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Next-generation sequencing of *BRCA1* and *BRCA2* in breast cancer patients and control subjects

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Abstract. Breast cancer is currently the most common type of cancer in females. The majority of the hereditary forms of breast cancer are caused by mutations in the *BRCA1* and *BRCA2* genes, whose main function is the DNA repair of double-strand breaks. Genetic testing of females with a family history of breast cancer is recommended to determine their hereditary predisposition for this type of cancer. The variants with no clear clinical significance may represent a diagnostic challenge when performing targeted resequencing. In this study, DNA samples were obtained from 24 breast cancer patients (mean age, 35±10 years) with a positive family history and from 71 age-matched healthy controls. Informed consent was obtained from all the subjects. Sequence-targeted *BRCA1* and *BRCA2* libraries were prepared using the TruSeq Custom Amplicon method and sequenced on the Illumina MiSeq system. A wide range of variants were identified in the *BRCA1* and *BRCA2* genes. Two pathological/presumably pathological variants were detected in the breast cancer patient group: a mutation in *BRCA2* at the chromosomal (chr) position chr13:32890665, which affected the first position of the 5' splice region following exon 2; and a mutation in *BRCA1* at chr17:41219635, causing an in-frame triple nucleotide deletion of valine 1688 (8.3%). In the patient and control groups, 7 likely polymorphic variants and 13 common variants were detected in the *BRCA1* and *BRCA2* genes. To the best of our knowledge, this study was the first to identify 3 common polymorphisms in *BRCA2,* characteristic solely of the Bulgarian population, including chr13:32973737, T/-, a single-nucleotide polymorphism (SNP) within the 3'-UTR of exon 27; chr13:32973280, A/-, a mononucleotide deletion within the 5'-UTR of exon 27; and chr13:32973924, T/-, a mononucleotide deletion downstream of the gene sequence. To the best of our knowledge, this study was the first to apply next-generation sequencing of the *BRCA1* and *BRCA2* genes in a Bulgarian population, prompting further investigation for local founder mutations and variants characteristic for this particular region.

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Introduction

Breast cancer is currently the most common type of cancer in females. Approximately 5-10% of oncological cases are due to inherited genetic defects in germ cells (1). The hereditary forms of breast cancer are mainly caused by mutations in the *BRCA1* and *BRCA2* tumor-suppressor genes, resulting in the production of non-functional proteins (2,3).

Sequencing of the *BRCA1* and *BRCA2* genes is currently considered the gold standard method for determining the mutation status in breast cancer patients. Due to the high prevalence of breast cancer, *BRCA1* and *BRCA2* are currently among the most sequenced genes worldwide (4). *BRCA1* and *BRCA2* are responsible for accurate DNA repair of double-strand breaks (5-7). In addition to the compromised DNA repair function, mutations in the *BRCA1* and *BRCA2* genes are likely to affect cell cycle regulation and transcriptional activity.

However, not all *BRCA1* and *BRCA2* mutations are pathological and their impact may vary depending on the extent to which the normal protein function is compromised. Furthermore, the frequency and type of mutations may vary among different populations (http://www.breastcancerdatabase.org/).

In order to determine the frequency and type of variants in the target exon sequences of the two genes, we sequenced and analyzed *BRCA1* and *BRCA2* using next-generation sequencing (NGS) technology in Bulgarian breast cancer patients and healthy controls. To the best of our knowledge, this study was the first to assess the genetic predisposition to breast cancer in the Bulgarian population. Elucidating the effects of *BRCA1* and *BRCA2* mutations is crucial for the prevention of breast cancer.

Materials and methods

Subjects. A total of 24 Bulgarian patients (mean age, 35±10 years) diagnosed with breast cancer and with a positive family history for this disease and 71 age-matched healthy controls without a positive family history were recruited in this study. DNA samples were collected from the subjects at the National Oncological Hospital (Sofia, Bulgaria) and the *BRCA1* and *BRCA2* genes were sequenced. Written informed consent was obtained from all subjects. The study was approved by the Ethical Comitee of Specialized Hospital for Active Treatment in Oncology, Sofia, Bulgaria.

NGS analysis. The first step for NGS technology is to use the TruSeq Custom Amplicon method to design oligo probes that are specific for the target regions of *BRCA1* and *BRCA2*, using Illumina DesignStudio (Illumina, Inc., San Diego, CA, USA). For each 150-bp sequence of the target region, a pair of oligo probes were synthesized to hybridize with the 5' and 3' ends of the sequence at one end (the other end was complementary to the polymerase chain reaction primers). These oligo probes were used to construct a library containing the necessary nucleotide sequences. The target regions were determined by selecting all exons of the *BRCA1* and *BRCA2* genes; however, in order to include sections of the intron-exon regions, the regions also included 50 nucleotides upstream and downstream of the exon.

Sequencing was performed using the NGS MiSeq Illumina sequencer (Illumina, Inc.). Obtained sequences were aligned to the reference genome (GRCh37/hg19) using MiSeq Reporter software (Illumina, Inc.), which detected discrepancies determining their type, such as deletions, insertions and SNPs. The sequences were analyzed using MiSeq software. As an acceptance threshold value we selected a Q-score of 30, corresponding to a 1:1,000 error rate.

Analysis of variants. In order to determine whether a given variant was situated in a coding or non-coding region, we used the University of California, Santa Cruz (UCSC) genomic browser (http://genome.ucsc.edu/). The mutation positions were identified by determining: i) whether the mutation was situated in an intron or an exon; ii) if it was situated in an intron, whether it affected the splice acceptor or donor, or the consensus splicing sequence; and iii) if it was located in an exon, whether it resulted in an alteration of the amino acid sequence.

The established variants were cross-checked with the Breast Cancer Information Core (BIC) database (http://lgdfm3.ncifcrf.gov/bic/BIC.html), which theoretically contains all identified *BRCA1* and *BRCA2* mutations. The variants were also cross-checked in the Database of Single-Nucleotide Polymorphisms (dbSNP) in order to verify our results. In order to elucidate the effects of the different variants with no clear clinical significance, we used the PROVEAN (8), PolyPhen-2 (9) and SIFT (10) web-based platforms.

Results

NGS analysis. NGS analysis identified several types of variants, which were classified according to their potential degree of pathogenicity as follows:

Class 5 (pathological). Variants harbouring mutations of verified clinical significance. These are usually non-sense mutations (causing truncation of the protein, as a portion of the amino acid sequence is lost), frame-shift, splice (causing incorrect splicing) and pathological missense mutations, experimentally verified to exert pathological effects.

Class 4 (presumably pathological). Variants harbouring mutations that are likely to exert negative pathological effects. For example, missense mutations have been identified in breast cancer patients, although they have not been verified as disease-causing mutations.

Class 3 [variants of unknown clinical significance (VUS)]. Variants harbouring rare missense mutations and triple nucleotide in-frame insertions and deletions. This class also includes variants with mutations in the introns that are often overlooked as possible causes for cancer development (11). When deciding whether a mutation belongs to this class or whether it is a polymorphism, its conservation in among-species comparative analysis has to be considered.

Class 2 (likely polymorphic variants). Variants with no or marginal clinical significance. This class includes missense mutations that are rare, but with an observable frequency in the population.

Class 1 (common polymorphisms). Variants without clinical significance. These can be synonymous mutations, polymorphisms with high frequencies and missense variants, which were established as not exerting any pathological effects.

Pathological mutations. The only pathological mutation was identified a patient with breast cancer and early-age diagnosis. This mutation was identified in the *BRCA2* gene at the chromosomal (chr) position chr13:32890665 and affected the first position of the 5' splice region following exon 2 (Fig. 1). The consensus 5' GT sequence at the beginning of the intron was replaced by a 5' AT sequence. There are two possible outcomes in such a case: skipping exon 2 (Fig. 1A) or using an alternative cryptic donor locus (Fig. 1B).

However, exon 2 contains the start codon that initiates translation. Therefore, we cross-checked in the UniProt database and established that the next start codon was at position 124 (Me124) and embedded in exon 5. This codon serves as an initiator of the translation of the other transcript of *BRCA2* (ENST00000380152), although the latter is rarely expressed. In case exon 2 is skipped, mRNA translation may commence from this codon; however, the protein sequence will lack the first 123 amino acids, of which the first 40 are operational in the interaction with the PALB2 protein (partner and localizer of BRCA2). Single-nucleotide mutations in this region (G25R, W31C and W31R) were shown to disrupt the interaction between BRCA2 and PALB2 (12), which is a key factor for the effective repair of double-strand breaks through homologous recombination. If the cryptic splice donor locus is used, the effects may be less predictable, but will likely result in frame-shift mutations in the majority of cases. Even if the reading frame on the *BRCA2* gene remains intact, the N-terminal amino acids that are required for the interaction with PALB2 would be lost. Bonatti *et al* (13) demonstrated that this mutation indeed resulted in aberrant transcripts and a consequent full loss of function. This particular mutation,

Table I. Likely polymorphic variants in the *BRCA1* and *BRCA2* genes in patients and controls.

Gene	Position	Variant	Description
Patients			
BRCA1 (2 females)	41277354	G>A	5'-UTR variant in exon 1. The position is not conserved among mammals (PhyloP, GERP). The risk of abnormal translation of the transcript is low.
BRCA2	32889548	C>T	Variant upstream of exon 1, few bases upstream of the highly conserved region of the promoter. The base itself is not conserved among mammals (PhyloP, GERP). The risk of pathological changes in the transcription and expression of *BRCA2* is low.
Controls			
BRCA1	41246812	A>C	Leu246Val missense variant in exon 11; BIC-unknown effect, it was detected 70 times, mostly in individuals from Western Europe.
BRCA2	32973748	A>G	Variant in the 3'-UTR. The position is not conserved among mammals (PhyloP, GERP) and it is unlikely that the variant leads to a change in truncation of mRNA.
BRCA2	32973660	C>T	Variant in the 3'-UTR. The position has been moderately conserved among mammals (PhyloP, GERP). The risk for truncation of mRNA and destabilization of the transcript is low.
BRCA2	32889593	G>A	Variant upstream of exon 1, a CpG-rich region of the promoter. Optionally, binding site for transcription factors. The position is not conserved among mammals (PhyloP, GERP). The risk of abnormal expression of *BRCA2* is low.
BRCA2	32889548	C>T	Variant upstream of exon 1, few bases upstream of the highly conserved region of the promoter. The base itself is not conserved among mammals (PhyloP, GERP). The risk for pathological changes in the transcription and expression of *BRCA2* is low.

BIC, Breast Cancer Information Core; PhyloP, phylogenetic P-values; GERP, genomic evolutionary rate profiling. UTR, untranslated region.

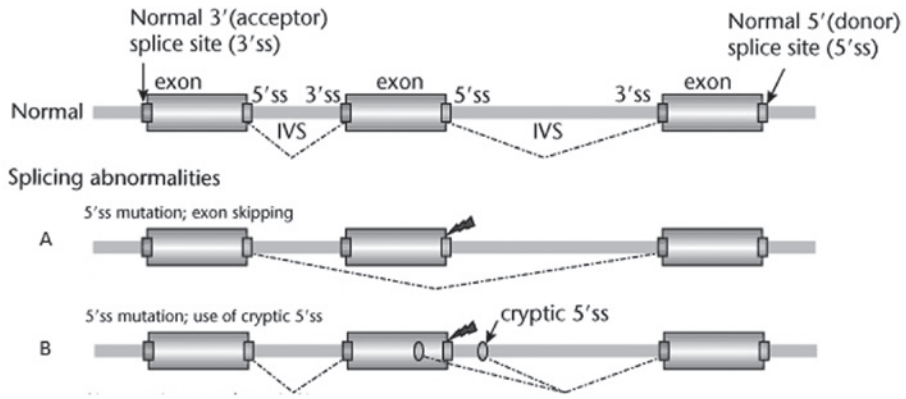

Figure 1. Mutations that may be identified in the 5' splice donor locus. (A) skipping the exon and (B) using an alternative cryptic donor locus (14).

c.67+1G>A, was also described in 5 patients, two of whom are from Western Europe (BIC database). The dbSNP identification number of the mutation is rs81002796 and it is described as 'pathological' in this database, meaning that this mutation has been verified to cause breast cancer. This finding was also confirmed by our study.

Possibly pathological mutations. This group includes mutations that are highly likely to exert a detrimental effect and have been identified in individuals with breast cancer, although without any direct disease-causing evidence. Such a mutation was detected in 1 patient in the *BRCA1* gene at position 41219635; it is an in-frame triple nucleotide deletion of valine 1688 in exon 17 and is classified in the BIC database as a mutation of unknown effect (14). The deleted amino acid is part of the BRCT 1 functional domain (1642-1736) and mutations in adjacent amino acids have been detected in patients with breast or ovarian cancer (T1685A, T1685I, M1689R, K1690Q, D1692N, C1697R and R1699W) (UniProt). Multivariate analyses predicted a pathological effect of this mutation (LOVD database) and, based on the multivariate analyses, we inferred that this mutation also exerted a pathological effect in our study.

VUS. Two VUS were detected in the control group. The first variant was located in exon 12 of *BRCA1* in position chr17:41234509, wherein the guanine was replaced by cytosine. This mutation resulted in a missense substitution at position 1423 of the protein, wherein the serine was replaced by arginine (Ser1423Arg). Serine 1423 is crucial, as this amino acid is phosphorylated by the protein kinase ataxia-telangiectasia

Table II. *BRCA1* common variants in Bulgarian females.

Start	SNP description	Total frequency (%)	Patients (%)	Controls (%)
41196408	G>A; SNP within 3'-UTR of exon 27, no effects, worldwide polymorphism	43.16	37.5	45.07
41197274	C>A; SNP within 3'-UTR of exon 24, no effects, worldwide polymorphism	43.16	37.5	45.07
41234470	A>G; Ser>Ser, synonymous SNP in exon 12, no effects, worldwide polymorphism	43.16	37.5	45.07

SNP, single-nucleotide polymorphism; UTR, untranslated region.

Table III. *BRCA2* common variants in Bulgarian females.

Start	SNP description	Total frequency (%)	Patients (%)	Controls (%)
32889792	A>G; upstream of gene sequence, within promoter sequence, binding site for transcription factors, polymorphisms observed at this site, no effects	22.11	20.83	22.54
32890572	G>A; SNP within 5'-UTR of exon 2, no effects, worldwide polymorphism	41.05	50.00	38.07
32929232	A>G; Ser>Ser, synonymous SNP in exon 14, no effects, worldwide polymorphism	34.74	33.33	35.21
32929387	T>C; Val>Ala, non-synonymous SNP in exon 14, similar (small, hydrophobic) amino acids substituted, no effects, worldwide polymorphism	97.89	91.67	100
32973276	A>G; SNP within 3'-UTR of exon 27, no effects, worldwide polymorphism	27.37	50.00	19.72
32973280	A/-, mononucleotide deletion within 5'-UTR of exon 27, polymorphism, no effects, undescribed, Bulgarian polymorphism	90.53	83.33	92.96
32973439	A>G; SNP within 3'-UTR of exon 27, no effects, worldwide polymorphism	33.68	33.33	33.80
32973737	T/-; SNP within 3'-UTR of exon 27, no effects, undescribed, Bulgarian polymorphism	78.95	95.83	73.24
32973924	T/-, mononucleotide deletion, downstream of gene sequence, no effects, undescribed, Bulgarian polymorphism	21.05	16.67	22.54
32973924	-/T, mononucleotide insertion, downstream of gene sequence, no effects	52.63	70.83	46.48

SNP, single-nucleotide polymorphism. UTR, untranslated region.

mutated (ATM) (15). Patients with this genotype have been identified worldwide and this mutation was located in the BIC database. However, such mutation variants may be generated by mutagenesis (16). It was demonstrated that, by exposing the cells to ionizing radiation, this mutation inhibited the insertion of *BRCA1* during the G2 phase and disrupted the repair of accumulated radiation-induced DNA damage. When serine 1423 is mutated, ATM cannot phosphorylate *BRCA1* and this modification is required for the activation of the G2/M checkpoint signaling pathway. Thus, the function of *BRCA1* in regulating the cell cycle is disrupted and the cell enters the M phase, despite the possible DNA damage. Furthermore, the area covering amino acids 1397-1424 is responsible for the interaction of BRCA1 with PALB2 (UniProt, 2013). The formation of the BRCA1-PALB2-BRCA2 complex is a repair mechanism for double-strand breaks by homologous recombination. PolyPhen-2 also suggested that the Ser1423Arg mutation was 'probably abnormal', while the SIFT algorithm considered the mutation as pathological and PROVEAN - as a common polymorphism.

The second VUS was detected in the *BRCA2* gene at position chr13:32930634, G>A. This is an Arg2502His missense mutation in exon 15 and the BIC database indicated 22 cases of unknown effect. The missense mutation and consequent amino acid replacement involves similar hydrophilic amino acids; however, the mutation has also been detected in patients with breast and ovarian cancer. PolyPhen-2, SIFT and PROVEAN predict a neutral effect.

Likely polymorphic variants. Likely polymorphic variants, as defined above, are presented in Table I for patients and controls.

Common polymorphic variants. The common polymorphic variants detected in our study are presented in Table II for *BRCA1* and in Table III for *BRCA2*.

Discussion

To the best of our knowledge, this study was the first to the perform *BRCA1* and *BRCA2* gene sequencing using NGS methods in 24 Bulgarian breast cancer patients with a family history of breast cancer and 71 healthy controls. A wide range of variants were detected in the *BRCA1* and *BRCA2* genes. In the patient group, we identified two pathological/presumably pathological variants, including a mutation in *BRCA2* at position chr13:32890665 that affected the first position of the 5' splice region following exon 2 and a mutation in *BRCA1* at position chr17:41219635, which was an in-frame triple nucleotide deletion of valine 1688 (8.3%).

According to a previous study, *BRCA1* and *BRCA2* mutations are responsible for 16% of breast cancer cases with a positive family history (17). We hypothesized that Bulgarian patients with a family history of breast cancer, but without verified pathological *BRCA1* and *BRCA2* mutations, may harbour mutations in other genes, including *CHEK2, PTEN, TP53, ATM, STK11, CDH1, NBS1, RAD50, BRIP1* and *PALB2* (18). Stratton and Rahman (19) classified the mutations responsible for the hereditary form of breast cancer into three categories: i) rare mutations in high-penetrance genes (*BRCA1* and *BRCA2*); ii) mutations in genes of moderate penetrance (*CHEK2, ATM, BRIP1* and *PALB2*); and iii) common mutations in a large number of low-penetrance genes. Whole-genome sequencing of patients with breast cancer and a positive family history that was not a result of mutations in the *BRCA1* or *BRCA2* genes may elucidate the genetic architecture that predisposes to the development of breast cancer. However, considering the various types of mutations, it is likely that environmental factors also modify the penetrance of this type of cancer.

In this study, we detected 2 VUS in the control group. The first variant was located in exon 12 of *BRCA1* at position chr17:41234509, wherein the guanine was replaced by cytosine, and the second was detected in *BRCA2* at position chr13:32930634, G>A, resulting in Arg2502His missense mutation in exon 15. Rare VUS mean that it is not possible to make an accurate clinical prediction. It is hypothesized that the genome of each individual contains a large number of rare missense alleles and it was estimated that 70% of these allelles may be of clinical significance (20). In such cases, co-segregation analyses are required to establish a correlation between the variant and the disease in large families.

In the patient and control groups, 7 likely polymorphic variants and 13 common variants were detected in the *BRCA1* and *BRCA2* genes. This study was the first to detect 3 common polymorphisms of *BRCA2*, characteristic solely of the Bulgarian population: position chr13:32973737, T/-, an SNP within the 3'-UTR of exon 27; position chr13:32973280, A/-, a mononucleotide deletion within the 5'-UTR of exon 27; and position chr13:32973924, T/-, a mononucleotide deletion downstream of the gene sequence.

In conclusion, the creation of a database for the type and frequency of *BRCA1* and *BRCA2* gene variants in the Bulgarian population is crucial, in order to enable accurate interpretation and genetic counseling regarding the genetic predisposition to breast cancer.

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