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ORIGINAL ARTICLE

Comprehensive *BRCA* mutation analysis in the Greek population. Experience from a single clinical diagnostic center

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Germline mutations in the *BRCA1* and *BRCA2* genes are associated with hereditary predisposition to breast and ovarian cancer. Sensitive and accurate detection of *BRCA1* and *BRCA2* mutations is crucial for personalized clinical management of individuals affected by breast or ovarian cancer, and for the identification of at-risk healthy relatives.

We performed molecular analysis of the *BRCA1* and *BRCA2* genes in 898 Greek families, using Sanger sequencing or Next Generation Sequencing for the detection of small insertion/deletion frameshift, nonsynonymous, truncating and splice-site alterations and MLPA for the detection of large genomic rearrangements.

In total, a pathogenic mutation was identified in 12.9% of 898 families analyzed. Of the 116 mutations identified in total 9% were novel and 14.7% were large genomic rearrangements.

Our results indicate that different types of mutational events in the *BRCA1* and *BRCA2* genes are responsible for the hereditary component of breast/ovarian cancer in the Greek population. Therefore the methodology used in the analysis of Greek patients must be able to detect both point and small frameshift mutations in addition to large genomic rearrangements across the entire coding region of the two genes.

Keywords *BRCA1*, *BRCA2*, Next Generation Sequencing (NGS), Multiplex Ligation-dependent Probe Amplification (MLPA), Greece

Introduction

Most cases of breast cancer are sporadic. However, it is more common in some families due to their genetic background. Approximately 5%–10% of all cancers are due to hereditary (germline) mutations.

Hereditary Breast and Ovarian Cancer (HBOC) Syndrome is caused by inherited mutations in the *BRCA1* or the *BRCA2* genes. In particular, germline mutations in *BRCA1* have been identified in 15%–20% of women with a family history of breast cancer and 60%–80% of women with a family history of breast and ovarian cancer (1,2). The lifetime risk of breast cancer in female carriers of a *BRCA1* mutation is 51%–87% while that of ovarian cancer is 23%–44% (3,4). The median age at diagnosis of breast cancer is 42 years, i.e. 20 years earlier than the median of unselected women in the USA and Western Europe (5). Female carriers of a *BRCA2* mutation have 28%–84% risk of developing breast cancer and up to 27% of developing ovarian cancer (4). Male carriers of *BRCA2*

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mutations have up to 8% risk of developing breast cancer and 20% risk of developing prostate cancer by the age of 80.

Both genes are very large and their protein products function in maintaining genomic integrity and in transcriptional regulation (6,7). The *BRCA1* gene has been shown to be mutated in ~50% of HBOC families, while a further 35% of the families carry germline mutations in the *BRCA2* gene (8,9). A multitude of mutations scattered throughout the coding sequence of the genes have been described with no obvious phenotype–genotype correlation. The majority of mutations identified are small insertions/deletions leading to frameshifts and truncated protein products. In addition, a large number of nonsense mutations or mutations affecting mRNA splicing have been identified (8,9). The percentage and type of mutations identified is largely dependent on the population studied, with strong founder effects evident in some populations (10–12).

While in the early studies for mutation detection only single point or small insertion/deletion mutations were screened for, recent studies have shown that large genomic rearrangements (LGRs) are also a common type of mutation of the two genes accounting for 10%–30% of all mutations identified in some populations (13–16). More specifically, studies of Greek, Italian and Dutch HBOC families have indicated that LGRs in *BRCA1* account for 17%, 23% and 27%–36% of the total *BRCA1* mutations detected, respectively (13–16), while in an analysis of a Danish HBOC cohort the percentage of LGRs was only 3.8% (17). LGRs are less commonly found to affect the *BRCA2* gene and have been shown to range from 0%–11% depending on the population studied (18–20).

The even distribution of mutations throughout the large coding regions of both *BRCA* genes (8,9) has made analysis expensive and time consuming. This in turn has led to the formulation of strict inclusion criteria of patients or families who should be screened for mutations in the two genes. In addition, analysis was largely focused on screening the two genes for point mutations and small insertions/deletions using PCR-based screening methods such as the Protein Truncation Test (PTT), Single Strand Conformational Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE), Heteroduplex Analysis (HA) and Denaturing High Performance Liquid Chromatography (DHPLC) with varying degrees of sensitivity for each method. Direct DNA sequencing was then used in order to confirm and characterize mutations detected by any of these approaches (21,22).

Technological advances in sequencing have led to the application of a new way of sequencing, generally referred to as Next Generation Sequencing (NGS) or Massively Parallel Sequencing (MPS) (23). Since the launch on the market of the first “next generation sequencing” platform, sequencing technology has undergone a rapid development (24). Introduction of NGS in laboratory practice allowed molecular diagnostic laboratories to dramatically increase the throughput, reducing turn-around times and costs. NGS can also be used to analyze multiple genes in a single run, facilitating the study of complex disease where Sanger sequencing is not technically or economically feasible (25–27).

Molecular diagnostic approaches must guarantee high-quality molecular testing, capable of detecting all types of mutations which may result in the deactivation of the *BRCA* genes. About 10%–36% of all *BRCA* mutations identified in some populations (13–20) are estimated to be LGRs, which cannot be easily detected by full gene sequencing. In this aspect, every comprehensive *BRCA* analysis offered must include a method such as quantitative multiplex PCR of short fluorescent fragments (28) or MLPA (29), capable of detecting this type of mutation.

The aim of this study was to further delineate the extent and nature of mutations in the *BRCA1* and *BRCA2* genes, responsible for hereditary breast and ovarian cancer in Greek families, unselected for family history.

Materials and methods

Study group

During the past 7 years, a total of 898 Greek unrelated families were referred to our center for genetic analysis of the *BRCA1* and *BRCA2* genes. These included 889 female and 9 male probands. The mean age of participants was 45 years (26-75). All samples were collected from the referring physicians during this study.

As this study took place in a private diagnostic laboratory, subjects were not selected by strict criteria for genetic analysis. All enrolled probands were informed about the significance of molecular screening and provided information about their personal and family history. All participants signed an informed consent form prior to molecular genetic testing and permission for the anonymous use of their data for research purposes and/or scientific publications. The study was approved by the ethics committee of Hellenic Breast Surgeons Society.

The *BRCA1* and *BRCA2* genes were analyzed using direct Sanger sequencing (345 samples) or Next Generation Sequencing (NGS) (553 samples). Multiplex Ligation-dependent Probe Amplification (MLPA) for analysis of large genomic rearrangements (LGRs) was carried out in all mutation negative cases. Biallelic mutations affecting both copies of *BRCA1* and *BRCA2* have been shown to produce a Fanconi aneamia phenotype which is distinct from that of HBOC. As such, when a single pathogenic variant was identified in either gene, MLPA analysis was not carried out for that patient.

DNA isolation

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (QIAGEN) and quantified using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific).

DNA Sanger sequencing

All exons and exon-intron boundaries of *BRCA1* and *BRCA2* were analyzed by conventional Sanger sequencing (primer sequences and conditions available upon request). PCR products were purified using NucleoFast® 96 PCR Clean-up kit (Macherey-Nagel GmbH and Co., Düren, Germany), according to the manufacturer’s instructions. Subsequently, 2 µl purified PCR product was used for each sequencing reaction, which was performed using the BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction products were purified prior to electrophoresis using the Montage™ SEQ96 Sequencing

Reaction kit (EMD Millipore Corp., Billerica, MA, USA). Electrophoresis of sequencing products was performed on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). The resulting sequences were compared to reference sequences (*BRCA1*: Genbank no: NM_007294; *BRCA2*: Genbank no: NM_000059) using the software SeqScape 2.7 (Applied Biosystems).

All clinically significant findings and Variants of Uncertain Significance (VUS) were confirmed on a new DNA from an alternative blood vial obtained from the patient.

Next Generation Sequencing (NGS)

Amplification of the entire coding region including the intron-exon boundaries of both *BRCA1* and *BRCA2* genes was carried out using the CE IVD BRCA MASTR Dx kit (MULTIPLICOM NV (27), according to the manufacturer's instructions (www.multiplicom.com). Briefly, the assay generates a library of 93 specific gene amplicons in two rounds of PCR: Initially, for each sample, 50 ng of DNA was used to perform 5 multiplex PCR to amplify the entire target region. This was followed by Universal PCR where the products of each multiplex PCR were used as template for a PCR using hybrid primers to univocally tag all the multiplexes from the same proband with a unique multiplex identifier (MID) and platform specific primer. The multiplexed tagged reactions from the same sample were pooled to create a library and were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Each library was quantified using the KAPA Library Quantification Kit for Next-Generation Sequencing on a Rotor-Gene 6000 system (Corbett Research, QIAGEN, Hilden, Germany). The products were subsequently analyzed by Next Generation Sequencing (NGS) using the Illumina platform, MiSeq, according to the manufacturer's instructions.

Analysis of the resulting sequences was carried out using the software suite SeqNext (JSI medical systems GmbH, Germany) using as reference sequences hg19/NM_007294 for *BRCA1* and hg19/NM_000059 for *BRCA2*. All sequence variants were annotated according to the nomenclature used by the Human Genome Variation Society (HGVS, http://www.hgvs.org/).The clinical significance of variants was further examined using standards and guidelines for the interpretation of sequence variants recommended by the American College of Medical Genetics and Genomics (ACMG Laboratory Quality Assurance Committee) and the Association for Molecular Pathology (30). Multiple lines of evidence were combined through a variant scoring pipeline to study the clinical meaning and significance of Variants of Uncertain Significance (VUS). The impact of missense substitutions on protein function or structure was analyzed using computational (*in silico*) predictive algorithms combined in the ensemble mutational impact score of MetaSVM (31) and the effect on splicing was computationally examined using Human Splicing Finder (32).

All clinically significant findings and VUS were confirmed on a new DNA preparation using Sanger sequencing.

Multiplex Ligation-dependent Probe Amplification (MLPA)

The presence of large genomic rearrangements (LGRs), in addition to the presence of the 1100delC mutation in the *CHEK2* gene was investigated by use of the method MLPA (Multiplex Ligation-dependent Probe Amplification, *BRCA1*: P002, *BRCA2*: P045, MRC Holland), according to the manufacturer's instructions. Electrophoresis was carried on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) and analysis was carried out using the Coffalyser.Net software.

All findings were confirmed on a new DNA preparation using alternative probe mixes (P087 for *BRCA1* and P077 for *BRCA2* gene) except for the deletion of *BRCA1* exon 20 and *BRCA2* exon 14, which are not detected by P087 and P077, respectively. Confirmation of these mutations was carried out using the P002 and P045 probe mixes, respectively, on new DNA preparations and Sanger sequencing of the probe hybridization sites in order to discount false positive findings.

RNA extraction and RT-PCR

Total RNA was extracted from peripheral blood lymphocytes using Trizol (Invitrogen, Paisley, UK) following standard protocol.

First strand synthesis was performed as previously described in Belogianni *et al*. (2004) (33). An aliquot of the resulting cDNA was amplified in a new PCR using the proper primer set (available upon request).

Results

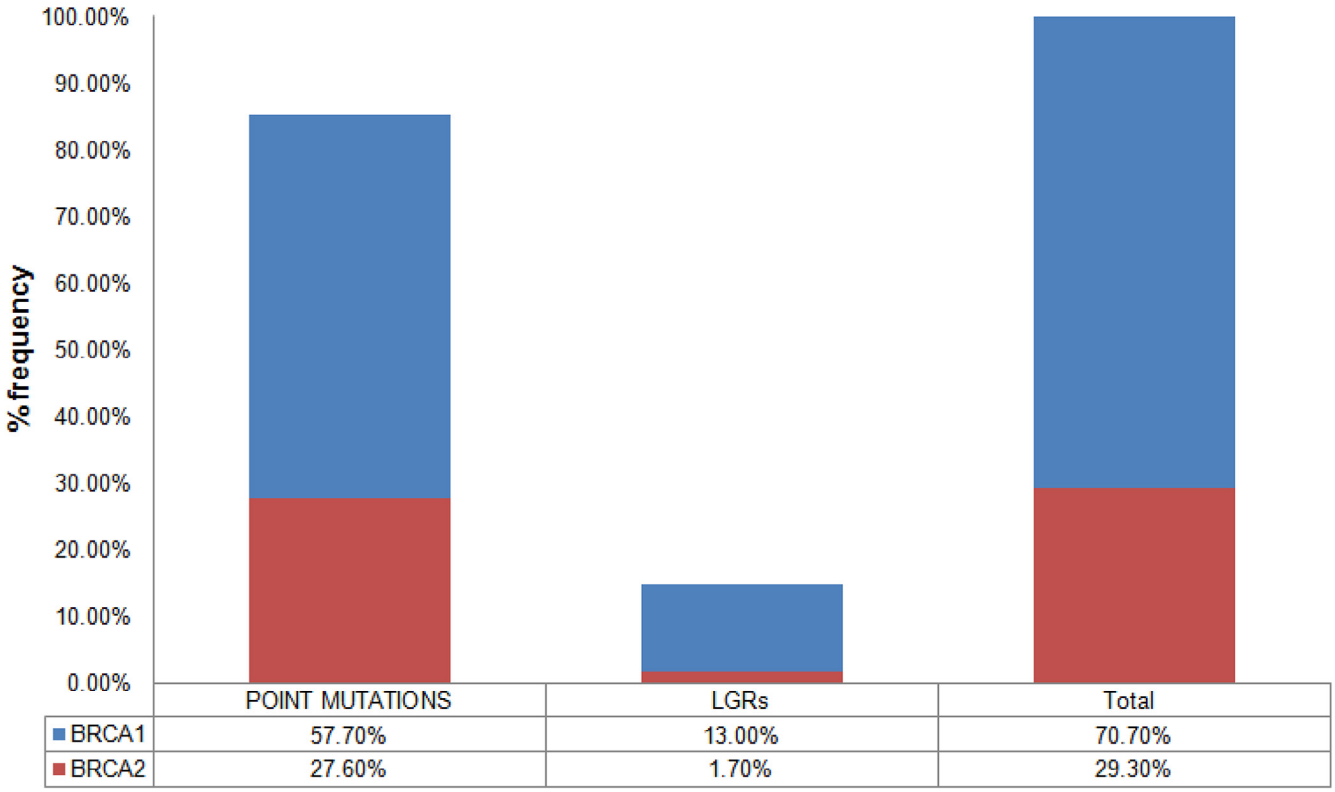
Validation and application of NGS

The increasing recognition by physicians of the importance of *BRCA1* and *BRCA2* mutational status in patient management has resulted in an increased need for diagnostic laboratories to be able to provide analyses faster and at reduced costs. The advent and rapid spread of NGS has allowed genetic testing facilities to achieve this goal. However, the transition from the "gold standard" method of Sanger sequencing to NGS has to be carefully standardized and tested before its use in clinical diagnosis. For this reason, a commercially available CE IVD marked kit was initially selected.

In order to validate this technology, 35 samples that had been previously fully analyzed by Sanger sequencing were subjected to NGS using the described methodology. Variants detected from the analysis with both methods were compared and found to be 100% concordant. For a further 35 samples analyzed by NGS, all variants detected, regardless of variant frequency were also analyzed by Sanger sequencing. This allowed both for the verification of the methodology and the determination of depth of coverage and variant allele frequency necessary.

As a result the depth of coverage deemed as necessary was determined to be 100 times per amplicon. As both *BRCA* genes are located on autosomes, and no cases of mosaicism have been described in the international literature inherited mutations are expected to be identified at a 50% frequency in a given sample. In reality this frequency was shown to range from 35%–60% which was the cutoff set for variant calling.

Deleterious *BRCA* gene mutations were identified in 116 (12.9%) probands. Of the mutations identified, 82 (70.7%) were located in the *BRCA1* gene and 34 (29.3%) in *BRCA2*. LGRs accounted for 14.7% (17 of 116) of all *BRCA* mutations: 18.3% (15 of 82) of the *BRCA1* mutations and 5.88% (2 of 34) of the *BRCA2* mutations (Figure 1).



	POINT MUTATIONS	LGRs	Total
BRCA1	57.70%	13.00%	70.70%
BRCA2	27.60%	1.70%	29.30%

Figure 1 Frequency of deleterious mutations in *BRCA1* and *BRCA2* genes in the 116 probands.

The *BRCA1* and *BRCA2* mutations (small insertion/deletion frameshift, nonsynonymous truncation and splice-site alterations) are shown in Tables 1 and 2, respectively.

In line with previous publications (34) c.5266dupC p.(Gln1756Profs) (15.6%), c.5251C>T p.(Arg1751*) (6.9%), c.5212G>A p.(Gly1738Arg) (6.1%) and c.181T>G p.(Cys61Gly) (3.5%) in the *BRCA1* gene and c.2339C>G p.(Ser780*) (3.5%) in the *BRCA2* gene, were the most commonly detected mutations (Figure 2).

Novel mutations

Eleven of the 116 deleterious mutations (9%) identified were novel. In *BCRA1* four of the identified mutations have not been previously described: c.65T>A p.(Leu22*), c.1154G>A p.(Trp385*), c.2933dupA p.(Tyr978*) and c.3132delT p.(Asn1045Metfs).

In the *BCRA2* gene the following novel mutations were identified: c.682-1 G > T, c.1057delT p.(Ser353fs), c.1821dupA p.(Asp608Argfs), c.4769delA p.(Lys1590Serfs), c.5206C>T p.(Gln1736*), c.7806-2A>T, c.9680delA p.(Ser3228Valfs). All these mutations were classified as "likely pathogenic" based on their expected effect on the protein produced by the mutated allele (30) (Table 3).

Large genomic rearrangements (LGRs)

The LGRs identified in the *BRCA1* gene were all deletions consisting of exon 19, 22, 22–23 and 23 (Figure 2 and Table 4). One patient carried a deletion of the entire *BRCA1* gene. As far as the *BRCA2* gene is concerned, deletion of the entire gene and deletion of exon 14 were identified in one family each (Table 4).

mRNA splicing variants

The mutation c.9501 + 1G>A in the *BRCA2* gene was observed in two unrelated probands: a male patient diagnosed with breast cancer and a 43-year-old female diagnosed with breast cancer at 41 whose mother was also diagnosed with breast cancer at 41.

This variant has been described in the mutation database ClinVar as VUS (https://www.ncbi.nlm.nih.gov/clinvar/variation/52853/). Algorithms developed to predict the effect of single nucleotide changes on mRNA processing, predict that this change may alter splicing of the resultant mRNA but this prediction has not been confirmed experimentally. Analysis of mRNA from these patients revealed that this mutation does indeed result in incorrect splicing, the resulting mRNA missing exon 25 (Figure 3). This results in a truncated protein, missing the last 332 amino acid residues including part of the *BRCA2*, oligonucleotide/oligosaccharide-binding, domain 3.

The novel variant c.682-1G>T was identified in the *BRCA2* gene in a woman diagnosed with breast cancer at the age of 34. This particular variant has not been described in the bibliography and is not present in mutation databases. However, a G > C change at the same location has been described as pathogenic/likely pathogenic (www.ncbi.nlm.nih.gov/clinvar/variation/52198/). Algorithms developed to predict the effect of single nucleotide changes on mRNA processing, predict that

Table 1 *BRCA1* mutations in the analyzed population

Exon/Intron	HGVS cDNA	HGVS Protein	dbSNP	No. of Carriers	Clinical Significance				
					BIC	UMD	ARUP	ClinVar	Additional Information
2	c.65T>A	p.(Leu22*)	-	1	-	-	-	-	Novel[a]
4	c.181T>G	p.(Cys61Gly)	28897672	4	Yes	-	Yes	Yes	-
6	c.329dupA	p.(Glu111Glyfs)	80357604	1	Yes	-	Yes	Yes	-
10	c.1059G>A	p.(Trp353*)	80356935	2	Yes	-	-	Yes	-
10	c.1154G>A	p.(Trp385*)	-	1	-	-	-	-	Novel[a]
10	c.1612C>T	p.(Gln538*)	80356893	1	Yes	Yes	Yes	Yes	-
10	c.1961delA	p.(Lys654Serfs)	80357522	1	Yes	Yes	Yes	Yes	-
10	c.2923C>T	p.(Gln975*)	80357497	1	Yes	-	-	Yes	-
10	c.2933dupA	p.(Tyr978*)	-	1	-	-	-	-	Novel[a]
10	c.3132delT	p.(Asn1045Metfs)	-	1	-	-	-	-	Novel[a]
10	c.3375_3376delTC	p.(Pro1126Ilefs)	80357828	1	Yes	-	Yes	Yes	-
10	c.3436_3439delTGTT	p.(Cys1146Leufs)	397509067	1	-	Yes	-	Yes	-
10	c.3607C>T	p.(Arg1203*)	62625308	1	Yes	Yes	Yes	Yes	-
10	c.3700_3704delGTAAA	p.(Val1234Glnfs)	80357609	3	Yes	Yes	-	Yes	-
10	c.3756_3759delGTCT	p.(Ser1253Argfs)	80357868	2	Yes	Yes	-	Yes	-
13	c.4391_4393delInsTT	p.Pro1464Leufs	273900730	1	-	Yes	-	Yes	-
IVS16	c.5075-1G>A	Splicing	1800747	1	Yes	-	Yes	Yes	-
17	c.5150delT	p.(Phe1717Serfs)	80357720	1	Yes	-	-	Yes	-
19	c.5212G>A	p.(Gly1738Arg)	80356937	7	Yes	Yes	Yes	Yes	-
19	c.5251C>T	p.(Arg1751*)	80357123	8	Yes	Yes	Yes	Yes	-
19	c.5266dupC	p.(Gln1756Profs)	397507246	18	Yes	Yes	Yes	Yes	-
IVS20	c.5333-1 G > A	Splicing	80358126	1	-	-	-	Yes	-
IVS22	c.5467 + 1G>A	Splicing	80358145	1	Yes	-	Yes	Yes	-
22	c.5467G>A	p.(Ala1823Thr)	80357212	3	Yes-	-	-	Yes	-
23	c.5497G>A	p.(Val1833Met)	80357268	1	Yes	-	-	Yes	-
23	c.5492delC	p.(Pro1831Leufs)	80357582	1	-	-	Yes	Yes	-
23	c.5503C>T	p.(Arg1835*)	41293465	2	Yes	Yes	Yes	Yes	-

As per human mutation nomenclature guidelines the asterisk signifies a STOP codon.

[a] Submitted to ClinVar, Organization ID: 505504.

Table 2 *BRCA2* mutations in the analyzed population

					Clinical Significance				
Exon/Intron	HGVS cDNA	HGVS Protein	dbSNP	No. of Carriers	BIC	UMD	ARUP	ClinVar	Additional Information
2	c.37G>T	p.(Glu13*)	80358622	1	Yes	-	Yes	Yes	-
8	c.658_659delGT	p.(Val220Ilefs)	80359604	2	Yes	Yes	Yes	Yes	-
IVS 8	c.682-1G>T	Splicing	-	1	-	-	-	-	Novel[a]
10	c.1057delT	p.(Ser353fs)	-	1	-	-	-	-	Novel[a]
10	c.1117C>T	p.(Gln373*)	397507572	1	Yes	-	Yes	Yes	-
10	c.1821dupA	p.(Asp608Argfs)	-	1	-	-	-	-	Novel[a]
11	c.2339C>G	p.(Ser780*)	587781471	4	-	-	-	Yes	-
11	c.2808_2811delTAAA	p.(Ala938Profs)	80359350	1	Yes	Yes	Yes	Yes	-
11	c.3554_3563delCAGTTGAAAT	p.(Thr1185Ilefs)	397507675	1	-	-	Yes	Yes	-
11	c.4769delA	p.(Lys1590Serfs)	-	1	-	-	-	-	Novel[a]
11	c.5206C>T	p.(Gln1736*)	-	1	-	-	-	-	Novel[a]
11	c.5681dupA	p.(Tyr1894Terfs)	80359527	1	Yes	-	Yes	Yes	-
11	c.6466_6469delTCTC	p.(Ser2156_Gln2157Asnfs)	879255330	1	-	Yes	-	Yes	-
11	c.6490delC	p.(Gln2164Serfs)	80359599	1	Yes	-	-	Yes	-
13	c.6941delC	p.(Thr2314Lysfs)	80359628	1	Yes	-	Yes	Yes	-
13	c.7007G>A	p.(Arg2336His)	28897743	1	Yes	Yes	Yes	Yes	-
IVS 16	c.7805 + 1G>A	Splicing	81002809	1	Yes	-	-	Yes	-
IVS 16	c.7806-2 A > T	Splicing	-	1	-	-	-	-	Novel[a]
22	c.8930delA	p.(Tyr2977Phefs)	869320799	1	-	-	-	Yes	-
23	c.9097dupA	p.(Thr3033Asnfs)	397507419	1	-	-	Yes	Yes	-
23	c.9098_9099dupA	p.(Gln3034Serfs)	80359747	1	Yes	-	-	Yes	-
24	c.9253dupA	p.(Thr3085Asnfs)	80359752	2	Yes	-	Yes	Yes	-
IVS 25	c.9501 + 1G>A	Splicing	397508058	2	-	-	-	Yes	-
25	c.9376C>T	p.(Gln3126*)	80359210	2	Yes	-	Yes	Yes	-
27	c.9680delA	p.(Ser3228Valfs)	-	1	-	-	-	-	Novel[a]

As per human mutation nomenclature guidelines the asterisk signifies a STOP codon.

[a] Submitted to ClinVar, Organization ID: 505504.

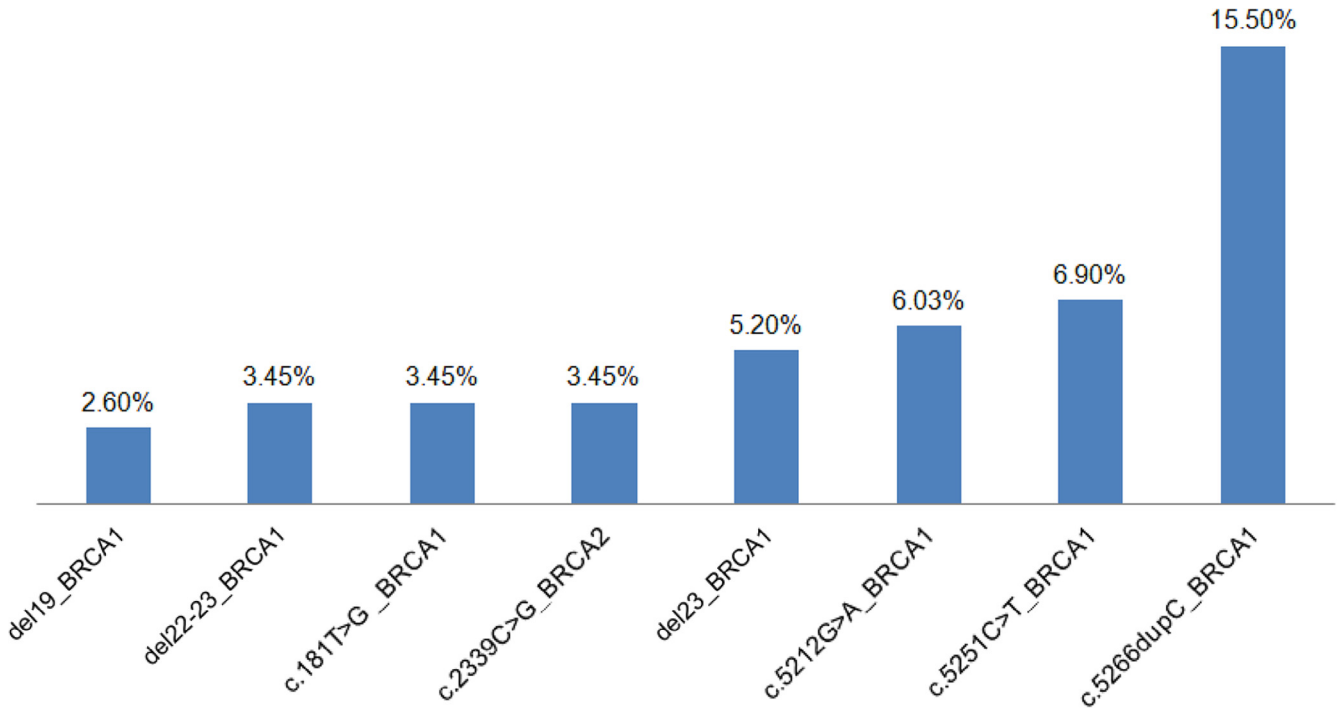


Figure 2 Most common pathogenic mutations in the *BRCA1* and *BRCA2* genes in the Greek population.

this change alters splicing of the resultant mRNA. RNA analysis demonstrated deletion of exon 9 of the *BRCA2* gene (Figure 4).

The mutation c.5333-1G>A in the *BRCA1* gene was identified in a female patient diagnosed with breast cancer at 45, as was her maternal grandmother at the age of 40. The patient's mother was diagnosed with asynchronous breast cancer at 37 and 47 years. This mutation is listed in ClinVar as "likely pathogenic" (https://www.ncbi.nlm.nih.gov/clinvar/variation/55533/). RNA analysis was performed for the better classification of this mutation.

RT-PCR analysis revealed two products, differing by 74 bp (Figure 5A). Sequencing analysis performed on the abnormal transcript demonstrated deletion of exon 21 of the *BRCA1* gene (Figure 5B). This results in a novel amino acid sequence after codon 1778 and the creation of a protein product lacking the last 35 amino acids which are part of the BRCT2 domain.

Table 3 Novel mutations identified in the Greek population

Gene	HGVS cDNA	HGVS Protein
BRCA1	c.65T>A	p.(Leu22*)
BRCA1	c.1154G>A	p.(Trp385*)
BRCA1	c.2933dupA	p.(Tyr978*)
BRCA1	c.3132delT	p.(Asn1045Metfs)
BRCA2	c.682-1G>T	Splicing
BRCA2	c.1057delT	p.(Ser353fs)
BRCA2	c.1821dupA	p.(Asp608Argfs)
BRCA2	c.4769delA	p.(Lys1590Serfs)
BRCA2	c.5206C>T	p.(Gln1736*)
BRCA2	c.7806-2 A > T	Splicing
BRCA2	c.9680delA	p.(Ser3228Valfs)

As per human mutation nomenclature guidelines the asterisk signifies a STOP codon.

Table 4 LGRs in the *BRCA1* and *BRCA2* genes

Gene	Exon Deletion	No. of Times an LGR Was Observed	% of Total Mutations
BRCA1	del19	3/17 (17.64%)	2.6%
BRCA1	del22	1/17 (5.88%)	0.9%
BRCA1	del23	6/17 (35.29%)	5.2%
BRCA1	del22–23	4/17 (23.52%)	3.4%
BRCA1	entire gene	1/17 (5.88%)	0.9%
BRCA2	entire gene	1/17 (5.88%)	0.9%
BRCA2	del14	1/17 (5.88%)	0.9%

Variants of uncertain significance (VUS)

In total 23 variants of uncertain clinical significance were identified in our study population in the *BRCA1* and *BRCA2* genes: 5 (21.7 %) VUS in *BRCA1* and 18 (78.3 %) VUS in *BRCA2* (Table 5).

Of those 19 were missense variants, 3 were synonymous changes which may affect splicing and 1 was a single base change at position +3 of intron 6 of the *BRCA1* gene which may also affect splicing.

In silico analysis showed that 6 of the missense variants (c.1510C>T p.(Arg504Cys) in *BRCA1*, and c.7481G>A p.(Arg2494Gln), c.7975A>G p.(Arg2659Gly), c.8360G>A p.(Arg2787His), c.9019A>G p.(Arg3007Gly), c.9104A>G p.(Tyr3035Cys) in *BRCA2* could have a damaging effect on the function of the corresponding protein.

The silent substitutions c.1881C>G p.(Val627Val) in *BRCA1* and c.963A>G p.(Ser694Ser) in *BRCA2* were *in silico* predicted to affect mRNA splicing through the creation of an exonic ESS site or the activation of an exonic cryptic acceptor site,

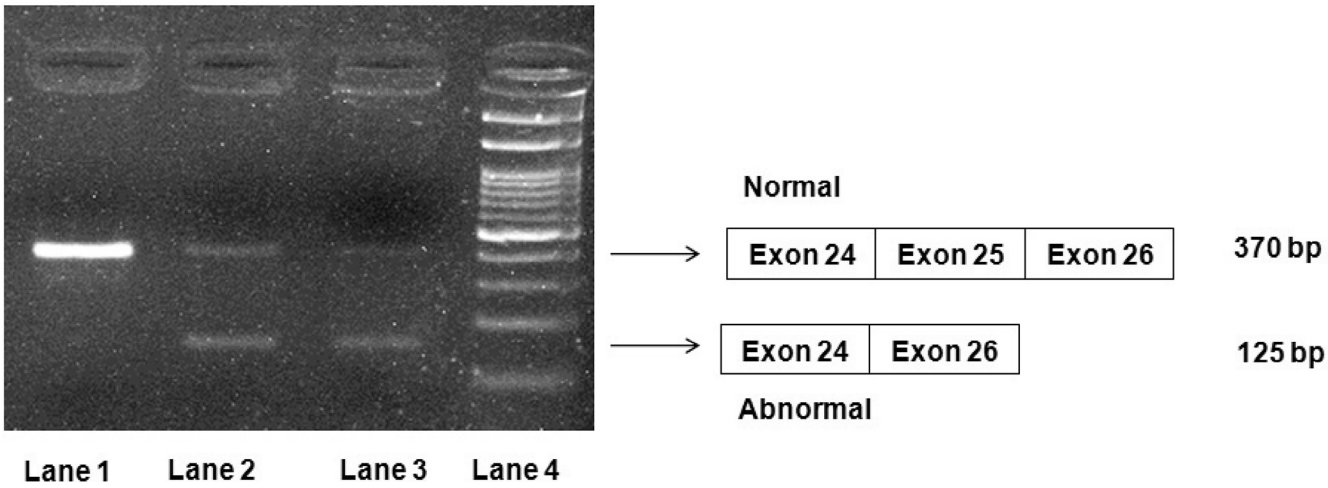


Figure 3 The normal splice variant (exons 24, 25, 26) and the abnormal variant missing exon 25 (exons 24, 26) of the *BRCA2* gene. RT-PCR products on 3% agarose gel are indicated by horizontal arrows. Lane 1: normal sample, Lanes 2 and 3: patient sample and Lane 4: 100 bp DNA Ladder (New England Biolabs). The 125 bp abnormal product is quite evident in the patient sample.

respectively, though these predictions have not been confirmed by experimental studies.

Finally, the novel variant c.442-3T>G was identified in intron 6 of the *BRCA1* gene in a patient diagnosed with breast cancer at the age of 30. This sequence change occurs at a position which is conserved in the human and other genomes and is involved in mRNA processing. This variant has not been reported in the literature or mutation databases (8,9). Algorithms developed to predict the effect of single base changes on mRNA splicing suggest that there is a high probability that this variant abolishes the function of the acceptor site, thus being pathogenic, but this prediction has not been proven by mRNA analysis.

Discussion

Regardless of heritability, *BRCA* genetic testing is one of the most common assays carried out to guide the clinical management of women suffering from breast and/or ovarian cancer. Knowledge of the *BRCA* mutational status of a breast/ovarian cancer patient can dramatically change clinical management and as a result the disease-free expectancy of the individual. As such, many national and international working groups have put forward Clinical Management Guidelines both in terms of surgical and chemotherapeutic approaches for the prevention and therapeutic management of breast/ovarian

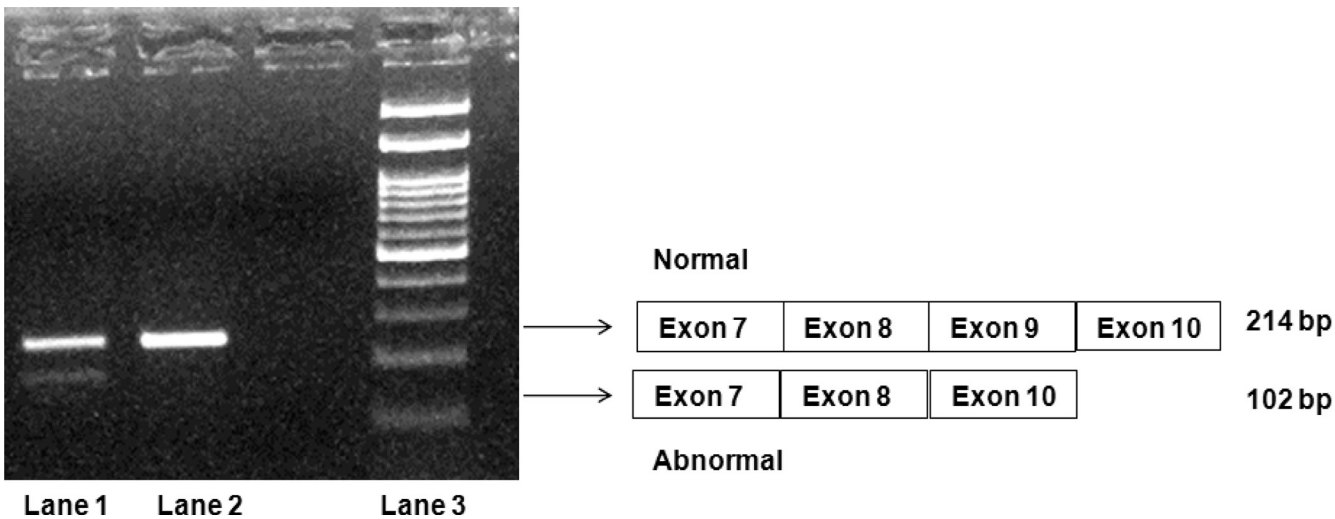


Figure 4 The normal splice variant (exons 7, 8, 9, 10) and the abnormal variant missing exon 9 (exons 7, 8, 10) of the *BRCA2* gene. RT-PCR products on 3% agarose gel are indicated by horizontal arrows. Lane 1: patient sample, Lane 2: normal sample and Lane 3: 100 bp DNA Ladder (New England Biolabs). The 102 bp abnormal product is quite evident in the patient sample.

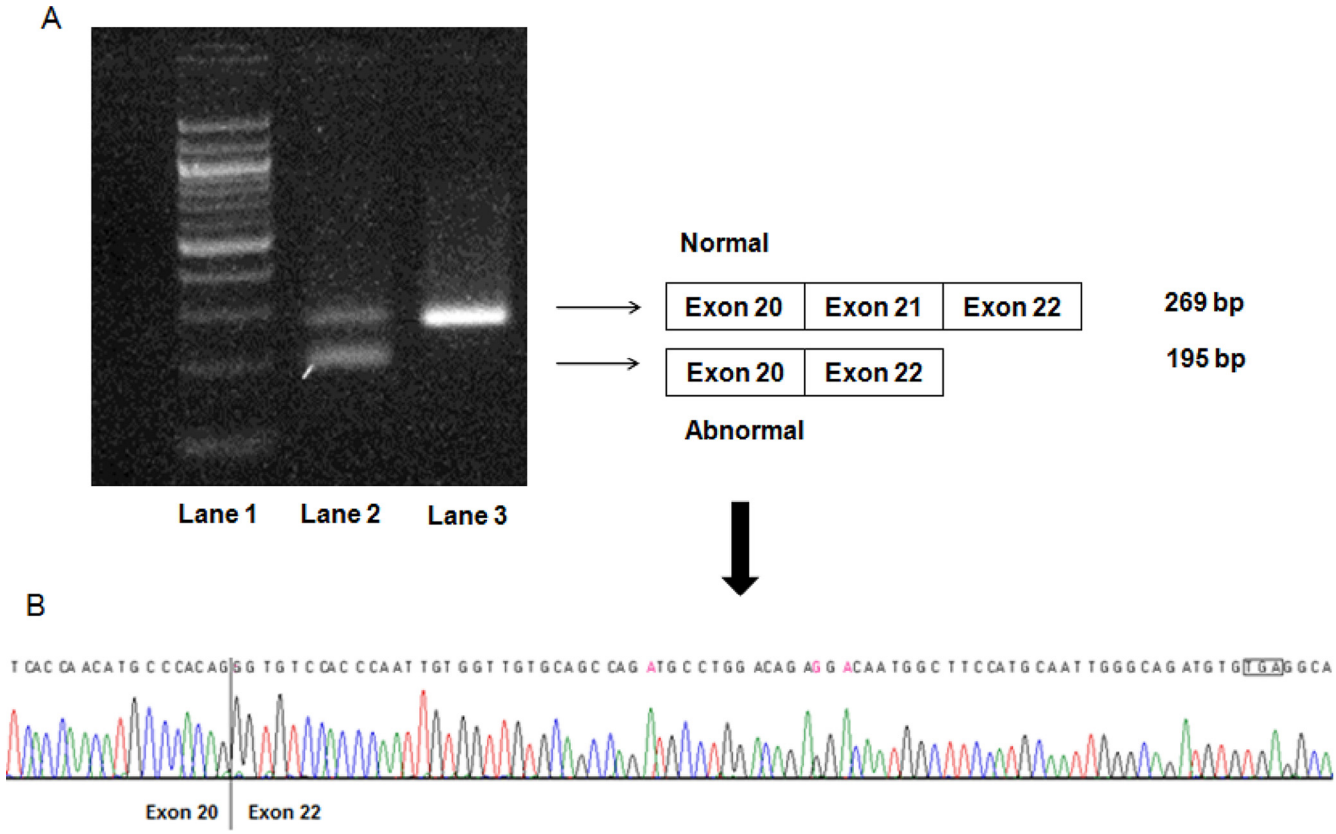


Figure 5 (A) The normal splice variant (exons 20, 21, 22) and the abnormal variant missing exon 21 (exons 20, 22) of the *BRCA1* gene. RT-PCR products on 3% agarose gel are indicated by horizontal arrows. Lane 1: 100 bp DNA Ladder (New England Biolabs), Lane 2: patient sample and Lane 3: normal sample. The 195 bp abnormal product is quite evident in the patient sample. (B) Chromatogram showing sequence of the abnormal transcript. Vertical line in chromatogram indicates the abnormal junction between exons 20 and 22 while the box indicates the STOP signal created by the frameshift.

cancer, based on *BRCA* mutation status [www.nccn.org]. More recently, the European Medical Agency has given declarative approval for the administration of PARP-1 inhibitors, conditioned to the presence of *BRCA* germline or somatic mutations. In this aspect, the need for rapid and sensitive testing of patients and their families is crucial.

Mutations leading to inactivation of the two genes have been shown to be evenly distributed throughout the large coding regions of both *BRCA* genes (8,9). DNA sequencing is used in order to characterize the mutations, traditionally using the Sanger method and more recently Next Generation Sequencing (NGS) (11–14). While in the early studies for mutation detection only single point or small insertion/deletion mutations were screened for, recent studies have shown that genomic rearrangements are also a common type of mutation in the two genes accounting for 10%–30% of all mutations identified in some populations (15–18).

Molecular diagnostic approaches must guarantee high-quality molecular testing, capable of detecting all types of mutations which may result in the deactivation of the *BRCA* genes. About 10%–36% of all *BRCA* mutations identified in some populations (13–20) are estimated to be LGRs, which cannot be easily detected by full gene sequencing. In this aspect, every comprehensive *BRCA* analysis offered must include a method such as quantitative multiplex PCR of short fluorescent fragments (28) or MLPA (29), capable of detecting this type of mutation.

In this study, we describe the application of available technologies in the molecular characterization of mutations in the *BRCA* genes. Analysis was initially carried out by conventional Sanger sequencing followed by MLPA analysis of *BRCA* mutation-negative patients. In the last 4 years, our group has made the transition from Sanger sequencing to NGS using the Illumina MiSeq Platform. This transition has allowed faster analysis of the two genes at a much lower cost. As a result, the inclusion criteria of patients/families analyzed by our group have become less stringent. In this respect, the results depicted in this study are more representative of the mutational spectrum and frequency in the Greek population, since many of the subjects included in the analysis do not meet inclusion criteria suggested by international working groups (www.nccn.org).

In total, a pathogenic mutation was identified in 12.9% of this unselected Greek population. This correlates well with other similar studies which report frequencies of 10%–16% (34–36). Of the mutations identified, the majority (70%) were found in the *BRCA1* gene and the remaining 30% in the *BRCA2* gene. In line with previous publications we found that 4 mutations in *BRCA1* and 1 mutation in *BRCA2* (Figure 2) accounted for the majority of point mutations detected in the Greek population (34).

Table 5 VUS in *BRCA1* and *BRCA2* genes

Gene	Exon/Intron	HGVS cDNA	HGVS Protein	dbSNP	*In Silico* Prediction Impact of Mutation
BRCA1	IVS 7	c.442-3T>G	-	-	Alteration of the WT acceptor site, most probably affecting splicing
BRCA1	11	c.1510C>T	p.(Arg504Cys)	80357445	Damaging
BRCA1	11	c.1881C>G	p.(Val627Val)	80356838	Creation of an exonic ESS site. Potential alteration of splicing
BRCA1	11	c.2561C>T	p.(Ala854Val)	80357315	Tolerated
BRCA1	11	c.3588A>C	p.(Thr191Thr)	-	Tolerated
BRCA2	2	c.191C>T	p.(Thr64Ile)	397507615	Tolerated
BRCA2	10	c.963A>G	p.(Ser694Ser)	-	Activation of an exonic cryptic acceptor site, with presence of one or more cryptic branch point(s). Potential alteration of splicing.
BRCA2	10	c.1181A>C	p.(Glu394Ala)	56016241	Tolerated
BRCA2	10	c.1219C>G	p.(Gln407Glu)	781079248	Tolerated
BRCA2	10	c.1289A>G	p.(Asp430Gly)	-	Tolerated
BRCA2	11	c.2221G>A	p.(Val741Ile)	-	Tolerated
BRCA2	11	c.3404A>G	p.(Tyr1135Cys)	-	Tolerated
BRCA2	11	c.3668A>G	p.(His1223Arg)	-	Tolerated
BRCA2	11	c.6613G>A	p.(Val2205Met)	80358889	Tolerated
BRCA2	14	c.7402G>A	p.(Val2468Ile)	730881553	Tolerated
BRCA2	15	c.7481G>A	p.(Arg2494Gln)	80358973	Damaging
BRCA2	17	c.7975A>G	p.(Arg2659Gly)	80359026	Damaging
BRCA2	19	c.8360G>A	p.(Arg2787His)	80359078	Damaging
BRCA2	20	c.8576A>G	p.(Gln2859Arg)	-	Tolerated
BRCA2	21	c.8881G>A	p.(Gly2961Ser)	878853614	Tolerated
BRCA2	23	c.9019A>G	p.(Arg3007Gly)	397507417	Damaging
BRCA2	23	c.9043A>G	p.(Lys3015Glu)	587781497	Tolerated
BRCA2	23	c.9104A>G	p.(Tyr3035Cys)	80359165	Damaging

Interestingly, 11 of the 116 (9%) pathogenic variants identified were novel mutations, not previously described in the bibliography (Table 3) or in mutation databases. Four of the novel variants were located in the *BRCA1* gene, while the remaining 7 were found in the *BRCA2* gene. All of them resulted in truncated protein products and were therefore classified as "likely pathogenic" according to the classification guidelines proposed by the American College of Medical Genetics and Genomics, the Association for Molecular Pathology (30) and the ENIGMA consortium (personal communication). Of the novel mutations identified, 4 were single base changes creating a premature translational stop codon, 5 were single base deletions/insertions resulting in frameshift, while the remaining 2, both located in *BRCA2,* were single base changes in the consensus splice sites and were both shown to result in the creation of a novel mRNA transcript lacking the relevant exon (Figure 3). Analysis of mRNA from the patients carrying these splicing mutations was deemed to be necessary in order to prove without any doubt the pathogenicity of these novel variants.

Another interesting point demonstrated by our data is the frequency and diversity of large genomic rearrangements detected in our population. In total, 17 of the 116 (14.6%) *BRCA*-mutated probands were found to carry one of 7 large genomic rearrangements. In line with previous studies (37,38), deletion of exon 19, 23 and 22–23 of the *BRCA1* gene were the most common rearrangements identified and accounted for 11.2% of the mutations identified. However, MLPA analysis of our cohort revealed three additional rearrangements: deletion of the entire *BRCA1,* deletion of the entire *BRCA2* and deletion of exon 14 of *BRCA2.* These findings indicate that LGRs are an important part of the Greek *BRCA* mutation spectrum. Furthermore, if only analysis of the founder LGRs was carried out, 3,4% of the mutations would have been missed.

The reliability of LGR detection by NGS is dependent on the library preparation method and the bioinformatics pipeline. We found that when target enrichment approaches based on hybridization and capture were used in addition to the appropriate bioinformatics pipeline screening for LGR was possible, though validation of the findings using orthogonal methods such as MLPA or qPCR was still necessary. However, when the NGS library preparation is amplicon-based LGR detection has a poor specificity with current bioinformatics pipelines. Therefore, MLPA still has a role in the testing algorithm and cannot be entirely replaced by NGS.

In addition to the pathogenic variants identified in our population, 23 Variants of Uncertain Clinical Significance (VUS) were identified in our population: 5 in *BRCA1* and 18 in *BRCA2*. Of those, eight (2 in *BRCA1* and 6 in *BRCA2*) have not, to our knowledge, been previously described in mutation or population databases or in the international bibliography. VUS identification poses significant diagnostic challenges, both in terms of clinical management and surveillance, as well as in risk assessment of at-risk relatives of the probands (39–41). It is estimated that 5%–10% of *BRCA* analyses identify VUS (41). With this in mind, it is crucial to use all available tools in an attempt to classify such variants. An integrated strategy, including *in silico* and co-segregation analyses, tumor

pathology data, as well as functional assays, is needed in order to complete a comprehensive assessment of pathogenicity. In this aspect participation of any diagnostic center in large collaboration efforts such as the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles), HVP (Human Variome Project) and others are becoming essential. In line with these efforts, all our "pathogenic" and "likely pathogenic" variants have been submitted to the central mutation database ClinVar (Organization ID: 505504).

In summary, we provide a comprehensive analysis of the *BRCA1* and *BRCA2* genes in a large series of Greek breast and/or ovarian cancer patients. In our cohort, pathogenic mutations were identified in 12.9% of probands tested. Nine percent of the deleterious mutations identified are novel and 14.6% are LGRs. This highlights the fact that "hot-spot" analysis of the *BRCA* genes is not applicable to the Greek population. Instead, the methodology used in the analysis of Greek patients must be able to detect both point and small frameshift mutations in addition to large genomic rearrangements across the entire coding region of the two genes.

Conflict of interest

None.

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