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Integrating Functional and Structural Analyses Improves the Assessment of *BRCA1* Missense Variants of Unknown Significance

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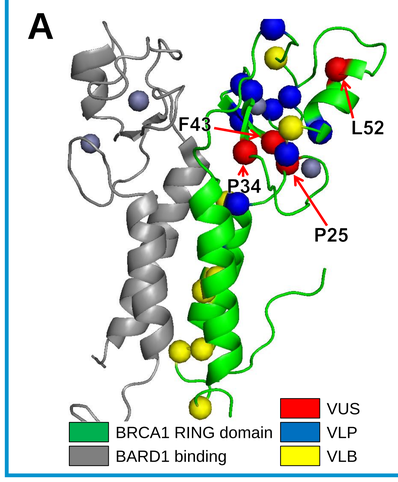
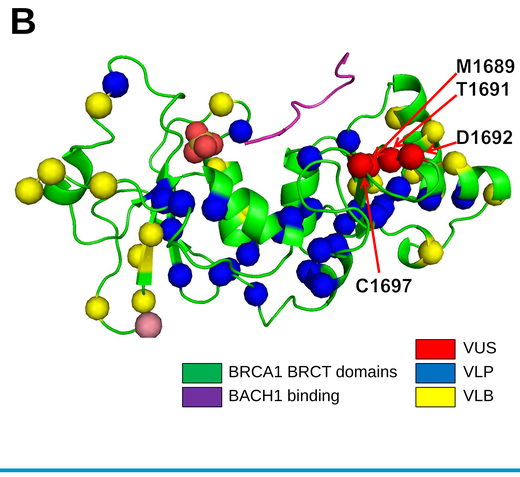
BACKGROUND

- ~0.5-2% of breast cancers are attributed to germline pathogenic alterations in *BRCA1.*[1,2]
- Up to 20% of *BRCA1/2* variants identified by genetic testing are variants of unknown significance (VUS).[3,4]
- Most VUS are missense variants due to the difficulty in predicting their clinical impact relative to other types of alterations.
- The majority of deleterious alterations in *BRCA1* are in the RING and BRCT domains which have been implicated in the HR function of the protein.
- Integrating functional assays with other evidence (eg, structural predictions, general population frequency, etc.) may improve the classification of *BRCA1* missense VUS.

SELECTING *BRCA1* MISSENSE VUS FOR FUNCTIONAL ANALYSIS



p.Variant	ΔΔG (kcal/mol)	Class
H41R	-1.66	VLP
I15L	-1.54	VLB
R71K	-0.50	VLP
C64Y	-0.47	VLP
C64G	-0.44	VLP
D67Y	-0.40	VLB
H41L	-0.29	VLP
I89M	-0.25	VLB
R71M	-0.24	VLP
H41N	-0.24	VLP
C64R	-0.18	VLP
R7H	-0.05	VLB
R71G	0.03	VLP
R43L	0.11	VUS
C39R	0.37	VLP
R7C	0.41	VLB
C61S	0.41	VLP
C39S	0.44	VLP
C61G	0.56	VLP
C39Y	0.59	VLP
Y21V	0.60	VLB
G44S	0.67	VLP
K45Q	0.70	VLB
C39Y	0.75	VLP
C44F	0.76	VLP
C47F	0.78	VLP
V8I	1.12	VLB
C44R	1.33	VLP
I52F	1.75	VUS
P34S	2.50	VUS
P25L	2.67	VUS
S4P	2.72	VLB
L22S	3.41	VLP
M18T	3.71	VLP
P34L	5.01	VUS
T37R	11.07	VLP

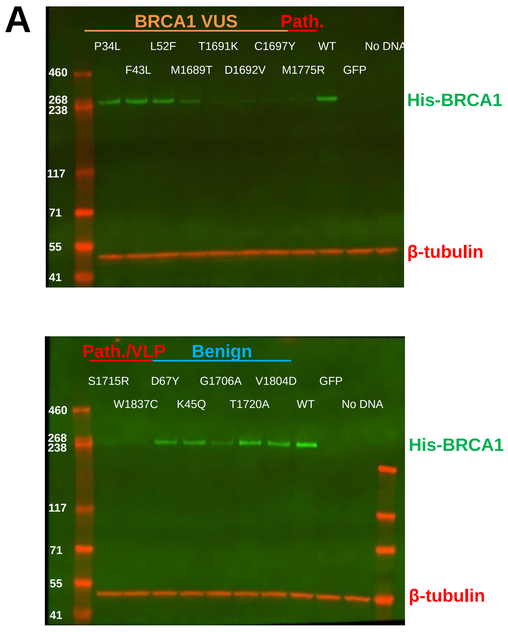


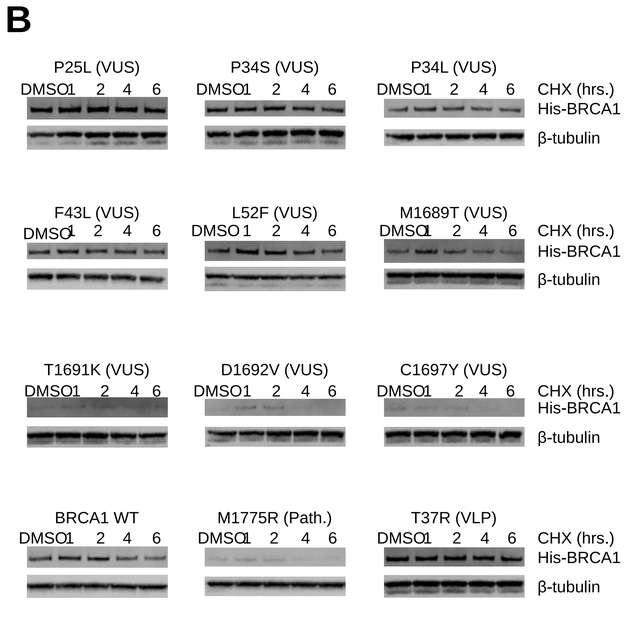
p.Variant	ΔΔG (kcal/mol)	Class
D1818G	-1.70	VLP
H1672Y	-0.52	VLB
T1720A	-0.41	VLB
T1675A	-0.32	VLB
I1858L	-0.23	VLB
N1819S	-0.03	VLB
T1773S	0.01	VLB
R1751Q	0.12	VLB
G1706A	0.13	VLB
P1859R	0.30	VLB
D1733G	0.44	VLB
V1804D	0.59	VLB
D1692N	0.62	VLP
I1807V	0.62	VLB
C1697S	0.85	VLP
K1759N	0.90	VLP
A1669S	1.00	VLB
P1856S	1.03	VLB
R1726G	1.18	VLB
L1664P	1.20	VLB
D1778G	1.30	VLB
V1736A	1.56	VLP
S1715C	1.71	VLP
H1686R	1.74	VLP
T1685A	2.05	VLP
D1692H	2.14	VLP
M1652I	2.20	VLB
R1699W	2.27	VLP
R1699Q	2.33	VLP
M1689R	2.47	VLP
F1662S	2.54	VLP
T1691I	2.58	VLP
P1806A	2.70	VLB

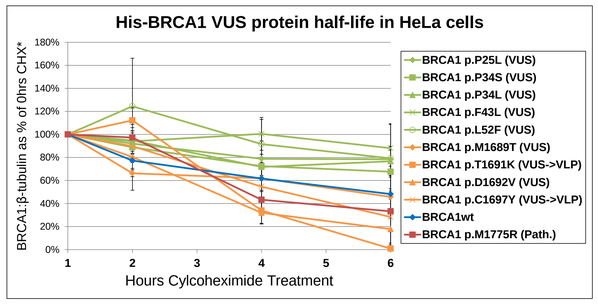
p.Variant	ΔΔG (kcal/mol)	Class
Y1853C	3.01	VLP
D1692V	3.04	VUS
M1783T	3.29	VLB
M1652T	3.51	VLB
G1801D	3.66	VLB
W1718C	3.83	VLP
M1775R	4.06	VLP
S1722P	4.08	VLP
I1766S	4.18	VLP
G1788C	4.28	VLP
V1714G	4.35	VLP
M1689T	4.47	VUS
R1751P	4.73	VLP
W1837C	4.93	VLP
G1788D	5.01	VLP
W1837G	5.35	VLP
A1843P	6.07	VLP
F1761S	6.41	VLP
W1837R	6.72	VLP
L1764P	6.83	VLP
A1708E	8.05	VLP
C1697Y	8.95	VUS
G1788V	9.23	VLP
C1697R	9.76	VLP
A1752P	9.94	VLP
T1691K	12.21	VLP
T1691K	12.21	VUS
G1748D	12.30	VLP
S1655F	12.90	VLP
G1706E	14.77	VLP
F1749R	18.12	VLP
S1715R	23.55	VLP
G1722F	30.76	VLP

Figure 1. A combination of clinical data, protein structure, *in silico* analyses, and population allele frequency was used to select *BRCA1* missense VUS for functional analysis. Structural models depict BRCA1 RING (A) and BRCT (B) domains (green) binding BARD1 (gray) and BACH1 (purple), respectively. Amino acids for previously known VLB (yellow) and VLP (blue) missense variants are colored in the models, and changes in folding free energy (ΔΔG, kcal/mol) are listed in tables. Five missense VUS in the RING domain and four missense VUS in the BRCT domain (red) were selected for functional analyses. These are labeled in the structural models with respective changes in folding free energy in tables.

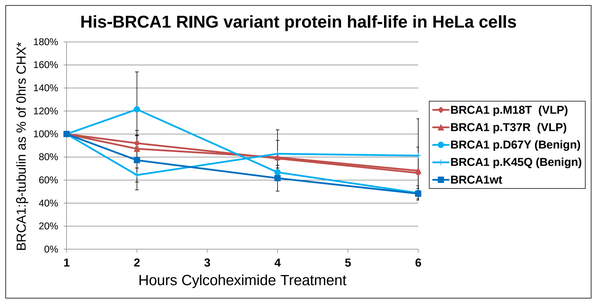
DELETERIOUS BRCT MISSENSE ALTERATIONS AFFECT BRCA1 PROTEIN HALF-LIFE







*Error bars indicate range of 2 biological replicates



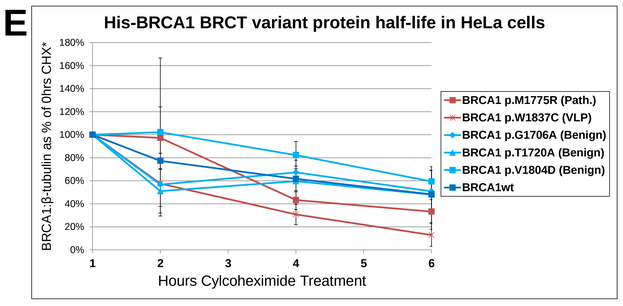


Figure 2. (A) Quantitative fluorescent western blot measures the steady-state expression of His-tagged BRCA1 (green) containing VUS, pathogenic, VLP, and benign missense alterations in transfected HeLa cells. β-tubulin (red) is measured as a loading control. (B) Half-life of His-BRCA1 variants and β-tubulin observed in HeLa cells treated with cycloheximide to block protein translation for the indicated times, or with DMSO as drug vehicle control. (C-E) quantification of His-BRCA1 expression normalized to β-tubulin of western blots (B, additional data not shown). BRCA1 RING (green) and BRCT (orange) missense VUS, wild-type (blue) and pathogenic M1775R (red) (C). BRCA1 RING (D) and BRCT(E) VLP/pathogenic (dark red), benign (pink), and wt (blue).

DELETERIOUS BRCT MISSENSE ALTERATIONS REDUCE BRCA1 HDR ACTIVITY

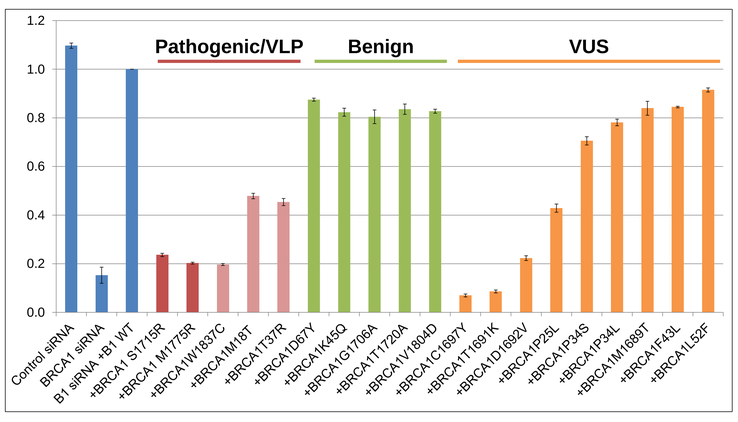


Figure 3. HDR activity measured by HDR assay[5] of cells transfected with Control siRNA, or *BRCA1* siRNA co-transfected with *BRCA1* VUS (orange), benign (green), VLP (pink), and pathogenic (red) missense variants. HDR activity was quantified as the fraction of %GFP+ cells relative to *BRCA1* siRNA +*BRCA1wt* cells. Results are from three independent experiments, with columns representing mean and error bars indicating standard error.

METHODS

- Folding free energy changes (ΔΔG, kcal/mol) were calculated using PDB structures 1JM7 and 1Y98 for BRCA1 RING and BRCT domains, respectively.
- Transfections were performed using pcDNA3-HBT-BRCA1 (Parvin Lab) containing the described missense alterations.
- Quantitative fluorescent western blots were performed using primary antibodies against His-tag (Qiagen) and β -tubulin (CST), and respective secondary antibodies (LI-COR). Blots were imaged using LI-COR Odyssey Sa and Image Studio.
- HDR assay was performed by the Parvin Lab using methods as described previously[5].

RESULTS

- Similar to BRCT pathogenic missense variants, the BRCA1 BRCT missense VUS T1691K, D1692V, and C1697Y proteins are expressed at lower steady-state levels and appear to degrade more rapidly than WT, and consistently show reduced HDR activity.
- *BRCA1* VUS M1689T does not affect protein half-life, and has similar HDR activity as WT, control siRNA, and benign *BRCA1* missense variants.
- These functional results in combination with other line of evidences allowed reclassification of T1691K and C1697Y to Likely Pathogenic alterations.
- These preliminary results suggest that the BRCA1 protein half-life assay may function as a screening method for evaluating missense variant deleteriousness.

TAKE-HOME POINTS

- The correct classification of BRCA1 missense variants presents a challenge to provide accurate genetic counseling and targeted cancer therapy.
- To improve the classification of these alterations, we propose an integrated approach: clinical data, protein structure, *in silico* analyses, and population allele frequency followed by protein half-life and HDR assay .
- This approach may also be used to assess additional missense VUS in other Hereditary Breast and Ovarian Cancer genes involved in the HDR pathway.

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