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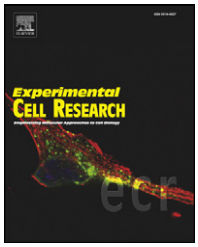

Research Article

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

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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 μM) 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.

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

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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.

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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 Materials and 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 catalog 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] (Fig. 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 and 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% confluence as determined by visual inspection, and separated from the flask using trypsin/ethylenediaminetetraacetic acid (EDTA)

Fig. 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.