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

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INTRODUCTION: Approximately 0.5-2% of breast cancers are attributed to germline pathogenic alterations in *BRCA1* (The Anglian Breast Cancer Study Group, *Br. J. Cancer,* 2000; Easton, *Breast Cancer Res.*, 1999). Cumulative breast and ovarian cancer risk estimates to age 70 years for carriers of *BRCA1* pathogenic alterations range from 40% to 87% and from 16% to 68%, respectively (Ford et al., *Am. J. Hum. Genet.*, 1998; Gabai-Kapara et al., *Proc. Natl. Acad. Sci.*, 2014). These cancers associated with *BRCA1* pathogenic alterations are selectively sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. However, up to 20% of *BRCA* variants identified in genetic testing are classified as variants of unknown significance (VUS) (Chenevix-Trench et al., *Cancer Res.*, 2006; Eccles et al., *BMC Cancer*, 2015). Most of these VUS are missense because the impact of single amino acid substitutions in the protein is more difficult to predict relative to other types of alterations, such as truncations. Therefore, the correct classification of *BRCA1* missense variants presents a challenge to provide accurate genetic counseling and targeted cancer therapy. Here we propose an integrated approach for assessing *BRCA1* missense variants that improves the classification of these alterations.

METHODS: We used a combination of clinical data, protein structure, *in silico* analyses, and population allele frequency in order to select *BRCA1* missense VUS for functional analysis. We then determined BRCA1 VUS protein function by measuring homology directed recombination (HDR) efficiency using HDR assay (Ransburgh et al., *Cancer Res.*, 2010) and quantitative infrared western blot analysis (LI-COR Odyssey Fc Imaging System).

RESULTS: Based on clinical data, protein structure, and *in silico* analyses, we selected 9 *BRCA1* missense VUS for functional analysis: p.P34L, p.F43L, p.L52F, p.P25L, p.P34S, p.M1689T, p.T1691K, p.D1692V, and p.C1697Y. The HDR assay indicated p.P25L, p.T1691K, p.D1692V, and p.C1697Y have deficient homologous recombination. Additionally, quantitative western blot analyses indicate p.T1691K, p.D1692V, and p.C1697Y have abnormal protein expression.

CONCLUSION: This approach provides a method for prioritizing missense *BRCA1* VUS for functional analysis. Ultimately this integrated approach can be used for the classification of *BRCA1* missense variants leading to the identification of clinically actionable alterations—information necessary for the indication of screening, prophylaxis, and treatment to the correct individuals. This approach may also be used to assess additional missense VUS in other

Hereditary Breast and Ovarian Cancer genes involved in the HDR pathway, providing valuable data for genetic counseling and targeted cancer therapy.