Published in final edited form as:

Exp Cell Res. 2011 September 10; 317(15): 2099–2109. doi:10.1016/j.yexcr.2011.06.010.

A new assay for functional screening of BRCA2 linker region mutations identifies variants that alter chemoresistance to cisplatin

Curtis R. Warren^{a,c,*}, Zohra Ali-Khan Catts^b, and Mary C. Farach-Carson^{a,c}

^aDepartment of Biological Sciences and Center for Translational Cancer Research, University of Delaware, Newark, DE 19717, USA

^bHelen F. Graham Cancer Center, Newark, DE 19718, USA

^cDepartment of Biochemistry and Cell Biology, Rice University, 6100 Main St., Houston, TX 77005, USA

Abstract

Variants of unknown significance (VUS) complicate the assignment of risk to new DNA sequence variants found in at-risk populations. This study focused on the poorly studied linker region of the cancer-associated BRCA2 protein encoded by exons twelve through fourteen of *BRCA2*. To develop a new method to characterize VUS in this region of *BRCA2*, we first chose to study 4 reported VUS occurring on evolutionarily conserved residues within the linker region. To determine if these VUS represent neutral changes or if they impact the function of the BRCA2 protein, we stably transfected expression plasmids encoding wild-type or each mutant peptide into T47D breast cancer cells, which are wild-type for *BRCA2*. Four mutant peptide expressing cell lines and a wild-type linker region expressing cell line next were studied by challenging transfected cell lines with the DNA crosslinking compound cisplatin (10 μ M) for 5 days. Expression of the wild-type linker region and certain mutant linker peptides (N2452D and I2285V) decreased apoptosis (as demonstrated by cell death detection assay) in transfected cell lines, indicating that the linker region peptide directly or indirectly affects the DNA damage repair pathway. By determining the cell survival and assaying the apoptotic index of treated cell lines, one could potentially use this screen to determine that a particular VUS has a functional impact on BRCA2 function, and hence is of functional significance. We conclude that this method is useful for screening the effect of linker region VUS on BRCA2 function, and to identify mutations for further testing. We also conclude that mutations in the linker region may have heretofore unappreciated roles in BRCA2 function.

Keywords

BRCA2; breast cancer; cisplatin; variants of unknown significance; mutation screen

© 2011 Elsevier Inc. All rights reserved.

*Address correspondence to: Mary C. Farach-Carson, Ph.D. Department of Biochemistry and Cell Biology, Rice University, 6100 Main St., Houston, TX 77005, USA. Phone number: +1-713-348-5052, Fax number: +1-713-348-5154, farachca@rice.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Screening for mutations in *BRCA1* and *BRCA2* is the standard of care for patients with family histories indicative of Hereditary Breast and Ovarian Cancer Syndrome (HBOC). A problem arises when patients present with what appears to be a familial cancer pedigree, yet sequence analysis reveals an uncharacterized missense mutation. Such missense mutations are termed “Variants of Unknown Significance” (VUS) because it is not known if these changes alter the function of the expressed protein *in vivo* [1]. For this reason, recognition of a VUS presents a conundrum to genetic counselors who cannot discern if mutations of this nature predispose their patients to cancer.

Such was the case when two previously uncharacterized VUS were discovered in a patient population being curated into a new High Risk Family Database (see methods). These VUS were both in the linker region of *BRCA2*, a region whose functional significance is unknown. The two variants were R2341C and N2452D, both carried by breast cancer patients whose family histories were indicative of HBOC. The Breast Cancer Information Core (BIC) [2], is a National Institutes of Health National Human Genome Research Institute initiative to catalogue cancer-associated mutations in *BRCA1* and *BRCA2*. At the time of this study, the BIC listed over 500 mutations reported to occur in the linker region; over 300 of these are VUS's. By mining the BIC, we identified 11 additional VUS occurring on evolutionarily conserved residues in the largely unannotated linker region of *BRCA2* (encoded by exons twelve through fourteen of *BRCA2*). Certain VUS have been successfully characterized in other systems by recombinantly expressing full-length *BRCA2* protein with VUS [3], and by expressing mutant full-length *BRCA2* in mouse embryonic stem cells[4]. This screen differs from these functional assays by recombinantly expressing only the linker region of the *BRCA2* protein, in stable cell lines.

The linker region is located between the two well-studied domains, one of which binds the recombinase RAD51 [5] and the other of which shows sequence similarity to single-stranded DNA-binding domains [6] (figure 1). One study reported an interaction with the meiosis-specific recombinase DMC1 near the C-terminal end of the linker region [7]. The function of the linker region remains unknown, and homology searches reveal that the linker region has no similarity to coding sequences of other known proteins. Important to this study are the roles of the *BRCA2* protein in the homologous recombinational DNA damage repair pathway (HRR) [5, 8] and the interstrand crosslink DNA damage repair pathway (ICLR) [9-12]. Cells derived from Fanconi Anemia (FA) patients are unable to repair these crosslink lesions [13, 14], and *BRCA2* is involved in the FA complex (ICLR) [9]. Thus if the linker peptide binds a partner in the ICLR, overexpression of the linker region peptides may increase sensitivity to treatment with DNA crosslinkers.

We studied the effect of overexpression of the linker region peptide on the DNA damage repair capabilities of T47D cells, which express wild-type *BRCA2*. We created a set of expression plasmids encoding the wild type linker region of the *BRCA2* cDNA and a panel of VUS chosen for this study. Stable transfection of these constructs into T47D cells led to constitutive expression of these linker region peptides. Our hypothesis was that constitutive expression of linker region peptides would impact cell survival in response to treatment with the DNA crosslink-inducing drug cisplatin (CDDP). To test this hypothesis, we treated the cells with a range of concentrations of the crosslink-inducing [15, 16] chemotherapeutic, cisplatin (CDDP). The effects of this treatment were measured by assaying cell survival with the crystal violet stain, and assaying apoptosis in attached cells by the cell death detection ELISA (CDDE). In this assay, cell death is indicative of a potentially detrimental mutation.

Materials & Methods

BRCA2 mutation mining and sequence alignment

The Ruth Ann Minner High Risk Family Cancer Registry (FCRR) and the National Human Genome Research Institute's BIC [2] were used to find a number of mutations in the linker region of *BRCA2*, many of which have been reported. The FCRR is a patient information and pedigree database maintained by the genetic counseling staff of the Helen F. Graham Cancer Center (Christiana Care Health Systems, Newark DE). The ClustalW tool of the Biology Workbench[®] (<http://seqtool.sdsc.edu/CGI/BW.cgi>) was used to align the primary amino acid sequences of the *Homo Sapiens*, *Mus Musculus*, *Rattus Norvegicus*, *Canis Lupus Familiaris*, and *Gallus Gallus* *BRCA2* linker region. Mutations of residues evolutionarily conserved amongst mammalian and avian genomes were chosen for study. The BIC lists over 300 patient-derived missense mutations in exons 12, 13 and 14, and 13 unique variants occur on evolutionarily conserved residues. Among these, four VUS were chosen for this proof-of-principle study.

This study was approved by the Institutional Review Board of Christiana Care Health Systems and the University of Delaware. Informed consent was obtained from all donors as required.

Cell lines

The breast ductal infiltrating carcinoma T47D cell line was purchased from ATCC (Manassas, VA) and maintained in Roswell Park Memorial Institute 1640 Medium (RPMI1640) supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. This cell line is derived from a breast ductal carcinoma [17] and expressed wild-type *BRCA2*, as shown by direct sequencing of a T47D cDNA library during this study (data not shown – Fox Chase Cancer Center – Philadelphia, PA). To maintain stable transfection, medium was supplemented with 1.2 mg/mL geneticin (Invitrogen – San Diego, CA). Cells were passaged at over 80 percent confluence as determined by visual inspection, and separated from the flask using trypsin/ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific – Pittsburgh, PA). Cells were incubated at 37° C with five percent CO₂. All media were purchased from Fisher Scientific.

Ribonucleic acid isolation

All RNA isolation was performed using the RNeasy Mini-prep[®] kit (Qiagen, Valencia, CA). The procedure for RNA isolation followed the manufacturers instructions. RNA was extracted from T47D cells after removal from tissue culture flasks and centrifuged at 2000 rpm for five minutes to create a cell pellet. This pellet then was disrupted with a detergent cell lysis buffer. Lysate then was homogenized using a Qias shredder[®] column before application of the lysate to the Qiagen RNA Isolation Column. This column was washed to remove protein and cell debris. RNA elution was performed using nuclease-free water. The collected eluate was treated for DNA contamination with the DNasefree kit and protocol from Ambion (Austin, TX). RNA concentration was assayed spectrophotometrically by measuring absorbance at 260 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR) and entry plasmid construction

Reverse transcription was performed using the Omniscript[™] kit and protocol from Qiagen. All reactions used 250 ng of RNA and resultant DNA was quantified spectrophotometrically as above. Polymerase Chain Reaction (PCR) was performed using GoTaq[®] Green Master Mix from Promega (Madison, WI) and following the manufacturer's 25 µL reaction volume guidelines. The thermal cycling conditions for the various reactions were as follows (all

reactions performed using the LongGene MG96G Gradient Thermocycler): for exons twelve through fourteen of BRCA2 – the linker region: five minutes at 94°C followed by thirty cycles of one minute at 94°C, ninety seconds at variable temperatures (Table 1) and ninety seconds at 72°C, followed by 10 minutes at 72°C.

The linker region PCR product was ligated into a TOPO TA vector (Invitrogen). The plasmid then was transformed into One Shot® TOP10 chemically competent *E. coli* following the protocol provided by Invitrogen. *E. coli* were selected for transformation using the recommended concentration of ampicillin. A Qiagen Miniprep® Kit was used to extract the plasmid intact from the *E. coli*. The plasmid extraction followed the manual provided with the kit. Ligation was confirmed by sequencing performed by Genewiz, Inc (La Jolla, CA). For ligation into the pENTR/D-TOPO plasmid (Invitrogen), primers were designed containing the 5' Kozak sequence and ending in-frame on the 3' end. The TOPO TA plasmid previously described was used in the PCR reaction, as described above. This PCR product was purified after gel electrophoresis using a Qiaquick® Gel Extraction Kit (Qiagen) in order to remove excess TOPO TA plasmid from the reaction mixture.

The purified PCR product was ligated into the pENTR/D-TOPO plasmid (Invitrogen) according to the procedure provided with the manual. This plasmid was transformed into *E. coli* using the previously described procedure and selected with the antibiotic, kanamycin. The plasmid was purified and sequenced using the same procedure described above.

Site-Directed Mutagenesis

The Quikchange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) was used to create a library of thirteen mutant plasmids in addition to the wild-type sequence in the pENTR/D-TOPO plasmid previously described. The kit follows a PCR-based mutagenesis protocol, and the thermal cycler amplification conditions used varied only in the primer annealing temperature for each set of primers. The core protocol used the following conditions: 95°C for one minute, eighteen cycles of 95°C for fifty seconds, variable temperatures (shown in table 1) for fifty seconds, and 65°C for five minutes, followed by an extension period of 68°C for seven minutes (reaction performed on the MG96G Gradient Thermal Cycler, LongGene, Hangzhou, P.R.China). These resultant wild-type and mutant-sequence plasmids were transformed into bacteria, purified and sequenced using the previously described procedure.

Recombination between Entr/D-Topo plasmids and pcDNA Dest40 expression plasmid

For transfer of sequences from the pENTR/D-TOPO plasmid into the pcDNA Dest40 Gateway™ Vector, the Gateway LR Clonase II® enzyme (Invitrogen) reaction was used according to the manual provided with both the entry plasmid and the pcDNA Dest40 Vector. The pENTR/D-TOPO plasmid (150 ng) and pcDNA Dest40 vector (150 ng) were used in the reaction. After incubation at 25°C for one hour, the LR Clonase II reaction mixture was transformed into *E. coli* and selected with the antibiotic ampicillin. Plasmid purification and sequencing were performed as described above.

Transfection

T47D cells were plated into each well of a six-well plate at 1×10^6 cells/well. The cells were incubated for 24 hours in antibiotic-free media in normal cell culture conditions. Thirty minutes prior to transfection, the cells were rinsed with PBS and then changed to Opti-Mem medium from Gibco (Invitrogen). The Lipofectamine™ 2000 reagent was used for transfection (Invitrogen) and the protocol provided by the manufacturer was followed. DNA (1 µg/well) was used with 4 µl of lipofectamine. After a 5 hour incubation, cells were switched to growth medium. 24 hours post transfection, cells were changed to selection

medium as described above. After three weeks of selection, colonies were picked to create stable expression clones. For the remainder of the study, neomycin selection (1.2 mg/mL) was continued on these stable clones to maintain plasmid integration.

Immunoblotting

Detection of Linker Region BRCA2 Peptides—Cells were grown to confluency and harvested by trypsin digestion. One fourth of the resultant cell pellet was treated with 200 μ L of radioimmunoprecipitation assay (RIPA) buffer supplemented with 4 μ L protease inhibitor (Sigma-Aldrich, St. Louis, MO) on ice, then gently agitated for 30 minutes. The mixture was centrifuged at 4°C for 10 minutes and the supernatant was stored at -20°C. Protein was quantified using the Biconchonic Acid protein assay kit from Pierce (Rockford, IL). Protein (15 μ L) was loaded onto a nitrocellulose membrane in a Bio-Rad Bio-Dot™ (Bio-Rad, Hercules, CA) apparatus and fixed by vacuum suction. Nitrocellulose blots were blocked overnight in 5% (w/v) bovine serum albumin (BSA) in PBSTween 20 (PBST20 - [0.01% (v/v)]) at 4°C. The Penta-His antibody (Qiagen) was used at a 1:1000 dilution in blocking buffer overnight at 4°C. Blots were washed three times in PBST20 for five minutes at room temperature with constant shaking. Sheep anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody from Jackson ImmunoResearch (West Grove, PA) was added at 1:200,000 in PBST20, 5% BSA and incubated for 45 minutes at room temperature with constant shaking. Blots were washed three times for 5 minutes each with constant shaking at room temperature in PBST20. Following washing, the blots were incubated for 5 minutes in SuperSignal® West Dura Extended Duration Substrate (Pierce). The blots were exposed to film for various durations. Total protein lysate from the untransfected T47D cell line served as the negative control.

Densitometric analysis of dot blots—Densitometry was performed to analyze protein levels detected by penta-his antibody in the dot blot assay used to screen stable cell lines. Pixel density on each blot was analyzed using the Dot Blot Analyzer plugin for ImageJ[18].

WST assay

This assay uses a reagent (WST-1, a tetrazolium salt) that is hydrolyzed by mitochondrial enzymes to serve as an indicator of cell number. Cells were plated into 96-well plates at 10,000 cells/well (in triplicate) in basic T47D growth medium without neomycin as above. Three wells containing no cells were filled with medium and served as a negative control. Cells were allowed to attach and grow for 48 hours at which point 20 μ L of WST-1 reagent (Roche Applied Science – Indianapolis, IN) were added to each well at the concentration provided. The plate was incubated at 37°C for one hour and then absorbance was read in a Dynex MRX plate reader (Pegasus Scientific, Rockville, MD) at dual wavelengths of 470 and 490.

DNA damage survival assay

Cells were plated into 24-well plates at 10,000 cells/well (in triplicate) or into 24-well plates at 10,000 cells/well in basic T47D growth medium without neomycin as previously described. After 24 hours, medium was replaced with the same growth medium supplemented with various concentrations of CDDP (figure 5). CDDP (American Pharmaceutical Partners, Schaumburg, IL) was provided by the HFGCC pharmacy and stored in the dark at room temperature. After five days, the cells were washed twice with PBS, then fixed for five minutes at room temperature in 10% (v/v) methanol and 10% (v/v) acetic acid. 1% (w/v) Crystal Violet in methanol was added for 1 minute. Dye was resolubilized for 1 hour at room temp in 0.5 mL 0.1% (w/v) SDS in methanol under mild

agitation. Resulting dye solution (150 μ L) was diluted 1:3 in methanol in 96 well plates and read in a Dynex MRX plate reader at 595 nm.

Cell death detection ELISA

The CDDE is a kit provided by Roche Applied Science measuring histones free in the cell, a marker of apoptosis. Cells were plated into 24-well plates at 10,000 cells/well (in triplicate) or into 24-well plates at 10,000 cells/well in basic T47D growth medium without neomycin. After 24 hours, medium was replaced with the same growth medium supplemented with CDDP at various concentrations as shown in figures. After five days, the sample preparation and ELISA assay procedure were followed as indicated in the manual provided (Roche). The medium supernatant was collected in 2 mL centrifuge tubes, and wells were washed for 5 minutes with 0.1 % (w/v) trypsin (200 μ L) as described above. Supernatant was used to wash cells off of the plate, and the cells were pelleted by spinning at 5,000 $\times$ G for 10 minutes. Medium was removed and cells were lysed with lysis buffer (200 μ L) (provided with the CDDE) for one hour at room temperature. This lysate was centrifuged for 10 minutes at 5000 $\times$ G and supernatants (20 μ L) were transferred to the ELISA plate. The provided Immunoreagent (consisting of monoclonal antibodies, biotin-conjugated anti-histone and peroxidase-conjugated anti-DNA) was added at the concentration suggested by the manufacturer (Roche). The plate was covered with an adhesive cover foil, then shaken on a DP MicroMix 5 at setting 7 for two hours at room temperature. The liquid was removed from the wells and each well was rinsed with the provided incubation buffer three times. The provided ABTS solution then was added to each well and absorbance was measured on the Dynex MRX plate reader at 405/495 dual filter absorbance.

Statistics

Significance of results was determined by use of the two-tailed T-test and ANOVA functions in GraphPad InStat (Graphpad software – La Jolla, CA). For the survival and apoptosis assays, absorbance of treated cells normalized to untreated cells was compared to the untransfected cell line. All survival assays and apoptosis assays were performed with triplicate experimental wells, in triplicate biological replicates (nine wells). Duplicate biological replicates of the WST assay were performed with triplicate experimental wells (six wells). Error bars represent standard error of each data point. Statistical analyses were performed on triplicate or duplicate data points gathered over the course of multiple replicates.

Results

Bioinformatic analysis of BRCA2 linker region

CLUSTALW analysis revealed a number amino acid residues that are evolutionarily conserved amongst mammalian and avian genomes in the linker region of BRCA2 (figure 2A). The databases we mined for VUS list over 300 unique mutations occurring in this 199 amino acid linker region, and we chose to study the thirteen VUS which occur on the aforementioned evolutionarily conserved residues (figure 2). One of the VUS (I2285V) was reported 79 times in the BIC, whereas others were reported as little as once [2]. A brief summary of results obtained in later experiments in relation to each VUS is presented in figure 2B.

Creation of stable expression clones for study of the linker region of BRCA2 and the panel of VUS

Expression of the linker region peptides (see figure 2) by non-isogenic stable transfectants was verified by dot blot analysis of total cell lysate (figure 3). 6x-His-tag antibody reactivity

with the linker region peptides was very high in many of the stable clones, but not existent in the untransfected T47D control and empty vector transfection control. The clones used in this study were found to express the linker region peptides by probing for the 6x-His-tag at the c-terminus of the peptides. The clones chosen for further study were selected for displaying qualitatively similar levels of expression by dot blot. Although stable cell lines were created expressing all 13 linker region peptides, four VUS-expressing, stably behaving cell lines were chosen for this study, for proof of concept.

Characterization of stable expression clones

Basal proliferation is unaffected by expression of wild-type or mutant linker region of BRCA2—

The WST assay demonstrated that the cell lines selected for use had similar proliferative capacity prior to treatment (figure 4). Occasional outliers were found in the absorbance readings, and this occurred in the several trials of this experiment; visually there appeared to be little difference in proliferation of the cell lines during the time domain of the WST assay. Cell proliferation was unaffected despite overexpression of the linker region peptides – all cell lines were no more than 1.27-fold more proliferative than untransfected cells (figure 4). No statistically significant differences between cell proliferation rates were found.

Overexpression of the linker region peptide increases survival in response to CDDP treatment—

Analysis of survival of stable expression clones treated with varying concentrations of CDDP shows varying survival capacity correlates with expression of different VUS in the linker region. During 1 μ M treatments with CDDP (Figure 5A), cells expressing the R2318Q VUS in the linker region peptide survived at a significantly lower rate than all other cell lines. Results also indicate decreased survival levels in the N2452D-expressing cell line, although statistical analysis did not reveal a difference. Treatment with CDDP (5 μ M - Figure 5B) revealed no significant difference ($p > .05$) between transfected and untransfected cell survival, although cell survival was globally decreased in all cell lines compared to 1 μ M treatment. When treated with 10 μ M CDDP (Figure 5C), there was no significant difference in survival of the cell lines. Cells stably expressing the wild-type peptide and R2318Q mutant peptide displayed similar levels of cell survival as the untransfected cell controls at all concentrations of CDDP. This effect was reproducible over several iterations of this experiment. The empty vector transfection controls and untransfected cells responded in the same way to CDDP treatment (Figure 5D). Cell lines were treated with CDDP vehicle (0.9% saline) for 5 days as a negative control (Figure 5E) to no effect.

Loss of the apoptotic pathway in stable transfectants—The CDDE was used to detect CDDP-induced apoptosis in cells treated with 10 μ M CDDP (Figure 6). Cells expressing the wild-type peptide and the N2452D and I2285V mutant peptides entered apoptosis in significantly smaller numbers compared to the untransfected T47D cells. I2285V mutant-expressing cells also entered apoptosis at a significantly lower rate than did F2293L mutant-expressing cells. There was no difference in apoptotic index between the empty vector transfection and untransfected cells. The R2318Q VUS changed the effect of the linker peptide on cell avoidance of apoptosis. Cells expressing this R2318Q mutant peptide entered apoptosis at the same rate as untransfected T47D cell controls, at a significantly higher rate than cells expressing the wild-type peptide and the N2452D and I2285V mutant peptides.

Discussion

To determine if VUS's in the linker region of *BRCA2* are associated with alterations in protein function, we chose to study a panel of VUS's occurring on evolutionarily conserved residues (figure 2A). The 4 mutations studied here are reported with varying frequency. One, I2285V, has been reported 79 times, whereas several others have been reported once each. Based on its high reporting incidence, I2285V could be a neutral mutation, or alternatively it could impact the function of *BRCA2*. Differentiating between neutral and function-impacting mutations in *BRCA2* is a goal of creating this screen. The VUS screen created in this study was used as a proof-of-principle study, developing a concept on which a quality controlled assay could be developed before being used to determine the effect of various VUS on protein function. The WST assay indicates that the stably transfected clones used in this study have similar growth rates in normal cell culture conditions. This affirms that the peptides by themselves do not change cell activity in the absence of a DNA damage insult.

To study functional consequences of *BRCA2* linker region variations, we created an assay based on site directed mutagenesis and functional testing. The data from the survival and apoptosis assays were used in conjunction to assess the affect of individual VUS on linker region function (see figure 7). Two of the cell lines expressing mutant peptides (N2452D and I2285V) survived a 10 μ M CDDP treatment. These cell lines (N2452D and I2285V) showed significantly decreased rates of apoptosis when treated with 10 μ M CDDP similar to the cell line expressing the wild-type peptide. These decreased apoptosis levels indicate that the VUS N2452D and I2285V did not change the function of the linker peptides— because their effect upon apoptosis levels is similar to that of the wild-type peptide (Figure 7A). R2318Q transfected cells behaved similarly to untransfected cells and the empty vector control in both the survival and apoptosis assays, which means that the mutation was interfering with the peptide's normal function (figure 7B). Although it would be theoretically interesting to knock down expression of these peptides using RNAi in stable cell lines, we believe the additional transfection of RNAi into the already transfected cell lines would likely lead to greater artifacts as a consequence of extra DNA.

Some inconsistencies were seen in results obtained during the Crystal Violet and apoptosis assays. Although for technical reasons we could not validate protein expression after each experiment, the stable clones chosen for this experiment were experimentally determined to express similar levels of the prescribed linker region peptides by dot blot. Additionally, neomycin selection was continued in cell culture throughout the study to maintain selective pressure. Though these clones are not isogenic, the chance of plasmid insertion into a site that would dramatically change cell behavior in these experiments is acceptably small, since we chose subclones with similar basal growth rates. Expression of the wild-type peptide did not increase survival of the cells as observed by the Crystal Violet assay, but did significantly decrease the level of apoptosis seen by CDDE. Lack of sensitivity in the Crystal Violet survival assay lead us to conclude that this is not the best possible method for use in similar studies in the future. As was seen in a head to head comparison of cytotoxicity of sodium lauryl sulfate [19], at low concentrations Crystal Violet failed to detect dying cells compared to either viability assay or neutral red, which measures lysosomal dye accumulation. Although Crystal Violet met our standards of an inexpensive and simple method for estimating cell number, it did not provide us with enough consistency to make conclusive observations about the activities of individual peptides at appropriate doses of CDDP. This may be compounded by the similarity in dye uptake for living cells and cells in early apoptosis.

Other laboratories have studied VUS's for their impact on the BRCA2 function. Full-length BRCA2 protein was expressed exogenously through a stable transfection in BRCA2 negative cells, and mutants representing patient-derived VUS's were introduced into this vector, allowing for a test of the mutation's effect on the BRCA2's various functional domains [3]. More recently, VUS have been characterized through the BAC-driven expression of mutant BRCA2 in conditional *BRCA2*-null mouse embryonic stem cells [4]. This technique has been successful and has since been applied to *BRCA1* VUS [20]. The current study had goals in addition to the characterization of the numerous VUS's. Studying the linker region as a separate domain made manipulation of the expression plasmid much easier than using a full-length *BRCA2* construct, which would also be more susceptible to spontaneous mutation during propagation in bacteria. This improvement thus is a step forward in creating a screen for linker region VUS that would be both high-throughput and affordable.

I2285V, one of the mutations tested in this study, was characterized by another laboratory using the BAC recombineering approach previously discussed [4]. This VUS was found to be a neutral mutation, leading to an alternative transcript of *BRCA2*, missing exon 12, but not changing protein function. This is consistent with our data, which suggest that I2285V mutant peptides behave similarly to wild-type linker peptides in the apoptosis assay. Another study determined that the peptide encoded by exon 12 of the *BRCA2* transcript is not essential for the function of the full-length protein [21] F2293L is the only other exon 12 mutation studied here, and produced results not significantly different from either cells expressing the wild-type peptide or the untransfected controls; this supports the notion that exon 12 is expendable for the function of the linker region as previously determined [21].

The data from the survival and cell death detection ELISA assays showed that some of these cell lines have avoided apoptosis and continued to proliferate in spite of DNA damage caused by CDDP. This change in behavior could stem from a number of effects of the peptides on the cell. Interpretation of these data is complicated by the fact that the function of the linker region remains unknown. Conservatively, we conclude that this model may in the future be a good method for screening the effect of VUS's on protein function. To better this screen, the nature of the peptide's effect on cell survival in response to treatment with CDDP and other DNA damaging agents must be studied by some assay that measures levels of DNA damage such as the COMET assay [22]. In addition, a more sensitive assay of cell number after treatment would be recommended in the place of the Crystal Violet survival assay. Recent studies presented at the American Association for Cancer Research (Orlando, FL) [23] examined *BRCA2* mutant's responsiveness to mytomicin C, ionizing radiation and CDDP. These studies indicate that *BRCA2*'s activity is the same in response to other DNA crosslinkers and double-strand breaks induced by ionizing radiation. To further validate the results from this screen in the future, a full-length construct of *BRCA2* could be studied in cells which are *BRCA2*^{-/-} as in referenced studies [3]. The full-length construct has already been used in such screens testing the effect of VUS' [24]. If the results of a screen using the full-length construct mirror those of this study, then it may be valid to use the cheaper, faster version of the screen using isolated regions of the protein. This type of screen, if commercialized, could provide an invaluable new tool in the arsenal of clinicians and genetic counselors to assist in discriminating function-altering mutations from function-neutral mutations in familial cancer syndromes.

Acknowledgments

This work was funded by the Center for Translational Cancer Research, PO1 NIH/NCI P01 CA098912, and the Cancer Genetics Fund from the Helen F. Graham Cancer Center (Christiana Care Health Systems, Newark DE). We are grateful to Dr. Nicholas Petrelli, Dr. Kristine Widders, and Ketan Patel for their assistance throughout this work. We thank Ms. Sharron Kingston for her assistance with the preparation and submission of the manuscript.

References

1. Szabo CI, Worley T, Monteiro AN. Understanding germ-line mutations in BRCA1. *Cancer Biol Ther.* 2004; 3:515–520. [PubMed: 15254424]
2. Brody L. Breast Cancer Information Core, National Institutes of Health - National Human Genome Research Institute. 2007
3. Carvalho MA, Couch FJ, Monteiro AN. Functional assays for BRCA1 and BRCA2. *Int J Biochem Cell Biol.* 2007; 39:298–310. [PubMed: 16978908]
4. Kuznetsov SG, Liu P, Sharan SK. Mouse embryonic stem cell-based functional assay to evaluate mutations in BRCA2. *Nat Med.* 2008; 14:875–881. [PubMed: 18607349]
5. Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol Cell.* 2001; 7:273–282. [PubMed: 11239456]
6. Yang H, Jeffrey PD, Miller J, Kinnucan E, Sun Y, Thoma NH, Zheng N, Chen PL, Lee WH, Pavletich NP. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. *Science.* 2002; 297:1837–1848. [PubMed: 12228710]
7. Thorslund T, Esashi F, West SC. Interactions between human BRCA2 protein and the meiosis-specific recombinase DMC1. *Embo J.* 2007; 26:2915–2922. [PubMed: 17541404]
8. Davies OR, Pellegrini L. Interaction with the BRCA2 C terminus protects RAD51-DNA filaments from disassembly by BRC repeats. *Nat Struct Mol Biol.* 2007; 14:475–483. [PubMed: 17515903]
9. Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, De Die-Smulders C, Persky N, Grompe M, Joenje H, Pals G, Ikeda H, Fox EA, D'Andrea AD. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science.* 2002; 297:606–609. [PubMed: 12065746]
10. Joenje H, Patel KJ. The emerging genetic and molecular basis of Fanconi anaemia. *Nat Rev Genet.* 2001; 2:446–457. [PubMed: 11389461]
11. Kitao H, Yamamoto K, Matsushita N, Ohzeki M, Ishiai M, Takata M. Functional interplay between BRCA2/FancD1 and FancC in DNA repair. *J Biol Chem.* 2006; 281:21312–21320. [PubMed: 16687415]
12. Wang X, Andreassen PR, D'Andrea AD. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol Cell Biol.* 2004; 24:5850–5862. [PubMed: 15199141]
13. Fujiwara Y, Nakamura M, Yokoo S. A new anticancer platinum compound, (-)-(R)-2-aminomethyl-pyrrolidine(1,1-cyclobutanedicarboxylato) platinum(II): DNA interstrand crosslinking, repair and lethal effects in normal human, Fanconi's anaemia and xeroderma pigmentosum cells. *Br J Cancer.* 1993; 67:1285–1292. [PubMed: 8512813]
14. Poll EH, Arwert F, Joenje H, Wanamarta AH. Differential sensitivity of Fanconi anaemia lymphocytes to the clastogenic action of cis-diamminedichloroplatinum (II) and trans-diamminedichloroplatinum (II). *Hum Genet.* 1985; 71:206–210. [PubMed: 4065892]
15. Pera MF Jr, Rawlings CJ, Shackleton J, Roberts JJ. Quantitative aspects of the formation and loss of DNA interstrand crosslinks in Chinese hamster cells following treatment with cis-diamminedichloroplatinum(II) (cisplatin). II. Comparison of results from alkaline elution, DNA renaturation and DNA sedimentation studies. *Biochim Biophys Acta.* 1981; 655:152–166. [PubMed: 6169370]
16. Zwelling LA, Bradley MO, Sharkey NA, Anderson T, Kohn KW. Mutagenicity, cytotoxicity and DNA crosslinking in V79 Chinese hamster cells treated with cis- and trans-Pt(II) diamminedichloride. *Mutat Res.* 1979; 67:271–280. [PubMed: 481452]
17. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, Brenner HJ. Establishment and characterization of a cell line of human breast carcinoma origin. *Eur J Cancer.* 1979; 15:659–670. [PubMed: 228940]
18. Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. *Biophotonics International.* 2004; 11:36–42.
19. Chiba K, Kawakami K, Tohyama K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. *Toxicol In Vitro.* 1998; 12:251–258. [PubMed: 20654407]

20. Chang S, Biswas K, Martin BK, Stauffer S, Sharan SK. Expression of human BRCA1 variants in mouse ES cells allows functional analysis of BRCA1 mutations. *J Clin Invest.* 2009; 119:3160–3171. [PubMed: 19770520]
21. Li L, Biswas K, Habib LA, Kuznetsov SG, Hamel N, Kirchhoff T, Wong N, Armel S, Chong G, Narod SA, Claes K, Offit K, Robson ME, Stauffer S, Sharan SK, Foulkes WD. Functional redundancy of exon 12 of BRCA2 revealed by a comprehensive analysis of the c.6853A>G (p.I2285V) variant. *Hum Mutat.* 2009; 30:1543–1550. [PubMed: 19795481]
22. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988; 175:184–191. [PubMed: 3345800]
23. Biswas, K.; Das, R.; Alter, BP.; Kuznetsov, S.; North, SL.; Stauffer, S.; Burkett, S.; Brody, LC.; Meyer, S.; Byrd, AR.; Sharan, SK. A comprehensive study to functionally classify the BRCA2 missense mutations found in Fanconi anemia patients. American Association for Cancer Research 102nd Annual Meeting; Orlando, FL. 2011.
24. Tavtigian SV, Simard J, Rommens J, Couch F, Shattuck-Eidens D, Neuhausen S, Merajver S, Thorlacius S, Offit K, Stoppa-Lyonnet D, Belanger C, Bell R, Berry S, Bogden R, Chen Q, Davis T, Dumont M, Frye C, Hattier T, Jammulapati S, Janecki T, Jiang P, Kehrer R, Leblanc JF, Mitchell JT, McArthur-Morrison J, Nguyen K, Peng Y, Samson C, Schroeder M, Snyder SC, Steele L, Stringfellow M, Stroup C, Swedlund B, Swense J, Teng D, Thomas A, Tran T, Tranchant M, Weaver-Feldhaus J, Wong AK, Shizuya H, Eyfjord JE, Cannon-Albright L, Tranchant M, Labrie F, Skolnick MH, Weber B, Kamb A, Goldgar DE. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. *Nat Genet.* 1996; 12:333–337. [PubMed: 8589730]
25. Sharan SK, Morimatsu M, Albrecht U, Lim DS, Regel E, Dinh C, Sands A, Eichele G, Hasty P, Bradley A. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature.* 1997; 386:804–810. [PubMed: 9126738]
26. Hughes-Davies L, Huntsman D, Ruas M, Fuks F, Bye J, Chin SF, Milner J, Brown LA, Hsu F, Gilks B, Nielsen T, Schulzer M, Chia S, Ragaz J, Cahn A, Linger L, Ozdag H, Cattaneo E, Jordanova ES, Schuurin E, Yu DS, Venkitaraman A, Ponder B, Doherty A, Aparicio S, Bentley D, Theillet C, Ponting CP, Caldas C, Kouzarides T. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. *Cell.* 2003; 115:523–535. [PubMed: 14651845]
27. Fuks F, Milner J, Kouzarides T. BRCA2 associates with acetyltransferase activity when bound to P/CAF. *Oncogene.* 1998; 17:2531–2534. [PubMed: 9824164]
28. Wong AK, Pero R, Ormonde PA, Tavtigian SV, Bartel PL. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J Biol Chem.* 1997; 272:31941–31944. [PubMed: 9405383]

Abbreviations

VUS	variants of unknown significance
CDDP	cisplatin
DDR	DNA Damage Repair
ELISA	enzyme-linked immunosorbent assay
CDDE	cell death detection ELISA
FCRR	Ruth Ann Minner High Risk Family Cancer Registry

Highlights

- Proof-of-principle new screen for BRCA2 VUS characterization.
- BRCA2 linker region influences cisplatin-induced cell death.
- VUS modify effect of BRCA2 linker region on cell response to cisplatin treatment.

Figure 1. A linear schematic of the BRCA2 protein including the linker region

Domains with known functions are indicated by their binding partner or cellular process. Amino acid residues attributed to these functional domains are indicated below the linear schematic. The protein is involved in DNA damage repair [25] and implicated in transactivation and histone modification through interactions with EMSY[26, 27]. The linker region lies between characterized functional domains, the RAD51-binding domain [28] and the single-stranded DNA binding domain [6]. The linker itself is encoded by exons 12, 13 and 14, comprising 198 amino acids.

A.

2281
 GEPSIKRNLLNEFDRIIENQEK
 SLKASKSTPDGTIKDRRLFMH
 HVSLEPITCVPFRTTKERQEIQ
 NPNFTAPGQEFLSKSHLYEHLT
 LEKSSSNLAVSGHPFYQVSAT
 RNEKMRHLITTGRPTKVFVPP
 FKTKSHFHRVEQCVRNINLEE
 NRQKQNI~~D~~GHGSDDSKNKIND
NEIHQFNKNNSNQAVAVTFTK
 CEEEPD
 2479

B.

Mutation designation	Entries in database	Source	Survival Assay	Apoptosis Assay
Wild-Type	N/A	N/A	=	-
P2283A	1	BIC	N/A	N/A
I2285V	79	BIC	+	-
F2293L	2	BIC	+	=
D2312V	5	BIC	N/A	N/A
R2318Q	4	BIC	=	=
P2329L	3	BIC	N/A	N/A
R2341C	1	HFGCC	N/A	N/A
P2347Q	1	BIC	N/A	N/A
P2352L	2	BIC	N/A	N/A
S2373T	2	BIC	N/A	N/A
F2406L	1	BIC	N/A	N/A
K2411T	6	BIC	N/A	N/A
N2452D	1	HFGCC	+	-

Figure 2. Primary structure of the linker region peptide and panel of VUS's created by site-directed mutagenesis

(A) Primary structure of the linker region of the BRCA2 protein. Underlined residues represent amino acids chosen for this panel of mutations for study. This linker region represents amino acid residues 2281 through 2479 of the full-length protein. (B) A table containing the mutations found in breast cancer patients on the residues highlighted in red in part (A). "Entries" column indicates how many separate times these variants have been reported to the BIC or HFGCC databases. The source column indicates what database the mutation was logged in. The Survival assay column indicates results obtained when testing expression of this peptide in the survival assay (+, higher survival; =, no difference in survival, when compared to untransfected control). The Apoptosis Assay column indicated results obtained when testing expression of this peptide in the apoptosis assay with 10 μ M CDDP treatment (-, lower apoptosis levels, =, no difference in apoptosis levels when compared to untransfected control). Bolded variants represent VUS chosen for this proof-of-principle study.

Figure 3. T47D cell expression clones produce recombinant linker region peptides as evidenced by C-terminal His-tag recognizing antibodies

(A) Penta-His antibody recognized the C-terminal His-tag of the recombinant linker region peptides in selected stable cell lines. (B) Untransfected T47D cells and cells resistant to neomycin but transfected with an empty vector served as negative controls. This blot was performed on cell extracts at the commencement of this study. (C) Densitometric analysis of dot blot for c-terminal His-tag in (B).

Figure 4. Proliferation of transfected cell lines does not differ from untransfected T47D cells
Results of the WST-1 assay graphed as a ratio of absorbances at 470 and 490 nanometers of the respective stably transfected cell line over that of the untransfected T47D cell line. No significant differences were detected by use of a one-way ANOVA followed by Tukey's multiple comparison test. Error bars represent standard error of the mean for two biological replicates of this experiment.

Figure 5. Crystal Violet assay demonstrated decrease in cell number in response to CDDP treatment

(A) 1 μ M CDDP treatment for 5 days and (B) 5 μ M CDDP treatment for 5 days result in similar survival among all cell lines. (C) 10 μ M CDDP treatment for 5 days. (D) Variable concentrations of CDDP do not elicit a difference in survival in untransfected cells when compared with cells transfected with an empty vector as a negative control. (E) 5 Day treatment of each cell line with CDDP vehicle (saline). Error bars represent standard error of the mean for each data point. These experiments were performed in triplicate experimental wells in three biological replicates. Results were not statistically significant unless so indicated. **p<.01, one-way ANOVA followed by Tukey's multiple comparison test.

Figure 6. Cell lines expressing recombinant linker region peptides avoid CDDP-induced apoptosis

Cells were treated with normal T47D growth medium supplemented with 10uM cisplatin for a 5-day period. Bar graphs represent the absorbance of duplicates of treated cells divided by the absorbance of untreated cells of the same cell line. The cell lines transfected with the empty vector and untransfected T47D cells served as controls. There was no difference between the empty vector cell line and untransfected cells. These experiments were performed in triplicate experimental wells in three biological replicates. Significance determined by one-way ANOVA followed by Tukey's multiple comparison test.

A. Wild-type BRCA2 linker function

B. Absent BRCA2 linker function

Figure 7. Schematic for analysis of peptide screen results

The linker region peptide screen can be interpreted by the cellular response to cisplatin treatment during the survival assay and CDDE. If the VUS does not interfere with linker region function, results will reflect those obtained when testing cells overexpressing the wild-type linker peptide (A), i.e. maintenance of protein function and cell survival. If the VUS does alter linker peptide function, cells will respond similarly to untransfected cells (B), i.e. loss of protein function and cell death in response to cisplatin treatment.

Table 1
Oligonucleotide Primers¹ used in RT-PCR and Site-Directed Mutagenesis

Name	Sequence	Product Size	Tm (°C)
BRCA2Exon12-14 F	AGAGCCCCCTTATCTTAGTGG	635	55
BRCA2Exon12-14 R	CTGAAGACTTGTAATTAAT		
BRCA212-14+KozakF	CACCATGGGAGAACCCTC	602	52
BRCA212-14+KozakR	TAAAGGTTCTTCTTCACACTTTGTG		
Site-directed mutagenesis primers			
F2293L F	CCTCAATCAAAAGAACTTATTAATGAATTAGACAGGATAATAG	N/A	53
F2293L R	CTATTATCCTGTCTAATTCATTTAATAAGTTTCTTTTGATTGAGG		
N2452D F	GATTAATGACGATGAGATTCACCAGTTTAACAAAAACAACCTCC	N/A	56.4
N2452D R	GGAGTTGTTTTTGTAAACTGGTGAATCTCATCGTCATTAATC		
I2285V F	CACCATGGGAGAACCCTCAGTCAAAAGAACTTATTAATG	N/A	57.2
I2285V R	CATTTAATAAGTTTCTTTGACTGAGGGTTCTCCCATGGTG		
R2318Q F	CTCCAGATGGCACAATAAAAGATCAAAGATIGTTTATGCATCATG	N/A	57.5
R2318Q R	CATGATGCATAAACAATCTTTGATCTTTTATTGTGCCATCTGGAG		
S2373T F	CATCTGACTTTGGAAAAATCTTCAACCAATTTAGCAGTTTCAGG	N/A	57.7
S2373T R	CCTGAAACTGCTAAATTTGGTTGAAGATTTTCCAAAGTCAGATG		
P2239L F	CATCATGTTTCTTTAGAGCTGATTACCTGTGTACCCTTTTCG	N/A	57.8
P2239L R	CGAAAGGGTACACAGGTAATCAGCTCTAAAGAAACATGATG		
K2411T F	CAACCAAAGTCTTTGTTCCACCTTTTACAACATAAATCGCATTTC	N/A	57.9
K2411T R	GAAATGCGATTTAGTTGTAAAAGGTGGAACAAAGACTTTGGTTG		
D2312V F	CTTCAAAAAGCACTCCAGTTGGCACAATAAAAGATCGAAG	N/A	58
D23122V R	CTTCGATCTTTTATTGTGCCAACTGGAGTGCTTTTGAAG		
R2341C F	CCCTTCGCACAATAAGGAATGTCAAGAGATACAGAATC	N/A	58
R2341C R	GGATTCTGTATCTCTTGACATTCCTTAGTTGTGCGAAAGGG		
P2352L F	GAGATACAGAATCCAAATTTTACCGCACTTGGTCAAGAATTTCTG	N/A	58.1
P2352L R	CAGAAATCTTTGACCAAGTGCGGTAAAATTTGGATTCTGTATCTC		
P2283A F	CCTTCACCATGGGAGAAGCCTCAATCAAAAGAAAC	N/A	58.2
P2283A R	GTTTCTTTTGATTGAGGCTTCTCCCATGGTGAAGG		
F2406L F	GCAGACCAACCAAGTCCTTGTTCACCTTTTAAAC	N/A	58.4
F2406L R	GTTTAAAAGGTGGAACAAGGACTTTGGTTGGTCTGC		
P2347Q F	GAACGTCAAGAGATACAGAATCAAAATTTTACCGCACCTGGTC	N/A	59
P2347Q R	GACCAGGTGCGGTAAAATTTGATTCTGTATCTCTTGACGTTT		

¹ All primers were designed originally for this project.