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Original paper

The molecular detection of germline mutations in BRCA1 and BRCA2 genes in a small group of patients from Romania

LILIANA-GEORGIANA GRIGORE[1,2,3], ATTILA CRISTIAN RAȚIU[2,3], CONSUEL SEVERUS IONICA[3,4], LORAND SAVU[3], ILEANA STOICA[2]

[1]University of Bucharest, Faculty of Biology, Doctoral School of Biology, Bucharest, Romania
[2]University of Bucharest, Faculty of Biology, Department of Genetics, Bucharest, Romania
[3]Genetic Lab, Bucharest, Romania
[4]"F.I. Rainer" Anthropology Institute, Bucharest, Romania

Abstract

Objective: The purpose of this study was to identify and classify the spectrum of small polymorphisms found in *BRCA1* and *BRCA2* genes in breast cancer risk-assessed patients.

Methods and results: The detection of germline mutations was performed by targeted amplification of 47 samples, followed by NGS and mutation data analysis. We identified 62 short polymorphisms that were classified according to their clinical significance reported in ClinVar database: three pathogenic, four with conflicting interpretations of pathogenicity, five likely benign, 46 benign, two without clinical evaluation and two not previously reported.

Conclusions: Among the detected polymorphisms a few are shared by most patients and might be prevalent in the Romanian population, a hypothesis that requires further investigation. The detection of only three pathogenic mutations suggests the need to extend the panel of breast cancer predisposing genes. It is conceivable that a mutational screening based primarily on risk factor evaluation would detect a lower number of pathogenic mutations than in confirmed breast cancer cases. Our results contribute to the setup of a *BRCA1* and *BRCA2* mutations database for the Romanian population.

Keywords Breast cancer, *BRCA1* gene, *BRCA2* gene, polymorphisms.

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*Corresponding author: ATTILA CRISTIAN RATIU, University of Bucharest, Faculty of Biology, Department of Genetics, 1-3 Aleea Portocalilor, 060101, Bucharest, Romania
E-mail: attila.ratiu@bio.unibuc.ro

Introduction

Worldwide breast cancer is one of the leading causes of mortality for women and the second most common type of cancer in both sexes (BECKER, 2015; MEHRGOU, 2016). Breast cancer is curable when discovered in the early stages of development and if access to quality surgical and medical treatment is provided. Breast cancer challenges the healthcare systems of the world and represents a considerable threat in Romania, accounting for 11.5% (9,629 as of 2018) of the total number of new cancer cases, regardless of sex and age (GLOBOCAN, 2019).

The hereditary breast cancer accounts for up to 10% of all breast cancer cases and it is mainly triggered by germline mutations (or inherited mutations) in *BRCA1 DNA repair associated* (*BRCA1*) and *BRCA2 DNA repair associated* (*BRCA2*) genes. These germline mutations play an important role in determining the risk of developing hereditary breast cancer. Major risk factors for developing breast cancer include: personal or familial history, non-cancer breast pathology, the use of estrogen and progesterone replacement therapies, early menarche, exposure to radiation, and physical inactivity (BAYRAKTAR, 2016). Genetic testing of *BRCA1* and *BRCA2* genes is particularly recommended for women with familial susceptibility to cancer, as well as for those pertaining to Ashkenazi Jewish, French-Canadian or Polish ethnic groups, which have a high prevalence of founder mutations (YOUNG, 2009; MAJEED, 2014).

To date (https://brcaexchange.org), the BRCA Exchange data base includes 12,628 polymorphisms for *BRCA1* gene (2,189 pathogenic, 605 benign, 441 likely benign, 9,390 not yet reviewed, and 3 with uncertain significance) and 13,747 polymorphisms for *BRCA2* gene (2,637 pathogenic, 639 benign, 738 likely benign, 9,729 not yet reviewed, and 4 with uncertain significance). These short polymorphisms are located in both intronic and exonic regions, being classified as missense (substitutions) and nonsense (stop-gain/stop-loss) single nucleotide polymorphisms (SNPs), and as small range deletions and insertions (InDels). Among the *BRCA1* pathogenic mutations carriers, the life-time risk to develop breast cancer is 70-80% and 50% to develop ovarian cancer, respectively. Patients harboring *BRCA2* pathogenic mutations have an estimated life-time risk of 50-60% to develop breast cancer and 30% for ovarian cancer (ROY, 2011).

According to data available at the International Agency for Research on Cancer, World Health Organization (2018), the top three most frequent cancers in both sexes, excluding non-melanoma skin cancer, are lung, colorectal, and breast cancers. The estimated cumulative risk of incidence and mortality for women up to 74 years old were 5.8 and, respectively, 1.7. In 2018, the estimated number of prevalent cases of breast cancer in women (5-year interval) per 100,000 was 19.9 (GLOBOCAN, 2019). In Romania, a large number of patients are diagnosed only in the late stages of breast cancer development because of the absence of a national or of local screening programs, due to a low level of health education and due to a poorly implemented healthcare system. Also, there are few interdisciplinary teams who can efficiently coordinate the diagnosis and treatment of the breast cancer patients. Implementing a national screening program as well as creating a national database of mutations for various genes responsible for breast cancer will potentially lead to a shorter diagnosis time-frame. Although steps have been taken to survey specific genes and mutations associated with various cancer types in the Romanian population (e.g. ROMCAN, https://eeagrants.org/archive/2009-2014/projects/RO14-0017), the results have yet to be transferred into a national database or a research collaborative resource. Localized academia research is gathering genetic data from subsets of patients referred to regional clinics (IRIMIE, 2010; IRIMIE, 2011; ENIU, 2017).

Our study aims to identify and classify the germline mutations polymorphisms in the exonic regions of *BRCA1* and *BRCA2* genes in a group of 47 patients, according to their frequency and clinical significance (ClinVar).

Materials and Methods

Biological samples consisted in peripheral blood collected from 47 female patients (age range 23-83, 43 median) referred to Genetic Lab diagnostic center, Bucharest, Romania. The analyses were performed at Genetic Lab and all the patients completed a written informed consent.

A number of 11 patients were evaluated with elevated risk or were previously diagnosed with breast cancer: four with invasive (breast) carcinoma of no special type (NST), one with ductal carcinoma in situ (DCIS), one with invasive lobular carcinoma, one with mixed invasive ductal and lobular carcinoma of the breast, one with invasive or in situ carcinoma, one with neoplasm of uncertain behavior of the breast, one with mucinous invasive carcinoma and one cured of metastatic breast cancer after completing treatment.

Five tested patients had family history of breast, tonsil, bone and other types of cancers. Two other subjects had no symptoms of breast cancer or recorded family history of cancer. Regarding the remaining 29 patients, we didn't have access to any clinical evaluation data (patients with not available clinical evaluation data – NACED patients).

Genomic DNA isolation was performed using the Favor Prep™ Blood DNA Extraction Mini Kit (FAVORGEN) following the manufacturer's protocol. The concentration of DNA elution was quantified using a Life Science UV/Vis spectrophotometer (Beckman Coulter, USA) and stored at -20ºC. Targeted amplification of exonic regions of *BRCA1* and *BRCA2* genes was

performed using two alternative approaches, dedicated for germline mutations identification: BRCA Mastr Dx kit (Multiplicom, Agilent) and NEXTflex BRCA1 & BRCA2 Plus-1 Amplicon Panel kit (Bioo Scientific, PerkinElmer). A total number of 32 samples were analyzed using BRCA Mastr Dx kit, while for the other 15 samples the NEXTflex procedure was employed. None of the DNA samples was processed using both approaches. Regardless of the chosen methodology, the quantity of DNA template/reaction was approximatively 35 ng. Specific amplicon libraries were obtained following the manufacturers' protocols. Before compiling the final targeted sequencing libraries, the concentrations of individual amplicon libraries were quantified using the High Sensitivity Qubit quantification kit (Life Technologies, Thermo Fisher Scientific, USA). Each final targeted sequencing library was diluted to a final working concentration of 10 nM in a total volume of 5 µl. For spike-in, we used a small amount (21 µl) of a PhiX solution of 20 pM. Paired-reads (500 cycles in total) were generated using the Illumina MiSeq sequencing platform with cMiSeq Reagent Nano Kit v2 (Illumina, San Diego, CA), according to the manufacturer's instructions. After the sequencing was completed and the adapter sequences were trimmed, a specific FASTQ file was generated for each patient.

Our dedicated bioinformatics workflow for NGS data analysis (Figure 1) included a standard procedure for reads quality assessment performed with FASTQC (ANDREWS, 2010), specific approaches in order to align the reads to the human genome assembly GRCh38 (hg38), automatic methods for the identification of small-scale DNA modifications and their classification according to the ClinVar database (version 2019.04.17). The aligning of nucleotide reads to the hg38 reference genome was performed with BWA-MEM algorithm (Li, 2013) thus generating the specific BAM files that were subsequently sorted and indexed using SAM tools (LI, 2009). The analysis followed the GATK Best Practices recommendations (VAN DER AUWERA, 2013) for clinical variant detection and filtering and was implemented in Ubuntu Linux bash on a dual e5-2620 v2 Intel Xeon workstation, using the Docker 2.0 environment and the official Broad Institute HaplotypeCaller 4 image. Resulting polymorphisms from the GATK pipeline, consisting in a VCF (Variant Call Format) file, were quality-filtered by adding the qualifier "PASS" or "FAIL" for each variant found, in order to remove sequencing errors and algorithm alignment biases. Criteria for quality passing strictly followed the "hard-filtering" GATK guideline. Good quality polymorphisms were imported in VarAFT v.2.15 software (DESVIGNES, 2018) and annotated regarding clinical significance and population frequency. Additionally, each variant in the raw VCF file, PASS or FAIL, was analyzed individually to avoid potential wrong categorization, and was further inspected visually in IGV software (ROBINSON, 2011). Finally, per-patient good-quality polymorphisms were exported in Excel for further analysis.

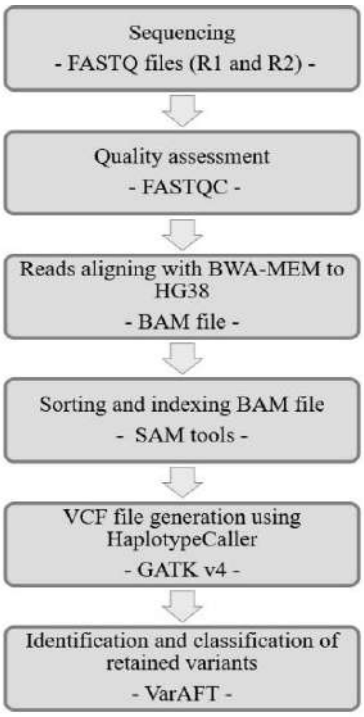

Figure 1. The workflow of data analysis. For quality assessment of the paired-end DNA sequencing reads (R1 and R2) we used FASTQC. BWA-MEM algorithm and SAM tools were employed for generating, sorting and indexing the BAM files. Haplotype Caller (GATK v4.0.1) was used for base quality recalibration, variant quality assessment and variant extraction. The detected polymorphisms were exported in VarAFT for annotation with clinical attributes and the final selection was exported in Excel.

Results

The number of polymorphisms identified for each patient ranged from 11 to 35 (when the Multiplicom kit was used) and from 5 to 23 (with the Bioo Scientific kit). Globally, we identified 62 distinct mutations that were classified according to ClinVar as pathogenic (3), benign (46), likely benign (5), polymorphisms without available clinical evaluation (2), polymorphisms with conflicting interpretations of pathogenicity (4); two polymorphisms were not previously reported. Every variant was located within the amplification genomic ranges indicated by the .bed files provided by the two kits used in the sequencing procedures. Three DNA polymorphisms were found in all of the patients included in our study, namely rs169547 (a missense variant classified as benign), rs206075 (synonymous variant – benign) and rs206076 (synonymous variant – benign). These polymorphisms are located within the exonic regions of *BRCA2* gene and correspond to the major alleles in the population, according to gnomAD. Regarding the remaining 59 polymorphisms, 43 were found in at least two patients, while 16 were found in single patients.

In *BRCA1* gene, we identified 25 distinct polymorphisms distributed as it follows: eight in exonic regions, nine in introns, seven within exon/intron junctions and one in intronic/upstream. Regarding their clinical significance, the 25 polymorphisms were classified as: pathogenic (rs80357906 and rs80357919), without clinical evaluation (rs72434991), likely benign (rs766938984), and benign (21 polymorphisms) (Table 1).

Table 1. The polymorphisms in ***BRCA1*** gene found in our group study. *HGVS nomenclature (DNA polymorphisms are numerated according to NCBI reference sequence); C.I.P – Conflicting interpretations of pathogenicity. For the last four columns the numbers represent relative frequencies.

HGVS nomenclature*	The rs code	Ref	Alt	Gene position	Protein change	Consequence	ClinVar	ExAC	gnomAD_ exome ALL	gnomAD_ genome	This study
c.999T>C	rs1060915	A	G	exonic	S1436S	synonymous variant	benign	0.3431	0.3493	0.3011	0.47
c.3113A>G	rs16941	T	C	exonic/intronic	E1038G	missense variant	benign	0.34287	0.349	0.3004	0.47
c.4837A>G	rs1799966	T	C	exonic	S1613G	missense variant	benign	0.3496	0.3546	0.3179	0.47
c.-19-115T>C	rs3765640	A	G	intronic; upstream	-	intron variant NBR2: 2KB upstream variant	benign	-	-	0.3166	0.47
c.787+1524T>C	rs16940	A	G	exonic/intronic	L771L	synonymous variant	benign	0.34196	0.3482	0.2998	0.45
c.787+1295C>T	rs1799949	G	A	exonic/intronic	S694S	synonymous variant	benign	0.34826	0.3533	0.316	0.45
c.5152+66G>A	rs3092994	C	T	intronic	-	intron variant	benign	-	-	0.3135	0.45
c.2612C>T	rs799917	G	A	exonic/intronic	P871L	missense variant	benign	0.41005	0.4032	0.4855	0.45
c.442-34C>T	rs799923	G	A	intronic	-	intron variant	benign	0.17379	0.1738	0.1545	0.45
c.407-58del	rs8176144	A	-	intronic	-	intron variant	benign	-	-	0.3005	0.45
c.3548A>G	rs16942	T	C	exonic/intronic	K1183R	missense variant	benign	0.349	0.3534	0.315	0.43
c.4485-63C>G	rs273900734	G	C	intronic	-	intron variant	benign	-	-	0.316	0.38
c.4097-141A>C	rs799916	T	G	intronic	-	intron variant	benign	-	-	0.4502	0.38
c.4987-92A>G	rs8176233	T	C	intronic	-	intron variant	benign	-	-	0.3123	0.38
c.4987-68A>G	rs8176234	T	C	intronic	-	intron variant	benign	-	-	0.3143	0.38
c.1067A>G	rs1799950	T	C	exonic/intronic	Q356R	missense variant	benign	0.04407	0.0467	0.0515	0.19
c.1644G>A	rs1799967	C	T	exonic	M1652I	missense variant	benign	0.01762	0.0182	0.0196	0.15
g.43104068_ 43104069del	rs72434991	A	-	intronic	-	intron variant	no clinical evaluation	-	-	-	0.09
c.2077G>A	rs4986850	C	T	exonic	D693N	missense variant	benign	0.05681	0.0587	0.054	0.09
c.5266dupC	rs80357906	-	G	exonic	Q1756fs	frameshift	pathogenic	0.00016	0.0002	0.0002	0.06
c.4875A>G	rs28897693	T	C	exonic	Q1604Q	synonymous variant	benign	0.00128	0.0011	0.0006	0.02
c.5265+24_5265+27del	rs766938984	CT CT	-	intronic	-	intron variant	likely benign	0.00003	3.59E-05	9.56E-05	0.02
c.2521C>T	rs1800709	G	A	exonic	R841W	missense variant	benign	0.00172	0.0017	0.0011	0.02
c.788-1380G>A	rs4986852	C	T	exonic	S1040N	missense variant	benign	0.01317	0.0132	0.0112	0.02
c.843_846del	rs80357919	TG AG	-	exonic/intronic	S282fs	frameshift	pathogenic	-	-	-	0.02

In *BRCA2* gene, we identified 37 distinct polymorphisms distributed as it follows: 23 in exonic regions, 12 in introns, one in the 3' UTR region and one in the 5' UTR region. Regarding their clinical significance, the 37 polymorphisms were classified as pathogenicity (rs276174878, rs76584943, rs730881599 and rs80358889), without clinical evaluation (rs77863879), likely benign (rs398122616, rs758307938, rs148607710 and rs750651726), and benign (25 polymorphisms) (Table 2). The c.503_504insCCA and c.1073T>C mutations were not previously reported.

Table 2. The polymorphisms in ***BRCA2*** gene found in our group study. *HGVS nomenclature (DNA polymorphisms are numerated according to NCBI reference sequence); C.I.P – Conflicting interpretations of pathogenicity. For the last four columns the numbers represent relative frequencies.

HGVS nomenclature*	The rs code	Ref	Alt	Gene position	Protein change	Consequence	ClinVar	ExAC	gnomAD_ exome ALL	gnomAD_ genome	This study
c.7397T>C	rs169547	T	C	exonic	V2466A	missense variant	benign	0.99372	0.995	0.9819	1.00
c.4563A>G	rs206075	A	G	exonic	L1521L	synonymous variant	benign	0.9931	0.9945	0.9799	1.00
c.6513G>C	rs206076	G	C	exonic	V2171V	synonymous variant	benign	0.993	0.9943	0.9798	1.00
c.7806-14T>C	rs9534262	T	C	intronic	-	intron variant	benign	0.5208	0.5224	0.547	0.74
g.32330812G>T	rs77863879	G	T	intronic	-	intron variant	no clinical evaluation	-	-	0.0042	0.68
c.68-7del	rs276174878	T	-	intronic	-	intron variant	C.I.P.	-	-	-	0.68
c.6938-120T>C	rs206080	T	C	intronic	-	intron variant	benign	-	-	0.9798	0.64
c.8755-66T>C	rs4942486	T	C	intronic	-	intron variant	benign	-	-	0.5258	0.53
c.3396A>G	rs1801406	A	G	exonic	K1132K	synonymous variant	benign	0.29449	0.2947	0.2984	0.51
c.3807T>C	rs543304	T	C	exonic	V1269V	synonymous variant	benign	0.18984	0.1747	0.1816	0.51
c.7008-62A>G	rs76584943	A	G	intronic	-	intron variant	C.I.P.	-	-	0.0034	0.45
c.7242A>G	rs1799955	A	G	exonic	S2414S	synonymous variant	benign	0.2244	0.225	0.2299	0.43
c.*105A>C	rs15869	A	C	3UTR	-	3 Prime UTR variant	benign	-	-	0.1563	0.40
c.-26G>A	rs1799943	G	A	5UTR	-	5 Prime UTR variant	benign	0.2465	0.2455	0.2208	0.40
c.631+183T>A	rs3752451	T	A	intronic	-	intron variant	benign	-	-	0.3386	0.40
c.1114A>C	rs144848	A	C	exonic	N372H	missense variant	benign	0.27793	0.2795	0.2218	0.38
c.6841+80_ 6841+83del	rs11571661	AA TT	-	intronic	-	intron variant	benign	-	-	0.2926	0.34
c.681+56C>T	rs2126042	C	T	intronic	-	intron variant	benign	-	-	0.2161	0.28
c.7435+53C>T	rs11147489	C	T	intronic	-	intron variant	benign	-	-	0.0303	0.06
c.5744C>T	rs4987117	C	T	exonic	T1915M	missense variant	benign	0.0179	0.0177	0.02	0.06
c.2971A>G	rs1799944	A	G	exonic	N991D	missense variant	benign	0.05341	0.054	0.0375	0.06
c.1365A>G	rs1801439	A	G	exonic	S455S	synonymous variant	benign	0.05178	0.0522	0.0306	0.06
c.2229T>C	rs1801499	T	C	exonic	H743H	synonymous variant	benign	0.05158	0.0526	0.0308	0.06
c.426-89T>C	rs3783265	T	C	intronic	-	intron variant	benign	-	-	0.0307	0.06
c.865A>C	rs766173	A	C	exonic	N289H	missense variant	benign	0.05178	0.052	0.0307	0.06
c.425+67A>C	rs11571610	A	C	intronic	-	intron variant	benign	-	-	0.0307	0.04
c.4258G>T	rs28897727	G	T	exonic	D1420Y	missense variant	benign	0.0068	0.0066	0.0081	0.02
c.7544C>T	rs28897744	C	T	exonic	T2515I	missense variant	benign	0.00075	0.0006	0.0007	0.02
c.9564T>C	rs398122616	T	C	exonic	D3188D	synonymous variant	likely benign	8.237E-06	4.06E-06	-	0.02
c.10093_10094 insTGAATTA TA	rs758307938	-	TGAA TTAT A	exonic	V3365del insVNYI	non-frameshift insertion	likely benign	0.00035	0.0002	0.0006	0.02
c.1167G>A	rs148607710	G	A	exonic	P389P	synonymous variant	likely benign	0.00005	5.20E-05	0.0002	0.02
c.6264T>C	rs750651726	T	C	exonic	T2088T	synonymous variant	likely benign	0.00007	6.25E-05	-	0.02
c.10095_10096 insT	rs730881599	-	T	exonic	V3365fs	stop gained	C.I.P.	0.00035	0.0002	0.0007	0.02
c.6613G>A	rs80358889	G	A	exonic	V2205M	missense variant	C.I.P.	0.00004	2.08E-05	-	0.02
c.9371A>T	rs28897759	A	T	exonic	N3124I	missense variant	pathogenic/likely pathogenic	8.248E-06	8.13E-06	-	0.02
c.503_504 insCCA	-	-	CCA	exonic	P168del insPQ	non-frameshift insertion	not reported	-	-	-	0.02
c.1073T>C	-	T	C	exonic	V358A	missense variant	not reported	-	-	-	0.02

We were also interested to ascertain the similarities and differences between the sequencing methodologies. First, we confirmed that the extended exonic regions of *BRCA1* and *BRCA2* genes targeted by the two alternative approaches are mostly overlapped. Consecutively, we were able to identify differences regarding the coverage, which is higher using the Multiplicom procedure, with an average value of 5000X, as opposed to the coverage of 3500X achievable with Bioo Scientific approach. Concerning the specificity, we noticed that the Bioo Scientific performance allows the generation of significantly more off-target reads compared with Multiplicom methodology.

The exonic DNA polymorphisms distribution inferred from our samples is similar with the known distribution of pathogenic and uncertain clinical significance mutations within *BRCA1* and *BRCA2* genes provided by the BRCA Mutation Database. More precisely, in *BRCA1* the most prominent mutational load was scored for exon 10, while in *BRCA2*, exons 10 and 11 were the most affected by mutations. This distribution could be indicative for the existence of putative mutational hot spots and maybe could highlight exons that encode for functional domains or active sites.

Discussion

The rs80357906 pathogenic variant from *BRCA1* gene is a frameshift mutation (c.5266dupC) that determines a protein truncation (p.Gln1756Profs) associated with breast and ovarian cancers. The mutation was found in three NACED patients; hence in rapport to our group of patients it has a relative frequency of 0.0638. According to different databases, there are several listed frequencies: 0.00018 (gnomAD Exomes), 0.00016 (gnomAD Genomes), and 0.00002 (TopMed). This mutation is considered to be a founder mutation in Ashkenazi Jews population, where it has a frequency of 0.0024, but it is also very common in non-Jewish Eastern European populations (FINKELMAN, 2012). The Eastern European countries in which the frequency of this mutation is particularly high are Poland (BROZEK, 2011; WOJCIK, 2016), Latvia (TIKHOMIROVA, 2005; PLAKHINS, 2011), Belarus (UGLANITSA, 2010), Lithuania (ELSAKOV, 2010), Russia (SOKOLENKO, 2006), Slovenia (STEGEL, 2011), Ukraine (GORODETSKA, 2015), and Czechia (MACHACKOVA, 2008). The mutation was also reported in Asian populations, mostly in Indians (CHAKRABORTY, 2013) and Turks (YAZICi, 2000). The estimated life-time risk for developing breast or ovarian cancer by the age of 70 for mutation carriers is 89%, and, respectively, 42% (FINKELMAN, 2012). Various studies have also found rs80357906 in Romanian population (BURCOS, 2013; Eniu, 2017; GOIDESCU, 2018).

The rs80357919 pathogenic mutation consists of a TGAG deletion in the 10th exon of *BRCA1* gene (c.843_846del) that determines a frameshift in genetic translation (p.Ser282fs) frequently linked with breast and ovarian cancers. Its global frequency is not known, but the relative frequency inferred from our study is 0.0212 (it was found in only one NACED patient). This mutation has been previously reported in association with the general population of Czechia (Machackova, 2008), Italy (CAPALBO, 2006), Germany (PERN, 2012; MUENDLEIN, 2015; MEISEL, 2017), USA (JANEZIC, 1999; SUSSWEIN, 2016), and Austria (MUENDLEIN, 2015).

The *BRCA1* intron variant rs72434991 consist in an adenosine deletion (g43104068_43104069del) and has no clinical evaluation on ClinVar database. The only reported frequency for this mutation (0.4213) is based on studies performed on Iranian population (AKBARI, 2017). This mutation's relative frequency in our group of patients is 0.0851 (4 NACED patients).

The rs28897759 pathogenic / likely pathogenic variant from *BRCA2* gene denotes a missense mutation (c.9371A>T) that determines a drastic modification of the protein conformation (p.Asn3124Ile). This mutation is associated with breast and ovarian cancer. Its relative frequency among the patients tested in our study is 0.0212, being found in one patient with family history of cancer. According to several databases, rs28897759 has the following global frequencies: 8.13E-06 (gnomAD Exome) and 0.00001 (TopMed). This mutation could lead to reduced homology-directed repair activity in *BRCA2* gene and was revealed in Germans (SUROWY, 2014) and as a recurrent pathogenic variant in the Polish population (KWIATKOWSKA, 2001; GUIDUGLI, 2013; WOJCIK, 2016). It was also identified in studies focused on Romanians (ENIU, 2017; GOIDESCU, 2018).

Two intron polymorphisms from *BRCA2* gene, rs276174878 and rs77863879, had a particularly high frequency in our group of patients, namely 0.6808. They were found in 32 patients, more specific in 18 NACED patients, 8 with elevated risk or previously diagnosed with breast cancer, four with family history of cancer, and two without known family history of cancer. The high frequency and non-discriminatory occurrence in the patients indicate that the polymorphisms might be common for Romanian population. It is worth mention that they were identified exclusively when employing the Multiplicom procedure.

The rs276174878 is a deletion mutation (c.68-7del) that has a conflicting interpretation of pathogenicity, i.e. two studies evaluated it as a benign mutation and two as with uncertain significance. The only reported frequency for this mutation (0.004023) is based on studies on Iranian population (AKBARI, 2017). The rs77863879 variant involves a substitution of guanosine to thymidine (g.32330812G>T) and has no clinical evaluation on ClinVar. According to several databases, its global frequency has the following frequencies: 0.0044 (gnomAD) and 0.00504 (TopMed).

Similarly, the rs76584943 intronic variant from *BRCA2* gene, which is a substitution mutation (c.7008-62A>G), was present in a mixed group of 20 patients with both high risk and unknown associations with breast cancer pathology. It was identified with Multiplicom procedure and could be a common polymorphism in Romanian population. Rs76584943 has a conflicting

interpretation of pathogenicity and its global frequencies are reported as following: 0.0033 (gnomAD) and 0.00511 (TopMed). This mutation was also reported by a study concerning the occurrences of *BRCA1* and *BRCA2* mutations in French families exhibiting hereditary breast and ovarian cancer (CAPUTO, 2012).

The exonic variant rs730881599 found in *BRCA2* gene is a frameshift mutation (c.10095_10096insT) that determines a premature Stop gain (p.Ser3366Ter). Its relative frequency in our group is 0.0212 (one NACED patient) and has a conflicting interpretation of pathogenicity: two studies evaluated it as a likely benign mutation and one study as with uncertain significance. According to several databases, its global frequency has the following frequencies: 0.00035 (ExAC), 0.00021 (gnomAD) and 0.0007 (gnomAD).

The exonic variant rs80358889 from *BRCA2* gene is a transition mutation (c.6613G>A) that changes the Valine from position 2,205 (p.Val2205Met). It has a conflicting interpretation of pathogenicity, since nine studies evaluated it with uncertain significance and one study evaluated it as a likely benign mutation. According to several databases, its global frequency has the following frequencies: 0.00004 (ExAC), 0.00002 (gnomAD Exome) and 0.00003 (TopMed). The relative frequency of this variant in our group is 0.0212 (one NACED patient). The mutation was also highlighted in a study performed in Spain that tested patients with confirmed breast or ovarian cancer for the detection of mutations in the *BRCA1* and *BRCA2* genes (RUIZ, 2014).

To the best of our knowledge, two of the polymorphisms identified in *BRCA2* gene, c.1073T>C (p.V358A) and c.503_504insCCA (p.P168delinsPQ) were not elsewhere reported. The first variant was identified in one NACED patient, while the second variant was found in a patient exhibiting NST invasive breast carcinoma.

A total of eight SNPs in *BRCA1* gene (rs1799967, rs4986852, rs16942, rs799917, rs16940, rs1799966, rs16941, and rs1060915) and one SNP in *BRCA2* gene (rs9534262) identified in the present study are included in the most common polymorphisms group worldwide (KARAMI, 2013). These mutations are associated with both NACED and breast cancer patients.

Some of the SNPs found in our group were also highlighted in patients from other countries: 16 SNPs found in Iranians (eight of them located in *BRCA1*, namely rs16941, rs1799966, rs799917, rs16942, rs4986850, rs1799949, rs16940, and rs1060915; the remaining ones are in *BRCA2*: rs1801406, rs543304, rs1799955, rs144848, rs1799944, rs1801439, rs1801499, and rs766173), four SNPs in *BRCA1* alleles found in Turkish (rs1799966, rs3092994, rs1799950, and rs799923), and one SNP in *BRCA2* that was reported in a study performed in India (rs1799943) (KARAMI, 2013; GEREDELI, 2019).

Several of the most common SNPs found in our study were described in another study performed on Romanian subjects (MIHALCEA, 2017). Some of these SNPs are located in *BRCA1* (rs1060915, rs16941, rs16940, rs16942, rs1799949, rs1799966, rs3765640, rs799917, and rs28897693), while others are in *BRCA2* (rs206075, rs169547, rs206076, rs144848, rs9534262, rs2126042, rs1801406, rs543304, rs4942486, rs1799955, and rs1799943). These apparently unexceptional SNPs appear to be prevalent in, and potentially specific for Romanian population, a hypothesis to be considered for further investigations.

Conclusions

Our investigation revealed a considerable number of SNPs and InDels within a limited sample of patients. The relatively high frequencies revealed for certain polymorphisms might be characteristic for the Romanian population, a hypothesis to be investigated in the future, along with the evaluation of putative founder mutations (ENIU, 2017). Furthermore, the fact that we successfully detected only three known pathogenic mutations in a group consisting of 47 samples may indicate the limitation of our approach. Addressing this later possibility, a study involving a more robust molecular diagnosis will necessarily include an extended panel of breast and ovarian cancer predisposing genes (e.g. *PALB2*, *CHEK2*, *ATM*, etc.). The results of our study also contribute to the growing data regarding *BRCA1* and *BRCA2* mutations, an addition to a much-needed genetic database for the Romanian population.

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