Identification of Breast Tumor Mutations in BRCA1 That Abolish Its Function in Homologous DNA Recombination

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Abstract

Effects of breast cancer–associated gene 1 (BRCA1) missense mutations on the function of BRCA1 protein in DNA recombination have been little studied. In this report, we adapted a homology-directed recombination (HDR) assay to analyze the effects of BRCA1 mutations on this function. Using a HeLa-derived cell line with a genomically integrated recombination substrate, we expressed an endonuclease creating a double-stranded break in the substrate that the HDR assay scores by generation of green fluorescent protein–positive cells. By combining RNA interference (RNAi) that targets cellular BRCA1 mRNA with expression of RNAi-resistant BRCA1 mutants, we could effectively substitute selected point mutants to test these in the cellular recombination assay. We found that ∼300 residues at both termini of the BRCA1 protein were essential for HDR. Whereas some mutations analyzed were neutral, mutations that altered any zinc-coordinating residue or generated M18T and T37R alterations were defective for recombination. This study established a robust assay system to analyze the function of BRCA1 in regulating homologous recombination, which is critical for its tumor suppressor function. Cancer Res; 70(3); 988–95. ©2010 AACR.

Introduction

The breast cancer–associated gene 1 (BRCA1) is a tumor suppressor that is specifically associated with familial cases of breast cancer and ovarian cancer (1). In familial breast cancer, a woman may inherit a BRCA1 allele with a mutation, and during her lifetime, there is a high likelihood that in at least one breast epithelial cell the second nonmutated allele of BRCA1 will be lost by deletion. Such a cell is hemizygous for BRCA1, and the one remaining allele has a mutation. If that mutation renders the BRCA1 defective for a key biological function, then that cell has increased likelihood of transforming into a breast tumor. The BRCA1 gene has been sequenced from many individuals with a family history of breast cancer, and many of the characterized mutations result in a frameshift or a stop codon and truncate the encoded BRCA1 protein. Clearly, such truncating mutations predispose to breast cancer. Missense mutations, however, can present a diagnostic dilemma because specific point mutations have low prevalence in the population and the disease association is often unclear. Several genetic and evolutionary analyses can predict whether a given point mutation is likely to predispose to breast cancer, but the interpretation can be ambiguous (2–4).

Although there have been many studies assessing the functional implication of BRCA1 mutations, there has been no systematic functional studies that are designed for analysis of the full-length protein or that assess a biological process such as homologous recombination. Several in vitro biochemical assays have tested the effects of point mutations in protein-protein interactions (5), in BRCA1-dependent ubiquitin ligase activity (6), and in the control of the centrosome (7). A tissue culture cell-based assay fuses 468 amino acids from the BRCA1 COOH terminus to a DNA-binding domain and analyzes the effect of the mutant on a transcription-based reporter assay (8). This latter assay has been very successful in assigning whether a missense mutation is associated with loss of transcription function, but the assay cannot evaluate BRCA1 sequences in the NH2-terminal three fourths of the protein, and the assay does not address nontranscription functions of BRCA1, such as regulation of DNA repair.

In this study, we have adapted the homologous recombination assay that BRCA1 regulates (9–11) to a robust method to analyze specific BRCA1 protein variants. The modified assay tests specific BRCA1 missense mutations in the homologous recombination process. We have characterized a set of point mutations in the NH2 terminus of BRCA1 in this assay as an initial step in determining whether a BRCA1 variant of unknown function may predispose to breast cancer.
Materials and Methods

Plasmids, small interfering RNA, and cell lines. The plasmid encoding the recombination substrate, pDR–green fluorescent protein (GFP), and the I-SceI expression plasmid, cBAS, were both kindly provided by Drs. K. Nakanishi and M. Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY). BRCA1 expression plasmid, pcDNA-5′ HA-BRCA1, has been previously described (12), and site-specific point mutations identified on the Breast Cancer Information Core (BIC) were generated using the QuikChange kit from Stratagene. Details of the subcloning are available on request. Plasmids for the expression of BRCA1 deletion mutants have been previously described (12, 13).

Sequences of the 3′-untranslated region (UTR) of BRCA1 were targeted with small interfering RNA (siRNA) oligonucleotides based on the sequence GCUCCUCUCACUCUCA-GU specific for nucleotides 80,780 to 80,798 of the BRCA1 gene (accession number AY273801). BRCA2 was targeted using siRNA based on the sequence UAAAUUUGGACAUAAG-GAGUCCUCC. The control siRNA, called GL2, targets the luciferase gene (14).

A stable derivative of HeLa cells was established by transfection with pDR-GFP using standard procedures and selection in 1.5 μg/mL puromycin. Puromycin-resistant cells were cloned by limiting dilution in 96-well plates, and an initial screen selected those colonies that had no detectable GFP fluorescence. Remaining colonies were replica plated and transfected in parallel with cBAS to express the I-SceI. The untransfected stocks of those clones with intense fluorescence in a high percentage of cells were selected and recloned by limiting dilution. The number of integration sites in the final cell line, HeLa-DR13-9, was determined by digesting genomic DNA with XhoI and analyzing by Southern blotting.

Homology-directed recombination assay. On day 1, HeLa-DR13-9 cells (~4.0 × 10⁴ in a 1.5-cm well) were transfected with 5 pmol of BRCA1 siRNA targeting the 3′-UTR of BRCA1 gene and 0.3 μg of the appropriate BRCA1 expression plasmid in the presence of 0.5 μL of Lipofectamine 2000 (Invitrogen). On day 2, the transfected cells were transferred to 3.5-cm well dishes. On day 3, the cells were transfected with 25 pmol of the BRCA1 siRNA, 0.75 μg of the appropriate BRCA1 expression plasmid, plus 0.75 μg of cBAS in the presence of 2.5 μL of Lipofectamine 2000. On day 6, cells were trypsinized and 10,000 cells from each well were counted by flow cytometry using a Becton Dickinson FACSCalibur instrument in the Ohio State University Comprehensive Cancer Center Analytical Cytometry shared resource.

All point mutants of BRCA1 analyzed were tested in triplicate, and the percentage of cells with recombined locus encoding GFP were normalized to the same percentage from the control siRNA–transfected cells. In the figures, the averages are shown with the SE depicted by error bars.

Immunoprecipitation analysis. HEK293T cells were transfected with the same BRCA1 variant expression constructs as used in the homology-directed recombination (HDR) assay. Whole-cell lysates were harvested 2 d after transfection and subjected to immunoadfinity purification using the HA.11 anti-hemagglutinin (HA) tag antibody (Covance) by standard procedures (12). Western blots were analyzed with anti-HA antibody and anti-BARD1 antibody H-300 (Santa Cruz Biotechnology) by standard methods.

Results

Both the NH₂ and COOH termini of BRCA1 are required for BRCA1 regulation of HDR. We adapted an established assay (15) for homologous recombination in which two
inactive alleles of GFP are integrated in a single locus in the genome of the cell. One allele contains the 18-bp recognition element for the I-SceI endonuclease. Transfection into the cells of a plasmid for expressing the I-SceI results in a double-strand break in one GFP allele, and this break can be repaired by HDR using the second inactive allele of GFP. (The plasmids encoding the recombination substrate and the I-SceI endonuclease were the gift of Dr. M. Jasin.) This repair results in gene conversion that creates a GFP allele that encodes an active protein, and the recombination can be detected by identifying green-fluorescing cells. This plasmid and strategy (Fig. 1A) has been successfully used in a variety of experiments in which a cell line carrying a mutant version of a protein under study has had this recombination substrate inserted in the cell, and the effect of the specific protein was then evaluated (15–17). We instead inserted a copy of this recombination substrate into HeLa cells and carefully selected a clone, called HeLa-DR13-9, which has no background GFP fluorescence, but following transfection of the I-SceI-expressing plasmid, there is a high level of GFP-positive cells. In many repeated experiments, between 10% and 20% of the cells will undergo recombination following I-SceI expression. This cell line is then useful for the analysis of RNA interference (RNAi) depletion of proteins and testing for their effects in HDR.

HeLa-DR13-9 cells were transfected with either a control siRNA or a siRNA specific to BRCA1. Two days later, these cells were retransfected with the appropriate siRNA plus the plasmid for expression of I-SceI, and 3 days later, the percentage of GFP-positive cells were determined by flow cytometry. The reduction in GFP-positive cells when BRCA1 is depleted is evident from inspection of the resultant monolayers (Fig. 1B) and is quantified by flow cytometry (10,000 cells counted per sample). In the experiment for Fig. 2, in the absence of transfected I-SceI expression plasmid, there are no GFP-positive cells (Fig. 2, lane 1). In the presence of I-SceI and a control siRNA, 16% of the cells were GFP positive (lane 2). Depletion of BRCA1 or BRCA2 reduced the number of cells with recombined GFP alleles by 8- or 40-fold, respectively. Similar reductions in HDR were observed with siRNAs targeting other BRCA1 and BRCA2 sequences, indicating that these results are not due to off-target effects of the siRNA (data not shown). Further, these results are consistent with published observations (9, 18). Importantly, when the siRNA targets the 3′-UTR of the BRCA1, expression of exogenous BRCA1 from a plasmid with a different 3′-UTR results in complete restoration of HDR activity (Fig. 2, lane 4). This result suggests that we have a robust assay for determining the effects of specific BRCA1 mutations in the regulation of the homologous recombination process.

We next assayed a series of synthetic BRCA1 deletion mutants for function in the HDR assay. In Fig. 3A, the four deletion mutants are diagrammed. Using the same time course as in Fig. 2, the endogenous BRCA1 protein was depleted by 3′-UTR–targeting siRNA, and the test BRCA1 protein was expressed from a cotransfected plasmid. Multiple repeat experiments were done, and in each experiment, the maximal recombination varied from 10% to 20% depending on transfection efficiency. Within an experiment, the ratio of GFP-positive cells in the BRCA1-depleted sample relative to the control RNAi was consistently 8- to 10-fold reduced. We thus normalized data in each experiment, allowing us to average the results from multiple experiments. Replacing the endogenous BRCA1 with ΔN-BRCA1, which has deleted residues 1 to 302, results in a 4- to 5-fold reduction in homologous recombination. Expression of ΔM1-BRCA1, which has deleted residues 305 to 770, reduced recombination by ~40%. Similarly, expression of ΔM2-BRCA1, which has deleted residues 775 to 1292, resulted in a decrease in recombination by ~60%. Expression of ΔC-BRCA1, which has deleted residues 1527 to 1863, resulted in a 10-fold loss of recombination. Clearly, the NH2 and COOH termini each had a significant role in controlling the recombination reaction. Expression of the two internal domains had intermediate effects. In each case, the concentrations of BRCA1 protein expressed from the transfected plasmid were at or higher levels than the endogenous BRCA1 protein (Fig. 3C), indicating that the reduction in HDR activity was not due to failure to express the test protein. Because the expression level of the ΔN, ΔM1, and ΔM2 mutants was significantly higher than the endogenous BRCA1 protein, it is possible that the magnitudes of the deficiencies in HDR activity with these BRCA1 deletion mutants were underestimated. We decided to focus on point mutations in the NH2 terminus.

Identification of BRCA1 point mutations that are critical for homologous recombination activity. We decided to test point mutations in the NH2-terminal 71-amino acid residues of BRCA1 that were derived from individuals with a family history of breast cancer and identified from the BIC database. The reasons for using mutations identified in breast or ovarian cancer clinics were 2-fold: first, this is the...
most direct way to identify candidate amino acid residues for critical function without performing saturating mutagenesis and testing a large number of variants, and second, the results of our functional tests might be useful in counseling such individuals who carry these mutations. Sixteen different variants were produced by site-directed mutagenesis of the BRCA1 cDNA-expressing plasmid. One of these variants, C27A, is a synthetic mutation and is not derived from a variant obtained from an individual with a family history with breast cancer. Rather, the C27A variant completes the set of eight different zinc-coordinating residues of BRCA1 in this analysis. The results of multiple experiments are shown in Fig. 4. Consistent with our previous experiments, depletion of BRCA1 by transfection of siRNA targeting the 3′-UTR and cotransfection of the empty plasmid vector resulted in a 10-fold reduction in GFP-positive cells relative to the control siRNA. Transfection of this BRCA1-specific siRNA along with the wild-type BRCA1 expression plasmid fully restored HDR (Fig. 4A, lane 3). Strikingly, transfection of each mutant either fully restored recombination to 100% or was fully negative, at the same level of homologous recombination as the vector-transfected control. We note that the deletion mutants used in Fig. 3 produced recombination levels that had intermediate results: the ΔM1 and ΔM2 deletions caused...
partial decrements in homologous recombination. Even deleting the NH₂ terminus, containing all of the residues being tested did not have as severe an effect on the HDR assay as did the point mutants. The expression levels of the point mutants of BRCA1 all were similar to the endogenous protein level (Fig. 4B, compare lane 3 with all other lanes), whereas the ΔN deletion, which encompassed all of these point mutants, was significantly overexpressed relative to the endogenous protein and perhaps causing a partial masking of the HDR defect. Alternatively, it is a formal possibility that the point mutations in the BRCA1 protein have both a loss-of-function phenotype and a dominant-negative phenotype.

Eight of the residues that were tested coordinate zinc ions in the RING domain: C24R, C27A, C39Y, H41R, C44F, C47G, C61G, and C64G. It is anticipated that substitution of any of these amino acid residues would have major structural consequences to the protein. Consistent with that concept, replacement of the endogenous BRCA1 with any of these BRCA1 molecules with mutated zinc-coordinating residues was nonfunctional in HDR (Fig. 4A, lanes 6, 7, 10, 11, 13, 14, 16, and 17).

Eight other substitution mutants were tested for function in the homologous recombination pathway. The M18T and T37R variants did not complement the HDR assay. Variants that could complement the HDR activity were I21V, I31M, I42V, L52, D67Y, and R71G.

We tested whether the mutant BRCA1 proteins used in this study could bind to BARD1 (Fig. 5). We transfected the HA epitope–tagged BRCA1 into HEK293T cells and purified proteins in complex with the variant BRCA1 protein by immunoprecipitation using antibody recognizing the HA tag. Purified proteins were analyzed by immunobLOTS specific for the HA epitope to evaluate the effectiveness of expression and purification of the mutant BRCA1 protein and specific for the endogenous BARD1 protein. We found that BRCA1 variant proteins I21V, I31M, I42V, L52F, D67Y, and R71G effectively purified cellular BARD1 protein. These are the same proteins that functioned in homologous recombination. Of note, the BRCA1 variant proteins M18T and H41R had detectable low level of BARD1 binding, but these BRCA1 variants did not complement the HDR assay.

Correlation of biological function with available clinical data for each BRCA1 missense mutant. We have summarized the available information for each mutant in Table 1, including results of HDR function and BARD1 binding (this study), E3 ubiquitin ligase activity and resistance to ionizing radiation (IR; ref. 6), and available clinical information. For many of these variants, family history was unavailable, and this limited the analysis we could do. Examples of available family data are supplied in Supplementary Table S1. We applied an algorithm called VUS Predict (19) to the 16 variants. VUS Predict calculates the odds of a variant being...
detrimental based on a variety of characteristics of tumors, including estrogen receptor, progesterone receptor, and Her2 status, tumor grade, histopathology, age of onset, and the position of the amino acid residue in a functional domain or evolutionary conserved sequence. If there were no clinical data, then the VUS Predict output was based on the single criterion of evolutionary conservation (asterisked values in Table 1).

Of the 16 BRCA1 variants in this study, 4 of the eight zinc-coordinating residues have been classified as deleterious when mutant (Table 1). One other variant, R71G, has been classified as deleterious (2, 21), and one, D67Y, has been classified as neutral (4). One of the substitution mutants, M18T, has a published odds ratio of being deleterious of 31:1 (4). Using VUS Predict, the M18T mutant has 170.8:1 odds of being deleterious. Although these analyses clearly indicate that the M18T variant has a trend as a deleterious allele, the magnitude of the ratio did not exceed the threshold of 1,000:1 for making a clinical classification. We find that this mutation of BRCA1 causes a complete loss of HDR activity. Although this substitution, when expressed in a truncated BRCA1 peptide in the absence of BARD1, did have ubiquitin ligase enzymatic activity, it was defective in protection of a cell against IR (6). In another study using the BRCA1/BARD1 heterodimer, the M18T variant was inactive as a ubiquitin ligase (21). The M18T substitution could affect the BRCA1-BARD1 heterodimerization interface (22), resulting in reduced BARD1 association with the BRCA1 variant (Fig. 5). Our results in the HDR assay are consistent with this substitution being deleterious.

Genetic analysis of one of these mutants, D67Y, has suggested that it is neutral with regard to cancer predisposition (4), and consistent with that notion, it has the same phenotype as does wild-type BRCA1 in the HDR assay. Although the R71G substitution is considered cancer promoting, the mutation is thought to affect the splicing of the mRNA (20), such a feature would be missed when expressing a cDNA as in this experiment. Mutant BRCA1(R71G) protein functioned in the HDR at similar levels as did wild-type BRCA1.

Among the remaining five variants of unknown clinical consequence, the T37R variant was defective in HDR (Fig. 4A, lane 9). In new research published while this study was being written has indicated that the similar T37K variant is likely to be deleterious based on clinical and evolutionary analysis (3). The T37R substitution was also found to be defective in providing IR resistance (6), consistent with our HDR results. Taken together, the Thr37 residue is likely critical to the homologous recombination process and, consequently, to radioresistance. The Thr37 side chain is solvent exposed but in a small cavity near the BRCA1-BARD1 heterodimerization interface (22). Perhaps the bulky lysine or arginine substitutions result in disruption of BRCA1-BARD1 binding, or alternatively, another protein important to HDR binds to this pocket.

**Discussion**

This study establishes a new assay for evaluating the function of BRCA1 protein variants. Critical components include the HeLa-derived cell line containing an integrated recombination substrate that is readily scored using flow cytometry. The very low background fluorescence in this cell, combined with the very high response to I-SceI expression, makes this cell line ideal for analyzing the homologous recombination process. Because HeLa cells are readily transfected, we routinely obtain >70% of the cells transfected in a monolayer (data not shown); this cell line is ideal for silencing a given gene by RNAi and reexpressing the gene via a RNAi-resistant plasmid expression vector. Although BRCA1 is associated with breast and ovarian cancers, this function for BRCA1 has been found in all cell types and is thus valid to study in the HeLa cell line. Although not high throughput, the assay is relatively quick once the mutant plasmids are prepared. Several variants can be analyzed simultaneously in a 1-week experiment. We plan to expand this analysis to several variants in the NH2 and COOH termini of the BRCA1 protein. This continuing effort will greatly expand the analysis of the functional consequences of missense mutations in BRCA1 function.

When a BRCA1 variant protein is defective in the homologous recombination process, is it cancer associated? Thus far, the results indicate a strong correlation with this HDR assay and cancer predisposition, but the analysis of more mutants will be important to determine whether there is an association. Conversely, are BRCA1 variants that have full HDR activity neutral mutations? The R71G variant has full HDR activity, but it is certainly cancer associated but via splicing (20), a mechanism that would not be identified in this assay using fully spliced cDNAs. Thus, we already have one example indicating that the converse is not true. In addition, we are developing a similar assay for centrosome control by BRCA1 variants, and initial results suggest that some of these variants do indeed function differently in the two assays.\(^5\)
Combining the functional analysis described in this study with genetic and clinical analysis of point mutants (3, 4) will undoubtedly be important for counseling women who carry missense mutations of \( \text{BRCA1} \). As the data become more complete, such results will likely become an excellent resource for guiding clinical decisions.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Table 1. Summary of biological functions of \( \text{BRCA1} \) proteins in the context of available clinical information for each variant

<table>
<thead>
<tr>
<th>( \text{BRCA1} ) missense change</th>
<th>Laboratory analysis of ( \text{BRCA1} ) variant protein</th>
<th>Clinical significance of ( \text{BRCA1} ) missense changes</th>
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<tbody>
<tr>
<td></td>
<td>HDR</td>
<td>BARD1 binding</td>
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<tr>
<td>M18T</td>
<td>−</td>
<td>Weak</td>
</tr>
<tr>
<td>I21V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C24R</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C27A</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>I31M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T37R</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C39Y</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H41R</td>
<td>−</td>
<td>Weak</td>
</tr>
<tr>
<td>I42V</td>
<td>+</td>
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<tr>
<td>C44F</td>
<td>−</td>
<td>−</td>
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<tr>
<td>C47G</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L52F</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C61G</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C64G</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D67Y</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R71G</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

NOTE: Mutants with similar function in the assay as the wild-type \( \text{BRCA1} \) are listed as “+" and those as deleterious in an assay as “−". Data for HDR activity and BARD1 binding are from this study. In cases where \( \text{BRCA1} \)-BARD1 binding was detected but at low levels, the result is indicated as weak. The ubiquitin ligase and IR resistance results were from Ruffner and colleagues (6). Blank spaces indicate that the indicated protein was not analyzed in that study. Available clinical information includes the number of times the variant has been reported, examples of a family with the variant, output from the VUS Predict algorithm, and other measures of clinical significance taken from published observations. VUS Predict classifications with an asterisk were performed only using evolutionary conservation and no clinical data. Blank cells represent the experiment has not yet been done. The clinical significance is “deleterious” if the odds of association with breast and ovarian cancer exceed 1,000:1, “VUS” if uncertain (odds >0.01:1 but <1,000:1), and “neutral” if not associated with increased cancer risk (odds <0.01:1). All variants of uncertain significance (VUS) were selected from the BIC database. Zinc-coordinating residues are indicated, and although they are mostly listed as VUS, such mutations would be expected to make large disruptions of the \( \text{BRCA1} \) structure.

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