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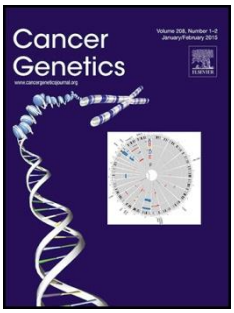

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Original Research

Cancer Genetics Journal

IN SILICO, *IN VITRO* AND CASE-CONTROL ANALYSES AS AN EFFECTIVE COMBINATION FOR ANALYZING *BRCA1* AND *BRCA2* UNCLASSIFIED VARIANTS IN A POPULATION-BASED SAMPLE.

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ABSTRACT

Ascertaining the clinical consequences of *BRCA1* and *BRCA2* variants of uncertain significance (VUS) is currently indispensable for providing effective genetic counseling and preventive actions for families with hereditary breast and ovarian cancer (HBOC). To this end, we conducted a combination of *in silico* prediction and cDNA splicing analyses of 13 *BRCA1* and 10 *BRCA2* VUS identified in our cohort as well as a case-control analysis in a population-based sample of 10 recurrent VUS. We observed consistent results between the *in silico* predictions and sequencing analyses for all analyzed VUS. An abnormal cDNA pattern was observed for variants c.212+1G>A and c.5278-1G>A in *BRCA1* and c.516+2T>A and c.8168A>G in *BRCA2* according to *in silico* splicing prediction.. A case-control study of VUS confirmed the polymorphisms of the c.67+62A>G, c.7008-62A>G and c.8851G>A *BRCA2* variants previously published. c.4068G>A in the *BRCA2* gene can also be considered a polymorphism due to its occurrence at a frequency greater than 1% in our population.

Our study shows that employing population-based analysis and a combination of several *in silico* methods yields highly accurate information, resulting in a reliable tool for selecting variants for cDNA sequencing analysis in routine cancer genetic counseling units.

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INTRODUCTION

The presence of a pathogenic mutation in the *BRCA1* or *BRCA2* gene in an individual confers a higher predisposition to developing hereditary breast and/or ovarian cancer (HBOC). Up to 15% of patients have been found to be carriers of variants in these genes that cannot be classified as benign or pathogenic and are defined as variants of uncertain significance (VUS).

BRCA VUS are currently mismanaged, hindering the clinical assessment of carriers, the determination of implications for cancer surveillance for patients and their family, decision making regarding risk-reducing surgeries and neonatal and prenatal testing and interventions [1].

Deleterious effects of VUS can be due to alterations in the coding sequence of the transcript or disruption of gene regulatory regions, such as promoters, untranslated regions, exons or introns, that modify the level of transcript expression [2].

It is widely accepted that one of the pathogenic pathways of unclassified variants is disruption of mRNA splicing of the *BRCA1* and *BRCA2* genes. In fact, it has been estimated that up to 60% of mutations that cause genetic diseases may alter splicing [3]. There are currently several available methods for analyzing the mRNA splicing processes of *BRCA1* and *BRCA2*. To standardize these methodologies across laboratories, the ENIGMA Consortium has provided guidelines and recommendations for the standardized clinical classification of *BRCA* gene sequence variation [4].

Several bioinformatic models have been proposed for prediction of the splicing effects of gene variants. The current gold standard method for variant classification is the multifactorial likelihood model that integrates clinical data from tumor pathology, segregation of the VUS with the disease, family history and co-occurrence with a deleterious mutation. This model derives a posterior probability of pathogenicity for each variant that is used as the basis for a 5-tier classification system with associated clinical recommendations [5]. However, there are some bottlenecks in the classification of *BRCA* sequence variants because this method requires large datasets, and tested individuals are not always available in many cancer genetic counseling units. Pathogenic mutations and VUS are often unique or only detected in a single or a few families, and family histories are limited due to reductions of the birth rate during the past 40 years. All of these factors make VUS classification according to current models that include segregation analysis difficult.

Therefore, the aim of the present study was to use the effectiveness of *in silico* splicing prediction tools, in combination with RNA analyses, to classify the VUS identified in our

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HBOC patients. We combined the two techniques because *in silico* predictors assess the impact of *BRCA* variants on splicing processes.

In addition, we performed a population-based case-control study of 10 recurrent *BRCA* VUS identified in the HBOC patients of our cohort, which were selected because they have been detected at a higher frequency in our population compared with that indicated in databases.

MATERIALS AND METHODS

Patients and control subjects

The 33 *BRCA1* and *BRCA2* VUS were detected in probands from 710 families undergoing genetic testing at the Genetic Counseling Unit of the Southern Catalonian Oncology Institute (IOCS) (Table 1). The screening criteria for the *BRCA* genes were set according to Spanish guidelines [6].

The control group was selected from a population-based sample recruited from a geographic region matching that of the patients and consisted of 793 males and females of self-reported European descent, between 18 and 77 years of age, whose parents and grandparents were also born in Spain.

The study was approved by the Ethics Committee of Clinical Research of Sant Joan University Hospital, and written informed consent was obtained from all participants.

DNA extraction and *BRCA1* and *BRCA2* analyses

Blood samples were collected from patients. The genomic DNA coding regions of the *BRCA1 and BRCA2* genes and approximately 100 intronic base pairs of the intron-exon boundary were sequenced on an ABI 3500 Genetic Analyzer (Life Technologies, Madrid, Spain), and an analysis of large rearrangements was performed via Multiplex Ligation-dependent Probe Amplification (MLPA;MRC-Holland, The Netherlands). Additional technical details and primers are available from the authors upon request.

RNA isolation, RT-PCR and cDNA sequencing

Following the recommendations of the ENIGMA Consortium [7] and the Clinical Molecular Genetics Society, patient blood samples were collected in PAXGENE tubes. Total RNA was isolated using a PAXGENE Blood RNA Kit (Qiagen, Hilden, Germany) and retrotranscribed using SuperScript II RT Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers. cDNA amplification reactions were performed for the flanking VUS regions using the AmpliTaq Gold Enzyme (Applied Biosystems, Madrid, Spain). The PCR

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primers used in this study were described previously [8,9]. The PCR products were separated on 1.5–3% agarose gels and visualized via SYBR Safe staining (Life Technologies, Madrid, Spain) with a Chemidoc XRS system (BioRad, Madrid, Spain). The PCR products were purified through ethanol precipitation and analyzed through direct sequencing on an ABI 3500 automated sequencer (Life Technologies, Spain). Finally, cDNA sequence analysis was performed using Sequencher® version 5.4.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA).

As a positive control, mRNA from three healthy subjects was included in the analysis of each variant.

Variants were selected without taking into account the *in silico* prediction results to determine the agreement between these tools and *in vitro* analyses.

Splicing prediction

The variants are named according to the recommendations of the Human Variation Society (HGVS), and numbering is based on the cDNA sequences of GenBank entries NM_007294.3 (*BRCA1*) and NM_000059.3 (*BRCA2*).

For bioinformatic splice site prediction and *in silico* analysis, we employed the integrative software Alamut visual version 2.7 (Interactive Biosoftware, Rowen, France), which includes several splice site prediction algorithms: Splice Site Finder-Like (SSF); MAxEntScan (MES); Gene Splicer (GS); Splice Site Prediction by Neural Network (NNSplice); and Human Splicing Finder (HSF). For DNA exonic variants that could alter putative exonic splicing enhancers, we used ESE Finder and RESCUE-ESE tools. We also employed *in silico* tools based on conservative and evolutionary analyses, including Polyphen, Align GVGD, SIFT and Mutation Taster, to predict the possible impact of the missense variants on the structure and function of the human BRCA1 and BRCA2 proteins.

Case-control analysis by quantitative real-time PCR (qPCR)

We selected 10 recurrent DNA variants that were detected in at least two unrelated families out of the 710 high-risk families fulfilling the HBOC criteria for genetic testing (Table 2). Among these variants, 4 could not be analyzed through cDNA sequencing due to their location in exon 11 of the *BRCA2* gene: c.-86C>T in the 5'UTR of *BRCA1*, c.4068G>A and c.4584C>T. The VUS c.8851G>A was not included because RNA was not available. Specific Custom TaqMan Genotyping assays (Applied Biosystems, Madrid, Spain) were designed for genotyping the 10 variants. Variants were assessed via qPCR using an ABI

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PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Madrid, Spain). SDS version 2.4 software (Applied Biosystems Madrid, Spain) was employed to automatically assign the allele calls. DNA samples presenting the rare-variant alleles were used as positive controls in each of the sample plates. Patients and control subjects were not matched for age or gender.

Statistical analyses

Fisher's exact test was used to test differences in allele frequencies between the HBOC patients and control subjects. Statistical analyses were performed with R (http://www.r-project.org) [10,11].

RESULTS

Comparison of *in silico* predictions and cDNA splicing analysis

Thirteen VUS located in the *BRCA1* gene and twenty located in the *BRCA2* gene were selected to determine the possibility of an aberrant splicing event (Table 1). The majority of the variants have been previously described in the international databases BIC (Breast Cancer Information Core) and LOVD (Leiden Open Variation Database) and are considered VUS.

BRCA1 c.212+1G>A was previously classified as a pathogenic variant in the databases because of its position at a canonical splice site, but its splicing effect according to *in vitro* analysis has not been reported [12]. *BRCA2* c.8168A>G [13] has been previously reported by several authors [12,14] and was classified as a class 5 variant by Lindor et al. cDNA analyses confirmed the splicing effects of both variants (data not shown).

The *in silico* analyses predicted an alteration of splicing due to interruption of the splice site or activation of a new acceptor site for seven variants (Table 1). For the c.212+1G>A *BRCA1* and the c.5152+5G>A *BRCA1* variants, at least four of the five programs predicted a 100% decrease in the donor site score and interruption of the intron-exon junction. For the c.5278-1G>A *BRCA1* variant, the five tools predicted a 100% decrease in the acceptor site. For c.4676-113C>G in *BRCA1*, the HSF, MES and SSF tools predicted activation of a new cryptic acceptor site. For c.516+2T>A in *BRCA2*, four tools predicted a 100% decrease in the donor site and an interruption of the intron-exon junction (see Supplementary Table 1). Therefore, all variants for which a 100% decrease was predicted by at least four of the *in silico* tools were analyzed through cDNA sequencing, and the

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prediction was confirmed by the presence of an aberrant transcript.

The *BRCA1* c.5152+5G>A VUS leads to complete skipping of exon 18 (r.5075_5152del), which at the protein level, is predicted to generate an in-frame deletion of 26 amino acids (p.Asp1692_Trp1718delinsGly) located in the BRCT functional domain. According to the ENIGMA consortium guidelines, in-frame deletions that remove amino acids located in important functional domains can be considered clinically relevant. The *BRCA1* c.5278-1G>A and *BRCA2* c.516+2T>A variants lead to deletion of the first seven bases of exon 21 (r.5278_5284del) and skipping of exon 6, respectively, generating an out-of-frame deletion in both cases and creating a premature stop codon (p.Ile1760Glyfs*3 and p.Val159Glyfs*10, respectively).

The remaining VUS included in the study had no apparent effect on mRNA splicing according to SYBR Safe stained agarose gel electrophoresis and sequencing analysis, as predicted by the *in silico* tools (data not shown). The *BRCA2* variant c.516+14C>T was first detected in co-occurrence with a well-established pathogenic mutation. However, this variant has already been classified as benign, and our results support this classification.

In summary, unrelated patients from 57 families were found to be carriers of 33 VUS. Of these VUS, six were associated with an alteration of the normal splicing pattern, resulting in deletion of a part of the gene (*BRCA1:* c.212+1G>A, c.4676-133C>G, c.5152+5G>A, 5278-1G>A. *BRCA2:* c.516+2T>A, c.8168A>G).

All of the bioinformatics predictions were confirmed through cDNA splicing analyses.

Case-control analyses of recurrent VUS

Ten recurrent VUS were selected for case-control analyses (Table 2). When these variants were analyzed by cDNA sequencing, none were found to be associated with modifications in splicing events. Four of these variants have not been reported in the BIC or LOVD database; two are considered VUS; and four have been recently classified as neutral by LOVD and/or the ENIGMA consortium.

Three of the variants, all of which were located in the *BRCA2* gene (c.4068G>A, c.7008-62A>G, c.8851G>A), were detected at a polymorphic frequency (>1%) in the control population-based sample (Table 2). Control carriers of these variants have no personal or family history of HBOC or related conditions. Variants c.7008-62A>G and c.8851G>A have been previously predicted as neutral. Furthermore, the c.7008-62A>G variant was detected in the homozygosis state in a control carrier (with no diagnosed Fanconi anemia).

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DISCUSSION

The aim of *BRCA1* and *BRCA2* mutational analysis in HBOC syndrome is to offer personalized clinical management for mutation carriers, but the reality is that a high proportion of the mutational results are non-informative due to the presence of VUS. Ascertaining the pathogenicity of VUS would aid in clinical management. mRNA splicing analyses combined with bioinformatic predictions have been proposed as an efficient method for identifying putative pathogenic effects of *BRCA* VUS according to a 5-tier scheme proposed by the IARC. *BRCA1* and *BRCA2* cDNA sequencing is a suitable methodology for studying mRNA splicing. As reported above, the ENIGMA Consortium established general guidelines and recommendations for best practices in mRNA assays that include PCR primer design, PCR conditions and product detection systems as well as other parameters [8]. The present study followed these recommendations for each point of the analysis, except for the characterization of naturally occuring alternative splicing events through semi-quantitative capillary electrophoresis, which was not performed.

The multifactorial model is a widely accepted method for classifying VUS. In addition, there are a wide range of classification algorithms, all of which are based on large datasets or tumor pathology information, which is not always available to genetic counseling units [15-18]. For this reason, it is important for each unit to be able to identify its own strategies for classifying the VUS with maximum accuracy using the available tools and according to published guidelines.

In the present study, we compared *in silico* tools and cDNA sequencing analysis based on RNA splicing results. We also performed cDNA analyses of VUS that were previously classified as pathogenic as an internal positive control for RNA splicing analyses. The results of the present work confirm current bioinformatic algorithms as powerful tools for predicting *BRCA* VUS splicing events. Concordant results of the two analyses were found for all of the analyzed variants. For all of the VUS predicted to disturb the natural splice site, the cDNA analysis results confirmed that the VUS can be considered pathogenic or likely pathogenic, with the exception of c.4676-113C>G, which resulted in skipping of only three nucleotides.

Conversely, according to the allele-based case-control analysis of the 10 recurrent VUS selected, in concordance with published databases, we considered four of the analyzed variants to be likely not pathogenic, as they were detected at a polymorphic frequency of at least 1% in our population. Following the classification rules [19] and the 5-tier system proposed for the IARC [20], VUS can be considered as class 1 (neutral or with subtle effects [21]) if the variant is reported to occur in a large outbred control reference group at

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an allele frequency ≥ 1%. The control groups used for such analyses are often datasets, such as those of the 1000 Genomes project and the Exome Variant Server. Our analysis was useful for determining the frequency in our control population specific to our demographic region, after the detection of variants at a frequency greater than that published in the databases.

In summary, based on the detection of a frequency higher than 1% in our demographic population, the *BRCA2* VUS c.7008-62A>G and c.8851G>A are likely neutral, and c.4068G>A can likely be considered a non-pathogenic variant. The frequency of the variant c.67+62T>G reported in public databases is 0.35, whereas in our population, we detected this variant at close to the 1% polymorphic frequency (0.9%); that threshold may have been achieved by increasing the sample size of our control subjects. Conversely, there were no significant differences in the presence of these variants between the cases and controls, allowing us to assume that these results add evidence in favor of neutrality.

In conclusion, the predictive ability of the *in silico* tools used in this study is high, and we are confident in using them as a filtering tool to select variants for *in vitro* analyses when a patient's blood sample is not available for RNA analysis, or a patient has a limited family history. Frequency population analysis is a good strategy for discarding VUS as pathogenic founder mutations based on their presence in control subjects with neither personal nor family antecedents of HBOC syndrome. In addition, we can conclude that the combination of these three tools is a good strategy for confirming VUS relevance in our population.

Conflicts of Interest: None

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REFERENCES

[1] Garcia C, Lyon L, Little RD, Powell CB. Comparison of risk management strategies between women testing positive for a BRCA variant of unknown significance and women with known BRCA deleterious mutations. Genet Med 2014;12:896-902. doi: 10.1038/gim.2014.48.

[2] Blencowe BJ. Alternative splicing: new insights from global analyses. Cell 2006;126:37-47. doi: 10.1016/j.cell.2006.06.023.

[3] López-Bigas N, Audit B, Ouzounis C, Parra G, Guigó R. Are splicing mutations the most frequent cause of hereditary disease? FEBS Lett 2005;579:1900–3. doi: 10.1016/j.febslet.2005.02.047.

[4] Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, *et al.* Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat 2008;29:1282-91. doi: 10.1002/humu.20880.

[5] Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro AN, Tavtigian SV, Couch FJ. Integrated evaluation of DNA sequence variants of unknown clinical significance: application to BRCA1 and BRCA2. Am J Hum Genet 2004;75:535-44. doi: 10.1086/424388.

[6] Graña B, Lastra E, Llort G, Brunet J, Isla D. SEOM clinical guidelines for hereditary cancer. Clin Transl Oncol 2011;13:580-6. doi: 10.1007/s12094-011-0701-2

[7] Whiley PJ, De La Hoya M, Thomassen M, Becker A, Brandão R, Pedersen IS, et al. Comparison of mRNA splicing assay protocols across multiple laboratories: Recommendations for best practice in standardized clinical testing. Clin Chem 2014;60:341–52. doi:10.1373/clinchem.2013.210658.

[8] Menéndez M, Castellsagué J, Mirete M, Pros E, Feliubadaló L, Osorio A, et al. Assessing the RNA effect of 26 DNA variants in the BRCA1 and BRCA2 genes. Breast Cancer Res Treat 2012;132:979–92. doi:10.1007/s10549-011-1661-5.

[9] Bonnet C, Krieger S, Vezain M, Rousselin A, Tournier I, Martins A, et al. Screening BRCA1 and BRCA2 unclassified variants for splicing mutations using reverse transcription PCR on patient RNA and an ex vivo assay based on a splicing reporter minigene. J Med Genet 2008;45:438–46. doi:10.1136/jmg.2007.056895.

[10] R Development Core Team. R: A language and environment for statistical computing, Vienna, Austria: R Foundation for Statistical Computing; 2008. ISBN 3-900051-07-0, http://www.R-project.org.

Accepted Manuscript

ACCEPTED MANUSCRIPT

[11] Fay MP. Confidence intervals that match Fisher's exact or Blaker's exact tests. Biostatistics 2010;11:373-4. doi: 10.1093/biostatistics/kxp050.

[12] Sanz DJ, Acedo A, Infante M, Durán M, Pérez-Cabornero L, Esteban-Cardeñosa E, *et al.* A high proportion of DNA variants of BRCA1 and BRCA2 is associated with aberrant splicing in breast/ovarian cancer patients. Clin Cancer Res 2010;16:1957-67. doi: 10.1158/1078-0432.CCR-09-2564.

[13] Théry JC, Krieger S, Gaildrat P, Révillion F, Buisine MP, Killian A, et al. Contribution of bioinformatics predictions and functional splicing assays to the interpretation of unclassified variants of the BRCA genes. Eur J Hum Genet 2011;9:1052-8.

[14] Walker LC, Whiley PJ, Couch FJ, Farrugia DJ, Healey S, Eccles DM, et al. Detection of splicing aberrations caused by BRCA1 and BRCA2 sequence variants encoding missense substitutions: Implications for prediction of pathogenicity. Hum Mutat 2010;31:E1484-E1505. doi:10.1002/humu.21267.

[15] Lindor NM, Guidugli L, Wang X, Vallée MP, Monteiro AN, Tavtigian S, *et al.* A review of a multifactorial probability-based model for classification of BRCA1 and BRCA2 variants of uncertain significance (VUS). Hum Mutat 2012;33:8-21. doi: 10.1002/humu.21627.

[16] Spearman AD, Sweet K, Zhou X-P, McLennan J, Couch FJ, Toland AE. Clinically applicable models to characterize BRCA1 and BRCA2 variants of uncertain significance. J Clin Oncol 2008;26:5393-400. doi: 10.1200/JCO.2008.17.8228.

[17] Karbassi I, Maston GA, Love A, DiVincenzo C, Braastad CD, Elzinga CD, *et al.* A Standardized DNA variant scoring system for pathogenicity assessments in Mendelian disorders. Hum Mutat 2016;37:127-34. doi: 10.1002/humu.22918.

[18] Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-23. doi: 10.1038/gim.2015.30.

[19] Spurdle AB, Healey S, Devereau A, Hogervorst FB, Monteiro AN, Nathanson KL, *et al.* ENIGMA--evidence-based network for the interpretation of germline mutant alleles: an international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. Hum Mutat 2012;33:2-7. doi: 10.1002/humu.21628.

[20] Walker LC, Whiley PJ, Houdayer C, Hansen TV, Vega A, Santamarina M, *et al.* Evaluation of a 5-tier scheme proposed for classification of sequence variants using bioinformatic and splicing

ACCEPTED MANUSCRIPT

assay data: inter-reviewer variability and promotion of minimum reporting guidelines. Hum Mutat 2013;34:1424-31. doi: 10.1002/humu.22388.

[21] Lindor NM, Goldgar DE, Tavtigian SV, Plon SE, Couch FJ. BRCA1/2 sequence variants of uncertain significance: a primer for providers to assist in discussions and in medical management. Oncologist 2013;18:518-24. doi: 10.1634/theoncologist.2012-0452.

Table 1. Description of unclassified *BRCA* variant predictions and results

Nucleotide Variant (HGVS)	N Families	Site	Interpretation Based on Splicing Prediction Tools	Database Classification (ClinVar, BIC, LOVD)	RNA Analysis	Protein Prediction
BRCA1						
c.212+1G>A	**2**	**D**	**Interruption of intron-exon junction**	**Pathogenic / VUS**	**r.135_212del**	**p.Phe46_Arg71del**
c.302-24_302-22del	2	A	No change	NR	r.=	p.=
c.4097-10G>A	1	D/A	No change	Benign / VUS / NR	r.=	p.=
c.4676-113C>G	2	A	Activation of a new cryptic acceptor site	NR	r.4358_4360del	p.Ala1453del
c.4676-105T>C	1	-	No change	NR	r.=	p.=
c.4766G>A	1	D	No change	VUS / Likely Benign	r.=	p.=
c.4974C>T	1	D	No change	NR	r.4674c>u	p.=
c.5074+6C>G	1	D	New donor site	Likely Benign / VUS	r.=	p.=
c.5074+29T>C	1	-	No change	NR	r.5193+29u>c	p.=
c.5152+5G>A	**1**	**D**	**Interruption of intron-exon junction**	**Likely Pathogenic / VUS**	**r.5075_5152del**	**p.Asp1692_Trp1718delinsGly**
c.5277+60_5277+61ins9	3	A	No change	VUS	r.=	p.=
c.5278-1G>A	**2**	**A**	**Interruption of intron-exon junction**	**Pathogenic / VUS**	**r.5278_5284del**	**p.Ile1760Glyfs*3**
c.5333-8C>T	1	A	Interruption of intron-exon junction	Likely Benign / VUS	r.5333-8c>u	p.=
BRCA2						
c.67+62T>G	2	A	No change	VUS	r.=	p.=
c.68-7T>A	3	A	Interruption of intron-exon junction	Likely Benign / VUS	r.=	p.=
c.438A>G	1	-	No change	NR	r.=	p.=
c.516+2T>A	**1**	**D**	**Interruption of intron-exon junction**	**VUS**	**r.476_516del**	**p.Val159Glyfsn*10**
c.516+14C>T	4	D	No change	Benign	r.516+14c>u	p.=
c.556G>C	1	A	No change	NR	r.=	p.=

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c.601C>G	1	A	No change	NR	r.=	p.=
c.680C>T	1	D	No change	VUS	r.680c>u	p.=
c.6928A>C	1	A	No change	NR	r.=	p.=
c.6938-26T>C	1	D/A	No change	NR	r.6938-26u>c	p.=
c.7007+22_7007+23dupCT	1	-	No change	NR	r.=	p.=
c.7008-62A>G	10	D	No change	Benign / VUS	r.=	p.=
c.7795_7797delGAA	1	A	No change	VUS	r.=	p.=
c.8168A>G	**1**	**D**	**Creation of new donor site**	**Pathogenic**	**r.8168_8831del**	**p.Asp2723Alafs*32**
c.8632+2T>G	1	D	Interruption of intron-exon junction	VUS	r.=	p.=
c.8632+18G>A	1	-	No change	VUS	r.=	p.=
c.8850G>T	1	A	No change	Benign / VUS	r.8850g>u	p.=
c.8854A>G	2	-	No change	Likely Benign / VUS	r.=	p.=
c.8953+98T>C	1	D	Interruption of intron-exon junction	Benign / VUS	r.=	p.=
c.9116C>T	3	D	No change	Likely Benign / VUS	r.9116c>u	p.=

Pathogenic mutations are highlighted in bold.

D: donor splice site, A: acceptor splice site, -: no predicted changes in donor or acceptor site.

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Table 2. Recurrent *BRCA1* and *BRCA2* variants analyzed in control subjects

Gene	Variant (HGVS)	rs (dbSNP)	BIC	LOVD Prediction	Population Frequency*		Patient Frequency		Control Frequency		P**
					N	%	N	%	N	%	
BRCA1	c.-86C>T	rs143160357	Nr	Nr	6/20450	0.029	2/710	0.3	2/793	0.3	1
	c.302-24_302-22delAAT	rs756577139	Nr	Nr	3/121402	0.025	2/710	0.3	0/793	0	0.223
	c.4676-113C>G	rs187218638	Nr	Nr	1/5008	0.02	2/710	0.3	4/793	0.5	0.6897
BRCA2	c.67+62T>G	rs11571574	VUS, 4 times	Neutral	14/5008	0.28	4/710	0.6	7/793	0.9	0.5542
	c.516+14C>T	rs182828913	Nr	Neutral	3/5008	0.06	4/710	0.6	4/793	0.5	1
	c.4068G>A	rs28897724	VUS, 9 times	VUS	2/5008	0.04	5/710	0.7	8/793	1.0	0.5871
	c.4584C>T	rs80359788	VUS, 2 times	VUS	2/5008	0.04	3/710	0.4	1/793	0.1	0.3492
	c.7008-62A>G	rs76584943	VUS, 5 times	Neutral	19/5008	0.38	10/710	1.4	17/793	2.1	0.3335
	c.8851G>A	rs11571769	Neutral, 43 times	Neutral	50/5008	0.99	3/710	0.4	10/793	1.3	0.09728
	c.8854A>G	rs397508016	Nr	Nr	Nr	0	2/710	0.3	0/793	0	0.223

*Population frequency derived from the 1000 Genomes database and ENSEMBL.

** P value for Fisher's exact test

HGVS: Human Genome Variation Society; BIC: Breast Cancer Information Core; LOVD: Leiden Open Variation Database; Nr: Not reported.

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