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Research Article

COMPUTATIONAL ANALYSIS OF MUTATIONS IN REALLY INTERESTING NEW GENE FINGER DOMAIN AND BRCA1 C TERMINUS DOMAIN OF BREAST CANCER SUSCEPTIBILITY GENE

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ABSTRACT

Objective: Breast Cancer 1 (BRCA1), Early Onset and Breast Cancer 2, Early Onset (BRCA2) genes are involved in pathways important for DNA damage recognition, double-strand break repair, checkpoint control, transcription regulation, and chromatin remodeling. These functions are essential and important for all cell types. Germline mutations in these genes increase the risk of breast and ovarian cancer in women. In this study, we did an analysis of the functional and structural impact of all known single nucleotide polymorphisms (SNPs) in BRCA1 and BRCA2 using publicly available computational prediction tools.

Methods: We analyzed the mutations using two mutation tolerance prediction approaches: Sorting intolerant from tolerant (SIFT), and polymorphism phenotyping (PolyPhen-2). In addition, stability of the protein was analyzed by I-Mutant. Affinity and stability of really interesting new gene (RING) and BRCA1 C-terminus (BRCT) domains were also analyzed by BioLuminate tool.

Results: Out of 486 SNPs in BRCA retrieved from functional SNP, a total of 10 SNPs were found to be deleterious by SIFT and PolyPhen. I-Mutant results indicate that C27F, A1708V could increase the stability of protein, whereas other mutations decrease the stability. Predicted changes in stability and affinity of RING and BRCT domains of BRCA were computed using residue scanning functionality in bioluminate for all 10 SNPs. The mutation C61R could affect the stability of RING domain and all mutations in BRCT domain were affecting the inter subunit affinity and stability of the complex.

Conclusion: The combination of computational methods provides a way in understanding the impact of deleterious mutations in altering the BRCA protein stability and affinity. Based on our investigation, we report potential candidate SNPs for future studies of BRCA mutations.

Keywords: Breast cancer susceptibility gene, BRCA1 C terminus domain, Zinc finger domain, Sorting intolerant from tolerant, Polymorphism phenotyping, I-mutant, BioLuminate.

INTRODUCTION

Breast cancer is the most common cancer in both more and less developed regions with slightly more cases in less developed (882,900 cases) than in more developed (793,700) regions. It is also the frequently diagnosed cancer and the leading cause of cancer death among females (in less developed regions - 324,300 deaths, 14.3% of total while in more developed regions it causes 197,600 deaths, 15.4%. Breast cancer ranks as the fifth common cause of death from cancer overall (521,900 deaths in 2012) [1].

Breast cancer is an inherited disease in 5-10% of all cases while the remaining is sporadic in nature. Of the hereditary type, 40-45% of cases are linked to the breast cancer susceptibility gene 1 (BRCA1) [2,3]. Germline mutations of the breast cancer 1 (BRCA1) gene are a major cause of familial breast and ovarian cancer. BRCA1 is a breast and ovarian tumor suppressor gene located on chromosome 17q21 and encodes a multi-domain protein of 1863 amino acids which is involved in important cellular functions such as in DNA repair, transcription, and cell cycle control through the DNA damage response [4].

The BRCA1 protein interacts with other proteins such as BRCA1-associated RING domain protein 1 (BARD1), cleavage stimulation factor 50 kDa subunit (CstF-50), and mRNA polyadenylation factor to form a complex. BRCA1 and BARD1 each contain a C-terminal BRCT domain and an N-terminal really interesting new gene (RING) domain. The RING domains govern the tight association between the two proteins through the formation of an extensive four-helix-bundle dimerization interface. Formation of the BRCA1-BARD1 complex is important for their mutual stability, their proper nuclear localization, and for maximal ubiquitin (Ub)-ligase (E3) activity [5].

The RING motif is characterized by a conserved pattern of 7 cysteine and one histidine residues arranged in an interleaved fashion forming two distinct Zn^{2+} binding sites (termed Sites I and II, Fig. 1) [6]. The key to structural characterization was the determination that the BRCA1-RING motif, which encompasses residues 24-64, is part of a larger domain (residues 1-109) that includes residues both N- and C-terminal to the core RING motif [5].

Brzovic *et al*. identified those single-site mutations that alter Zn^{2+} binding residues in Site II of the BRCA1 RING domain (Fig. 1) and Site II mutants not only fold and bind Zn^{2+} at Site I but also retain the ability to bind Zn^{2+} at Site II, albeit with reduced affinity. Furthermore, each of the BRCA1 mutants retain the ability to spontaneously form heterodimers with the RING domain of BARD1 [5].

We were interested to know whether mutations of these two domains (BRCT and RING) affect the stability and affinity of the protein. Hence, we did *in silico* analysis to identify the SNPs effect on these two domains. Further *in silico* mutational analysis was initiated by mapping the mutations onto the available protein data bank (PDB) structure (RING domain: 1JM7, BRCT: 1T29) with the help of BioLuminate tool. We also utilized different publicly available computational algorithms such as sorting intolerant from tolerant (SIFT), polymorphism phenotyping (PolyPhen), and function analysis and selection tool for single nucleotide polymorphisms (SNP) to identify deleterious effect of these mutations on protein structure and stability.

METHODS

The BRCA1 sequence was retrieved from UniProtKB: P38398. The SNPs were collected from functional SNP (F-SNP) (A collection of functional SNPs, specifically prioritized for disease association studies) http://compbio.cs.queensu.ca/F-SNP/ [7,8]. The protein structure for Zinc finger domain (1JM7) (Fig. 2) and BRCT domain (1T29) (Fig. 3) were retrieved from PDB [9].

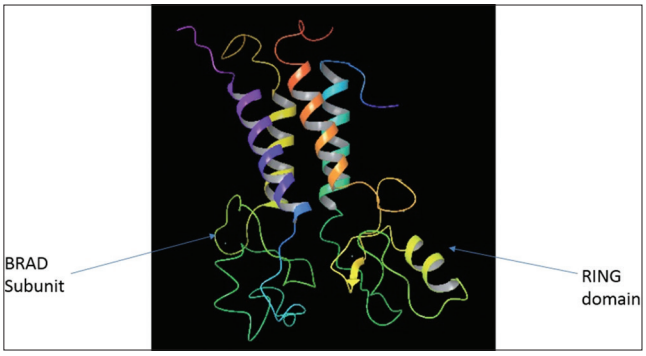

Fig. 1: Really interesting new gene domain of BRCA1 C terminus with BRAD

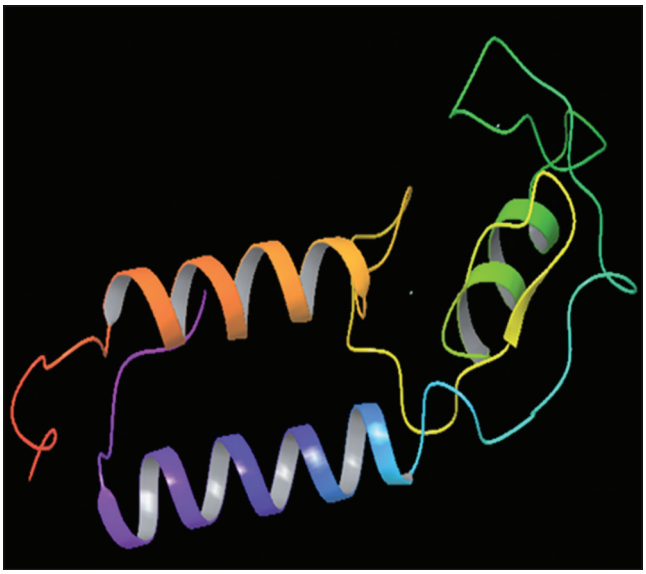

Fig. 2: 1JM7 (really interesting new gene domain)

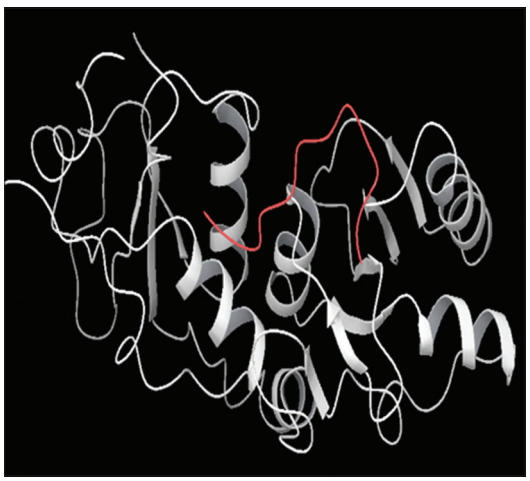

Fig. 3: 1T29 (BRCA1 C terminus domain)

Statistical inference of function through evolutionary relationships (SIFTER) http://sifter.berkeley.edu/about/ [10]

We did phylogeny based protein function prediction using SIFTER tool.

SIFT: http://sift.jcvi.org

SIFT is a computational tool which predicts whether an amino acid substitution affects protein function or not. Its prediction is based on sequence homology to predict tolerated and deleterious effects of every position of the query sequence. It assigns a tolerance index score ranging from 0 (deleterious) to 1 (neutral of a particular amino acid [11,12].

Polymorphism Phenotyping (PolyPhen): http://genetics.bwh.harvard.edu/pph2/index.shtml

PolyPhen is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using physical and evolutionary comparative considerations. PolyPhen 2 calculates and computes the difference in the position-specific independent count score of the two variants. It uses eight sequences based and three structure-based predictive features were selected automatically by an iterative greedy algorithm. The probabilistic score ranges from 0 (neutral) to 1 (deleterious), and functional significance is categorized into benign (0.00-0.14), possibly damaging (0.15-0.84), and probably damaging (0.85-1) [13,14].

I-Mutant: http://folding.biofold.org/i-mutant/i-mutant2.0.html

Stability of mutated protein was measured by computing the change in its Gibbs free energy upon folding. We used a computational tool I-Mutant to predict the protein stability after a substitution of single amino acid. Single amino acid change in a protein sequence can result in a significant change in protein stability (ΔΔG): Positive value represents a destabilizing effect and negative value represents a stabilizing effect on mutation [15]

BioLuminate: http://www.schrodinger.com/BioLuminate

Residue scanning wizard of Bioluminate 1.0 in Schrodinger suite [16-18], was used to study the structural effects of mutations on RING domain and BRCT domain. The difference in the stability of mutant compared to wild-type (WS) protein was calculated for individual mutations. Similarly, the difference in binding affinity of each mutant for the BRCT domain and the peptide, corresponding wild-type, was also analyzed.

RESULTS

SIFTER provides a list of predicted functions from the GO molecular function ontology for BRCA1 (P38398) along with confidence score (Table 1). The score is between 0 and 1 and it was determined based on the number of candidate functions, the family size, the frequency and distribution of GO terms. The most prominent function of BRCA1 is ubiquitin protein ligase binding with a confidence score of 0.89.

We collected 486 SNPs from F-SNP by submitting the gene name (BRCA1) in the query form "Query by Gene." The functional effects are predicted and indicated at the splicing, transcriptional, translational, and post-translational level. As such, the F-SNP database helps to

Table 1: SIFTER: GO molecular function ontology

GO term	Protein prediction	Confidence score
GO: 0031625	Ubiquitin protein ligase binding	0.89
GO: 0050681	Androgen receptor binding	0.89
GO: 0044212	Transcription regulatory region DNA binding	0.88
GO: 0008270	Zinc ion binding	0.88
GO: 0003713	Transcription coactivator activity	0.82
GO: 0003684	Damaged DNA binding	0.79

SIFTER: Statistical inference of function through evolutionary relationships

identify and focus on SNPs which has the potential pathological effect to human health.

Among 486 SNPs SIFT predicted, we choose 10 SNPs which are present in zinc finger domain [4] and in BRCT domain [6] are deleterious. All 10 SNPs are identified as deleterious with tolerance score either 0 or 0.01 (Table 2).

PolyPhen 2.0 evaluates the location of the amino acid replacement within the identified functional domains and 3D structures. PolyPhen 2.0 predicted that all SNPs are damaging (Table 3).

I-Mutant results indicate that C27F, A1708V could increase the stability of protein, whereas other mutations decrease the stability (Table 4).

RING domain

We analyzed Zinc finger domain using Bio-Luminate residue scanning tool. The stability of the protein was increased in C27F, K38N, C61G, C61S, whereas the protein stability decreased when cysteine residue mutated to arginine (Table 5). Polar cysteine residues are mutated to hydrophobic (C61G, C61F), charged C61R and to polar C61S residues.

These mutations increase the hydrophobicity and also affect the solvent accessible area. The root mean square deviation indicated that there is a slight variation in the positions of amino acids present in mutation site. Hydrogen bonds formed in Cys61 in wild type lost in C61R and new bonds formed between Thr37 and Asp67. No changes in hydrogen bonds were found in the remaining two mutants (C61S, C61G) (Fig. 4).

K38 not forming any hydrogen bond, whereas the mutation K38N forms hydrogen bond with Asp67 (Fig. 5).

BRCT domain

BRCT domain of stability and affinity was analyzed. All mutations showed positive values of delta stability, indicates that the mutations play as structure destabilizing factor (Table 6).

Estimation of binding affinities of mutant BRCT domain with peptide indicates a reduction in affinity of mutants such as F1662C, F1662S, A1708E, and V1809F. Majority of the mutations were affecting the inter subunit affinity and stability of the complex. Mutations in BRCT domain decrease the hydrophobhicity, and there is a change in the total surface area.

The values of root mean square deviation show slight variation in the mutant structure when compared with wild type. F1662WT and F1662C form hydrogen bonds with T1658, Y1666 **(**Fig. 6a and b). However, the mutant F1662S forms two hydrogen bonds with T1658 and two more with V1665 and Y1666 (Fig. 6c).

A1708E form extra hydrogen bond with glutamine (Fig. 7).

The hydrogen bond interaction between amino acid residues Q1857 and F1821, I1824, G1825 was lost in mutation Q1857H (Fig. 8). No change in other mutant V1809F (Fig. 9).

DISCUSSION

Breast and ovarian cancer are the most common diseases linked to BRCA1 and BRCA2 changes, but mutated forms of the BRCA genes may increase people's risk for other cancers as well. The list of mutations in the database increases rapidly. These mutations induce structural changes in the protein. Proper folding and assembly is necessary for protein activity and also for structural stability. Phylogeny based protein function prediction showed that BRCA1 sequence has the most prominent GO biological term such as the ubiquitin protein ligase binding and Zinc ion binding. Zn finger domain of BRCA1 binds with ubiquitin ligase for ubiquitination. According to SIFT analysis all mutations are identified as deleterious with tolerance index score ≤0.05. Except F1662S and F1662C other mutations showed a highly deleterious tolerance score 0.00. These two mutations showed tolerance index scores of 0.01. All 10 mutations were submitted to the PolyPhen server. Four SNPs in the Zinc finger domain were scored

Table 2: List of SNPs analyzed by SIFT

SNP	Allele	AA_pos	wild_AA	mutant_AA	Score	Prediction
RING domain						
rs1800062	C|A	38	K	N	0	Damaging
rs28897672	A|G	61	C	R	0	Damaging
rs28897672	A|T	61	C	S	0	Damaging
rs28897672	A|C	61	C	G	0	Damaging
BRCT domain						
rs28897695	A|G	1662	F	S	0.01	Damaging
rs28897695	A|C	1662	F	C	0.01	Damaging
rs28897696	G|A	1708	A	V	0	Damaging
rs28897696	G|T	1708	A	E	0	Damaging
rs28897698	C|A	1809	V	F	0	Damaging
rs28897699	C|A	1857	Q	H	0	Damaging

SIFT: Sorting intolerant from tolerant, SNP: Single nucleotide polymorphisms, BRCT: BRCA1 C-terminus, RING: Really interesting new gene

Table 3: List of SNPs analyzed by PolyPhen

SNP	Allele	AA_pos	wild_AA	mutant_AA	Score	Prediction
RING domain						
rs1800062	C|A	38	K	N	0.891	Probably damaging
rs28897672	A|G	61	C	R	0.99	Probably damaging
rs28897672	A|T	61	C	S	0.96	Probably damaging
rs28897672	A|C	61	C	G	0.99	Probably damaging
BRCT domain						
rs28897695	A|G	1662	F	S	0.227	Possibly damaging
rs28897695	A|C	1662	F	C	0.381	Possibly damaging
rs28897696	G|A	1708	A	V	0.634	Possibly damaging
rs28897696	G|T	1708	A	E	0.993	Probably damaging
rs28897698	C|A	1809	V	F	0.438	Possibly damaging
rs28897699	C|A	1857	Q	H	0.014	Benign

PolyPhen: Polymorphism phenotyping, SNP: Single nucleotide polymorphisms, BRCT: BRCA1 C-terminus, RING: Really interesting new gene

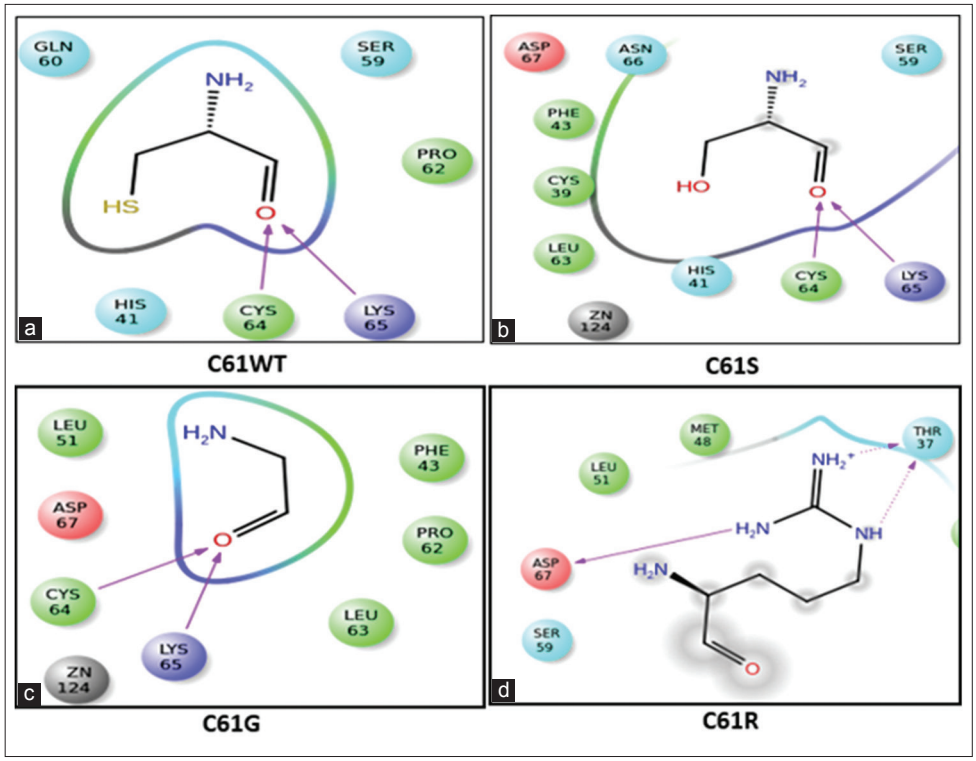

Fig. 4: Interactions of cysteine residue (a) wild type (b) mutant C61S (c) mutant C61G (d) C61R

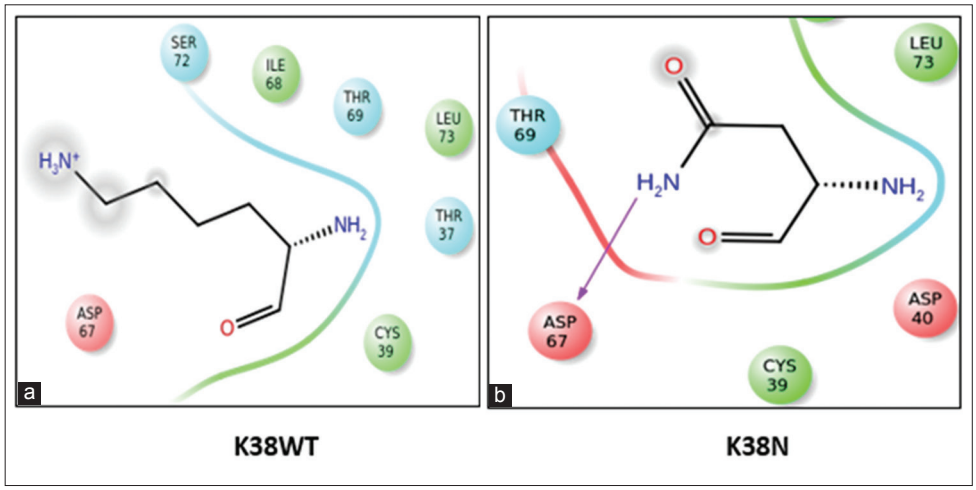

Fig. 5: Interaction of lysine (a) wild type (b) mutant K38N

Table 4: I-Mutant result

Position	WT AA	Mutated AA	DDG	pH	T	RSA
38	K	N	−1.29	7.0	25	38.9
61	C	G	−1.85	7.0	25	0.0
61	C	S	−0.76	7.0	25	0.0
61	C	R	−1.17	7.0	25	0.0
1662	F	S	−2.73	7.0	25	33.4
1662	F	C	−2.65	7.0	25	33.4
1708	A	V	0.22	7.0	25	0
1708	A	E	−1.19	7.0	25	0
1809	V	F	−2.44	7.0	25	0.0
1857	Q	H	−0.87	7.0	25	33.0

as "probably damaging" and five of BRCT domain SNPs as "possibly damaging" and one SNP Q1857H showed "Benign." PolyPhen uses sequence information and structural information of the protein to predict the effect of SNPs, suggesting that these SNPs may disrupt both the protein structure and function. The protein stability was predicted using support vector machine based tool I-Mutant for all 10 mutations. The energy difference between wild type and mutant protein was calculated based on Gibbs-free energy value. Except A1708V all other mutations stabilize the protein.

Mutations of the cysteine residues in RING domain that binds with Zn^{2+} ions have been reported as clinically important indicating that they result in altered function and an increased risk of cancer [19,20]. Studies suggest that mutation of cysteine residues may affect BRCA1 ubiquitin ligase activity [21]. Mutations of cys61 have a structural effect and also alter the proteolytic susceptibility of Zinc. Cys39, His41, cys61 and cys64 interacts with Zn^{2+} and forms a RING domain. Studies show that Cys-X2-Cys motif makes up tetrahedral Zn^{2+} binding site [5]. Mutation of Cys61 to G, S or R affects the coordination of Zinc ion. This would surely affect the ability of RING domain to form complexes

Table 5: Stability of RING domain using bioluminate tool

Position	WT	New	Delta stability	Delta prime energy	Delta hydropathy	Delta SASA	RMSD A°
38	K	N	−28.25	−29.12	0.13	−8.67	0.021
61	C	G	−7.24	−7.044	0	0	0.009
61	C	S	−4.301	−6.111	0	0	0.008
61	C	R	241.447	219.247	0	−0.005	0.036

RING: Really interesting new gene

Table 6: Stability of BRCT domain using BioLuminate tool

Position	WT	NEW	Delta stability	Delta affinity	Delta prime energy	Delta hydropathy	Delta SASA (total)	RMSD A°
1662	F	C	10.466	0.09	7.556	−0.173	28.072	0.003
1662	F	S	10.121	0.079	9.49	−0.605	27.739	0.003
1708	A	E	157.044	4.578	136.532	−0.003	0.117	0.042
1708	A	V	44.224	−0.367	50.647	0.001	0.377	0.014
1809	V	F	113.33	0.042	102.132	−0.001	0.368	0.020
1857	Q	H	19.403	−0.017	53.946	−0.266	−5.536	0.008

BRCT: BRCA1 C-terminus

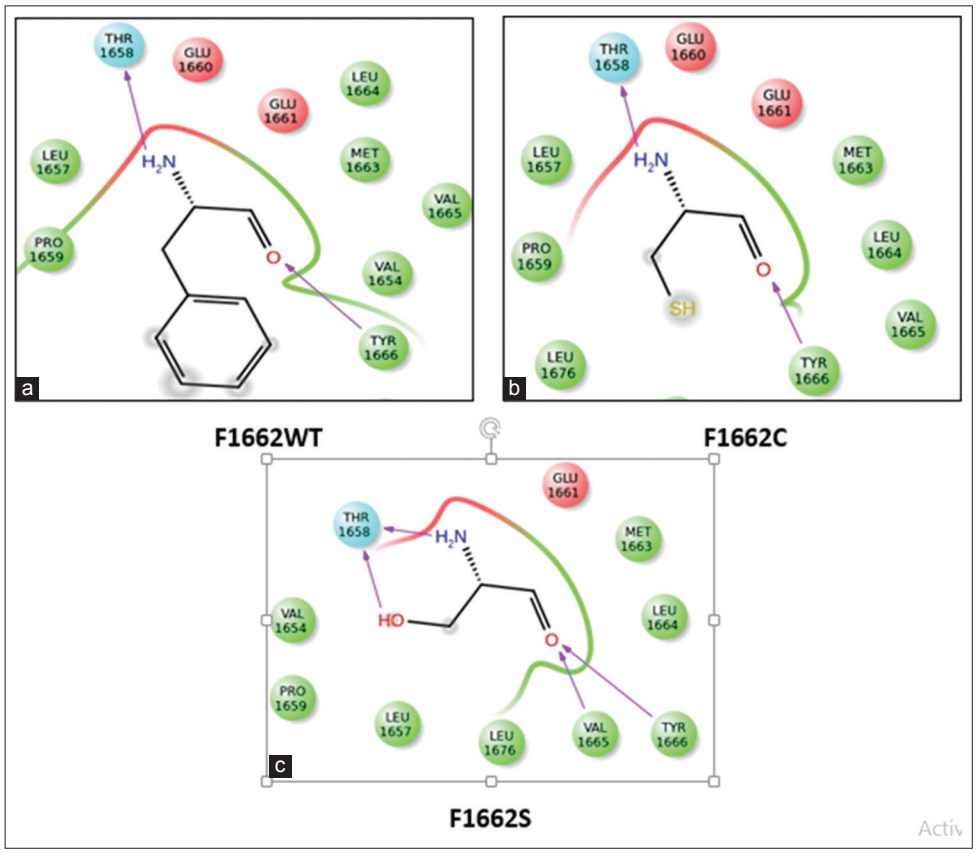

Fig. 6: Interaction of phenyl alanine (a) wild type (b) mutant F1662C (c) mutant F1662S

with interacting proteins. The Ring finger mutant C61R showed the greatest increase in energy and decrease in stability of the protein. C61G mutant does not alter the protein's size [22] but it disrupt the interaction between BRCA1 and its partner protein BARD1 [23]. Zinc finger domains are typically found in clusters within a single protein and structurally stabilize the protein for interaction with other proteins and biomolecules such as DNA and RNA [24]. Mutations within the RING finger domain could affect the interaction with other proteins and with biomolecules.

The charged amino acid K38 is mutated to polar residue K38N. Asn forms hydrogen bond with Asp67 and increases stability of the protein. Hydrogen bonds are responsible for stability of the protein and also provide specificity required for selective macromolecular interactions. However, these mutations affect folding kinetics of protein or lead to impaired biological function.

BRCA1 interacts with DNA damaging proteins through its BRCT domain. Mutations within BRCT domain inhibit BRCA1 from interacting with various DNA damage proteins through its BRCT domain, so damaged DNA escapes from checkpoints during the G2/M phase of the cell cycle. As a result, DNA repair cannot occur and these mutations are passed to daughter cells. The results obtained from our analysis indicated that native BRCT domain is more stable than mutated proteins. Phenylalanine mutation with cysteine (F1662C) and serine (F1662S) affects both stability and affinity of the protein. The arrangement of

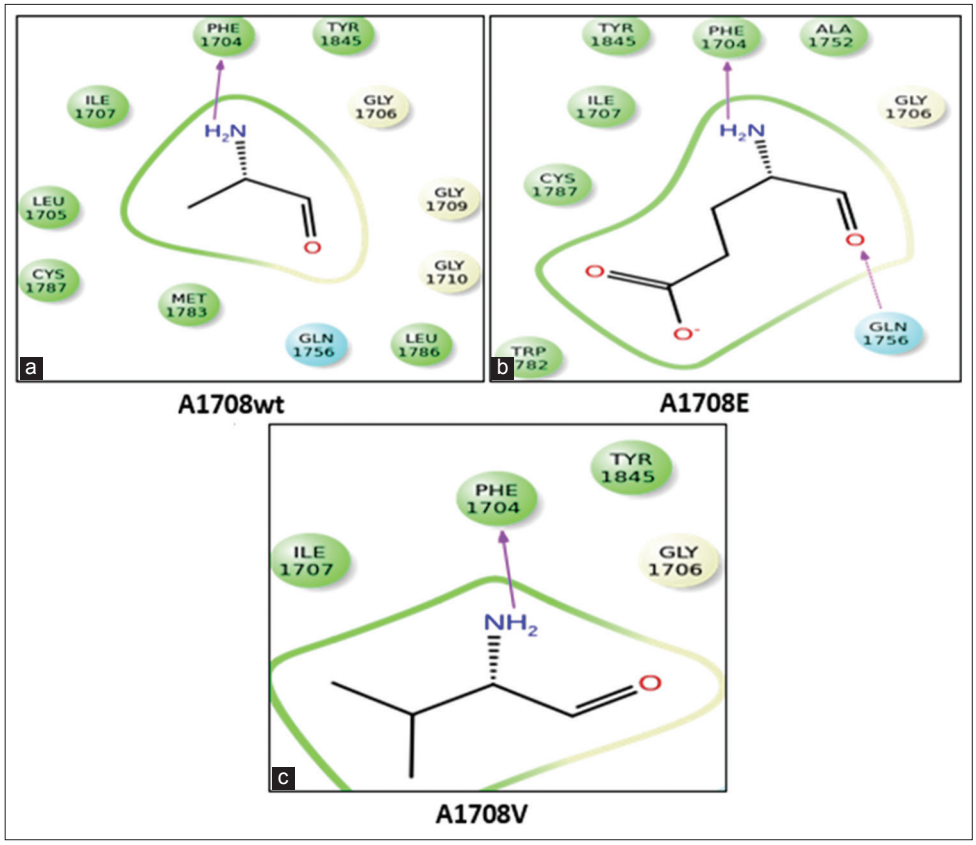

Fig. 7: Interaction of alanine (a) wild type (b) mutant A1708E (c) mutant A1708V

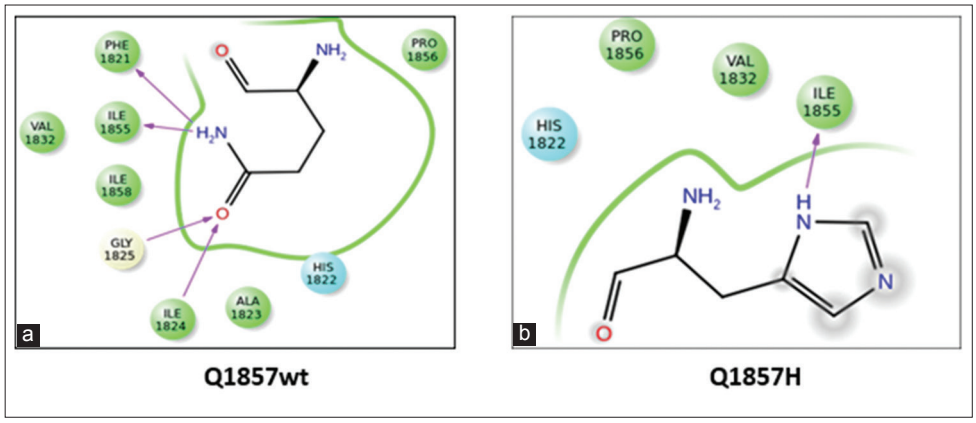

Fig. 8: Interaction of glutamine (a) wild type (b) mutant Q1857H

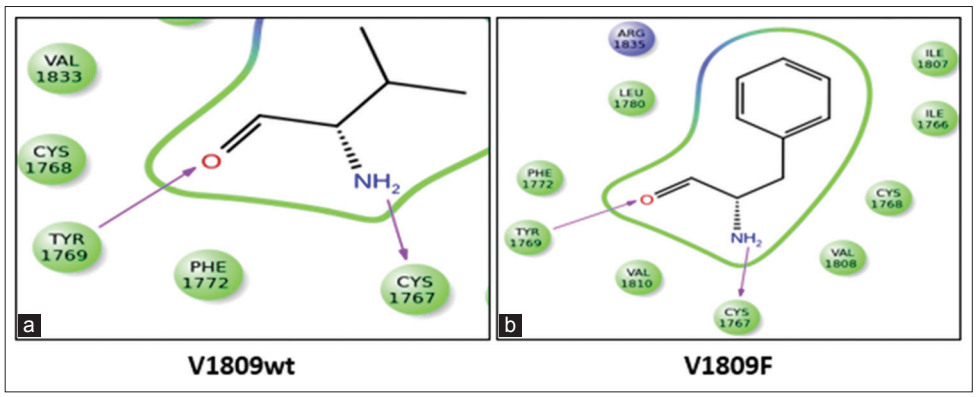

Fig. 9: Interaction of valine (a) wild type (b) mutant V1708F

α helix and β sheet in BRCT domain is maintained by hydrophobic residues such as phenylalanine. Mutation of phenylalanine to Cys or Ser will disturb the packing due its small size which affects the structural stability and folding of BRCT domain. Substitution of valine for Ala1708 showed slightly elevated binding affinity of BRCT domain, whereas Glu for Ala1708 decreases the affinity of the protein. Alanine side chain is non-reactive so rarely directly involved in protein function whereas valine is hydrophobic and usually buried in protein hydrophobic cores and is more difficult to adapt an α- helical structure. Being charged and polar, glutamate generally prefers to be on the surface of the proteins and exposed to the aqueous environment. Hanafusa *et al*. observed in their experiments that in mammalian and yeast cells, A1708E mutation destabilizes interaction with DNA binding proteins which impair the ability to activate transcription of genes involved in suppression of transformation [25].

V1809F affects both stability and affinity of the BRCT domain. The deleterious effect of this mutation may be due to the bulky aromatic phenylalanine. V1809 in BRCT domain contribute to intra β sheet packing, and substitution of this residue with phenylalanine is highly destabilizing [26]. Gln (Q) 1857His effects stability but not affinity, since histidine contains an imidazole ring it may participate in protein stacking interactions. Hydrogen bond formation from the crystal structure is clear that glutamine 1857 interacts with phenylalanine 1821, Ile1824 and Gly1825. Replacing 1857 with mutant histidine residue, which contains imidazole ring, will create structural restraints thereby affecting the BRCT domain stability. Thus, *in silico* analysis of the 3D structure of RING and BRCT domain, their physicochemical properties and energy calculations, show that we can successfully predict molecular effects due to these mutations. Such an analysis helps better understand how these mutations affect the structure, stability and affinity of the protein. We also expect that the results from the current computational analysis on BRCA1 domains with suitable experimental validation in near future will aid in understanding the effect of individual drug response, for rapid diagnosis, prognosis, and treatment of diseases.

CONCLUSION

As the majority of disease-causing mutations affect protein stability and affinity, we analyzed the BRCA1 protein mutations and compared them with the native protein to evaluate stability changes. A total of 10 SNPs were found to be damaging by both SIFT and PolyPhen tools. The structural analysis of RING and BRCT domains of BRCA1 results showed that the amino acid residue substitutions had the greatest impact on the stability of the BRCA protein and hydrogen bond interaction explaining the functional deviations of mutated protein from wild type. From our study, we would suggest that all mutations have a great impact on protein stability and affinity. Further structural and functional studies of Ring finger domain and BRCT domain of BRCA1 are essential for understanding disease pathogenesis and the discovery of novel therapeutics.

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