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Functional Investigation of the Novel *BRCA1*variant (Glu1661Gly) by Computational Tools and Yeast Transcription Activation Assay

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Abstract

Introduction: Mutations in the *BRCA1* gene are major risk factors for breast and ovarian cancers. However, the relationship between some *BRCA1* mutations and cancer risk remains largely unknown**.** Cancer risk predictions could be improved by evaluation of the impairment degree in the *BRCA1* functions due to a specific mutation. This study aimed to assess the functional effect of a novel variant (Glu1661Gly) in *BRCA1* gene by a combination of in silico tools, structural analysis, and also experimental functional assay based on yeast transcription activation.

Methods: Computational tools including PROVEAN, PolyPhen2, Align-GVGD, Mutation Taster, and also structural analysis were used for prediction of the impact of Glu1661Gly on protein function. To perform the yeast functional assay, the *BRCA1* C-terminal (BRCT domain) was cloned into pLexA plasmid in-frame with the DNA-binding domain of LexA to generate a functional transcription activator. The resulted construct was transformed into EGY48/pRB1840 yeast and positive colonies were assayed for β-galactosidase activity. Wild-type *BRCA1* and Ser1613Gly were used as positive controls and Met1775Arg as negative control.

Results: The Glu1661Gly variant was predicted to be neutral by PROVEAN, disease-causing by Mutation Taster, probably damaging by Polyphen2, and intermediate effect by Align-GVGD. The yeast functional assay revealed that Glu1661Gly activity was comparable to wild-type BRCA1.

Conclusions: Observed discrepancies between in silico tools make it difficult to interpret the results. Based on structural analysis, the Glu1661Gly on α1 helix of the C-terminal domain does not seem to impair function due to α1 helix is far from the BRCT-BRCT interface and phosphopeptide-binding site. This variant was also classified as neutral; using yeast functional assay.

INTRODUCTION

Germline mutations in the tumor suppressor BRCA1 gene strongly predispose the carriers to breast, ovary, pancreas and several other types of cancerss [1, 2]. To date, nearly 600 different

BRCA1 missense variants have been reported in the Breast Cancer Information Core (BIC) database. The clinical significance of a large proportion of mutations in the *BRCA1* gene is unclear (Variant of Uncertain Significance; VUS).

Classification of mutations according to population-based studies is usually challenging. For *BRCA1*, this problem is more complicated because the prevalence of germline *BRCA1* mutation carriers in the general population is very low [3, 4]. So, there is a serious need in medicine to employ techniques that can facilitate the classification of mutations. The distinction of the deleterious from neutral nsSNPs by computational analysis is a simple and cost-effective method to explore the structure-function relationship. Beside computational methods, a variety of functional assays were developed; based on identified protein functions [5].

BRCA1 encodes a protein of 1863 amino acids that contains 1) N-terminal RING domain binding to BARD1 [6] 2) a domain in the middle of the structure; interacting with DNA repair protein RAD51 [7, 8], and 3) two tandem BRCA1 C-terminal (BRCT) domains in the C-terminus that are involved in tumor suppression, growth inhibition, and transcription activation [9-11]. RING finger and BRCT domains are the most conserved regions of *BRCA1*. *BRCA1* mutations, and especially those in the BRCT and RING finger domains, are associated with hereditary breast and ovarian cancers [12-15]. The BRCT domain of BRCA1 binds to phosphopeptides such as BACH1 and CtIP and regulates DNA damage responses [16-24]. BRCA1 phosphopeptide binding defect leads to increased susceptibility to breast and ovarian cancers [18, 25-28].

Studies have shown that the BRCT domain activates transcription in the yeast and mammalian cells by fusion of this domain to a heterologous DNA-binding domain [29-31]. According to this finding, a functional assay called transcription-activation assay has been designed. In this study, a novel germline variant with unknown clinical significance; Glu1661Gly; was identified in one of the breast cancer patients of Motamed Cancer Institute-Breast Cancer Research Center [32]. This variant was located in the BRCT domain of *BRCA1*. Computational analysis and transcription activation assay were used to investigate the impact of this variant on BRCA1 protein function.

METHODS

A novel variant; Glu1661Gly; was found in one of the breast cancer patients of Motamed Cancer Institute-Breast Cancer Research Center [32]. This variant is located on exon 16 (c.4982A>G; NM_007294.3) of *BRCA1* BRCT domain. Ethical approval was obtained from the Ethics Review Committee for Medical Research of Avicenna Research Institute (ethical code: IR.ACECR.Avicenna.REC.1396.24). Written informed consent was obtained from all patients before entering the biobank.

Computational Analysis

Computational tools were used from different information such as sequence, sequence/structure and/or functional parameters to predict whether a mutation is deleterious or neutral. In this study, computational analysis was performed by a combination of methods with different features, including PROVEAN [33], PolyPhen2 [34], Mutation Taster [35], and Align-GVGD [36, 37]. The details of each method are summarized in Table 1.

Modeling

The X-ray crystal structure of the BRCA1/BACH1 (PDB code: 1T15) [38] was obtained from the

Table 1: The Details of Used Computational Tools in This Study

	Patients, No. (%)	Prediction Result	URL
PROVEAN	sequence and evolutionary conservation	neutral and deleterious	http://provean.jcvi.org/index.php
Align-GVGD	sequence evolutionary along with the physicochemicalprop-erties	C0 (likely neutral), C15, C25, C35, C45, C55 and C65 (most likely deleterious)	http://agvgd.iarc.fr/
PolyPhen2	protein sequence and structure	probably damaging, Possibly damaging and neutral	http://genetics.bwh.harvard.edu/pph2/
Mutation Taster	evolutionary conservation, mutation frequency, protein sequence annotations	disease-causing, polymor-phism	http://www.mutationtaster.org/

Protein Data Bank. Missing residues identified in this structure were modeled; using Modeller v9.12. Structural analysis was performed; using the final modeled structure.

Yeast Functional Assay

Constructs

The Glu1661Gy mutation was introduced by site-directed mutagenesis with SOEing PCR [39]. Briefly, plasmid plex9-*BRCA1* (wt) (gift from Dr. Monteiro; exons16-24) was used as a template in the first and second PCR reactions. The first PCR was performed; using the primer pairs of E1661GF and BRCAR. The second PCR was performed; using the primer pairs of BRCAF and E1661GR. All primer sequences are listed in Table 2. Finally, the two PCR products and primer pairs (BRCAF/BRCAR) were used for SOEing PCR; generating a 928-bp product. The PCR fragment was cloned into the pTZ57RT vector (Thermo Fisher). The pTZ57RT-Glu1661Gly variant was digested with EcoRI and BamHI enzymes. The purified Glu1661Gly *BRCA1* fragment was cloned into the pLexA plasmid vector in-frame with the LexA DNA-binding domain. The plasmid construct was confirmed by sequencing.

Plasmid constructs containing wild-type *BRCA1* (exons 16-24), as well as the neutral mutation (Ser1613Gly) and deleterious mutation (Met1775Arg) were provided by Dr. Monteiro [40]. All mutations were confirmed by sequencing.

Table 2: Primers Used for Site-Directed Mutagenesis by SOEing PCR [a]

	Sequences (5'-3')
E1661G-F	CCAGAAG**G**ATTTATGCTCGT
E1661G-R	ACGAGCATAAAT**C**CTTCTGG
BRCAF	ACTTGGAATTCGAGGGAACCCCTTACCTG
BRCAR	GTTTGGATCCTCAGTAGTGGCTGTGGGGGAT

[a] Restriction sites are underlined (EcoRI and BamHI); Single nucleotide changes are in red.

Transcription Assay in Yeast

EGY48 strain [MATa,ura3, trp1, his3, 6 lexA operator-LEU2] [41] was transformed with the lacZ reporter plasmid pRB1840 [41, 42]; using the lithium acetate method (Clontech). Positive colonies were selected on medium lacking uracil**.** The yeast cells (EGY48/pRB1840) were separately transformed with pLexA plasmid; encoding wild-type BRCA1, Ser1613Gly, Met1775Arg, and Glu1661Gly. [41, 42]. All transformations were confirmed by colony PCR and sequencing. Each variant was assayed for β-galactosidase activity using ONPG [43]. Experiments were carried out in triplicates. The activity was determined by comparing the results with the positive controls (Wild-type *BRCA1* and Ser1613Gly) and negative control (Met1775Arg).

RESULTS

Structural Analysis of Glu1661Gly Variant

The BRCT domain of BRCA1 contains two BRCT repeats (N-terminal BRCT repeat and C-terminal BRCT repeat). The BRCT repeat structure was conserved in different protein families, composed of four parallel β-strands that are flanked on one face by two α-helices (α1 and α3), and a single alpha helix (α2) on the opposite face [44]. Glu 1661 was located on α1 helix in N-terminal BRCT repeat. The Glu1661 residue formed a salt bridge with a lysine residue at codon 1690 [27]. A mutation from glutamic to glycine disrupted this interaction (Figure 1). However, the effect of this interaction on BRCA1 protein function is unclear.

Phosphopeptides are recognized by a hydrophobic pocket at the interface between the two BRCT repeats and a phosphopeptide binding pocket in the N-BRCT domain [27]. The hydrophobic pocket of BRCA1 is composed of residues including Leu1701, Phe1704, leu1780, Met1783, Arg1835, and Leu1839 which form conserved interactions with phosphopeptides. The phosphopeptide-binding pocket includes key residues (Ser 1655, Gly 1656, and Lys 1702) that make direct interactions with phosphate moiety [27]. Mutations lead to a defect in the BRCA1 binding to phosphopeptides; resulting in impaired transcriptional activation**.** The Glu1661 was located on α1 helix in N-terminal BRCT repeat. The α1 helix in N-terminal BRCT domain of BRCA1 lies far from phosphopeptide-binding pocket and BRCT-BRCT interface. So, Glu1661Gly variant does not seem to affect the transcriptional activation of BRCA1. However, molecular dynamics

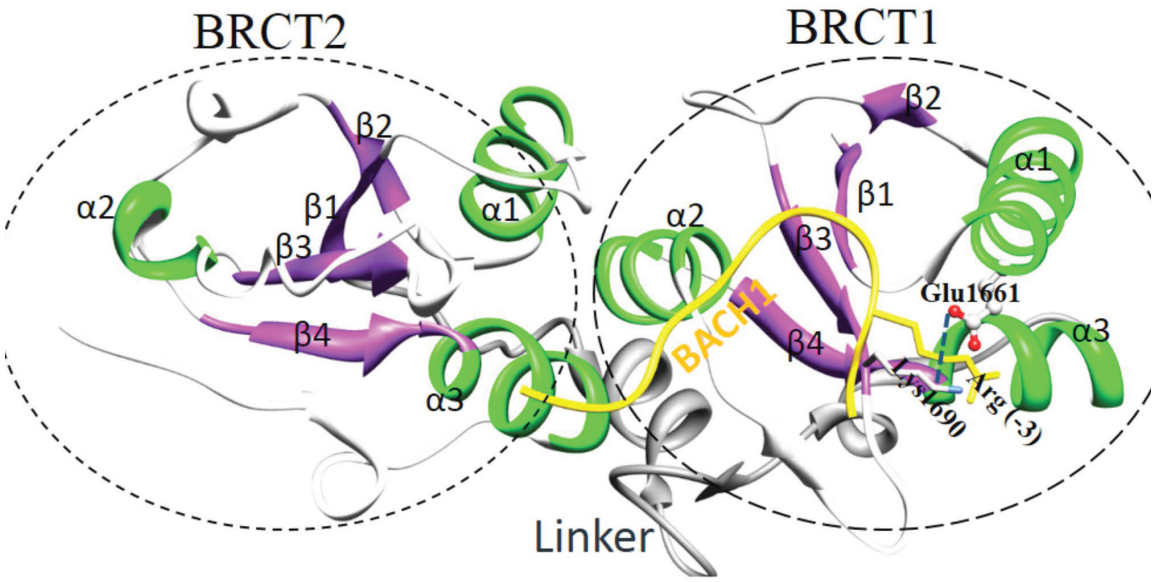

Figure 1: 3D Structure of the BRCT Domain of *BRCA1* (Alpha–Helices Are Green and Sheets Are Purple) Glutamate 1661 in the α1 helix of N-BRCT hydrogen bonded with Lys1690 residue.

simulation is required to better understand the effect of the mutation on protein structure.

Computational Prediction of Glu1661Gly

The software online tools including PROVEAN [33], PolyPhen2 [34], Align-GVGD [36, 37], and Mutation Taster [35] were used to predict effects of mutation on the structure and function of the protein.Glu1661Gly was predicted to be neutral by PROVEAN, disease-causing by Mutation Taster, probably damaging by PolyPhen2, and an intermediate effect (C35) by Align-GVGD (Table 3).

Table 3: Predicted Effect of the Glu1661Gly Variant by Align-GVGD, PROVEAN, PolyPhen2 and Mutation Taster

	Score	Interpretation
Align-GVGD	C35	intermediate effect
PROVEAN	-1.2	neutral
PolyPhen	0.972	probably damaging
Mutation Taster	-	disease-causing

Transcription Activation Assay

Glu1661Gly variant with unknown clinical significance was located in the BRCT domain of BRCA1. To examine the functional consequences of the Glu1661Gly on the transcriptional activity of BRCA1 protein, we performed a yeast-based transcriptional activity assay.

In this study, wild-type and neutral variant (Ser1613Gly) were used as positive controls and cancer-related mutation (Met1775Arg) as the negative control [29-31]. As expected, wild-type BRCA1 protein significantly activated transcription in the yeast system. The positive control (Ser163Gly) revealed activity similar to the wild-type level, whereas the negative control (Met1775Arg) mutation lost its ability to activate transcription (Figure 2).

DISCUSSION

In this study, a novel germline variant with unknown clinical significance (Glu1661Gly) in one breast cancer patients was investigated [32].Computational tools, structural analysis, and transcription activation assay were used to investigate the impact of this variant on BRCA1 protein function.

Genome-wide association studies (GWAS) have resulted in a significant increase in the identification of various single nucleotide polymorphisms (SNPs) in *BRCA1* and *BRCA2* genes. However, the clinical significance of many of these nucleotide changes is still unclear. Detecting VUSs in *BRCA* genes imposes a big challenge for BRCA genetic counseling and clinical decision making. The classification of mutations based on population studies is time-consuming and expensive. So, the development of new methods for separating deleterious mutations from neutral variants is required.

Nowadays, a variety of methods, including computational analysis and functional assays are used to determine the functional significance of VUSs. Transcription assay is perhaps the most commonly used assay for BRCT domain integrity

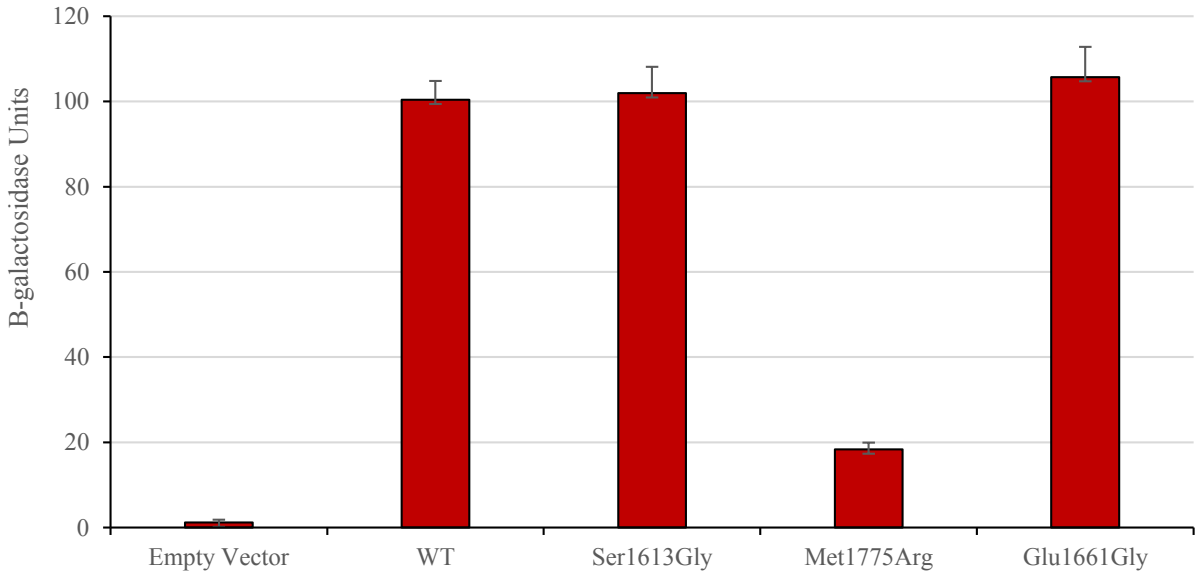

Figure 2: Transcriptional Assay of Wild-Type *BRCA1* and Variants (Met1775Arg, Ser1613Gly, Glu1661Gly, and empty vector)
The Ser1613Gly variant showed activity equal to or slightly higher than the wild-type. Transcription activation was significantly decreased in Met1775Arg relative to the wild-type. Glu1661Gly variant with unknown significance showed transcriptional activity nearly equal to the wild-type.

testing in BRCA1. This method is successfully used for classification of mutations in the carboxyl-terminus of *BRCA1* [23, 45-47].

Computational tools provide conflicting results for predicting the functional consequence of Glu1661Gly variant. It is therefore difficult to draw a clear conclusion from predictions generated by computational tools. Structural analysis showed that Gu1661 is located in the N-terminal BRCT domain of BRCA1. The α1 helix lies far from the phosphopeptide-binding pocket and BRCT-BRCT interface. So, Glu1661Gly variant does not seem to affect the transcriptional activation of BRCA1. This suggestion was confirmed by the results of yeast transcription assay. Other Mutations on the α1 helix including F1662S, M1663L, A1669S, M1663K, L1664P, and V1665M have been previously assessed by transcription activation assay [40, 48]. The results indicated that all mentioned mutations had transcriptional activity equivalent to wild-type in yeast and mammalian cells [40, 48]. These findings are consistent with clinical data and there was also no report about the pathogenicity of these mutations in ClinVar.

In this study, the impact of a novel germline variant with unknown clinical significance; Glu1661Gly; was investigated by computational tools, structural analysis, and yeast functional assay. Based on structural analysis, the Glu1661Gly variant seems to have no effect on BRCA1 activity. Transcription activation assay shows that this variant had activity comparable to the wild-type BRCA1 in yeast. In total, this method can be used as an alternative way to evaluate the effects of variants of unknown significance in the BRCT.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

ETHICS APPROVAL

This study was approved by the Ethics Committee for Medical Research of Avicenna Research Institute.

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