

Open Access

Pathogenicity of Mutations Discovered in BRCA1 BRCT Domains is Characterized by Destabilizing the Hydrophobic Interactions

Dilip C Badgujar, Ulka Sawant, Hafiza Mahadik, Nikhil Gadewal and Ashok K Varma*

Tata Memorial Centre, Advanced Centre for Treatment, Research and Education in Cancer, Kharghar, Navi Mumbai - 410 210, India

Abstract

Background: Breast and ovarian cancer are the most common cancers among hetrogenetically diversified women. It is quite difficult to categorize the population at a high risk of breast cancer using peer genetic information because one particular mutation can be found in the same or in different families. Several mutations have been discovered across the full length of *BRCA1* gene, and categorizing their pathogenicity is a major challenge. Carriers of *BRCA1* mutations have an increased risk of developing cancer. In the breast cancer database BIC, approximately 1,500 genetic variants have been reported. It is very difficult to characterize each of the reported mutations. Given the complexities in characterizing the mutations, we decided to investigate functional basis associated with the mutations, rather than looking at each mutation.

Materials and methods: *BRCA1* BRCT domains were cloned, expressed, and purified using e-coli bacterial expression system. Mutations were generated using site-directed mutagenesis techniques, and all the mutations were sequence verified. The secondary structure of the mutant was characterized by Circular dichroism (CD) and Fluorescence spectroscopy. Molecular dynamics simulations were performed using Desmond software. Hydrophobic interactions and hydrogen bonding of docked molecules were compared using the LigPlot program.

Results: Genetic mutations were discovered throughout *BRCA1*, and most of the pathogenic mutations were buried in the hydrophobic core and destabilized the *BRCA1* BRCT domain. This unstable BRCT domain destabilized the full-length *BRCA1*, resulting in a loss of function. We conclude that the pathogenicity of each of the mutations in the BRCT domain can be categorized on the basis of its ability to destabilize the hydrophobic interactions. Although such instability is not sufficient to predispose someone to cancer, it provides a basis for formulating a concept for genetic counseling and targeted therapy.

Keywords: BRCA1 BRCT; Pathogenicity; Hydrophobic interactions; Genetic counseling; Targeted therapy

Introduction

BRCA1, a breast and ovarian cancer gene, comprises 1,863 amino acids, and it has multifunctional cellular domains involved in the maintenance of genomic integrity [1,2]. Genetic mutations discovered in the BRCA1 gene from a large cohort of patients are helping in clinical management [2-4]. Somatic mutations are present in most patients. Some patients also possess germ-line mutations, which they inherited from their parents [5]. Genetic testing for cancer predisposing germline mutations can help clinicians to select treatment options when performing surgery, radiotherapy, and chemotherapy [6]. Providing information on the importance of genetic testing to high-risk women can help in detecting breast and ovarian cancer at an early stage and, therefore, increase the probability of recovery [7]. At present, an important challenge in genetic counseling is to evaluate the pathogenicity of mutations discovered throughout of the BRCA1 gene. The risk of developing cancer due to carrier of BRCA1 mutations is 40-87% for breast cancer and 16-68% for ovarian cancer [8]. Albeit, there are several other factors such as lifestyle, environmental changes, and hormonal factors that may influence the risk of cancer associated these mutations.

The management of mutations discovered across the world in cancer patients is also a major challenge. Human mutation databases compile information on mutation under one umbrella, e.g., http:// www.biologie.uni-hamburg.de/b-online/library/genomeweb/ GenomeWeb/human-gen-db-mutation.html. The database of the Breast Cancer Information Core (BIC) also serves as a repository of genetic mutations and variants observed in the *BRCA1* and *BRCA2*

J Cancer Sci Ther ISSN:1948-5956 JCST, an open access journal

genes: http://research.nhgri.nih.gov/bic/. Over 1,500 genetic variants have been reported in the BRCA1/2 genes. These are classified as either known deleterious mutations, polymorphisms without clinical significance, or sequence variations of unknown significance. Several groups have deposited BRCA1/2 mutations in the BIC's database and categorized their pathogenicity. However, a significant number of reported mutations need further evaluation [9]. Considering the importance of mutations in targeted therapy and drug discovery, we decided to determine the possible locations of pathogenic mutations and evaluate these using multimodel-based structural, in-silico, and biophysical approaches. The challenge is to identify the mutations that cause breast cancer and develop a possible diagnostic marker for the disease. Investigators across the world have different views on mutation-based targeted therapy and biomarkers [10,11]. There are several messages to the scientific community but challenges are in studying cancer biology [12]. For example, are we targeting the

*Corresponding author: Ashok K Varma, PhD, Principal Investigator & Scientific Officer 'E', Tata Memorial Centre, Advanced Centre for Treatment, Research and Education in Cancer Kharghar, Navi-Mumbai, Maharashtra State 410 210, India, Tel: +91-22-2740 5112; Fax: +91-22-2740 5085; E-mail: avarma@actrec.gov.in

Received October 16, 2012; Accepted October 27, 2012; Published October 29, 2012

Citation: Badgujar DC, Sawant U, Mahadik H, Gadewal N, Varma AK (2012) Pathogenicity of Mutations Discovered in BRCA1 BRCT Domains is Characterized by Destabilizing the Hydrophobic Interactions. J Cancer Sci Ther 4: 386-393. doi:10.4172/1948-5956.1000172

Copyright: © 2012 Badgujar DC, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

correct target in cancer therapy and are we focusing on the mutationassociated function of the protein. Such questions are a direct result of the number of mutations discovered throughout the 1,863 amino acids of the *BRCA1* gene and the approaches various groups have adopted to explore these mutations left several unanswered questions.

Another major question is whether mutations discovered in cancer patients should be used for medical management and whether there are any functional aspects associated with these mutations that can help in targeted therapy or finding biomarkers. To answer these questions, we have evaluated reported structures of *BRCA1* BRCT extensively and compared the structure-function association with already reported pathogenic mutations. We found that most of the pathogenic mutations in the *BRCA1* BRCT domains are located in the hydrophobic core of BRCT. Furthermore, we evaluated the pathogenicity of the mutations using *in-vitro* and *in-silico* approaches.

BRCA1 is a well-known tumor suppressor gene, which performs cellular functions through its interaction with proteins participating in DNA repair, cell cycle checkpoint control, and ubiquitination [13,14]. BRCA1 has a ubiquitin-interacting motif at its N-terminus, a DNAbinding domain in the middle, and a transcriptional activation BRCT domain at the C-terminus. The C-terminus of BRCA1 has two tandem repeats of BRCT, each comprising ~90-100 amino acids. The protein data bank (www.pdb.org) lists 124 structures for BRCT. Structurally, both the repeats of BRCA1 BRCT domains are packed together in a head-to-tail manner [15]. There are ~100 BRCT-containing proteins, and some of the BRCTs' repeats adopt a similar conserved conformation, with a central β -sheet stabilized by four parallel β -sheets (β 1- β 4) and three α -helices (α 1- α 3) on each arm of the repeats (Figure 1). BRCA1 BRCT adopts similar secondary structure conformations in the C-terminal of XRCC1 [16], 53 BP1 [17], MDC1 [18], and BARD1 [19]. However, the rmsd of each amino acid reveals that the conserved region of the central β -sheets and the α -helices (α 1 and α 3) are super-imposable and stabilized by a number of hydrogen bonds and hydrophobic interactions, whereas others such as $\beta 1-\alpha 1$, $\beta 2-\beta 3$, and $\beta 3-\alpha 2$ and the location of $\alpha 2$ in the central β -sheets are not super-imposable. These variations highlight the fact that the BRCTs of different proteins may have different binding motifs unlike BRCA1



Figure 1: Superimposed structure of BRCA1 BRCT (pdb id: 1JNX, pink in color), BARD1BRCT (pdb id: 2NTE, marine in color), MDC1 BRCT (pdb id: 2ETX, magenta in color). The putative phosphopeptide binding site is encircled.

BRCT [20]. Most of the BRCTs do not have much sequence similarity but share three-dimensional structural folding and functions.

The interaction of the C-terminal domain of BRCA1 with phosphopeptide-containing protein targets is well studied [20]. The crystal structure of BRCT repeats that bind to phosphoserine-specific peptides like DNA helicase BACH1 [21-23], CtIP (CtBP-interacting proteins) [24], and ACC1 (acetyl-CoA carboxylase) [25] through its conserved binding pocket has been determined. In all reported complex structures of BRCA1 BRCT, binding to phosphopeptides promiscuous about the role of BRCT in the repair of DNA double-strand breaks, cell cycle checkpoint control, and tumor suppressor function. Earlier reported structures of BRCT complexes with BACH1, CtIP have predicted that the pocket near the N-terminus of BRCT harbors phosphoserine and that the interface between repeats has a hydrophobic groove, which stabilizes phenylalanine [21-24,26,27]. Phosphoserine at the zero position (pS_0) and phenylalanine at the plus three position $(F_{1,2})$ in the consensus sequence X-pS₀-X-X-F₂-X, (X-can by any amino acids) binds to BRCT at the dimer interface (BRCT1 and BRCT1) in a two-sided manner. In most BRCT structures, the phosphate group of phosphoserine makes salt bridges and hydrogen bonds with the amide terminus repeats of BRCT. Phenylalanine from the C-terminus of the peptide stabilizes the hydrophobic pocket located at the interface of the repeats and the C-terminus of BRCT. Recent results also predict that Abraxas, which has the sequence motif X-pS-X-pS-X-F, binds to BRCA1 BRCT through the mutual exclusion of binding conformation of earlier reported structures of BACH1 and CtIP [28]. This indicates that a dual phosphopeptide recognition site is also present in the BRCT domain and that it may help in maintaining its structural integrity [28].

To understand the molecular mechanism of the conserved region of the phosphopeptide's recognition site and the hydrophobic core of BRCTs in evaluating the pathogenicity of mutants for clinical management and targeted therapy, a complete structural and biophysical comparison of *BRCA1* BRCT and clinically significant mutants is presented here.

Materials and Methods

Gene- cloning and site-directed-mutagenesis

The gene encoding BRCA1 BRCT domain (1646-1859) was PCR amplified using BRCT construct in pGEX-kT(generous gift from John A A Ladias). NdeI site was added in foreword primer (5'-ATCATATGGCC ATG GTC AAC AAA AGAATGTCC-3') and BamHI in reverse primer (5'-TAGGATCC TCACTA GGG GAT CTGGGGTAT CAGG-3'). The amplicon was first cloned in pJET blunt end ligation vector and later sub-cloned in pET3a Vector using Quick T4 DNA ligase from NEB. Site directed mutagenesis for H1686Q (Forward: 5'-CTGAAGAGACTACTCAGGTTGTTATGAAAAACAG-3' Reverse: 5'-CTGTTTTCATA ACAACCTCAGTAGTCCTTCAG-3', P1749R (Forward: 5'-GAAACCACCAAGGTCGAA AGCGAGCAAGAG-3' Reverse: 5'-CTCTTGCTCGCTTTCGACCTTGGTGGTTTC-3'),

S1715 R (Forward: 5'-GAAAATGGGTAGTTAGGTATTTCT-GGGTGACC-3', Reverse: 5'-GGTCACCCAGAAATACCTAAC-TACCCATTTTC-3') and M1775R Forward: (5'-CCCTT CACCAACAGGCCCACAGATCAAC-3') Reverse: (5'-GTTGATCTGTGGGCCTGTT-GGTGAAGGG-3') M-1775-K Forward: (5'-CCCTTCACCAACAGGCCCACAGATCAAC-3') Reverse: (5'-GTTGATCTGTGGGCCTGTT-GGTGAAGGG-3') was carried out by amplification using pfu polymerase

Citation: Badgujar DC, Sawant U, Mahadik H, Gadewal N, Varma AK (2012) Pathogenicity of Mutations Discovered in BRCA1 BRCT Domains is Characterized by Destabilizing the Hydrophobic Interactions. J Cancer Sci Ther 4: 386-393. doi:10.4172/1948-5956.1000172

and digestion of template DNA by DpnI. All site-directed- mutations were confirmed by DNA sequencing.

Protein expression and purification

The BRCA1 BRCT variants (H1686Q, P1749R and S1715R) was incorporated in BL21 (pLysS) for overproduction of protein. Cells were grown in LB medium containing 100 mg/ml Ampicillin and 34 mg/ml chloramphenicol at 37°C till OD reaches A600 between 0.6-0.8, then the culture was induced by 0.4 mM IPTG at 18°C, incubated at 250 rpm for 16 hours. All the further protein purification steps were carried out at 4°C. The induced culture were harvested in Sorvall SLC-3000 rotor at 6000 rpm for 10 min. harvested induced cells was resuspended in lysis buffer (20 mM Sodium phosphate pH- 6.0, 50 mM NaCl, 1 mM EDTA, 1 mM β ME, 1 mM PMSF and 0.5% Triton X-100) and sonicated for 5-8 times with 50 duty cycles. Cell lysate was centrifuged at 18000 rpm for 30 min. BRCA1 BRCT Mutants S1715R and P1749R were not much soluble and folded properly. However, we could proceed with BRCA1 BRCT variants H1686Q for further purifications. Cleared bacterial cell lysate of BRCA1 BRCT H1686Q was bound to SP sepharose resins pre -calibrated by buffer B (20 mM Sodium phosphate pH- 6.0, 50 mM NaCl, 1 mM EDTA, 1 mM BME, 1 mM PMSF). Bound protein was eluted with a gradient of NaCl (100 mM - 800 mM). The fractions which showed proteins were pooled and concentrated till 2 ml and buffer exchanged using G-50 desalting column to sodium borate buffer of pH 8.7. Variant is now applied on Q sepharose calibrated by 10 mM Sodium borate buffer. Bound protein was eluted using NaCl gradient (100 mM-500 mM). Protein expression and purification was monitored by loading the fractions on SDS PAGE.

Gel filtration chromatography

Gel filtration chromatography was done using superdex-75 Hiload column from GE Healthcare. The elution fractions from Q sepharose which shows protein were pooled down and concentrated till 2 ml and applied on pre-calibrated superdex-75 column using buffer (10 mM Sodium borate pH 8.7, 100 mM NaCl). Fractions were collected by monitoring the absorbance at 280 nm.

Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectra were collected using JASCO J-715 spectropolarimeter (Jasco, Easton, MD), in the far-UV region (180–260 nm). The JASCO J-715 is well equipped with a Jasco PTC 348 WI temperature controller and sealed quartz cuvettes. In the far-UV region (180–260 nm) BRCA1 BRCT (in buffer 10 μ M solution of BRCA1 BRCT and BRCT H1686Q (10 mM Borate buffer, pH 9.0, 300 mM NaCl) was loaded onto a 0.1 cm path-length quartz cuvette (Helma). Seven spectra were accumulated and averaged for each experiment, with a resolution of 1 nm at a scan speed of 50 nm/min and a response time of 1 s. Buffer blank spectra, obtained at identical conditions, have been subtracted from the raw data. The results in all experiments have been expressed as ellipticity [θ] (°cm² dmol⁻¹).

Fluorescence spectroscopy

Emission spectrums were collected on HORIBA FL3-21 spectrofluorometer. The variant and wild type BRCA1 BRCT of 10 μM concentration taken in 1 mm cuvette was excited at 280 nm and checked its emission maxima.

Align-GVGD

Align-GVGD was carried out using multiple sequence alignment available for BRCA1 from human to frog. The grade of pathogenicity is

categorised in different classes beginning from C0, C15, up to C65. Any mutation with category C0 is less likely and C65 most likely pathogenic.

In-silico modelling

Mutational analysis was performed by building the four mutants (H1668Q, S1715R, M1775R and P1749R) on reported structure BRCA1 BRCT (pdb id: 1Y98), to study their molecular interactions by MD Simulation using Desmond 2010 software package [1]. Optimized Potentials for Liquid Simulations (OPLS) all-atom force field was used to analyze model stability. The protein structures were solvated with the Monte Carlo simulated SPC water model using orthorhombic box. The system was then neutralized replacing water molecules with sodium and chloride counter ions. Subsequently, a maximum of 2,000 energy minimization steps were carried out for all complexes using a steepest descent algorithm with a tolerance of 1000 kJ mol⁻¹ nm⁻¹. A twin-range cutoff was applied to long-range interactions (1.0 nm for van der Waals and electrostatic interactions) using Particle Mesh Ewald (PME). These minimized and solvated systems were considered reasonable structures in terms of geometry and solvent orientations and used in further simulation steps. Equilibration MDS for both temperature (300 K) and pressure (1 atm) was carried out for 100 ps. These pre-equilibrated systems were subsequently used in the 1ns production MDS with a time-step of 2 fs. Structural coordinates were saved every 1.2 ps and analyzed using the analytical tools in the Desmond package. The lowest energy structures were obtained for each mutant structure and molecular interactions of wt and mutant were plotted using Ligplot [2].

Results and Discussion

BRCA1 is one of the most studied genes for breast and ovarian cancer. Several investigators have tried to focus on different domains of BRCA1 to characterize its associated functions [29]. Structurally, the C-terminus of BRCA1 has been the focus of much attention to evaluate the pathogenicity of mutants discovered in a large cohort of patients and to facilitate genetic counseling and targeted therapy [30-32]. Most work has concentrated on the conserved phosphoserine-binding motif located at the interface of the BRCTs. However, the association of BRCA1 BRCT with doubly phosphorylated Abraxas indicates the presence of other domains within the BRCT that require further evaluation [33]. Thus, we attempted to study the phosphopeptide binding and the hydrophobic pocket of BRCT wild-type and reported pathogenic mutants. Most of the mutations are categorized as highgrade pathogenic with A-GVGD prediction grade of C65. Although H1686Q has been reported to be pathogenic [34], its A-GVGD value GV (0.00), GD (24.08) and prediction class C15 suggests that this is less likely pathogenic [35]. All these A-GVGD values are in agreement with results reported in this paper. Most of the pathogenic mutations destabilized the hydrophobic core of the BRCA1 BRCT domain, which may prove useful in designing small molecules for targeted therapy.

Structural basis of the BRCT domains

Mostly, BRCT domains are found in proteins involved in the mediation of DNA damage repair, cell cycle checkpoint control, and tumor suppressor function. There are several deletion/mutations in the BRCT domain that give rise to deleterious/truncated mutants, which, in turn, lead to breast and ovarian cancer. *BRCA1*, *BARD1*, and *MDC1* have tandem repeats of BRCTs (Figure 1) whereas, *PARP1*, *XRCC1* and DNA ligase III have a single domain. The tandem repeats of *BRCA1* BRCT are known to interact with phosphospecific binding partners, where phosphoserine occupies the first N-terminus domain of BRCT and, mostly, phenylalanine occupies the second repeat at

the hydrophobic core of C-terminus BRCT [21]. The phospospecific binding domains are present on only selected tandem repeats of BRCT. Despite having the same three-dimensional structural folding, there are no reported binding partners for the single domain of BRCT, but tandem repeats of BRCA1 BRCT and MDC1 BRCT have phosphospecific binding domains. BARD1 BRCT does not have any structurally reported binding partners, whereas in-silico and in-vitro results indicate binding between BARD1 BRCT and its phosphospecific target [36] (Figure 2A & 2B). Recently, reports suggest that Abraxas, which is a double phosphopeptide, binds to BRCA1 BRCT [28]. All these results have lead to much confusion in the scientific community in terms of translational research. Although the three-dimensional structural folding of BRCA1, MDC1, and BARD1 is very similar, the sequences holding the binding domains are drastically different. Furthermore, the functional aspects of BRCT are characterized by direct interactions of these domains with several cellular binding partners during cell cycle progression. Looking at the tandem repeat structure of BRCT and the single domain of BRCT, it can be concluded that not only protein-protein interactions, but also intramolecular interactions play a vital role in determining the protein's functions.

It is well known that weak intramolecular interactions play an important role in protein folding [37]. Furthermore, it is well established that hydrogen bonding and hydrophobic interactions play a major role in protein stability [38]. Within the available complex structure of BRCT, we analyzed hydrogen bonding and hydrophobic interactions for mutations discovered to predispose patients to cancer. However, how these weak interactions are associated with the pathogenicity of mutants and targeted therapy has not been explored earlier.

Phosphospecific binding pocket

There are several BRCT complex structures available in the protein data bank (www.pdb.org). The phosphoserine binding pocket is conserved and stabilized by a number of weak inter-and intramolecular interactions. The C-terminal domain of BRCA1 interacts with cellular proteins like phospho-BACH1(DNA helicase BACH1), phospho-CtIP (CtBP interacting proteins) [23,24], and phospho-ACC1 [39-41] through its conserved binding pocket in a structurally diverse manner. Although the pS-X-X-F motif is more or less conserved, the different conformational orientation of the N-terminus and the C-terminus of the binding partners bestow a multifaceted role of the BRCT repeats. The BACH1/CtIP phosphate group of Ser interacts with Lys 1702, Ser 1655, and Gly 1656 and stabilizes this pocket through hydrogen bonding and hydrophobic interactions. The hydrophilic environment around phosphoserine plays a very important role in stabilizing this pocket. Water-mediated hydrogen bonds hold BRCT1 and BRCT2 together (Arg 1699 and Glu 1836 are held together in the same way as the phosphobinding pocket pS-X-X-F holds BRCT1 and 2 together). The N-terminal residues of the phosphospecific peptide bind to the N-terminus of the protein, and the C-terminus of each peptide binds to the C-terminus of the protein, contributing to stabilizing the binding pocket [42].

The overall location and orientation of the phosphopeptide binding motif of MDC1 BRCT with γ -H2AX is similar to that in *BRCA1* BRCT (Figure 2C). The phosphoserine is largely buried in the hydrophilic environment of the protein. γ -H2AX phosphoserine, located at position 139, forms a hydrogen bond with Thr 1898, Gly 1899, and Lys 1936. This binding is further stabilized by intramolecular hydrophobic interactions of the residues Gln 140 and Ala 138 of γ -H2AX. Both the proteins bind the phosphate moiety through direct interactions with



side- and main-chain atoms of the structurally conserved residues: Lys 1936, Thr 1898, and Gly 1899 are found in *MDC1* BRCT, whereas Thr 1898, Lys 1936, and Arg 1933 are found in BRCA1 BRCT [43].

The sulfate ion is accommodated in the same location in BARD1 BRCT as the phosphoserine of BACH1/CtIP in the BRCA1 BRCT complex structure, and shows similar kinds of interactions and atomic orientations. The sulfate group forms hydrogen bonds with Ser 575, Thr 617, and Lys 619, which is an anticipated phosphospecific binding pocket of BARD1. The sulfate ion might have enthroned its position during crystallization [19]. On the other hand, the active and proper folded dimer structure of BARD1 BRCT is situated at the phosphospecific binding motif, which inhibits the binding with sequences of pS-X-X-F. The first molecule of the BARD1 dimer (pdb id: 2NTE, chain A, residues from 686-HHKFTGWLY-678) can be superimposed on the phosphospecific CtIP peptide, which is bound to the BRCA1 BRCT (pdb id: 1Y98) structure (Figure 2A & 2B). This may either be an active dimer of BARD1 BRCT that does not have the same consensus sequence binding motif as BRCA1 BRCT, or it may have a monomeric structure at low temperature, supporting the concept of binding with BRIP1 [36]. Structural analysis of BARD1 BRCT at low temperature may help in unraveling the exact molecular mechanism associated with the BARD1 BRCT phosphospecific binding motif.

Hydrophobic phenylalanine binding pocket

The hydrophobic phenylalanine at the +3 position located at the peptide side contains the *BRCA1* BRCT repeats. Phe (+3) is fully buried in the hydrophobic environment, which comprises Leu 1701, Phe 1704, Met 1775, and Leu 1839 (Figure 3A). Binding between phenylalanine and *BRCA1* BRCT has been reported to be associated with the transcriptional activation function. It is very important to mention that the supramolecular arrangement due to Phe (+3) binding plays a very important role in characterizing the function of the associated

domains. Phe (+3) forms hydrophobic interactions and stabilizes the domains. It is very well known that the transactivation function of BRCA1 BRCT is due to binding with the phosphospecific BACH1 [21] and CtIP [24]. However, binding does not occur unless the aromatic ring of Phe is at the +3 position . This provides strong support for the ability of weak intermolecular interactions to shed light on the functional characteristic of proteins. The binding motif is conserved for all the reported peptide complexes, and Arg1699, Asn 1774, Met 1775, Phe 1704, Leu 1701, and Arg 1835 all stabilize the hydrophobic pocket of BRCT. Hydrophobic interactions and hydrogen bonding at the binding interface stabilize the genomic integrity of BRCA1 [42]. Comparing the structure of BRCA1 BRCT with MDC1 BRCT, we found that the Phe +3 hydrophobic pocket is conserved (Figure 3A & 3B). However, thus far, there have been no reports of a complex structure for BARD1 BRCT containing the consensus sequences of the BRCA1 BRCT binding peptide (Figure 3C). This suggests that the mutations that occur at the hydrophobic core of BARD1 BRCT either have different pathological significance than BRCA1 BRCT or may bind to other consensus sequences. The hydrophobic pocket of BARD1 is stabilized by Ser 616, Met 621, His 685, His 686, and Ile764 [19]. Interestingly, MDC1 BRCT senses tyrosine at the +3 position [43]. 53 BP1 BRCT (pdb id: 1GZH) does not have phospho-Serine at putative pSer position (Figure 3D). This indicates that even if the threedimensional folding is the same, the genomic integrity of each BRCT may differ.

Hydrophobic interactions and mutagenesis of pathogenic residues

It is rather difficult to characterize the large number of mutations available in cancer populations using structural biology and bioinformatics tools. In the C-terminal of *BRCA1* BRCT, ~100 mutations are reported in the BIC's database [44]. Some of these are predicted to be pathogenic mutations with no transcription, and some are characterized but have unknown functions. Most reported pathogenic mutations Met 1775 Arg (Figure 4 i & ii), Met 1775 Lys of *BRCA1* BRCT diminished the binding capacity between *BRCA1* BRCT and *BACH1*, CtIP by destabilizing the BRCT domain. Most



Figure 3: Protein-Protein interactions between phosphoserine of (A) BRCA1 BRCT-CtIP complex (B) MDC1 BRCT- γ H2AX complex, (C) BARD1 BRCT-sulphate complex, (D) 53BP1 BRCT-p53 tumor suppressor complex.

strikingly, His 1686 Gln is a reported novel pathogenic mutation that has different binding affinity to phosphopeptide target proteins than the BACH1 and CtIP [34]. These findings leave many questions unanswered regarding the functionality of this gene, which predispose high-risk individuals to cancer. Looking at different levels of accuracy in predicting the pathogenicity of mutations to provide novel concepts, which may help in predicting mutation-based biomarkers or designing novel drug leads. We have performed a multimodel *in-silico*, *in-vitro*, and biophysical based study to answer major challenging questions pertaining to human genomics.

Proline 1749 to arginine: A structural change in proline (P) from a medium-sized, rigid, and hydrophilic residue to a large-sized and positively charged basic amino acid arginine (R) has been reported to be associated with ovarian cancer, with unknown pathological significance. Proline at position 1749 in BRCT (pdb: 1y98) is >15 Å away from pSer₍₀₎ and $F_{(+3)}$ of CtIP and surrounded by hydrophobic interactions of Lys 1750, Glu 1735, Gln 1747, Gly 1748, and Val 1736; a hydrogen bond with Arg 1753 is also present (Figure 4iii). However, mutant BRCT1749 arginine has re-oriented the hydrophobic pocket by forming hydrophobic interactions with Lys 1750, His 1746, Val 1713, and Ile 1707 and by forming hydrogen bonds with Gly 1738, Asp 1739, Tyr 1707, Ala 1843, and Gly 1748 (Figure 4 iv). The structural changes around the hydrophobic environment point to the pathogenicity of the proline to arginine mutation.

Histidine 1686 to glutamine: This mutation has been discovered in Italian breast and ovarian cancer families [34]. It is located on exon 17 of BRCA1 and characterized as pathogenic. Structurally, *BRCA1* His 1686 is stabilized by four hydrophobic interactions of Met, 1650, Met 1652, Val 1687, and Trp 1712 and four hydrogen bonds of Met 1650, Thr 1685, Val 1653, and Glu 1731 (Figure 4v) . However, BRCA1-1686Q forms a single hydrophobic interaction with Val 1687 and four hydrogen bonds with Thr 1685, Val 1653, Ser 1651, and Glu 1731 (Figure 4vi). These characteristics differentiate it from the wild-type. It also exhibits significant differences in its three-dimensional structure and genomic stability around the hydrophobic core, pointing to the pathogenicity of the mutation.

Serine 1715 to arginine: Ser 1715 is located on exon 18 of *BRCA1*. It has been reported on one occasion in a Danish patient, with unknown clinical significance [45]. Mutation from a small size and polar Ser (S) to a large size and basic Arg (R) on that BRCA1-Ser1715 is ~13Å away from $pSer_{(0)}$ and 19 Å from $Phe_{(+3)}$ in BRCA1 CtIP (pdb id: 1Y98) complex structure. There is not much conformational changes occur due to mutation (Figure 4vii & viii). It is stabilized by three hydrophobic interactions Val 1714, Tyr 1716, and Phe 1695 and three hydrogen bonds with Phe 1734, Trp 1718, and Thr 1691. BRCA1-Ser 1715 is located at the head of parallel β -sheets and on the tail of the α -helix. A superimposed model of BRCA1-S1715R destabilized the turn site, moving away from the CtIP binding sites and resulting comparatively less association with the BRCT binding partners.

Comparing the in-vitro results, we used FPLC purified BRCA1 BRCT (1646-1859) His 1686 Gln mutation (Figure 5A & 5B), however Ser 1775 Arg, and Pro 1749 Arg were not soluble and stable. Therefore we have characterized BRCA1 (1646-1859) His 1686 Gln mutation using Circular dichroism (CD) and Fluorescence spectroscopy and found little change in the secondary structure of the wild-type and the mutants (Figure 5C). Tryptophan is buried in the hydrophobic environment of BRCT structure (Figure 5D). Thus, we can conclude Citation: Badgujar DC, Sawant U, Mahadik H, Gadewal N, Varma AK (2012) Pathogenicity of Mutations Discovered in BRCA1 BRCT Domains is Characterized by Destabilizing the Hydrophobic Interactions. J Cancer Sci Ther 4: 386-393. doi:10.4172/1948-5956.1000172



Figure 4: Schematic representations of weak intramolecular interactions between BRCA1 BRCT wt and reported pathogenic mutations (i) BRCA1 BRCT M1775 (ii) BRCA1 BRCT M1775 (iii) BRCA1 BRCT P1749 (iv) BRCA1 BRCT P1749R (v) BRCA1 BRCT H1686 (vi) BRCA1 BRCT H1686Q (vii) BRCA1BRCT S1715 (viii) BRCA1 BRCT S1715R. for clarity in Ligplot, the mutants are shown as B chain in this figure only.



Figure 5: Purification profiles of BRCA1 BRCT H1686Q mutation (A) FPLC chromatogram (B) FPLC purified fractions on SDS PAGE gel (C) secondary structure analysis using Circular Dichroism (D) Fluorescence spectra at 280 nm.

that the grade of pathogenicity of the BRCT domain cannot be defined based on the protein's secondary structure or its three-dimensional folding levels. We can also conclude that hydrophobic interactions play a very important role in unraveling the functional complexities of BRCA1 BRCT mutations. Structural folding, weak intermolecular interactions and microarray analysis have already been reported for the pathogenic mutations M1775R [46] and M1775K [47], and to our conclusion we have found these mutations are not only impairing transactivation function but also destabilizing the hydrophobic interactions of BRCA1 BRCT.

Conclusions

Based on a large cohort of patients, breast and ovarian cancer is thought to be genetically heterogeneous. This genetic heterogeneity is indicated by familial occurrences of cancer, with mutations in a particular exon of BRCA1 involved in the same or in different families .Mutations at more than one locus can be found in different families or even in the same case. Based on our multimodal-based approach to characterizing the pathogenicity of mutations discovered in BRCA1 BRCT domains, we can conclude that genomic characterization alone is insufficient for clinical management of high-risk patients predisposed to cancer. There is also still a lack of consensus about whether genetic information can be used as a sole tool for targeted therapy or diagnostic biomarkers. We found that most of the mutations in the BRCA1 gene are located at the hydrophobic core of BRCT. The evaluation of pathogenicity of the mutations depends on how they destabilize the hydrophobic environment of the protein structure. As the hydrophobic region is buried inside the BRCT domains, all the reported pathogenic mutations destabilize the hydrophobic core, thereby further destabilizing the full-length BRCA1. The destabilization of three-dimensional folding as a result of the mutations leads to the loss of hydrophobic interactions and hydrogen bonding. It would be impossible to obtain essential information on the pathogenicity of the mutants without characterizing the associated protein functions. By looking at the structural characteristics, we found that the mutations that destabilize the hydrophobic core evaluate its pathogenicity. The next aim should be to look for small molecules that stabilize the hydrophobic environments of BRCT. These small molecules, which should target specific functions, i.e., causes of cancer, could serve as potential drug leads for targeted therapy. This will help in designing small-molecule compounds that may also help in stabilizing the genomic integrity and reducing the pathogenicity of the mutations.

Acknowledgement

Funding for this study was supported by DBT (No. BT/PR10765/BRB/664/2008 and BT/PR12565/BID/07/303/2009), Seed in Air fund from TMC. We thank DBT-BTIS facility at ACTREC for providing necessary software to this study. Startup funds of Varma Lab at Tata Memorial Centre, Advanced Centre for Treatment, Research and Education in Cancer, Kharghar to AKV. Hafiza Mahadik thanks Seed in Air fund from TMC for fellowship.

References

- Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266: 66-71.
- Futreal PA, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, et al. (1994) BRCA1 mutations in primary breast and ovarian carcinomas. Science 266: 120-122.
- Ford D, Easton DF, Bishop DT, Narod SA, Goldgar DE (1994) Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Lancet 343: 692-695.
- 4. Berchuck A, Cirisano F, Lancaster JM, Schildkraut JM, Wiseman RW, et al.

- de Pauw A, Stoppa-Lyonnet D (2011) [Genetic consultation: hereditary cancer risk?]. Rev Prat 61: 538-541.
- Trainer AH, Lewis CR, Tucker K, Meiser B, Friedlander M, et al. (2010) The role of BRCA mutation testing in determining breast cancer therapy. Nat Rev Clin Oncol 7: 708-717.
- Katapodi MC, Northouse L, Pierce P, Milliron KJ, Liu G, et al. (2011) Differences between women who pursued genetic testing for hereditary breast and ovarian cancer and their at-risk relatives who did not. Oncol Nurs Forum 38: 572-581.
- Barnes DR, A.C Antoniou (2012) Unravelling modifiers of breast and ovarian cancer risk for BRCA1 and BRCA2 mutation carriers: update on genetic modifiers. J Intern Med 271: 331-343.
- Shen D, Vadgama JV (1999) BRCA1 and BRCA2 gene mutation analysis: visit to the Breast Cancer Information Core (BIC). Oncol Res 11: 63-69.
- Yap TA, Gerlinger M, Futreal PA, Pusztai L, Swanton C, et al. (2012) Intratumor heterogeneity: seeing the wood for the trees. Sci Transl Med 4: 127ps10.
- Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, et al. (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366: 883-892.
- Malacrida S, Agata S, Callegaro M, Casella C, Barana D, et al. (2008) BRCA1 p.Val1688del is a deleterious mutation that recurs in breast and ovarian cancer families from Northeast Italy. J Clin Oncol 26: 26-31.
- Deng CX (2006) BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res 34: 1416-1126.
- Starita LM, Parvin JD (2003) The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair. Curr Opin Cell Biol 15: 345-350.
- Williams RS, Green R, Glover JN (2001) Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1. Nat Struct Biol 8: 838-842.
- Zhang X, Moréra S, Bates PA, Whitehead PC, Coffer AI, et al. (1998) Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. EMBO J 17: 6404-6411.
- Derbyshire DJ, Basu BP, Serpell LC, Joo WS, Date T, et al. (2002) Crystal structure of human 53BP1 BRCT domains bound to p53 tumour suppressor. EMBO J 21: 3863-3872.
- Singh N, Wiltshire TD, Thompson JR, Mer G, Couch FJ (2012) Molecular basis for the association of microcephalin (MCPH1) protein with the cell division cycle protein 27 (Cdc27) subunit of the anaphase-promoting complex. J Biol Chem 287: 2854-2862.
- 19. Birrane G, Varma AK, Soni A, Ladias JA (2007) Crystal structure of the BARD1 BRCT domains. Biochemistry 46: 7706-7712.
- Rodriguez M, Yu X, Chen J, Songyang Z (2003) Phosphopeptide binding specificities of BRCA1 COOH-terminal (BRCT) domains. J Biol Chem 278: 52914-52918.
- Shiozaki EN, Gu L, Yan N, Shi Y. (2004) Structure of the BRCT repeats of BRCA1 bound to a BACH1 phosphopeptide: implications for signaling. Mol Cell 14: 405-412.
- Botuyan MV, Nominé Y, Yu X, Juranic N, Macura S, et al. (2004) Structural basis of BACH1 phosphopeptide recognition by BRCA1 tandem BRCT domains. Structure 12: 1137-1146.
- Clapperton JA, Manke IA, Lowery DM, Ho T, Haire LF, et al. (2004) Structure and mechanism of BRCA1 BRCT domain recognition of phosphorylated BACH1 with implications for cancer. Nat Struct Mol Biol 11: 512-518.
- Varma AK, Brown RS, Birrane G, Ladias JA (2005) Structural basis for cell cycle checkpoint control by the BRCA1-CtIP complex. Biochemistry 44: 10941-10946.
- Shen Y, Tong L (2008) Structural evidence for direct interactions between the BRCT domains of human BRCA1 and a phospho-peptide from human ACC1. Biochemistry 47: 5767-5773.
- 26. Li S, Chen PL, Subramanian T, Chinnadurai G, Tomlinson G, et al. (1999) Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription

regulation of p21 is disrupted upon DNA damage. J Biol Chem 274: 11334-11338.

- 27. Yu X, Wu LC, Bowcock AM, Aronheim A, Baer R (1998) The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. J Biol Chem 273: 25388-25392.
- Wang B, Matsuoka S, Ballif BA, Zhang D, Smogorzewska A, et al. (2007) Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. Science 316: 1194-1198.
- Chen Y, Borowicz S, Fackenthal J, Collart FR, Myatt E, et al. (2006) Primary structure-based function characterization of BRCT domain replicates in BRCA1. Biochem Biophys Res Commun 345: 188-1896.
- Plevova P, Hladikova A (2012) Genetic counselling in male carriers of BRCA1 and BRCA2 gene mutations. Klin Onkol 25: S67-73.
- 31. Halbert CH, Kessler L, Collier A, Weathers B, Stopfer J, et al. (2012) Low rates of african american participation in genetic counseling and testing for BRCA1/2 mutations: racial disparities or just a difference? J Genet Couns 21: 676-683.
- Millot GA, Carvalho MA, Caputo SM, Vreeswijk MP, Brown MA, et al. (2012) A guide for functional analysis of BRCA1 variants of uncertain significance. Hum Mutat 33: 1526-1537.
- Solyom S, Aressy B, Pylkäs K, Patterson-Fortin J, Hartikainen JM, et al. (2012) Breast cancer-associated Abraxas mutation disrupts nuclear localization and DNA damage response functions. Sci Transl Med 4: 122-123.
- 34. Giannini G, Capalbo C, Ottini L, Buffone A, De Marchis L, et al. (2008) Clinical classification of BRCA1 DNA missense variants: H1686Q is a novel pathogenic mutation occurring in the ontogenetically invariant THV motif of the N-terminal BRCT domain. J Clin Oncol 26: 4212-4214.
- Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, et al. (2006) Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. J Med Genet 43: 295-305.
- 36. Thanassoulas A, Nomikos M, Theodoridou M, Yannoukakos D, Mastellos D, et al. (2010) Thermodynamic study of the BRCT domain of BARD1 and its interaction with the -pSER-X-Phe- motif-containing BRIP1 peptide. Biochim Biophys Acta 1804: 1908-1916.

- Genoni A, Morra G, Colombo G (2012) Identification of domains in protein structures from the analysis of intramolecular interactions. J Phys Chem B 116: 3331-3343.
- 38. Patil R, Das S, Stanley A, Yadav L, Sudhakar A, et al. (2010) Optimized hydrophobic interactions and hydrogen bonding at the target-ligand interface leads the pathways of drug-designing. PLoS One 5: e12029.
- Brunet J, Vazquez-Martin A, Colomer R, Graña-Suarez B, Martin-Castillo B, et al. (2008) BRCA1 and acetyl-CoA carboxylase: the metabolic syndrome of breast cancer. Mol Carcinog 47: 157-163.
- Moreau K, Dizin E, Ray H, Luquain C, Lefai E, et al. (2006) BRCA1 affects lipid synthesis through its interaction with acetyl-CoA carboxylase. J Biol Chem 281: 3172-3181.
- 41. Magnard C, Bachelier R, Vincent A, Jaquinod M, Kieffer S, et al. (2002) BRCA1 interacts with acetyl-CoA carboxylase through its tandem of BRCT domains. Oncogene 21: 6729-6739.
- 42. Williams RS, Lee MS, Hau DD, Glover JN (2004) Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1. Nat Struct Mol Biol 11: 519-525.
- 43. Stucki M, Clapperton JA, Mohammad D, Yaffe MB, Smerdon SJ, et al. (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell 123: 1213-1226.
- 44. Mirkovic N, Marti-Renom MA, Weber BL, Sali A, Monteiro AN (2004) Structurebased assessment of missense mutations in human BRCA1: implications for breast and ovarian cancer predisposition. Cancer Res 64: 3790-3797.
- 45. Easton DF, Deffenbaugh AM, Pruss D, Frye C, Wenstrup RJ, et al. (2007) A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. Am J Hum Genet 81: 873-883.
- Iofrida C, Melissari E, Mariotti V, Guglielmi C, Guidugli L, et al. (2012) Effects on human transcriptome of mutated BRCA1 BRCT domain: a microarray study. BMC Cancer 12: 207.
- 47. Tischkowitz M, Hamel N, Carvalho MA, Birrane G, Soni A, et al. (2008) Pathogenicity of the BRCA1 missense variant M1775K is determined by the disruption of the BRCT phosphopeptide-binding pocket: a multi-modal approach. Eur J Hum Genet 16: 820-832.