Title: Characterization of *BRCA1* and *BRCA2* variants found in a Norwegian breast or ovarian cancer cohort.

Authors: Elisabeth Jarhelle[1, 2], Hilde Monica Frostad Riise Stensland[1, 3], Lovise Mæhle[4] and Marijke Van Ghelue[1, 2, 3].

Affiliations:
1. Department of Medical Genetics, Division of Child and Adolescent Health, University Hospital of North Norway, Tromsø, Norway;
2. Department of Clinical Medicine, University of Tromsø, Tromsø, Norway;
3. Northern Norway Family Cancer Center, University Hospital of North Norway, Tromsø, Norway;
4. Department of Medical Genetics, Oslo University Hospital, Oslo, Norway.

Correspondence: Marijke Van Ghelue, tlf. +47 776 46859,
e-mail: marijke.van.ghelue@unn.no

Abstract

Germline mutations in *BRCA1* and *BRCA2* cause hereditary breast and ovarian cancer (HBOC). Molecular screening of these two genes in patients with a family history of breast or ovarian cancer has revealed pathogenic variants as well as genetic variants of unknown significance (VUS). These VUS may cause a challenge in the genetic counseling process regarding clinical management of the patient and the family. In this study, we further characterized 32 variants previously detected in 33 samples from patients with a family history of breast or ovarian cancer. cDNA was analyzed for alternative transcripts and selected missense variants located in the BRCT domains of *BRCA1* were assessed for their trans-activation ability.

Although an extensive cDNA analysis was done, only three of the 32 variants appeared to affect the splice-process (*BRCA1* c.213-5T>A, *BRCA1* c.5434C>G and *BRCA2* c.68-7T>A). In addition, two variants located in the BRCT domains of BRCA1 (c.5075A>C p.Asp1692Ala and c.5513T>G p.Val1838Gly) were shown to abolish the BRCT domain trans-activation ability, whereas *BRCA1* c.5125G>A (p.Gly1709Arg) exhibited equal trans-activation capability as the WT domain. These functional studies may offer further insights into the pathogenicity of certain identified variants; however, this assay is only applicable for a subset of missense variants.

Key words: BRCA1, BRCA2, cancer, cDNA-analysis, functional-assay

Introduction

The *BRCA1* gene consists of 23 exons and encodes a 208 kDa protein encompassing 1863 amino acids (aa) [1]. N-terminally, BRCA1 has a RING-domain (aa 8-96) and two nuclear localization signals (aa 200-300) [2]. It also contains a phosphorylation site for Checkpoint Kinase 2 (CHEK2) protein at Ser988, a coiled coil domain (aa 1364-1437), followed by several phosphorylation sites for Ataxia Telangiectasia Mutated protein (ATM) (between aa 1280-1524) and two trans-activating BRCT-domains (aa 1646-1859) [2]. BRCA1 has several interactions partners, for instance BRCA1 associated RING domain 1 (BARD1) protein, which interacts with the RING-domain during homologous recombination repair (HRR) [2].

The *BRCA2* gene consists of 27 exons and encodes a 384 kDa protein encompassing 3418 aa [1]. BRCA2 has eight BRC-repeats spaced evenly from aa 1009-2083, a helical domain, three oligonucleotide binding folds and a tower domain [2]. C-terminally, BRCA2 has two nuclear localization signals and a Cyclin Dependent Kinase 2 (CDK2) phosphorylation site at Ser3291 [2]. N-terminally, BRCA2 has the ability to interact with Partner And Localizer of BRCA2 (PALB2) at aa 21-39, overlapping with exon 3 (aa 23-106) [3]. The physical connection between BRCA2 and PALB2 is important because PALB2 links BRCA2 and BRCA1 during HRR, at the coiled coil domain of BRCA1 [2].

Together, mutated *BRCA1* and *BRCA2* are responsible for about 15-25% of familial breast and ovarian cancer cases [4, 5]. Pathogenic variants in *BRCA1* and *BRCA2* are estimated to give a 40-87% risk of breast cancer and a 11-68% risk of ovarian cancer by age 70 [6]. Since the identification of *BRCA1* and *BRCA2*, many pathogenic variants have been reported in these two genes. The Breast cancer information core (BIC) database includes over 1700 distinct variants in *BRCA1* and approximately 2000 in *BRCA2* (https://research.nhgri.nih.gov/projects/bic/). However, many of these variants are classified as variants of unknown significance (VUS) and include synonymous, missense, intronic and in-frame deletions/insertions. Missense mutations have the capacity to affect protein function; additionally they may also disturb mRNA splicing. Similarly, synonymous variants, intronic variants outside the consensus splice sites (ss) and deletions/insertions may also cause aberrant splicing. This has been reported for several genes including *BRCA1* and *BRCA2* [7-9].

Several normal alternative transcripts have been reported both for *BRCA1* and *BRCA2* [10-13]. The Evidence based Network for the Interpretation of Germline Mutation Alleles (ENIGMA) consortium reported 63 splicing events in *BRCA1* and 24 in *BRCA2* [11, 13]. Ten of the 63 *BRCA1* alternative splicing events and four of the *BRCA2* alternative splicing events were considered major splicing events, thus complicating the investigation of

aberrant splicing [11, 13]. In this study we assessed the consequences of some of the variants detected in a Norwegian breast and ovarian cancer cohort, both by performing cDNA analysis, as well as evaluating the functional consequences of variants located in the BRCA1 C-Terminal (BRCT) domains (aa 1646-1859) using a trans-activation assay [14, 15].

Materials and Methods

Patients and samples

Thirty-three whole-blood samples collected in RNA preserving tubes (PAXgene tubes) were obtained from the University Hospital of Oslo, Norway. The samples were collected from unrelated patients who were carriers of sequence variants in *BRCA1* or *BRCA2* (Table 1). All patients had a family history of breast or ovarian cancer. Complete sequencing of the coding regions, corresponding exon-intron borders and parts of the 5'and 3' untranslated regions in *BRCA1* and *BRCA2* and multiplex ligation-dependent probe amplification (MLPA) were previously performed for all patients. In total, these patients carried 18 variants in *BRCA1* and 14 variants in *BRCA2* (Table 1). As controls, samples from individuals without a family history of breast- and ovarian cancer were used.

RNA isolation and cDNA synthesis

RNA was isolated from the PAXgene tubes using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's protocol. cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Waltham, MA USA).

Nomenclature

Variants were named following Human Genome Variation Society (HGVS) nomenclature [16]. Reference sequences for *BRCA1* and *BRCA2* were NM_007294.3 and NM_000059.3, respectively. Custom numbering was used for *BRCA1*.

Bioinformatic tools

Primers were designed using the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) [17, 18]. *In silico* evaluation of the variants was done with Alamut Visual version 2.7 (Interactive Biosoftware, Rouen, France), which includes the missense prediction programs Align GVGD, SIFT, MutationTaster and PolyPhen-2. Alamut also contains the splice prediction tools SpliceSiteFinder-like (SSF), MaxEntScan (MES), NNSPLICE, GeneSplicer (GS) and Human Splicing Finder (HSF), where the thresholds were set to zero for all prediction tools.

Alamut also includes results and/or links to the following databases investigated in this study: the Exome Aggregation Consortium (ExAC), the Exome Variant Server (EVS), the Single Nucleotide Polymorphism Database (dbSNP), ClinVar, Human Gene Mutation Database (HGMD) and Breast Cancer Information Core (BIC).

Compliance with Ethical Standards

All participants gave written informed consent for diagnostical testing. The project was submitted to the appropriate regional ethics committee, however, since the samples were tested with a diagnostically purpose the regional ethical committee waved the need for ethical approval based on the Norwegian regional health organization law § 2 and § 9 and the Norwegian research ethical law § 4.

cDNA analysis

The variants were investigated for their effect on splicing. Primers were positioned in flanking exons, preferentially so PCR-products covered at least one exon on either side of the exon containing the variant of interest (Table 2). Due to the size of the large exons 11 of *BRCA1* and *BRCA2*, alternative strategies were used. For these exons, the corresponding PCR-products did not contain the entire exon 11, as one of the primers in each set was located in exon 11 (Table 2). The PCR-products were visualized on agarose gels, sequenced using Sanger sequencing and evaluated in Sequencher® version 5.3 (Gene Codes Inc. [19]). All exonically located variants were used as markers for biallelic expression. All PCR-reactions were repeated using a second cDNA preparation as template (prepared from the same RNA sample).

Trans-activation (TA) assay

Plasmids, mutagenesis and transformation. A fusion construct containing GAL4 DBD:BRCA1 (amino acids 1396-1863) WT and the known neutral variant c.4837A>G (p.Ser1613Gly) sub-cloned into pcDNA3 were kindly provided by Alvaro N.A. Monteiro [15]. As an internal transfection control, the phRG-TK vector was used. The phRG-TK contains a *Renilla-luciferase* gene under the control of a constitutive TK-promoter. The pGAL4-e1b-Luc containing the *Firefly-luciferase* gene was used as a reporter for measuring the trans-activating ability (Figure 2a). Variants c.5075A>C (p.Asp1692Ala), c.5125G>A (p.Gly1709Arg), c.5513T>G (p.Val1838Gly), and the pathogenic control c.5324T>G (p.Met1775Arg)[15], were introduced in pcDNA3 GAL4 DBD:BRCA1 (amino acid 1396-1863) WT using the QuikChange XL Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA USA) according to the manufacturer's protocol. Mutant plasmids were transformed into XL-10 Gold or Top10 competent cells and successful mutagenesis was verified by Sanger sequencing.

Transfection and harvesting. Both BHK-21 and HEK293 cells (ATCC, www.atcc.org) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Waltham, MA USA) with 10% Fetal Bovine Serum (Life Technologies) and 60 U/ml Penicillin-Streptomycin (Life Technologies). Approximately 150 000 BHK-21 and 300 000 HEK293 cells were transferred to each well of a 6-well plate and grown overnight before transfection. One µg of pcDNA3 GAL4 DBD:BRCA1 was co-transfected with one µg of pGAL4-e1b-Luc and 100 ng phRG-TK (internal transfection control). Fugene® HD Transfection Reagent (Promega, Madison, WI USA) was used as transfecting agent according to the protocol recommended by the supplier. Untransfected cells, cells transfected exclusively with the reporter plasmids (pGAL4-e1b-Luc and phRG-TK) and cells transfected with the plasmid containing the *BRCA1* WT, the p.Ser1613Gly (neutral) and p.Met1775Arg (pathogenic) variants, were used as controls. Cells were harvested 24 hours post-transfection. The transfection experiments were repeated three times.

Luciferase measurements. The Dual-Luciferase Assay System (Promega) was used to measure the trans-activation activity. In short, 50µl Luciferase Assay Reagent II (LARII) was injected into wells containing 20µl cell lysate. The amount of light produced was measured and subsequently 50µl Stop & Glo Reagent was injected. A CLARIOstar (BMG LABTECH, Ortenberg, Germany) was used for injections and recordings. For each lysate, both Renilla- and Firefly-luciferase activities were measured in triplicates. The data are presented as ratios of Firefly- to Renilla-excitation values. The activity-ratios obtained from cells transfected with only the reporter plasmid were defined as background and thus subtracted from the activity-ratios obtained from the BRCT containing plasmids. For each WT lysate/triplicates, the average was calculated. All luciferase measurements within the same transfection set-up were then calculated as the percentage of the corresponding WT average. Values were combined, before the average and standard deviations were calculated.

Western blot. Lysates from one of the HEK293 transfections and one of the BHK-21 transfections were used for western blot analysis to confirm the presence of fusion proteins. The amount of light produced by the internal transfection control (Renilla luciferase) was used for normalization of samples. Samples were loaded on NuPAGE 4-12% Bis-Tris pre cast gels (Life Technologies) and the proteins were separated for 1.5 hours at 200V and 120mA. Proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Life Technologies) (1.5 hours at 25V and 160mA), blocked for one hour in phosphate buffered saline (PBS) with 5% nonfat dried milk powder (PanReac AppliChem, Darmstadt, Germany) and incubated overnight with 1:200 dilution of BRCA1 (C-20) primary antibodies (Santa Cruz Biotechnology, Dallas, Texas USA). Membranes were incubated for one hour with HRP-Chicken anti-rabbit secondary antibodies (1:50 000) (Santa Cruz Biotechnology)

followed by treatment with Signal® West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA USA). The ImageQuant Las4000 (GE Healthcare Life Sciences, Buckinghamshire, U.K.) was used to capture images.

Results

cDNA analysis

Eighteen *BRCA1* variants, comprising three intronic and 15 exonic variants, and 14 *BRCA2* variants, comprising one intronic variant and 13 exonic variants were investigated (Table 1 and 3). All variants, except *BRCA1* c.3418A>G and *BRCA2* c.4068G>A (which were earlier identified as benign variants [20, 21]), were screened for their effect on splicing. In addition, all exonic variants (including *BRCA1* c.3418A>G and *BRCA2* c. 4068G>A) were used as markers to investigate biallelic expression.

In the performed cDNA analysis, three variants appeared to cause alterations in the normal splicing. *BRCA1* c.213-5T>A (intron 5) resulted in inclusion of 59 nucleotides of the 3'-end of intron 5, leading to a frame-shift introducing an early stop-codon (r.212_213ins213-59_213-1 p.Arg71Serfs*11) (Figure 1a). *BRCA1* c.5434C>G (exon 23) induced skipping of exon 23, also leading to a frame-shift and subsequently an early stop-codon (r.5407_5467del p.Gly1803Glnfs*11) (Figure 1b). *BRCA2* c.68-7T>A (intron 2) appeared to increase skipping of exon 3 (Figure 1c). Skipping of exon 3 is an in-frame deletion (r.68_316del p.Asp23_Leu105del) which was also detected in controls. Splice site predictions for these three variants can be seen in Table 4.

Heterozygous positions identified in gDNA that appear homozygous when cDNA is investigated suggest the loss of expression from one of the alleles or alternative splicing in the investigated region. The majority of patients with an exonic variant were confirmed to have both alleles transcribed (exception marked in Table 1).

Trans-activation assay

Seven patients were carriers of variants in the BRCT domains of *BRCA1* (c.5075A>C, c.5096G>A, c.5117G>C, c.5123C>T, c.5125G>A, c.5434C>G and c.5513T>G). Of these, three variants were novel (c.5075A>C p.Asp1692Ala, c.5125G>A p.Gly1709Arg and c.5513T>G p.Val1838Gly). The consequences of these three variants were further investigated for their trans-activation ability. For the remaining variants c.5434C>G, c.5096G>A, c.5117G>C and c.5123C>T, we were able to confirm that the sequence variant c.5434C>G caused aberrant splicing, hence this variant was not included in the TA assay. Variants c.5096G>A (p.Arg1699Gln),

c.5117G>C (p.Gly1706Ala) and c.5123C>T (p.Ala1708Val) had previously been evaluated by trans-activation assays and were also not included in the TA assay [22-24].

BRCA1 p.Asp1692Ala and p.Val1838Gly were unable to induce transcription of the firefly luciferase, equal to the known pathogenic variant p.Met1775Arg, which was apparent in both BHK-21 and HEK293 cells (Figure 2b). BRCA1 p.Gly1709Arg however, showed trans-activation activity similar to the WT and the known benign variant p.Ser1613Gly (Figure 2b).

Western blot results indicated an equal expression of the plasmid constructs in the BHK-21 cells, but showed some variation in HEK293 cells despite adjusting the protein concentrations according to the transfection control, Renilla luciferase (Figure 2c). However, the BRCT mutants were expressed in both cell types, indicating that the reduced values were due to reduced trans-activation ability and not due to variations in expression/stability.

Discussion

Prophylactic mastectomy and salphingo-oophorectomy are potent, but invasive risk reducing managements for carriers of pathogenic *BRCA1/2* variants. Accordingly, identifying a VUS pose a considerable challenge for genetic counsellors and medical geneticists in advising clinical management. In this study, we characterized some of the variants detected in a Norwegian breast and ovarian cancer cohort, both by cDNA analysis and analysis of the trans-activation ability of variants located in the BRCT domains.

cDNA analysis

Alternative splicing allows for a more diverse expression of mRNA, and can regulate localization, enzymatic properties and different interaction properties of proteins [25]. The majority of variants located in the consensus ss (GT-AG in position +/- 1, 2) lead to abnormal splicing [26], but the effects of variants at positions further away from the exon-intron border are more difficult to predict. In addition, both missense variants and silent exonic variants might affect splicing [27], both by creating cryptic ss, remove binding sites for exonic splicing enhancers (ESE) or create binding sites for exonic splicing silencers (ESS). However, normal alternative splicing can counteract the effect of some variants leading to aberrant splicing[28]. De La Hoya *et al.* (2016) recently reported a variant leading to *BRCA1* Δex10 (out-of-frame), that were rescued by in-frame Δex9,10 [28].

In the current study, three of the 32 variants had a consequence on pre-mRNA splicing.

***BRCA1* c.213-5T>A,** a novel variant located in intron 5, resulted in usage of a cryptic ss 59 nucleotides upstream of the original site. Three splice prediction tools, SSF, MES and HSF anticipated a 3'ss at the original position. The variant led to reduced predictions of the original ss (Table 4) and the cryptic ss 59 bases upstream was strongly predicted by all prediction programs (also in the WT sequence). Inclusion of 59 nucleotides causes a frame-shift, introducing a premature stop-codon after 75 codons. Another variant in this region, *BRCA1* c.213-11T>G, has previously been shown to lead to the use of the same cryptic ss [8]. The presence of a premature stop-codon likely activates the nonsense-mediated mRNA decay pathway [29]. However, variants in *BRCA1*, which introduce a stop-codon before position c.297, are presumed to allow re-initiation of translation at the AUG at this position [30]. A re-initiation at c.297 would lead to BRCA1 proteins lacking the RING-finger motif located at the N-termini (amino acids 8-96)[14]. Binding of the BRCA1 RING-domain to BARD1 protein seems to be essential for tumor suppression [31], accordingly, variants lacking this domain are expected to be of clinical importance.

***BRCA1* c.5434C>G** is located in exon 23 and was previously reported by Gaildrat *et al.* (2010) to cause skipping of exon 23 [7]. It has been experimentally demonstrated that the variant most likely affects a splice regulatory element (SRE), either by removal of an ESE or introducing an ESS [7]. This demonstrates the importance of experimentally assessing the effect of exonic variants on splicing. ***BRCA2* c.68-7T>A** in intron 2 had previously been reported by Vreeswijk *et. al.* (2009) and Sanz *et. al.* (2010), who performed mini-gene assays that revealed partial skipping of exon 3 (p.Asp23_Leu105del) [32, 33]. Prediction programs suggested a reduced strength of the downstream original 3'ss in the presence of the variant (Table 4). The cDNA analysis indicated that the variant led to increased exon 3 skipping. However, the skipping of exon 3 gives an in-frame alternative transcript, also present in normal controls (albeit at lower levels). Exon 3 in *BRCA2* encodes the part of BRCA2 that interacts with PALB2 [34], however, the consequence (if any) of reduced interaction with PALB2 is currently unknown. Santos and colleagues have shown that in two families, *BRCA2* c.68-7T>A did not segregate with the disease, suggesting the variant is neutral [35].

Recently, De La Hoya *et al.* (2016) [28] suggested that variants in *BRCA1* not leading to more than 70-80% loss of functional transcripts from one of the alleles still can show tumor suppressor haplosufficiency, implicating the importance of knowing normal alternative splicing events in the genes investigated.

Splice predictions as cDNA analysis inclusion criteria

In 2012, Houdayer *et al.* introduced specific criteria for selection of variants which should be tested for splicing [36]. They concluded that as long as the original splice site in *BRCA1* or *BRCA2* has a prediction value over three

for the MES prediction tool and over 60 for the SSF prediction tool, a reduction of 15% and 5%, respectively, was sufficient to include variants for cDNA analysis. Both *BRCA1* c.213-5T>A and *BRCA2* c.68-7T>A would have been included using these criteria. However, *BRCA1* c.5434C>G would have been omitted from cDNA analysis, since this variant most likely affects an SRE. Splicing regulatory element predictions were assumed to be unreliable and therefore not used [36]. In summary, although prediction programs can indicate that some variants can cause aberrant splicing, the true outcome can only be identified experimentally.

Trans-activation assay

We investigated three novel *BRCA1* variants for their effect on BRCA1's trans-activation activity (Table 1). Two of the three variants (*BRCA1* c.5075A>C p.Asp1692Ala and c.5513T>G p.Val1838Gly) showed a clear loss of activity (Figure 2b). *BRCA1* p.Asp1692Ala exchanging the highly conserved aspartate to an alanine and *BRCA1*p.Val1838Gly, substituting the highly conserved valine to a glycine, are both predicted to be pathogenic by the missense prediction tools Align GVGD, SIFT and mutationTaster. However, PolyPhen-2 only predicts p.Val1838Gly to be damaging. Both these variants result in changes in the BRCT domains and our functional study indicated their pathogenicity by loss of trans-activation activity (Figure 2b). Other variants have been reported at the same positions; p.Asp1692His, p.Asp1692Asn, p.Asp1692Tyr and p.Val1838Glu have all previously been shown to have a functional impact using the TA-assay, indicating the importance of the conserved amino acids at these positions [37, 38]. *BRCA1* c.5125G>A p.Gly1709Arg however, substituting the highly conserved glycine with arginine, is predicted differently by Align GVGD, SIFT, Mutation taster and PolyPhen2 (Table 3). Even though some of the prediction programs indicated pathogenicity, p.Gly1709Arg displayed normal trans-activation activity.

Although the *in vitro* trans-activation studies suggest the pathogenicity of *BRCA1* c.5075A>C and c.5513T>G, we only investigated a limited part and the BRCA1 protein. Further assessment including segregation studies in families with these variants are needed to establish their classification.

Several *BRCA1* variants in our cohort are classified as either likely pathogenic, likely benign or benign based on cDNA analysis, functional studies, segregation analysis, frequency in control populations, among others (Table 1 and 3). However, some remain classified as VUS. Two variants identified in our cohort (*BRCA1* c.734A>T and c.1419C>T) have not been previously reported in the literature and both are reported with a low frequency in the ExAC database [39], accordingly, the clinical significance is uncertain (Table 1). *BRCA1* c.3708T>G and c.5123C>T were previously reported in both the literature and with low frequencies in databases (Table 3).

In *BRCA2* none of the variants identified in our cohort were classified as likely pathogenic. One variant (c.4068G>A) was classified as benign and five variants (c.750G>A, c.2680 G>A, c.3568C>T, c.6100C>T and c.6821G>T) were classified as likely benign (Table 1). Eight variants remained classified as VUS; The *BRCA2* c.40A>G has not been reported in the investigated databases nor in the literature (Table 3), while the *BRCA2*c.8323A>G have not been reported in the literature and only with low frequency in the ExAC database (Table 3). The five remaining variants, c.4828G>A, c.5272_5274delAAT, c.7301A>C, c.8177A>G and c.9116C>T, have been reported in the literature, but with low frequencies in the investigated databases (Table 3). *BRCA2* c.8177A>G is however not reported in the ExAC database (Table 3). Our current study was unable to disclose new variants located in regulatory sequences, affecting the expression of one of the alleles.

Conclusion

In the current study, we identified three variants leading to abnormal splicing of pre-mRNA; Two variants located intronically, *BRCA1* c.213-5T>A and *BRCA2* c.68-7T>A and one exonic variant, *BRCA1* c.5434C>G. In addition, functional studies assessing the trans-activation activity of the BRCT domains resulted in identification of two variants, c.5075A>C p.Asp1692Ala and c.5513T>G p.Val1838Gly, which lacked trans-activation activity. The use of partial proteins can lead to further understanding of how variants may affect protein function, however, the use of full-length proteins would be preferable in functional studies.

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Figure and table legends:

Figure 1 cDNA analysis. At the top of each image the wild type (WT) sequence is shown, followed by the alternative sequences observed in the patient samples. At the bottom the electropherograms are displayed. (a) *BRCA1* c.213-5T>A resulted in an inclusion of 59 nucleotides from the 3'end of intron 5 (r.212_213ins213-59_213-1 p.Arg71Serfs*11). (b) *BRCA1* c.5434C>G resulted in skipping of exon 23 (r.5407_5467del p.Gly1803Glnfs*11). Electropherogram displayed with sequences from the reverse primer. (c) *BRCA2* c.68-7T>A resulted in increased skipping of exon 3 (r.68_316del p.Asp23_Leu105del), which is a normal alternative splicing event.

Figure 2 Trans-activation assay. a) A simplified view of the assay set-up; Plasmids with constructs encoding a DNA binding domain (DBD) and the C-terminal of BRCA1 (amino acids 1396-1863) were co-transfected into HEK293 and BHK-21 cells with a reporter plasmid containing firefly luciferase. If the plasmids with the C-terminal part of BRCA1 have trans-activation activity, they will activate transcription of firefly luciferase, luciferase activity is measured and quantitated. b) The dual luciferase reporter assay (Promega) was used to evaluate the trans-activation activity of *BRCA1* BRCT variants in BHK-21 cells and HEK293 cells. The first three columns represent controls: wild type (WT) BRCA1, a neutral polymorphism (p.Ser1613Gly) and a pathogenic variant (p.Met1775Arg), respectively. p.Asp1692Ala (*BRCA1* c.5075A>C) and p.Val1838Gly (*BRCA1* c.5513T>G) had no trans-activation activity, whereas p.Gly1709Arg (*BRCA1* c.5125G>A) showed normal activity. c) Western blot results from proteins isolated from one of the transfections in BHK-21 cells and HEK293 cells. Samples were normalized according to renilla expression measured by CLARIOstar (BMG LABTECH).**Table 1.** The variants/samples investigated in this study. VUS = Variant of unknown clinical significance. Variants marked in bold have not previously been reported in the literature.

Table 2. List of primers for each sequence variant and the size of the PCR-products without alternative splicing.

Table 3. Predictions, database results and literature for each variant included in the study. Six databases were explored, the Exome Aggregation Consortium (ExAC), the Exome Variant Server (EVS), the Single Nucleotide Polymorphism Database (dbSNP), ClinVar, the Human Gene Mutation Database (HGMD) and the Breast Cancer Information Core (BIC). HD=HumDiv, HV=HumVar, NFE=European (non-Finnish), EA=European American, AA=African American, DM=Disease-causing Mutation, DM?=Conflicting evidence for Disease-causing Mutation.

Table 4. Splice prediction information for variants with abnormal splicing. Predictions were gathered from the nearest predicted splice site (ss) change where predictions from several programs (at least two) were made, for these three variants, only 3'ss were identified. An exception was made for c.213-5T>A, where also the ss at c.213-59 was included in the table. Threshold was set to zero for all four programs. "Pos. ss"= Position of splice site in regards to sequence variant. Numbers are nucleotides to the splice junction, meaning -0 is right upstream of the variant, while +0 is right downstream. "NP"=Not predicted, "-"=No change in prediction, "New"=not predicted in the WT sequence and "Lost"=Not predicted in the variant sequence.

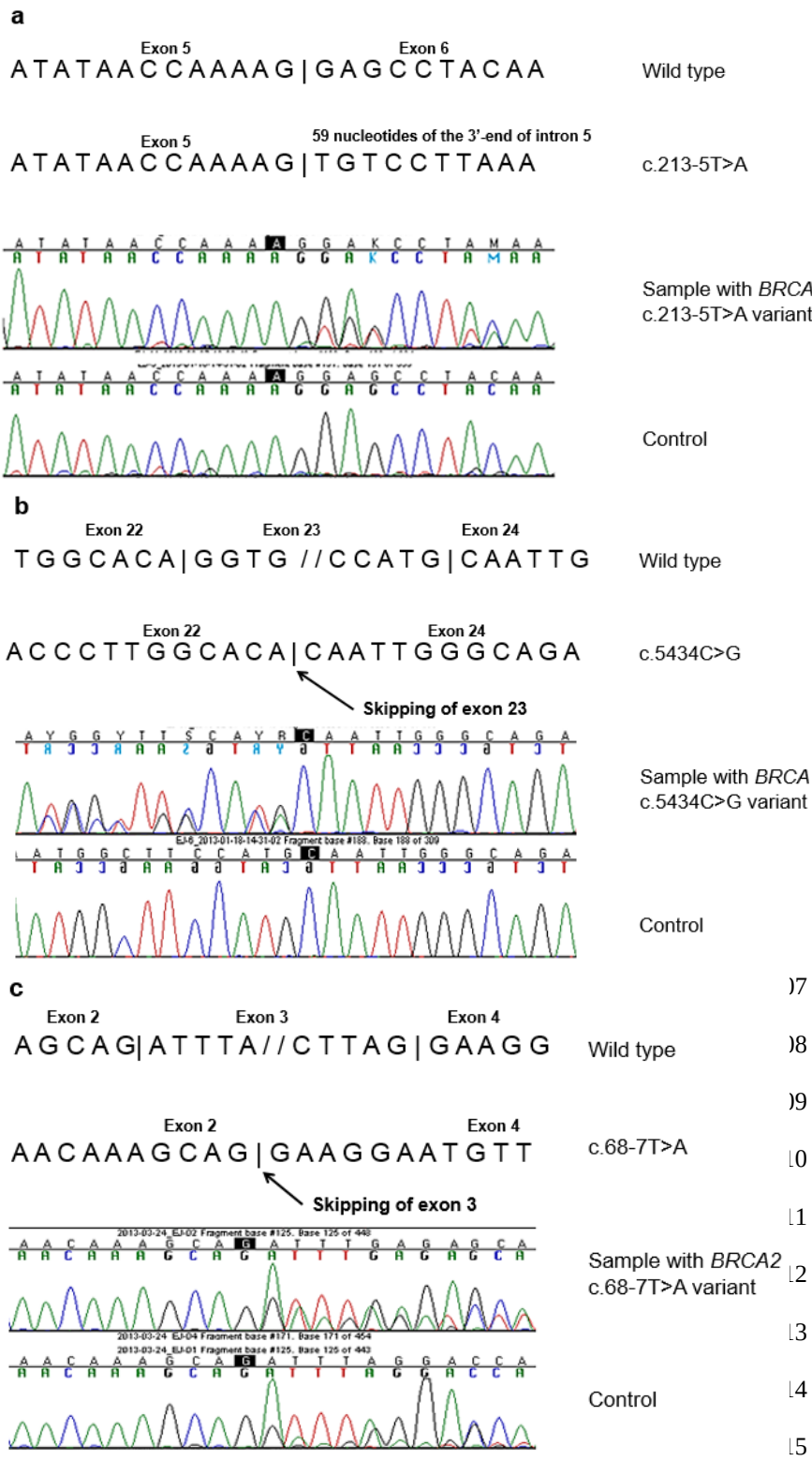
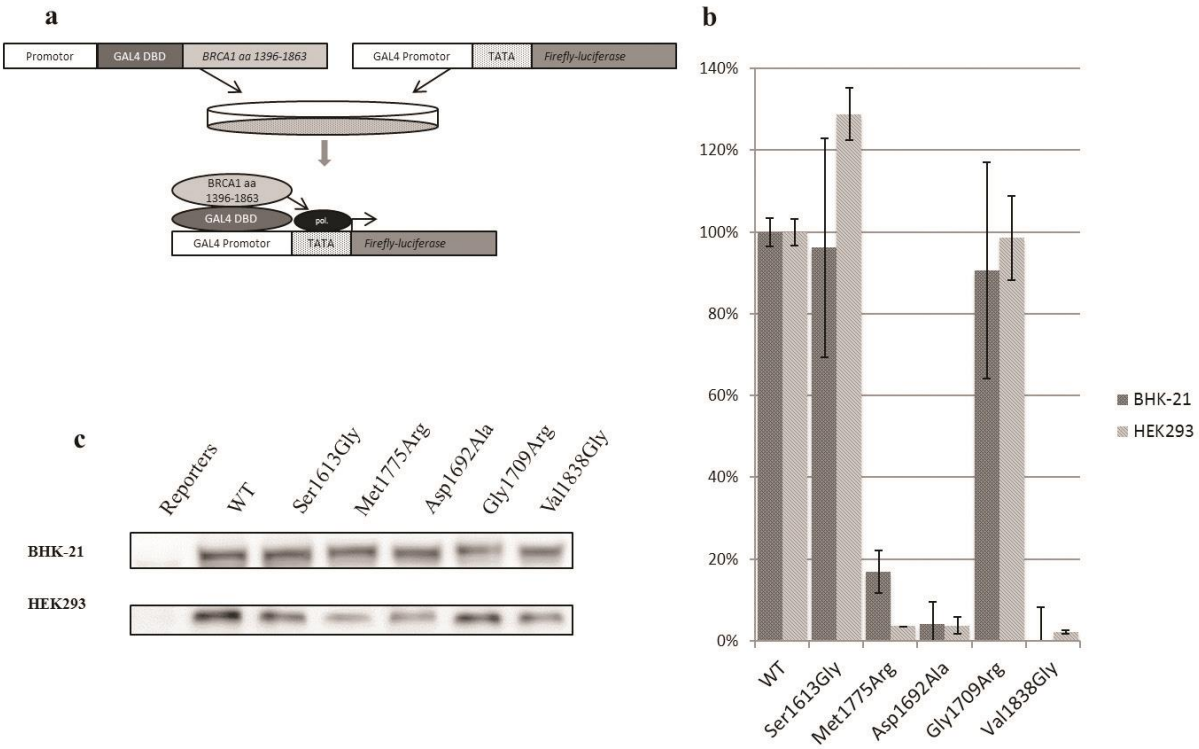

a
Exon 5
Exon 6
A T A T A A C C A A A A G | G A G C C T A C A A
Wild type
Exon 5
59 nucleotides of the 3'-end of intron 5
A T A T A A C C A A A A G | T G T C C T T A A A
c.213-5T>A
Sample with BRCA1
c.213-5T>A variant
Control
b
Exon 22
Exon 23
Exon 24
T G G C A C A | G G T G / / C C A T G | C A A T T G
Wild type
Exon 22
Exon 24
A C C C T T G G C A C A | C A A T T G G G C A G A
c.5434C>G
Skipping of exon 23
Sample with BRCA1
c.5434C>G variant
Control
c
Exon 2
Exon 3
Exon 4
A G C A G | A T T T A / / C T T A G | G A A G G
Wild type
Exon 2
Exon 4
A A C A A A G C A G | G A A G G A A T G T T
c.68-7T>A
Skipping of exon 3
Sample with BRCA2
c.68-7T>A variant
Control

Figure 1



518

519 Figure 2

Table 1Mutation	Location	Protein	Patient	Final Classification
BRCA1				
c.-20+521_-20+525delAAAAA	Intron 1	-	1	2 – likely benign
c.140G>T	Exon 5	p.Cys47Phe	2	4 – likely pathogenic
c.213-5T>A [a]	Intron 5	-	3	4 – likely pathogenic
c.486G>T	Exon 8	p.= (p.Val162Val)	4	2 – likely benign
c.548-17G>T	Intron 8	-	5	2 – likely benign
c.734A>T	Exon 11	p.Asp245Val	6	3 – VUS
c.1419C>T	Exon 11	p.= (p.Asn473Asn)	7	3 –VUS
c.1487G>A	Exon 11	p.Arg496His	8	2 – likely benign
c.2521C>T	Exon 11	p.Arg841Trp	9	2 – likely benign
c.3418A>G [b]	Exon 11	p.Ser1140Gly	10	1 – benign
c.3708T>G	Exon 11	p.Asn1236Lys	11	3 – VUS
c.5075A>C [c]	Exon 18	p.Asp1692Ala	12	3 – VUS
c.5096G>A	Exon 18	p.Arg1699Gln	13	4 – Likely pathogenic
c.5117G>C	Exon 18	p.Gly1706Ala	9	2 – likely benign
c.5123C>T	Exon 18	p.Ala1708Val	14	3 – VUS
c.5125G>A [c]	Exon 18	p.Gly1709Arg	15	3 – VUS
c.5434C>G [a]	Exon 23	p.Pro1812Ala	16	4 – likely pathogenic
c.5513T>G [c]	Exon 24	p.Val1838Gly	17	3 – VUS
BRCA2				
c.40A>G	Exon 2	p.Ile14Val	18	3 – VUS
c.68-7T>A [a]	Intron 2	-	19	2 – likely benign
c.750G>A	Exon 9	p.= (p.Val250Val)	20, 33	2 – likely benign
c.2680G>A	Exon 11	p.Val894Ile	21	2 – likely benign
c.3568C>T [d]	Exon 11	p.Arg1190Trp	22	2 – likely benign
c.4068G>A [b,	Exon 11	p.= (p.Leu1356Leu)	23, 10	1 – benign
c.4828G>A	Exon 11	p.Val1610Met	24	3 – VUS
c.5272_5274delAAT	Exon 11	p.Asn1758del	25	3 – VUS
c.6100C>T	Exon 11	p.Arg2034Cys	26	2 – likely benign
c.6821G>T	Exon 11	p.Gly2274Val	27	2 – likely benign
c.7301A>C	Exon 14	p.Lys2434Thr	28	3 – VUS
c.8177A>G	Exon 18	p.Tyr2726Cys	29	3 – VUS
c.8323A>G	Exon 18	p.Met2775Val	30	3 – VUS
c.9116C>T	Exon 23	p.Pro3039Leu	31, 32	3 – VUS

[a] Affects pre-mRNA splicing

[b] Reported homozygote in ExAC

[c] Part of the BRCT dual luciferase reporter assay

[d] Not able to confirm biallelic expression

Table 2.

	Mutation	Location	Forward primer	5' → 3'	Reverse primer	5' → 3'	Amplicon size (bp)
BRCA1	c.-20+521_-20+525delAAAAA	Intron 1	BRCA1 ex1.F	CTCGCTGAGACTTCCTGGAC	BRCA1 ex3.R	TGTGGAGACAGGTTCCTTGA	227
	c.140G>T	Exon 5	BRCA1 ex2.F	GCTCTTCGCGTTGAAGAAGT	BRCA1 ex7.R	GAAGTCTTTTGGCACGGTTT	400
	c.213-5T>A	Intron 5	BRCA1 ex2.F	GCTCTTCGCGTTGAAGAAGT	BRCA1 ex7.R	GAAGTCTTTTGGCACGGTTT	400
	c.486G>T	Exon 8	BRCA1 ex6.F	CAGCTTGACACAGGTTTGGA	BRCA1 ex11a.R	TTTCTGGATGCCTCTCAGCT	499
	c.548-17G>T	Intron 8	BRCA1 ex6.F	CAGCTTGACACAGGTTTGGA	BRCA1 ex11a.R	TTTCTGGATGCCTCTCAGCT	499
	c.734A>T	Exon 11	BRCA1 ex8.F	GAGGACAAAGCAGCGGATAC	BRCA1 ex11.1R	GCTGTAATGAGCTGGCATGA	359
	c.1419C>T	Exon 11	BRCA1 ex8.F	GAGGACAAAGCAGCGGATAC	BRCA1 ex11.2R	CCGTTTGGTTAGTTCCCTGA	1,124
	c.1487G>A	Exon 11	BRCA1 ex8.F	GAGGACAAAGCAGCGGATAC	BRCA1 ex11.2R	CCGTTTGGTTAGTTCCCTGA	1,124
	c.2521C>T	Exon 11	BRCA1 ex11.F	CAGCATTTGAAAACCCCAAG	BRCA1 ex13.R	ATGGAAGGGTAGCTGTTAGAAGG	1,879
	c.3418A>G	Exon 11	BRCA1 ex11.1F	TAGGGGTTTTGCAACCTGAG	BRCA1 ex13.R	ATGGAAGGGTAGCTGTTAGAAGG	1,039
	c.3708T>G	Exon 11	BRCA1 ex11.1F	TAGGGGTTTTGCAACCTGAG	BRCA1 ex13.R	ATGGAAGGGTAGCTGTTAGAAGG	1,039
	c.5075A>C	Exon 18	BRCA1 ex16.F	GGGAGAAGCCAGAATTGACA	BRCA1 ex20.R	CTCGCTTTGGACCTTGGTG	354
	c.5096G>A	Exon 18	BRCA1 ex16.F	GGGAGAAGCCAGAATTGACA	BRCA1 ex20.R	CTCGCTTTGGACCTTGGTG	354
	c.5117G>C	Exon 18	BRCA1 ex16.F	GGGAGAAGCCAGAATTGACA	BRCA1 ex20.R	CTCGCTTTGGACCTTGGTG	354
	c.5123C>T	Exon 18	BRCA1 ex16.F	GGGAGAAGCCAGAATTGACA	BRCA1 ex20.R	CTCGCTTTGGACCTTGGTG	354
	c.5125G>A	Exon 18	BRCA1 ex16.F	GGGAGAAGCCAGAATTGACA	BRCA1 ex20.R	CTCGCTTTGGACCTTGGTG	354
	c.5434C>G	Exon 23	BRCA1 ex21.F	TTCAGGGGGCTAGAAATCTG	BRCA1 ex24.R	AAGCTCATTCTTGGGGTCCT	289
	c.5513T>G	Exon 24	BRCA1 ex21.F	TTCAGGGGGCTAGAAATCTG	BRCA1 ex24.R	GGGGTATCAGGTAGGTGTCC	289
BRCA2	c.40A>G	Exon 2	BRCA2 ex1.F	AGCGTGAGGGGACAGATTTG	BRCA2 ex4.R	GTGGACAGGAAACATCATCTGC	519
	c.68-7T>A	Intron 2	BRCA2 ex1.F	AGCGTGAGGGGACAGATTTG	BRCA2 ex4.R	GTGGACAGGAAACATCATCTGC	519
	c.750G>A	Exon 9	BRCA2 ex7.F	AGGAGCTGAGGTGGATCCTG	BRCA2 ex11.R1	TCAGAATTGTCCCAAAAGAGCT	1,451
	c.2680G>A	Exon 11	BRCA2 ex10.F	GTTCAGCCCAGTTTGAAGCA	BRCA2 ex11.R2	TGACACTTGGGTTGCTTGTT	980
	c.3568C>T	Exon 11	BRCA2 ex10.F	GTTCAGCCCAGTTTGAAGCA	BRCA2 ex11.R3	CTTGAGCTTTCGCAACTTCC	2,343
	c.4068G>A	Exon 11	BRCA2 ex10.F	GTTCAGCCCAGTTTGAAGCA	BRCA2 ex11.R3	CTTGAGCTTTCGCAACTTCC	2,343
	c.4828G>A	Exon 11	BRCA2 ex11.F1	CAATGGGCAAAGACCCTAAA	BRCA2 ex13.R	CGAAAGGGTACACAGGTAATCG	2,324
	c.5272_5274delAAT	Exon 11	BRCA2 ex11.F2	TTTGATGGTCAACCAGAAAGAA	BRCA2 ex13.R	CGAAAGGGTACACAGGTAATCG	1,916
	c.6100C>T	Exon 11	BRCA2 ex11.F3	CGCAAGACAAGTGTTTTCTGA	BRCA2 ex13.R	CGAAAGGGTACACAGGTAATCG	1,023
	c.6821G>T	Exon 11	BRCA2 ex11.F3	CGCAAGACAAGTGTTTTCTGA	BRCA2 ex13.R	CGAAAGGGTACACAGGTAATCG	1,023
	c.7301A>C	Exon 14	BRCA2 ex11.F4	TGTCCCGAAAATGAGGAAATGG	BRCA2 ex16.R	TGTGAAACTGAAAAGACTCTGCA	925
	c.8177A>G	Exon 18	BRCA2 ex16.F	GGTGGATGGCTCATACCCTC	BRCA2 ex20.R	TTTGCTGCTTCCTTTTCTTCC	809
	c.8323A>G	Exon 18	BRCA2 ex16.F	GGTGGATGGCTCATACCCTC	BRCA2 ex20.R	TTTGCTGCTTCCTTTTCTTCC	809
	c.9116C>T	Exon 23	BRCA2 ex21.F	GAAGAATGCAGCAGACCCAG	BRCA2 ex25.R	TGTCTCTTGAAAGTGGCCCT	751

Table 3.

Mutation	Location	Protein	Prediction programs				Databases						Ref.
			Align GVGD	SIFT	Mutation taster	PolyPhen2	ExAC	ESP/EVS	dbSNP	ClinVar	HGMD	BIC	
BRCA1													
c.-20+521_-20+525delAAAAA	Intron 1	-	-	-	-	-	-	-	-	-	-	-	-
c.140G>T	Exon 5	p.Cys47Phe	C65	Deleterious	Disease causing	HD: POSSIBLY DAMAGING HV: BENIGN	-	-	rs80357150	RCV000111876.1 RCV000047469.2	CM032549 (DM)	x2 VUS	[36, 40, 41]
c.213-5T>A	Intron 5	-	-	-	-	-	-	-	-	-	-	-	-
c.486G>T	Exon 8	p.= (p.Val162Val)	-	-	-	-	-	-	-	-	-	-	-
c.548-17G>T	Intron 8	-	-	-	-	-	ALL:T=0.017%NFE:0.023%	-	rs80358014	RCV000197647.2 RCV000123884.2 RCV000031256.6[a]	-	x31 VUS	[20, 26, 42]
c.734A>T	Exon 11	p.Asp245Val	C0	Deleterious	Disease causing	HD: PROBABLY DAMAGING HV: POSSIBLY DAMAGING	ALL:T=0.00084% NFE:0.0015%	-	rs80356865	RCV000049112.4 RCV000129392.2 RCV000112778.1	-	x1 VUS	-
c.1419C>T	Exon 11	p.= (p.Asn473Asn)	-	-	-	-	ALL:T=0.0025% NFE:0.0045%	-	-	RCV000165155.1	-	-	-
c.1487G>A	Exon 11	p.Arg496His	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	ALL:A=0.047% NFE:0.077%	EA: T=0.09% AA: T=0.00%	rs28897677	RCV000120286.3 RCV000111630.5[a] RCV000034727.3 RCV000047494.5 RCV000162601.1	CM014323 (DM?)	x86 VUS	[20, 43]
c.2521C>T	Exon 11	p.Arg841Trp	C15	Deleterious	Polymorphism	HD: BENIGN HV: BENIGN	ALL:T=0.17%NFE:0.22%	EA: A=0.31% AA: A=0.09%	rs1800709	RCV000120283.3 RCV000034733.3 RCV000047867.5 RCV000019251.10[a] RCV000162566.1	CM004236 (DM?)	x119 VUS	[20, 44-46]
c.3418A>G	Exon 11	p.Ser1140Gly	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	ALL:G=0.31%NFE:0.013%	EA: C=0.01% AA: C=3.09%	rs2227945	RCV000112092.5[a] RCV000048187.5 RCV000157733.1 RCV000162594.1 RCV000034741.3 RCV000120277.6	-	x29 VUS	[20, 47]
c.3708T>G	Exon 11	p.Asn1236Lys	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	ALL:G=0.024% NFE:0.027%	EA: C=0.03% AA: C=0.00%	rs28897687	RCV000120300.3 RCV000083197.5 RCV000131695.3 RCV000048292.6 RCV000148395.2	CM994631 (DM?)	x35 VUS	[23, 48]
c.5075A>C	Exon 18	p.Asp1692Ala	C65	Deleterious	Disease causing	HD: BENIGN HV: BENIGN	-	-	-	-	-	-	-
c.5096G>A	Exon 18	p.Arg1699Gln	C35	Deleterious	Disease causing	HD: PROBABLY DAMAGING HV: PROBABLY DAMAGING	ALL:A=0.0025% NFE:0.0045%	-	rs41293459	RCV000195350.2 RCV000131564.2 RCV000048790.4 RCV000031217.11	CM034007 (DM)	x11 VUS	[20, 22, 23, 36, 49]

c.5117G>C	Exon 18	p.Gly1706Ala	C55	Deleterious	Disease causing	HD: POSSIBLY DAMAGING HV: BENIGN	ALL:C=0.0041% NFE:0.0030%	EA: G=0.01% AA: G=0.00%	rs80356860	RCV000195322.1 RCV000048801.5 RCV000077598.8[a] RCV000162991.1	CM030790 (DM?)	x6 VUS	[20, 23, 50, 51]
c.5123C>T	Exon 18	p.Ala1708Val	C65	Deleterious	Disease causing	HD: PROBABLY DAMAGING HV: POSSIBLY DAMAGING	ALL:T=0.0033% NFE:0%	EA: A=0.01% AA: A=0.05%	rs28897696	RCV000048803.4 RCV000031221.4 RCV000148393.1 RCV000131166.2	CM065004 (DM)	-	[24, 52]
c.5125G>A	Exon 18	p.Gly1709Arg	C15	Deleterious	Disease causing	HD: POSSIBLY DAMAGING HV: BENIGN	-	-	-	-	-	-	-
c.5434C>G	Exon 23	p.Pro1812Ala	C0	Tolerated	Disease causing	HD: BENIGN HV: BENIGN	-	-	rs1800751	RCV000031251.5 RCV000048994.2	CM032862 (DM)	X2 VUS	[7, 36, 53]
c.5513T>G	Exon 24	p.Val1838Gly	C35	Deleterious	Disease causing	HD: PROBABLY DAMAGING HV: PROBABLY DAMAGING	-	-	-	-	-	-	-
BRCA2													
c.40A>G	Exon 2	p.Ile14Val	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	-	-	-	-	-	-	-
c.68-7T>A	Intron 2	-	-	-	-	-	ALL:A=0.24% NFE:0.30%	EA: A=0.15% AA: A=0.02%	rs81002830	RCV000074550.4 RCV000045051.5 RCV000077384.6 RCV000168529.2	CS033491 (DM?)	x7 VUS	[35, 36, 54, 55]
c.750G>A	Exon 9	p.= (p.Val250Val)	-	-	-	-	ALL:A=0.0052% NFE:0.0096%	EA: A=0.01% AA: A=0.00%	rs143214959	RCV000144219.1 RCV000123940.3 RCV000122928.3 RCV000162788.1	-	-	-
c.2680G>A	Exon 11	p.Val894Ile	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	ALL:A=0.0042% NFE:0.0060%	EA: A=0.05% AA: A=0.02%	rs28897715	RCV000160217.2 RCV000077283.6[a] RCV000044037.6 RCV000162506.1	-	x17 VUS	[20, 26]
c.3568C>T	Exon 11	p.Arg1190Trp	C15	Deleterious	Polymorphism	HD: POSSIBLY DAMAGING HV: BENIGN	ALL:T=0.011% NFE:0.0015%	-	rs80358604	RCV000160220.2 RCV000113191.2[a] RCV000044223.4 RCV000162698.1	-	x12 VUS	[26, 56]
c.4068G>A	Exon 11	p.= (p.Leu1356Leu)	-	-	-	-	ALL:A=0.30% NFE:0.47%	EA: A=0.47% AA: A=0.02%	rs28897724	RCV000044340.5 RCV000168569.2 RCV000162367.1 RCV000123968.2 RCV000113269.4	-	x9 VUS	[21, 57]
c.4828G>A	Exon 11	p.Val1610Met	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	ALL:A=0.013% NFE:0.023%	EA: A=0.02% AA: A=0.00%	rs80358705	RCV000074530.5 RCV000044498.3 RCV000130783.2 RCV000031508.5	-	x7 VUS	[58]
c.5272_5274del AAT	Exon 11	p.Asn1758del	-	-	-	-	ALL:0.0050% NFE:0.0091%	-	-	RCV000165160.1 RCV000122916.2	CD1410479 (DM)	-	[59]
c.6100C>T	Exon 11	p.Arg2034Cys	C0	Tolerated	Polymorphism	HD: POSSIBLY DAMAGING HV: BENIGN	ALL:T=0.32% NFE:0.49%	EA: T=0.51% AA: T=0.18%	rs1799954	RCV000120331.4 RCV000113532.6[a] RCV000044844.5 RCV000034452.3 RCV000162509.1	CM994286 (DM?)	x104 VUS	[20, 60]
c.6821G>T	Exon 11	p.Gly2274Val	C0	Tolerated	Disease causing	HD: PROBABLY DAMAGING HV: POSSIBLY DAMAGING	ALL:T=0.14% NFE:0.12%	-	rs55712212	RCV000077387.6 RCV000074551.6 RCV000131679.2 RCV000045064.3	-	x15 VUS	[20, 52]

c.7301A>C	Exon 14	p.Lys2434Thr	C0	Tolerated	Polymorphism	HD: BENIGN HV: BENIGN	ALL:C=0.0049% NFE:0%	-	rs80358954	RCV000045182.4 RCV000113743.1	CM142736 (DM?)	x2 VUS	[61]
c.8177A>G	Exon 18	p.Tyr2726Cys	C65	Deleterious	Disease causing	HD: PROBABLY DAMAGING HV: PROBABLY DAMAGING	-	EA: G=0.01% AA: G=0.00%	rs80359064	RCV000077430.4 RCV000130671.2 RCV000045442.4	-	x1 VUS	[62, 63]
c.8323A>G	Exon 18	p.Met2775Val	C0	Tolerated	Disease causing	HD: POSSIBLY DAMAGING HV: POSSIBLY DAMAGING	ALL:G=0.00084% NFE:0.0015%	-	-	-	-	-	-
c.9116C>T	Exon 23	p.Pro3039Leu	C0	Deleterious	Disease causing	HD: POSSIBLY DAMAGING HV: BENIGN	ALL:T=0.0086% NFE:0.0048%	EA: T=0.01% AA: T=0.00%	rs80359167	RCV000083154.4 RCV000045720.3 RCV000131718.2	CS020529 (DM?)	x6 VUS	[36, 64, 65]

[a] Classified by the ENIGMA expert panel as benign

Table 4.

BRCA1	Location	Patient number	Pos. ss	Splice predictions				
				SSF [0-100]	MES [0-16]	NNSPLICE [0-1]	GS [0-15]	HSF [0-100]
BRCA1 c.213-5T>A	Intron 5	3	+4	-7.6%	-52.1%	Lost 0.1	NP	-4.0%
			-54	-	-	-	+3.3%	-
BRCA1 c.5434C>G	Exon 23	16	-0 /	+7.1%	+20.9%	New 0.6	-	+5.2%
			+3	NP	200%	NP	NP	0.7%
BRCA2 c.68-7T>A	Intron 2	19	+6	-5.7%	-23.9 %	-27.3%	-	-2.7%