DSB. Indeed, in general attenuation of the HDR pathway sensitized cells to cisplatin-induced crosslinks in the DNA. Only variants p.Arg2842Cys and p.Asp2913Gly (>50% HDR reduction) showed no increased sensitivity to cisplatin. In contrast, impairment of HDR only occasionally resulted in sensitivity to treatment with a PARP inhibitor. Only variant p.Trp31Cys, and to a lesser extent p.Arg2784Gln, showed substantial sensitivity in this context.

In this study we performed experiments to assess the functional impact of *BRCA2* variants on HDR, which is thought to be the most prominent tumor suppressor function of BRCA2. However, BRCA2 has been suggested to be involved in additional genome maintenance processes like centrosome amplification and replication fork stabilization, which might also be important for tumor suppression.[18,38] For this reason we formally cannot exclude that carriers of variants that do not show an effect on HDR activity have no elevated cancer risk. Future studies have to demonstrate whether HDR independent functions of BRCA2 are relevant for tumor suppression.

In conclusion, the functional analysis described here provides insight into the functional consequences of *BRCA2* variants and will be applied to a much broader spectrum of variants, including those that might influence RNA splicing. In the mESC assay, the functional impact of variant-associated alternative splicing can be analyzed in a mono-allelic manner, which is currently impossible using other methods. Future work in collaborative studies within the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) Consortium will be focused on the translation of functional results into associated cancer risk. The ultimate aim is to incorporate the functional read-outs into a multifactorial model in which the functional and clinical data on a specific VUS are merged to arrive at a decisive posterior probability of pathogenicity. These efforts will enable accurate cancer risk estimation and support clinical decision making in the absence of sufficient genetic data.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1038/s41436-018-0052-2) contains supplementary material, which is available to authorized users.

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AUTHOR CONTRIBUTIONS

MV and HV designed and supervised the project. RM, FC, and BMo were responsible for data acquisition and RM analysed the results. HV, MV, PD, CA, and RM were involved in data interpretation. BMi performed the bioinformatics analysis. RM, MV, and HV wrote the manuscript and CA, GH, and PD revised the manuscript. All authors read and approved the final manuscript.

DISCLOSURE

The authors declare no conflicts of interest.

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