Functional Analysis of BRCA1 variants: a snapshot of the linker region

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INTRODUCTION: *BRCA1* is a tumor suppressor gene crucial for the maintenance of genome integrity. Germline mutations that lead to a dysfunctional protein can increase the risk of breast and ovarian cancer. Functional assays represent an important tool to contribute to pathogenicity classification of low frequency variants, mostly missense. **OBJECTIVES:** Since 2007, our group have been working a functional assay that correlates BRCA1 transcriptional activity and its C-terminus integrity (TA assay). In this work, we focused on the region that links the two tandem BRCT domains (linker).

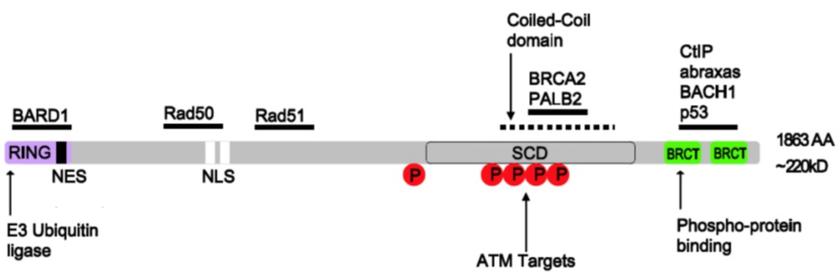

Figure 1 – Schematic representation of full full-length BRCA1 protein. N-terminal region contains a RING-finger domain (purple square) and a the nuclear export signal (NES) (black square) and C-terminal region contains N- and C-BRCT domains (green squares). NLS, nuclear localization signals are represented in white, and red circles represent phosphorylation sites. SCD – Serine rich region. BRCA1 interaction partners are indicated above each interaction region. Adapted from Clark et al, 2012.

MATERIAL AND METHODS: Using bioinformatics strategies (Align-GVGD, SFIT and PolyPhen-2) we predicted and scored all possible mutations located in the linker region (136 mutants) but variants already studied/classified (16 mutants). Only 23 variants were predicted in *silico* as cancer associated by all methods. We generated and cloned the 23 variants predicted as pathogenic into pCDNA3 vector, coding a fusion protein (DNA binding domain fused to BRCA1 C-terminus). The constructs were used to assess functional data through the TA assay (using Dual-Luciferase Reporter Assay System, Promega).

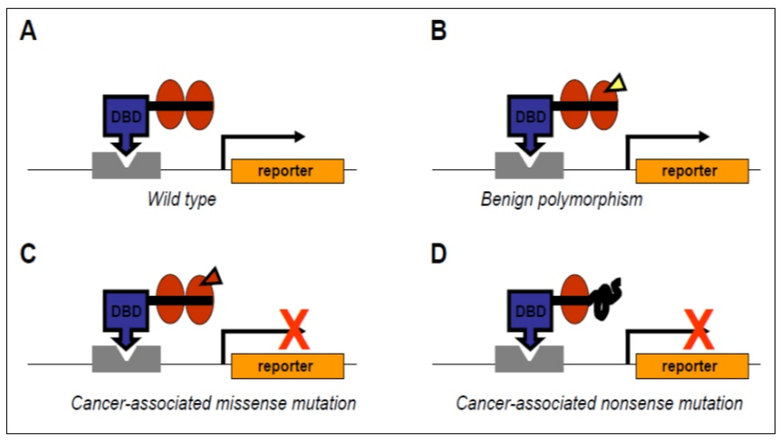

Figure 2 – Outcome from BRCA1 TA assay. Human cells are co-transfected with a Gal4 responsive luciferase reporter gene and a Gal4 DBD fusion to residues 1396 to 1863 of WT BRCA1 or the same fragment carrying the variants (Gal4 DBD:BRCA1). **(A)** The wild type sequence or **(B)** a non-pathogenic variant recruit the RNA polimerase II, thus transcripts the reporter gene. Cancer associated mutation disrupts tBRCT structure, therefore transcription does not occur **(C)** pathogenic missense variant e **(D)** pathogenic nonsense variant. Adapted from Monteiro et al, 2000.

RESULTS AND DISCUSSION: Assays were originally conducted at 37ºC. Most variants (20 mutants) behaved similar to pathogenic controls, whilst 3 variants (all in 1740 position) appear to behave as positive controls. Variants in 1740 position demonstrate a significant variation in transcription activity (replicates and in different experiments), featuring a possible thermosensitive property. Therefore, all variants were tested at 30ºC. The 1740 variants sustained a non-pathogenic behavior, however other 14 variants displayed an increase in transcriptional activity. We did not observe any significant change in the linker secondary structure due to nucleotide change using the Ramachandran diagram approach. **CONCLUSIONS:** The differences in transcription activity observed 37ºC and 30ºC suggest that the linker region is possibly a thermosensitive segment in BRCA1 structure. We also generated data that will support mathematical models to predict the pathogenicity of BRCA1 variants.

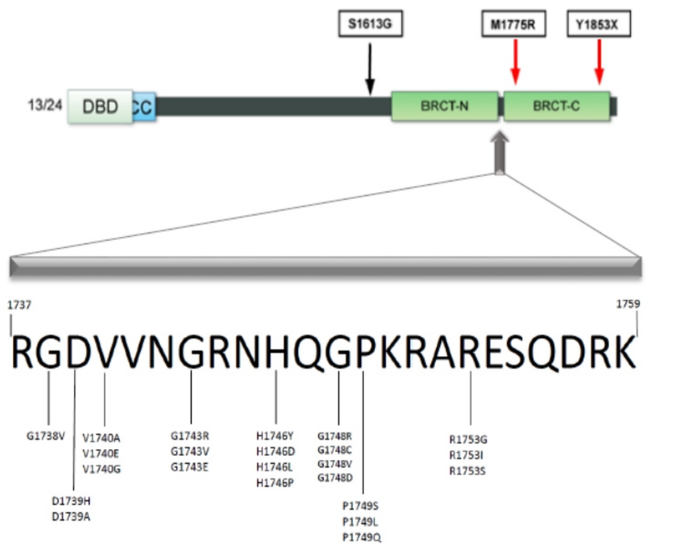

Figure 3 – Schematic representation of GAL4DBD:BRCA113/24 protein and localization of studied variants and controls. White rectangle represents Gal4 DBD; blue rectangle represents coil-coiled (CC) domain; the BRCT domains are designate by green rectangles. Thin arrows indicates controls position, the black arrow indicates the positive control (missense non-pathogenic variant) and red arrows indicate negative controls (missense pathogenic variant and nonsense pathogenic variant, respectively). Grey arrow indicates the linker region (region of interest in this study, amino acids 1737 to 1759). The 23 amino acids from linker are represented by their correspondent letters and the variants studied are located above the sequence.

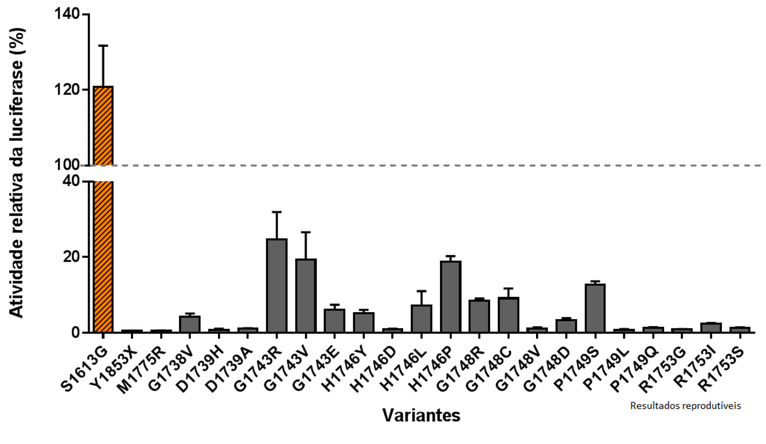

Figure 4 – BRCA1 variants transcriptional activity at 37ºC. Quantitative transcriptional assay performed in human cells, HEK293-FT. WT BRCA1 activity is represented as reference (100%). Assays were conducted in quadruplicates, an internal control was used for data normalization. The yellow bar represents neutral control (S1613G) and red bars represent negative controls (Y1853X and M1775R).

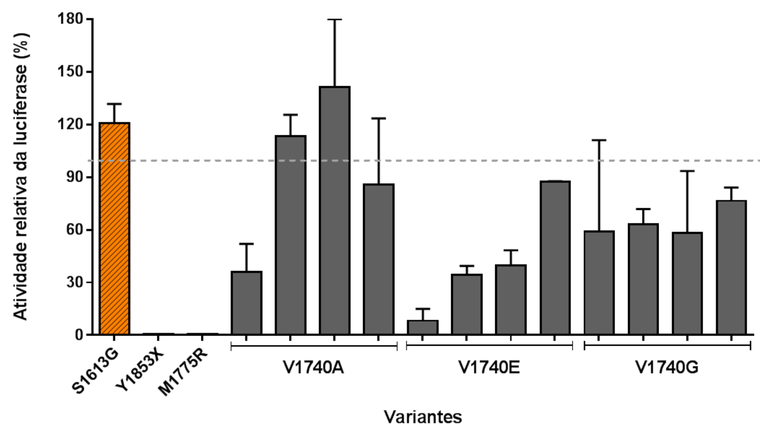

Figure 5 – BRCA1 variants transcriptional activity localized on amino acid 1740 at 37ºC. Quantitative transcriptional assay performed in human cells, HEK293-FT. WT BRCA1 activity is represented as reference (100%). Assays were conducted in quadruplicates, an internal control was used for data normalization. The yellow bar represents neutral control (S1613G) and red bars represent negative controls (Y1853X and M1775R). The red bar indicate neutral activity (up 50%).

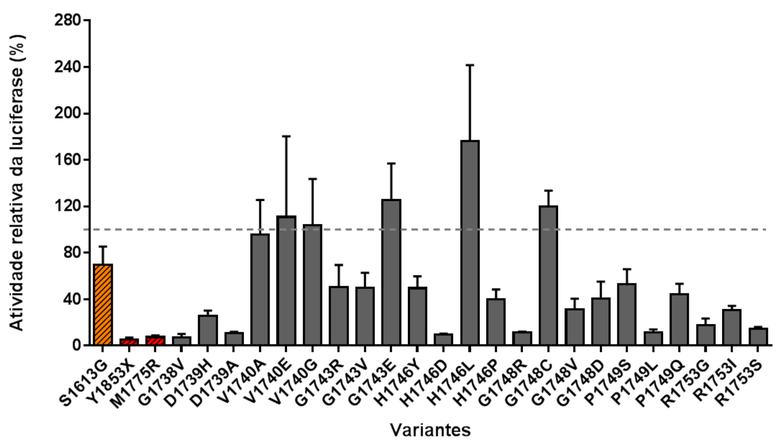

Figure 6 – BRCA1 variants transcriptional activity at 30ºC. Quantitative transcriptional assay performed in human cells, HEK293-FT. WT BRCA1 activity is represented as reference (100%). Assays were conducted in quadruplicates, an internal control was used for data normalization. The yellow bar represents neutral control (S1613G) and red bars represent negative controls (Y1853X and M1775R). The red bar indicate a temperature dependent activity (up 30%).

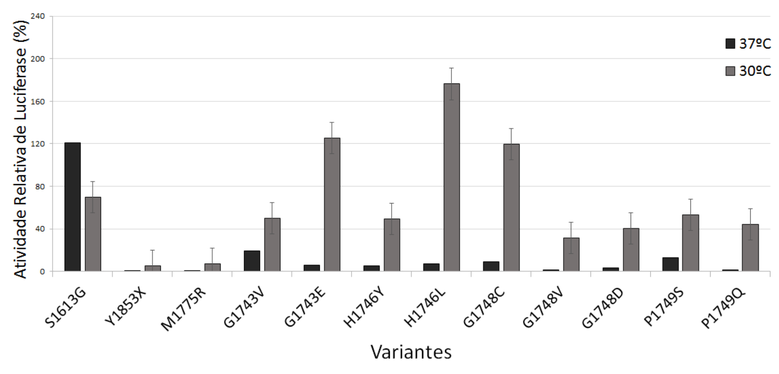

Figure 7 – Comparison BRCA1 variants transcriptional activity at 37ºC and 30ºC. Quantitative transcriptional assay performed in human cells, HEK293-FT. WT BRCA1 activity is represented as reference (100%). Assays were conducted in quadruplicates, an internal control was used for data normalization. Only temperature sensitivity variants are represent (variants with activity up to 30% at 30ºC and luciferase activity different statistically at 37ºC and 30ºC. The yellow bar represents neutral control (S1613G) and red bars represent negative controls (Y1853X and M1775R). The red bar indicate a temperature dependent activity (up 30%).

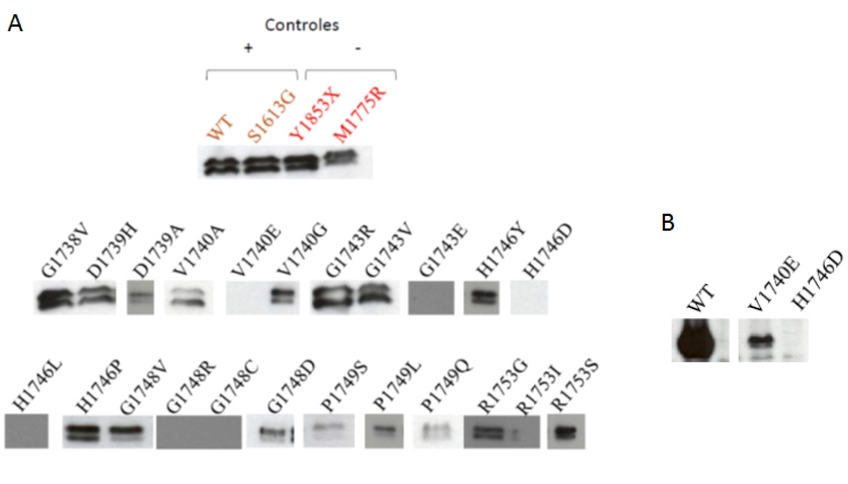

Figure 8 – BRCA1 protein expression levels (variants and controls). To control for possible variations in protein expression levels, samples **(A)** maintained at 37ºC were analyzed by immnoblotting 24 hours after transfection using anti-GAL4 DBD monoclonal antibody. **(B)** V1740E and H1746D variants were also evaluated at 30ºC, 48 hours after transfection.

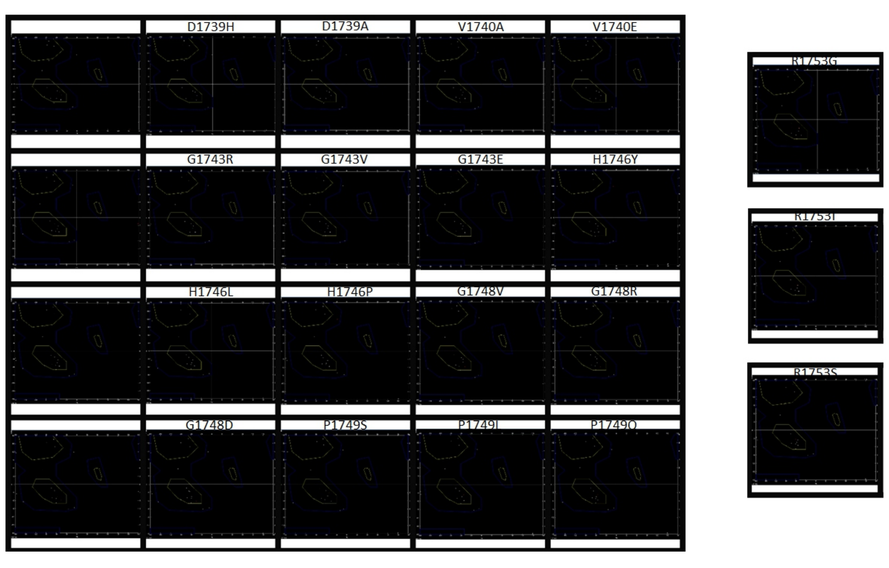

Figure 9 – BRCA1 variants Ramachandran plots. All amino acids alterations were tested in Ramachandran plots to verify a possible change in the ligation angles oh the central carbon. No alteration were observed.

Projeto Gráfico: Serviço de Edição e Informação Técnico-Científica / INCA