Functional Evaluation and Cancer Risk Assessment of BRCA2 Unclassified Variants

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Abstract

The influence of germ line BRCA2 unclassified variants (UCV), including missense mutations and in-frame deletions and insertions on BRCA2 function and on cancer risk, has not been defined although these mutations account for 43% of all identified BRCA2 sequence alterations. To investigate the effects of UCVs on BRCA2 function, we compared mutant and wild-type forms of BRCA2 using assays of cellular survival and viability, homologous recombination repair, and genome instability. We confirm that the effects of known deleterious mutations can be distinguished from neutral polymorphisms and wild-type BRCA2 in these assays, and we characterize the influence of a series of UCVs on BRCA2 function. We also describe how the results from the assays can be combined with data from analysis of cosegregation of the UCVs with cancer, co-occurrence of the UCVs with other deleterious mutations, and interspecies sequence variation in a comprehensive framework in an effort to better distinguish between disease predisposing and neutral UCVs. This combined approach represents a useful means of addressing the functional significance and cancer relevance of UCVs in BRCA2. (Cancer Res 2005; 65(2): 417-26)

Introduction

Germ line mutations in the BRCA2 gene on chromosome 13q12-13 (1) that truncate the BRCA2 protein are associated with a 60% to 85% lifetime risk of breast cancer, a 15% to 30% lifetime risk of ovarian cancer (2), and predisposition to pancreatic cancer (3). These mutations are readily classified as cancer predisposing because they uniformly truncate BRCA2 prior to the COOH-terminal nuclear localization signals (4), resulting in exclusion of the mutant proteins from the nucleus and inactivation of all associated nuclear functions. However, 43% of all sequence alterations that have been detected in the BRCA2 gene during clinical mutation screening, excluding common polymorphisms, do not truncate the encoded protein (5). These unclassified variants (UCV) are predominantly missense mutations and in-frame deletions and have been detected in 13% of women undergoing clinical screening for mutations (5). Indeed, >800 unique BRCA2 UCVs have been described (Breast Cancer Information Core, http://research.nhgri.nih.gov/bic/). To date, it has proved difficult to discriminate the disease predisposing/causing UCVs from the neutral/benign UCVs. This inability to determine which mutations are disease causing has led to significant problems in risk evaluation, counseling, and preventative care of thousands of carriers of these variants.

A number of methods for discriminating deleterious/high-risk from neutral/low-risk UCVs in BRCA2 and other genes have been proposed. Analysis of cosegregation of the UCVs with cancer in families (6) is a powerful approach based on the cancer phenotype. Approaches based on sequence conservation and the physicochemical properties of amino acid changes (7, 8) have also been used. Similarly, functional studies that measure the influence of mutations on the wild-type activity of a protein, such as ATM or BRCA1, in assays based on the known functions of the protein have been used with some success (9–11). Whereas all of these approaches have limitations, the combination of these approaches is likely to provide some insight into which missense mutations are cancer causing (6).

Importantly, little has been done in terms of functional analysis of BRCA2 missense mutations. Although the function of BRCA2 is not fully defined at the biochemical level, there is strong support for a role in DNA damage repair and in maintenance of genomic integrity (12). Specifically, BRCA2 binds to the Rad51 DNA recombination enzyme and regulates formation of the rad51 nucleoprotein filament that is required for homologous recombination repair of DNA damage (13, 14). Indeed, BRCA2 null cells show a reduced efficiency of homologous recombination mediated double-strand break repair (15), and brca2 null or mutant cells exhibit hypersensitivity to DNA damage (15–24). BRCA2 also seems to have a distinct role in mediation of chromosomal integrity, as evidenced by its association with BRF35 chromatin complexes during chromosome condensation (25), and the observation that brca2 null or mutant cells display a high frequency of broken and deformed chromosomes, micronuclei, and centrosome amplification (18, 20, 23, 24, 26).

Here we describe for the first time, the evaluation of the effects of BRCA2 UCVMs on BRCA2 function using a series of in vitro assays based on these known activities of BRCA2. We show that the assays can clearly discriminate between known deleterious mutations and neutral variants/polymorphisms. In addition, we report on the evaluation of a number of UCVs and the finding that certain UCVs alter BRCA2 function. Furthermore, we combine the results from these assays with data from a disease causality prediction algorithm for the UCVs that includes genetic segregation data from high-risk breast cancer families, observations of co-occurrence of UCVs with other deleterious mutations, and interspecies sequence variation (6), to confirm that the results from the functional analysis of BRCA2 UCVMs are consistent with this “genetic” data.

Materials and Methods

Mutation Frequency. DNA from 476 controls with no personal or family history of cancer were genotyped for each of 11 BRCA2 mutations by dHPLC analysis. Briefly, exons containing the relevant mutations were
PCR amplified using intronic primers, products were denatured and heteroduplexed, and evaluated for the presence of the mutations by dHPLC using product-specific melting and solvent conditions. Details are available from the authors.

**Site-Directed Mutagenesis.** Nucleotide changes were incorporated into five partial *BRCA2* cDNA pCR3.1 and pcDNA3.1 subclones, defined by unique restriction enzyme sites in the *BRCA2* cDNA, using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primer sequences and conditions are available from the authors. Mutant partial cDNA fragments were subcloned into the green fluorescent protein (GFP)-tagged full-length *BRCA2* cDNA in the EGFP-C2 plasmid and the untagged *BRCA2* cDNA in pCR3.1. The presence of the mutations was confirmed by sequencing and the stability of the constructs was established by a series of restriction enzyme digestions (data not shown).

**Cell Culture, Transfection, and Stable Cells.** *BRCA2*-deficient VC8 cells (23) were maintained in DMEM-F12 (BioWhittaker, Walkersville, MD) supplemented with 10% bovine calf serum (HyClone, Logan, UT), 2 mmol/L l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. 293T and 293 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM (Life Technologies, Gaithersburg, MD) with 10% bovine calf serum. Transfections were done using FuGENE-6 reagents according to the manufacturer's protocol (Roche, Indianapolis, IN). VC8 *BRCA2* stable cells were selected with G418 (400 μg/mL).

**Immunoprecipitation and Immunoblotting.** Cell lysates were prepared, *BRCA2* protein was immunoprecipitated with anti-BRCA2 Ab-1 antibody (Oncogene Research, Boston, MA), and *BRCA2* protein was immunoblotted using the anti-BRCA2 Ab-2 antibody (Oncogene Research) as described previously (27).

**Immunofluorescence and Confocal Microscopy.** Cells were cultured on glass coverslips and transfected with GFP-BRCA2 constructs. For subcellular localization experiments, the cells were fixed with cold methanol, stained with 1 μg/mL Hoechst (Molecular Probes, Eugene, OR), and imaged 48 hours post-transfection. For indirect immunofluorescence, cells were fixed, permeabilized, and stained with anti-pericentrin (1:800) antibodies, Texas red goat anti-rabbit antibodies (1:800), and Hoechst 4 days after transfection (28). Centrosome numbers were counted in ~300 total cells from two independent experiments. All images were acquired with a Zeiss LSM510 confocal microscope.

**Clonogenic Survival and Trypan Blue Viability Assays.** Forty hours after transfection with GFP-tagged constructs, VC8 cells were flow sorted for GFP-positive cells. For clonogenic survival assay, 2,000 GFP-positive cells were seeded into 10-cm dishes, incubated overnight, and treated with 1, 1.75, or 2.5 ng/mL mitomycin C (MMC) for 72 hours. In parallel, 500 cells were incubated in the absence of MMC. Cells were rinsed with fresh medium incubated for 4 days, stained with 0.2% Coomassie brilliant blue-R250 solution and visible colonies were enumerated. Stable cell lines were also irradiated with γ-irradiation (0, 2, 4, or 6 Gy) using a 3Cs source, incubated, and stained as before. For trypan blue exclusion assays, 4,000 GFP-positive or stable VC8 cells were plated, treated with MMC (0, 1, 2.5, or 5 ng/mL) for 72 hours, harvested, resuspended in 50% trypan blue solution, and the living cells were enumerated using a hemocytometer. Survival was calculated by plating the number of surviving colonies or cells from MMC or radiation treated and untreated cells.

**Homologous Recombination Assay.** Stable incorporation of DR-GFP (15) in VC8 cells was established by selection with 2.5 μg/mL puromycin. Southern blotting of genomic DNA from clones with a DR-GFP probe was done and a low copy number clone (VC8-DR-GFP) was selected for use in the assay. VC8-DR-GFP cells were transfected with either pCR3.1 vector, pcBASE1 vector containing the SceI restriction endonuclease gene, or pcBASE1 plus various *BRCA2* pCR3.1 constructs. The percentage of cells that were GFP positive was quantitated by flow cytometric analysis 5 days after transfection.

**Multiple Sequence Alignment.** A multiple sequence alignment of full-length *BRCA2* cDNA sequences was generated using the T-coffee program (29). The alignment was restricted to *BRCA2* cDNA sequences derived from vertebrates in an effort to avoid using sequences that are evolutionarily related but are not direct homologues of the *BRCA2* gene. The Tetraodon *BRCA2* sequence was obtained from unpublished sources, and other *BRCA2* protein sequences were obtained from Genbank.

**Segregation Analysis.** For the analysis of cosegregation, we assumed an allele frequency of the variant of 0.0001 and a penetrance model with separate age-specific risks of breast and ovarian cancer for *BRCA2* based on the meta-analysis estimates of Antoniou et al. (30), with pooling across age groups (6). For *BRCA2* variants, we assumed relative risks in carriers relative to noncarriers of 11.5 for breast cancer, independent of age, whereas for ovarian cancer these relative risks were 4.8 for ovarian cancer below age 50 and 13.1 for ovarian cancer at ages ≥50 years. The corresponding cumulative risk of breast or ovarian cancer was 51% by age 70. In cases where full pedigrees were unavailable, we relied on family history information and reconstructed pedigrees by creating individuals of unknown phenotype and genotype to connect the individuals in the pedigree. These assumptions were applied to the model of Thompson et al. (31) for estimation of the likelihood of disease causality associated with *BRCA2* UCVs, as recently outlined (6).

**Results**

**Frequency in Control Populations.** The goal of this study was to show how functional assays can discriminate between disease-causing and neutral UCVs in the *BRCA2* gene and to outline how data from functional and genetic studies can be combined to predict the disease causality of *BRCA2* UCVs. For the purposes of the study, we selected 11 *BRCA2* mutations (Table 1). Three encoded amino acid alterations in the NH2-terminal region (Y42C, N372H, and E462G), two were in the BRCA repeats (T1302del and E1382del), and four were in the COOH-terminal DNA binding domain (T2515I, R2659K, D2723H, and V2908G). The *BRCA2* truncating 6174delT Ashkenazi Jewish founder mutation associated with a breast cancer risk of 70% by age 70 (32) was also included as a positive/activating control, whereas the K3326X truncating variant that does not segregate with disease (33) was used as a negative/wild-type control.

Initially, the frequency of all 11 mutations in normal controls was assessed to determine if any of the mutations were frequently observed in normal controls and were therefore likely to be neutral/low-risk mutations. Germ line DNAs from 476 healthy Caucasians of varying ages (18-65 years) undergoing routine colonooscopy at the Mayo Clinic were evaluated for the presence of the mutations using dHPLC analysis. All UCVs were at a frequency of 0 in 476 except for N372H and K3326X, which were found in 183 and 4 individuals, respectively. As only these two mutations detected in the control population can be excluded as inactivating and deleterious, the majority of the UCVs under study here remain as candidate deleterious mutations based on these data.

**In vitro Mutagenesis of BRCA2 cDNA.** In order to conduct *in vitro* assays to distinguish missense mutations and in-frame point deletions/insertions (IFD/I) that alter BRCA2 function from those with no effect, full-length *BRCA2* cDNA expression constructs encoding the wild-type and the 11 mutant forms of *BRCA2* were cotransfected into 293T cells. As only these two mutations detected in the control population were categorized as deleterious mutations based on these data, we have used a BRCA2 expression construct containing a 171-bp in-frame deletion to correctly reflect this exon skipping.

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4 S.V. Tartgian, unpublished data.
BRCA2 Expression and Localization. Ectopic expression of the GFP-tagged BRCA2 proteins in 293T cells after transient transfection was verified by immunoblotting with anti-BRCA2 antibodies (Fig. 1B). Immunoblotting of nontagged versions of the BRCA2 proteins detected similar levels of protein, suggesting that the GFP tag did not have a significant effect on protein stability. Protein stability analysis in the presence of cycloheximide also showed that the mutations had no effect on the half-life of the BRCA2 protein (data not shown).

Subcellular localization of the GFP-tagged BRCA2 proteins was evaluated by fluorescence confocal microscopy. The localization of BRCA2 and 6174delT to the nucleus and cytoplasm (Table 2; Fig. 1C), respectively, was consistent with the C-terminal location of the human BRCA2 nuclear localization signals (4). As expected, the Y42C, N372H, E462G, T1302del, and E1382del mutants were localized predominantly in the cytoplasm in >90% of transfected cells. However, the D2723H and R2659K BRCA2 mutants were localized predominantly in the cytoplasm in >90% of transfected cells (Table 2; Fig. 1C). Interestingly, the T2515I mutant was found in the nucleus (58%), the cytoplasm (26%), and both (16%). The influence of this partial relocalization of the protein on function is unclear. Mislocalization of these mutant proteins relative to the other mutants and wild-type BRCA2 was confirmed in HeLa cells (data not shown). As these mutations do not directly disrupt the known nuclear localization signal motifs (4), the results suggest an effect either on previously undetected sites that mediate BRCA2 localization or an effect on BRCA2 protein folding.

MMC Hypersensitivity. The BRCA2 deficient VC8 (23) cell line is extremely sensitive to cross-linking agents such as MMC. Stable reintroduction of the BRCA2 gene by chromosomal transfer or by bacterial artificial chromosome significantly reduces this MMC sensitivity (23), suggesting that it is possible to discriminate between wild-type BRCA2 and inactivated mutant BRCA2 based on the MMC sensitivity of VC8 cells. To begin to evaluate this possibility, we generated clonal isolates of VC8 cells stably transfected with wild-type BRCA2 and confirmed BRCA2 expression by immunoblotting of immunoprecipitated BRCA2 (Fig. 2A). The integrity of wild-type BRCA2 function in these cells was established by demonstrating that reconstituted BRCA2 facilitated the formation of Rad51 nuclear foci in response to γ-irradiation (Supplementary Fig. 1). Subsequently, we used clonogenic survival assays to show that constitutive expression of wild-type BRCA2 in VC8 cells reduced MMC sensitivity (Fig. 2B). In addition, we showed that wild-type BRCA2 expressing VC8 cells were less sensitive than vector transfected cells to γ-irradiation (Fig. 2B). In light of these observations, we also evaluated the effects of BRCA2 UCVs on MMC sensitivity. Stable expression of E462G, D2723H, and T2515I BRCA2 in VC8 cells was established and verified by immunoprecipitation/Western blot (Fig. 2A), and the cells were used in MMC-dependent clonogenic survival assays. The E462G variant suppressed hypersensitivity to MMC and γ-irradiation similarly to wild-type BRCA2, whereas the T2515I and D2723H variants seemed to inactivate BRCA2 and had no ability to reduce the cellular sensitivity to these agents (Fig. 2B).

As we were unable to generate stable cell lines constitutively expressing other BRCA2 UCVs, we developed a strategy for evaluation of MMC and radiation sensitivity of VC8 cells based on transient transfection of VC8 cells with wild-type and mutant forms of BRCA2. VC8 cells transiently transfected with GFP vector, GFP-tagged wild type, and GFP-tagged 6174delT human BRCA2 expression constructs were flow sorted for GFP-positive cells and BRCA2 expression was verified by immunoprecipitation/Western blot. The selected cells were evaluated for MMC sensitivity using clonogenic survival assays and cell viability assays. Wild-type BRCA2 significantly decreased cellular sensitivity to MMC relative to vector or the 6174delT truncation mutant (Fig. 2C). Similarly, wild-type BRCA2 enhanced cell viability relative to controls in response to MMC (Fig. 2C). Subsequent analysis of flow-sorted VC8 cells expressing GFP-tagged Y42C, N372H, E462G, V2908G, and K3326X showed 5-fold enhanced survival (Fig. 2D) and 12- to 14-fold improved viability (Fig. 2D) in response to MMC, similarly to wild-type BRCA2 expressing cells. In contrast, GFP-tagged T2515I, R2659K, D2723H, T1302del, and E1382del mutants did not reduce sensitivity to MMC. As these mutants all expressed equivalently to wild-type, the suggestion is that these UCVs inactivate BRCA2 function and that transiently transfected and flow sorted GFP-BRCA2 expressing cells can be used in MMC sensitivity assays to evaluate the influence of UCVs on BRCA2 function.

**Table 1. Sequence conservation and GMS for BRCA2 UCVs**

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<th>Amino acid variant</th>
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<th>Cat</th>
<th>Dog</th>
<th>Puffer fish</th>
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**NOTE:** Δ, deleted; —, not applicable. Rat brca2 cDNA (AB107955.1), mouse brca2 cDNA (MMU82270), chicken brca2 cDNA (AB066374.1 and AY083934.1), cat brca2 cDNA (AB107955.1), and dog brca2 cDNA (AB043895.2).
A mutant protein was visualized by confocal microscopy.

wild-type and mutant BRCA2 protein in 293T cells. GFP-tagged wild-type and mutant Capan-1 cells and mouse embryo fibroblasts was recently obtained using an I-SceI–dependent DR-GFP reporter assay (15, 35). Mutant BRCA2 in promoting homology-directed repair (HDR) in BRCA2 wtBRCA2 immunoblots of ectopically expressed wild-type (WT) and mutant BRCA2 protein from 293T cells.

Expression of BRCA2 wild-type and mutant proteins.

Figure 1. Expression of BRCA2 wild-type and mutant proteins. A, schematic of the BRCA2 protein: location of specific motifs and the BRCA2 UCVs. B, immunoblot of ectopically expressed wild-type (wtBRCA2) and mutant BRCA2 protein from 293T cells. C, subcellular localization of ectopically expressed wild-type and mutant BRCA2 protein in 293T cells. GFP-tagged wild-type and mutant protein was visualized by confocal microscopy.

Homology-Directed Repair. Direct evidence for a role of BRCA2 in promoting homology-directed repair (HDR) in BRCA2 mutant Capan-1 cells and mouse embryo fibroblasts was recently obtained using an I-SceI–dependent DR-GFP reporter assay (15, 35). In this reporter, a GFP gene is inactivated by the introduction of an I-SceI recognition site, whereas an adjacent GFP gene is differentially mutated. After the introduction of a DNA double-strand break at the I-SceI site, the GFP gene can be reconstituted by HDR using the downstream-inactivated GFP gene as a template. To evaluate the influence of UCVs on HDR using this system, the DR-GFP reporter construct was first stably integrated into VC8 cells and the presence of the construct at low copy number in the genome was verified by Southern blotting. These DR-GFP VC8 cells were not GFP positive in the absence of I-SceI indicating that spontaneous gene conversion in DR-GFP-VC8 cells was rare (Fig. 3A). Similarly, spontaneous recombination in the presence of I-SceI was only detected in 0.35% of cells (Fig. 3A). In contrast, coexpression of I-SceI and non-GFP tagged wild-type BRCA2 resulted in 8-fold more GFP-positive cells (Fig. 3A and B), indicating that wild-type BRCA2 can promote recombinational repair of Sce1-mediated DNA double-strand breaks. This approach has also been successfully applied to the evaluation of the role of the 53BP1 protein in HDR of double-strand breaks (36). Subsequent evaluation of the effects of BRCA2 mutants on the ability of BRCA2 to mediate recombinational DNA repair revealed that the Y42C, N372H, E462G, T1302del, and K3326X mutations all functioned similarly to wild-type BRCA2 (Fig. 3B). In contrast, levels of HDR equivalent to those obtained with vector alone were associated with expression of the E1382del, R2659K, D2723H, and 6174delT mutants, whereas T2515I showed a slight reduction in HDR relative to wild-type (Fig. 3B). In parallel, confocal microscopy and semiquantitative reverse transcription-PCR were used to verify that all constructs had similar transfection efficiencies and levels of expression in the DR-GFP-VC8 cells (Fig. 3C). Taken together, the data indicate that transient transfection of BRCA2 expression constructs into DR-GFP-VC8 cells can be used to evaluate the effects of BRCA2 UCVs on the HDR activity of BRCA2.

Induction of Centrosome Amplification. BRCA2 mutant mouse embryo fibroblasts, Capan-1, and FANC-D1 cells, and BRCA2-deficient VC8 cells all display extensive centrosome amplification (23, 24, 26), suggesting that the chromosomal instability observed in BRCA2 mutant cells (23, 26) either in part results from aberrant centrosome function and abnormal numbers of centrosomes, or causes centrosome amplification. Alternatively, BRCA2-associated centrosome amplification may result from uncoupling of DNA replication and centrosome duplication in S phase. In either case, centrosome amplification is a marker of cell cycle disruption and chromosomal instability and can be used as a measure of BRCA2 function. Indeed, Kraakman-van der Zwaet et al. (23) showed that reconstitution of wild-type BRCA2 through chromosomal transfer or bacterial artificial chromosome clones in VC8 cells caused a significant reduction in the proportion of cells with centrosome amplification and chromosomal instability.

To investigate the influence of BRCA2 UCVs on BRCA2 function using this approach, we quantified centrosome amplification in VC8 BRCA2 wild-type and UCV stable cell lines. Cells containing a vector control or D2723H BRCA2 did not have a reduced frequency of centrosome amplification, whereas the proportion of wild-type BRCA2 and the E462G UCV expressing cells with amplification was reduced from 80% to 40% to 50% (Fig. 4A). These findings indicate that plasmid-based expression of BRCA2 can rescue centrosome amplification and that centrosome amplification can be used to evaluate the influence of UCVs on BRCA2 function. However, because of the difficulty in establishing VC8 BRCA2 stable cell lines, we also developed a transient transfection-based approach. We used 293T cells because they are readily transfected by BRCA2 expression constructs, have a normal complement of centrosomes, and display centrosome amplification in response to mutant forms.
of BRCA2 (Fig. 4B and C). Indeed, only 5% to 6% of 293T cells ectopically expressing GFP-tagged wild-type BRCA2 or GFP vector have centrosome amplification (Fig. 4B), whereas 18% to 20% of cells expressing the 6174delT truncation mutant display centrosome amplification. Similarly, 17% to 20% of 293T cells ectopically expressing GFP-tagged T1302del, R2659K, and D2723H contained amplified centrosomes, whereas expression of several other UCVs had no effect on centrosome number (Fig. 4B). Similar results were obtained in 293 and HeLa cells (data not shown), suggesting that mutant forms of BRCA2 can induce centrosome amplification and that the T1302del, R2659K, and D2723H UCVs all inactivate BRCA2 function. It should be noted that these effects are likely the result of competitive dominant negative effects of the overexpressed mutants. However, the results are consistent with those from stable cell lines and seem to reflect the role of BRCA2 in regulation of cytokinesis (37) and centrosome number (data not shown).

Cross-Species Multiple Sequence Alignment. Interspecies multiple protein sequence alignments provide an evolutionary perspective to identification of important functional motifs and amino acids within proteins (38, 39). In addition, the Grantham chemical difference matrix (40) quantifies physicochemical differences between mutant and wild-type amino acids in humans and can be used to correlate alterations with changes in phenotype and function, as recently shown for BRCA1 (7, 8, 40). Here, we use multiple sequence alignment and Grantham matrix scores (GMS) to predict whether the BRCA2 UCVs are important for BRCA2 function, as previously described (6). As shown in Table 1, the E1382, R2659, and D2723 residues are fully conserved across all species including the relatively distantly related pufferfish, Tetraodon nigriviridis. This strong conservation results in odds of ∼5:1 in favor of the UCVs being deleterious (Table 1) and suggests that these UCVs are important for BRCA2 function, although the GMS for the associated UCVs in humans were not high. The Y42, N372, E462, T1302, T2515, and V2908 residues were divergent across species and even deleted from the tetraodon BRCA2 sequence and are likely not important for function (Table 1). However, it remains possible that BRCA2 has acquired functions during evolution and that the NH2-terminal residues that are not present in tetraodon but are present in mammals are important for function. Overall, the odds of causality based on alignment and the GMS (Table 1) are insufficient for classification of the disease causality of the UCVs, but they do provide support for the outcomes of the functional assays.

Cosegregation Analysis. To further evaluate the ability of the functional assays to discriminate between deleterious and neutral UCVs, we compared the results from the functional assays with the predicted disease causality of the UCVs. Disease causality of the UCVs was defined using a recently developed likelihood model that establishes the odds of disease causality for mutations in BRCA1 and BRCA2 based on available family data (6, 31). Information on segregation of the UCVs in families was provided by Myriad Genetics Laboratories, Inc. (Salt Lake City, UT) and the odds in favor or against disease causality for each UCV were determined (Table 2). Segregation analyses for the N372H and K3326X mutations were not done because both mutations have been detected many times in combination with a large variety of truncating BRCA2 mutations and it is clear that these variants are associated with little or no risk of cancer. We assign odds of <1 × 10−3 for disease causality for these variants in Table 2 to reflect the known neutrality of the mutations. In contrast, segregation was evaluated in 10 of the 24 known families that carry the D2723H UCV. All pedigrees showed complete cosegregation with breast and ovarian cancer, yielding overall odds of 13,723:1 in favor of disease causality (6). Similarly, T2515I and R2659K were evaluated in 9 and 3 pedigrees, respectively. Information was limiting and the odds in favor of disease causality were low. Similarly, one pedigree with the E1382del variant generated odds in favor of causality of 1:65:1. In contrast, 17 pedigrees containing the Y42C variant and 20 containing the E462G variant yielded highly significant odds against causality (Table 2). Whereas it was only possible to classify
Y42C, N372H, E462G, D2723H, and K3326X by this method, it is important to note that the results matched the outcome from the functional assays (Table 2).

Co-occurrence with Deleterious Mutations. Given that biallelic inactivation of BRCA2 is associated with either embryonic lethality or Fanconi anemia, it is unlikely that UCVs that inactivate BRCA2 function can be found in trans with other truncating BRCA2 mutations in non-Fanconi anemia adults. Similarly, two inactivating BRCA2 mutations have never been found on the same allele. This suggests that BRCA2 UCVs that co-occur with known deleterious BRCA2 mutations are unlikely to inactivate BRCA2 function or confer a high risk of cancer. By applying co-occurrence data from 20,000 individuals tested at Myriad Genetics Laboratories to a likelihood model that predicts disease causality based on co-occurrence in families (6) we evaluated the disease causality of the UCVs described above (Table 2). The N372H and K3326X variants co-occurred with many truncating mutations suggesting that these variants do not alter BRCA2 function sufficiently to predispose to cancer. Likewise, the Y42C UCV co-occurred with eight different BRCA2 truncating mutations and can be excluded as disease causing, whereas E462G UCV co-occurred with a Y3098X truncating mutation, and is likely not a deleterious mutation. In contrast, the T2515I, D2723H, and V2908G missense mutations and the T1302del, E1382del, and R2659K IFDs did not co-occur with other deleterious mutations (Table 2). However, the absence of co-occurring mutations does not verify that the UCVs are disease causing because the limited number of families with these UCVs restricts the possibility of observing co-occurrences. Whereas this model is most useful for frequently observed UCVs, the odds from the model can be combined with those from cosegregation, sequence conservation, and Grantham matrix analysis to give an overall likelihood of disease causality (Table 2; ref. 6); thus, it remains useful to conduct these analyses. Using odds of 1,000:1

![Figure 2](image-url)
in favor and 100:1 against as thresholds for disease causality, we noted that the functional assay data mirrors the results from the individual and combined likelihood analyses.

Discussion

Although many missense mutations and in-frame insertions and deletions in BRCA2, collectively termed unclassified variants, have been identified in high-risk breast and ovarian cancer patients (affected with breast or ovarian cancer and with a family history of breast or ovarian cancer), it is not known if any of these alter BRCA2 function sufficiently to predispose carriers of these mutations to cancer. Thus, the carriers of these mutations receive limited cancer risk assessment. A number of approaches involving analysis of high-risk breast cancer families carrying these mutations have been used to address the relevance of these mutations to cancer. These include analysis of cosegregation of variants with cancer in families and evaluation of the co-occurrence of variants with other known deleterious mutations (6, 41). However, these approaches can be limited by the frequency of the variant and the availability of family data. Thus, several lines of investigation must be considered to classify BRCA2 variants as deleterious/high risk or neutral/low risk (6, 41, 42).

In this study, we describe the analysis of the effects of several UCVs on BRCA2 function using in vitro assays that evaluate BRCA2 subcellular localization, MMC sensitivity, homologous recombinational DNA repair, and centrosome amplification. We show that each assay can discriminate between the BRCA2 6174delT truncation mutant and wild-type BRCA2, and that a series of BRCA2 UCVs can be separated into groups that inactivate or have no effect on the cellular response to BRCA2. The results from the functional studies were generally consistent and led to the prediction that D2723H and R2659K are inactivating and disease causing mutations. Whereas these mutations inactivated the cell survival, homologous recombination repair, and centrosome regulatory functions of BRCA2, their ability to inactivate these putatively independent functions of the protein in unison was unexpected, as each mutation alters only a single residue in BRCA2. However, the finding that both D2723H and R2659K are excluded from the cell nucleus and cannot perform the predominantly nuclear functions of BRCA2 provides a likely explanation for these effects. The mechanism by which these mutant forms of BRCA2 are restricted to the cytoplasm is

![Figure 3. HDR analysis of BRCA2 UCVs.](https://www.aacrjournals.org)
unknown. However, the nuclear exclusion of these proteins and the associated inactivation of all measured BRCA2 functions suggests that evaluation of the cellular localization of ectopically expressed UCV mutants of BRCA2 may be useful as a prescreen for identifying inactivating UCVs. We also identified a number of UCVs that influenced a subset of BRCA2 functions or only partially inactivated BRCA2 function. The T2515I UCV localized to both the cytoplasm and the nucleus and seemed to partially inactivate the homologous recombination and centrosome regulatory functions of BRCA2 and completely ablated the cell survival activity of BRCA2. The partial inactivation of these functions by T2515I may be in response to the lower levels of BRCA2 in the nucleus resulting from mislocalization of a significant amount of BRCA2 to the cytoplasm, but clearly suggests that T2515I has a relatively subtle effect on BRCA2 function. Likewise, the E1382del variant located in the BRC repeats of BRCA2 inactivated BRCA2 in the cell survival and HDR assays suggesting that the UCV specifically alters Rad51 binding by BRCA2 resulting in loss of DNA repair activity. However, it would seem that E1382del does not confer a conformational change on BRCA2 that leads to inactivation of all BRCA2 functions because the UCV had no influence on centrosome number. This supports the notion that BRCA2 is a multifunctional protein that is not only involved in DNA repair but may also contribute to cell cycle and/or mitotic regulation in an as yet undefined manner. Similarly, the T1302del in-frame deletion influences specific functions of BRCA2. In this case, only cell survival, as previously reported (43), and centrosome amplification effects were observed, whereas the variant had no effect on HDR. This suggests that the influence of BRCA2 on survival and viability in response to MMC damage is independent of its homologous recombination activity. In contrast, the Y42C, E462G, and V2908G UCVs and the frequently observed N372H and K3326X variants had no effect on BRCA2 function in any assay. Importantly, when considering all of the data, it is clear that homologous recombination, cell survival, and centrosome regulation are all independent functions of BRCA2. This observation validates our use of three independent assays for assessment of BRCA2 function in this study.

We also used this series of assays to evaluate BRCA2 function because they measure the cellular, as opposed to biochemical effect, of BRCA2 UCVs and may be more relevant to cancer development. Another important feature of this study is that full-length BRCA2 proteins were used to assess the effects of the UCVs on BRCA2 function. Whereas partial proteins containing specific functional domains can be useful for evaluation of protein function in certain circumstances, we believe that it is best to place mutations in the context of a complete protein especially when a cell cycle rather than biochemical assays are being applied.

In an effort to apply the results from the functional assays to an evaluation of the disease causality/predisposition associated with the BRCA2 UCVs we combined these data with results from genetic analyses of the UCVs (in a comprehensive approach to classification of BRCA2 UCVs (6). Specifically, we used the likelihood of disease causality based on cosegregation with cancer in families (Table 2), evidence of co-occurrence with other deleterious mutations (Table 2), and sequence conservation
of the relevant amino acids (Table 1), and physicochemical differences in wild-type and mutant amino acids (Table 1) as previously outlined by Goldgar et al. (6). The results from the functional assays were remarkably consistent with those from the individual likelihood models and with the combined overall odds of causality and suggest that these assays can discriminate between deleterious/high-risk and neutral/low-risk UCVs. Indeed, when combining these data, it is apparent that the D2723H UCV is a disease-causing mutation in BRCA2 (6). This is an important finding when considering that 24 families are known to carry this mutation. The data are less compelling for the R2659K UCV because the limited number of families carrying the mutation result in equivocal odds of causality in the cosegregation model and in the combined odds model. In this situation, the functional assays are the only means of defining the disease causality associated with the variant, and they suggest that R2659K is disease causing. The likelihood models also are consistent with the functional assays in defining Y42C (6) and E462G as neutral/low-risk variants. In both cases, the combined odds against causality are highly significant (Table 2), and agree completely with the finding that both variants have no effect on BRCA2 function. Whereas we cannot completely rule out that the UCVs are associated with a low risk of cancer, it is possible to classify them as not clinically relevant. Similar statements can be made about the common K3326X and N372H variants. Conversely, whereas the V2908G UCV might be classified as neutral/low risk based on a high GMS, evolutionary divergence, and limited influence on BRCA2 function in the functional assays, it remains possible that not all functions of BRCA2 are being evaluated in these assays. Thus, the definition of its role in cancer predisposition at a clinical level should await further experience in interpretation of these assays. Similarly, the T1302del, E1382del, and T2515I variants must remain unclassified in terms of disease causality because of equivocal odds of causality in the likelihood model due to limited numbers and availability of pedigrees, and because it is not yet clear that inactivation of specific functions of BRCA2 as defined by the functional assays can increase predisposition to cancer.

The power in combining various UCV classification methods (6) is evident from our ability to establish the role of the D2723H, R2659K, Y42C, and E462G mutations in cancer predisposition. This suggests that many other BRCA2 UCVs might be classified in much the same manner leading to improved risk evaluation for UCV carriers and enhanced identification of individuals at risk or not at elevated risk of cancer. Importantly, this study also suggests that functional assays may also prove useful for classification of BRCA2 UCVs when cosegregation or co-occurrence data are limiting, as is the case for the majority of identified UCVs. However, a more complete understanding of all of the functions of BRCA2 will be needed so that mutations are not excluded as deleterious on the basis of incomplete information. In addition, a careful determination of the sensitivity and specificity of each assay will be needed before this approach to classifying variants can be utilized for clinical purposes.

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