

## Rapid screening test of most frequent BRCA1/BRCA2 pathogenic variants in the NGS era

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The average risk of breast cancer in general Slovak population of women is 4–5% and the risk of ovarian cancer is 2%. Probability of breast/ovarian cancer development is higher in individuals carrying a causative germline DNA variant in *BRCA1* or *BRCA2* gene responsible for hereditary breast/ovarian cancer (HBOC). Although a major proportion of inherited breast/ovarian cancers are due to the mentioned causal mutations, a number of new genes have emerged. Here we describe a rapid, multiplex and comprehensive approach for the detection of pathogenic variants in *BRCA1* and *BRCA2* genes which most frequently occur in Slovak HBOC population. Analysis comprises the combination of mutation specific methods. Fluorescent PCR amplification followed by fragment analysis for detection of insertions/deletions in exon 11 of *BRCA1* gene. Second method is SNaPshot analysis for detection of the most frequent missense and ins/del variants in exons 2, 5, 13, 20 of *BRCA1* and exons 11, 23 and 25 of *BRCA2* gene. Altogether, we have analyzed 687 samples, 86 (12.5%) in group 1, which fulfilled indication criteria based on the positive family/personal history. Group 2 involved 601 (87.5%) cases, who did not meet the indication criteria and only the screening test was recommended. Using the combined approach, we have identified 47 mutated samples (6.8%), 40 in group 1 (46.5%) and 7 in group 2 (1.1%). However, the presented screening test would not provide complex results of *BRCA1/2* gene analysis, it offers testing accessible to a broader spectrum of individuals under the threshold of indication for whole gene analysis. This approach may provide valuable information even in the NGS analysis era.

*Key words: BRCA1, BRCA2, screening test, SNaPshot analysis, pathogenic variant*

Breast cancer is one of the most common cancers and one of the leading causes of cancer-related death among women [1]. It has been estimated that 10–12% of women will develop breast cancer over the course of their life [2]. The average risk of breast cancer in general Slovak population of women is 4–5% and of ovarian cancer 2% [3].

In general, the presence of breast cancer in any first-degree female relative nearly doubles the risk for an individual and the risk gradually increases with the number of affected relatives [4]. It is estimated that approximately 5–10% of all patients with breast cancer and approximately 20% of all patients with ovarian cancer exhibit a monogenic predisposition to breast and ovarian cancer [5]. Currently, more than 25 genes have been associated with familial breast and/or ovarian cancer, but still *BRCA1* and *BRCA2* are the most frequently affected ones [6]. *BRCA1* and *BRCA2* are the tumor-suppressor genes located on chromosomes 17q21 and

13q12, respectively, that encode proteins playing vital roles in securing the stability of the DNA. Presence of inactivating variant in these genes may therefore result in improper repair of a DNA damage [7]. The frequency of pathogenic variants in both genes varies among populations worldwide, being the highest in the Ashkenazi Jewish population, in which they affect 2.5% of the general female population [8, 9]. The result of genetic testing for the *BRCA* variant is important for the management of breast cancer. Individuals with hereditary or familial breast and ovarian cancer are likely to benefit from strategies including prevention, screening and targeted treatment. Lack of mutational hot spots, presence of different types of variants, genomic rearrangements, and the sheer size of the genes are major challenges in the timely and efficient identification of mutant carriers.

Here we describe a rapid, multiplex and comprehensive screening approach for the detection of most frequent

*BRCA1* and *BRCA2* variants occurring in Slovak HBOC population, which may prove to be valuable even in the era of NGS analysis. The *BRCA1/2* screening test consists of two particular methods, SNaPshot analysis of selected variants in *BRCA1/2* genes and fragment analysis targeted for the insertions/deletions (ins/del) in exon 11 of *BRCA1* gene. Currently, we are able to detect approximately 75% of all *BRCA1/2* causal variants reported in Slovak HBOC population, namely 13 variants in *BRCA1* and 4 in *BRCA2* gene.

## Patients and methods

In this retrospective study, we have analyzed **687 DNA samples** sent to the laboratory of Department of Clinical Genetics, St. Elizabeth Cancer Institute for *BRCA1/2* screening test between 2012 and 2016. All analyzed individuals signed an informed consent and based on the family history, the samples were classified into two groups: those, who met minimally one of the below indication criteria (group 1, n=86) and those who did not (group 2, n=601).

Indication criteria:

- at least 2 patients with breast/ovarian cancer and 1 case before 50 years
- at least 3 patients of breast/ovarian cancer in one lineage
- one patient with breast/ovarian cancer and 1 case of associated carcinoma, 1 case before 50 years
- bilateral breast/ovarian cancer under 50 years
- sporadic breast/ovarian cancer under 40 years
- triple negative breast cancer under 50 years
- breast cancer in male patient.

Group 1 consists of individuals indicated for complex *BRCA1/2* analysis and was comprised of 48 patients with diagnosed cancer disease and 38 healthy probands. The group of patients was composed of 38 breast cancer cases, 4 bilateral breast cancer cases, 2 ovarian cancer cases, 2 breast and ovarian cancer cases and 2 cases with other type of cancer.

Group 2 consists of individuals indicated only for the *BRCA1/2* screening testing and was represented by 487 healthy individuals and 114 patients with diagnosed cancer but not fulfilled indication criteria. This set was composed of 90 breast cancer cases, 4 ovarian cancer cases, 8 cases with breast fibroadenomas and 12 cases with other type of cancer.

**Genomic DNA** was extracted from 2 ml of peripheral blood using Genomic DNA Large Volume Whole Blood Kit (MagCore). **Multiplex PCR amplification** was performed using Kapa2G Robust HotStart Mastermix (Kapa Biosystems), mix of 8 primer pairs and 100 ng of DNA template (Table S1). Samples were subsequently analyzed by **SNaPshot analysis**, which detected point variants in exon 2 (c.68\_69del2), 5 (c.181T>G), 13 (c.4243delG), 20 (c.5266dupC) of *BRCA1* and in exon 11 (c.3076A>T, c.5645C>A), 23 (c.9098dupA) and 25 (c.9403delC) of *BRCA2* gene (Table 1). The primers for SNaPshot analysis were designed directly before or on the mutated nucleotide. SNaPshot analysis was performed

using the SNaPshot Multiplex Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations (Figure 1, part A). **Fluorescent PCR amplification and fragment analysis** was performed using Kapa2G Robust HotStart Mastermix (Kapa Biosystems), each primer and 60 ng of DNA template. Fragment analysis was performed for the detection of the most relevant ins/del variants in three regions of exon 11 of *BRCA1* gene (Table 1). Migration of the fluorescent labeled fragments was compared to the size standard and the wild-type sample (Figure 1, part B).

**Statistical analysis.** All individuals' data were statistically described, analyzed for between-group differences, and summarized as means with respective standard deviations (SD) for continuous variables, or in contingency tables as counts or percentages. Agreement analysis was used to evaluate the degree of agreement and disagreement between both methods for the detection of variants in *BRCA1/2* over categories in 2x2 tables. Diagnostic performance of the screening test was evaluated using the reference test (sequencing analysis). Cohen's kappa, Maxwell's chi-square and McNemar's statistics were used to test the agreement, disagreement and significant differences. The accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the screening test were calculated. Diagnostic odds ratios (OR) for having the variant were estimated using the sample and the general population prevalence. All estimates are presented along with the respective 95% confidence intervals (95% CI). Statistical analyzes were performed using StatsDirect 3.0.161 software (Stats Direct Ltd., Cheshire, UK). All reported p-values were two sided, and significance was set to  $p < 0.05$ .

Variants are reported in the context of a reference sequence, *BRCA1* gene RefSeq NM\_007294.3 and *BRCA2* gene RefSeq NM\_000059.3.

## Results

In the screening test, we have used the combination of two detection methods. The SNaPshot analysis was used for the detection of specific variants in exons 2, 5, 13, 20 of *BRCA1* and exons 11, 23, 25 of *BRCA2*. The fragment analysis was used for the detection of ins/del in exon 11 of *BRCA1* gene (Table 1, Figure 1).

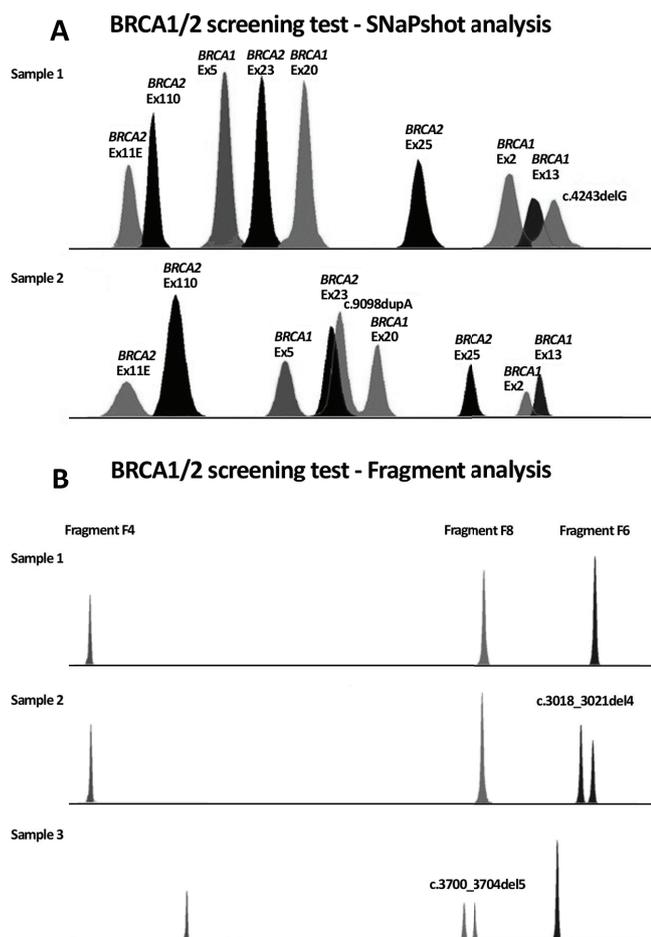
We have analyzed 687 patients, 86 of them (12.5%) met the indication criteria for the complex analysis of *BRCA1/2* genes based on the positive family and personal history (group 1). We used the screening test to expedite the analysis and release of the results. The samples with negative results from screening were submitted for complex *BRCA1/2* analysis. The other set of samples (group 2) consisted of 601 individuals (87.5%) who did not meet the indication criteria for complex *BRCA1/2* analysis, but the screening for the most frequent variants was recommended. Both groups of individuals were balanced with regard to age ( $p=0.77$ ; two-sample t-test). The overall average age was calculated at 52.23 years

**Table 1. Top:** Characterization of the PCR products and SNaPshot primers. The mutation type, PCR product size, and type of wild-type (wt) and mutant (mut) nucleotide are indicated. **Bottom:** Characterization of the fragment analysis with mutations, fluorescent label, size and specified localization indicated.

Multiplex PCR				SNaPshot analysis			
Exon	Gene	Concentration	Length	Mutation	Concentration	Primer length	Wt/mut
2	BRCA1	20.0 μM	257 bp	c.68_69delAG	1.5 μM	47 bp	A>T
5		7.5 μM	200 bp	c.181T>G	0.5 μM	33 bp	T>G
13		5.0 μM	332 bp	c.4243delG	0.5 μM	49 bp	G>A
20		5.0 μM	249 bp	c.5266dupC	0.5 μM	41 bp	C>A
11-E	BRCA2	5.0 μM	319 bp	c.3076A>T	0.75 μM	25 bp	A>T
11-O		7.5 μM	302 bp	c.5645C>A	0.5 μM	32 bp	C>A
23		7.5 μM	264 bp	c.9098dupA	0.5 μM	38 bp	A>C
25		10.0 μM	252 bp	c.9403delC	0.5 μM	45 bp	C>T

Exon 11	Gene	Concentration	Size	Mutations	Label	cDNA
F4		2.5 μM	489 bp	c.1938_1947del10 c.1953_1956del4 c.2068delA	PET/ red	c.1632_c.2104
F6	BRCA1	3.0 μM	655 bp	c.3016_3019del4 c.3018_3021del4 c.3700_3704del5	FAM/ blue	c.2523_c.3169
F8		1.5 μM	609 bp	c.3770_3771del2 c.4065_4068del4	VIC/ green	c.3588_c.4096+78



(range 20–87 years), the average age of group 1 was calculated at 52.75 years (range 22–85) and group 2 average age at 50.75 (range 20–87). Presence of all detected variants was independently confirmed using the Sanger sequencing. This combined approach allowed us to identify 47 mutated samples (6.8%), 40 in the group 1 (46.5%) and 7 in the group 2 (1.1%). Samples from group 1 were further submitted for complex analysis of *BRCA1/2* by massive parallel sequencing, which revealed 5 additional mutated samples (Table 2).

The subgroup of 7 positive samples of group 2 consisted of 6 healthy individuals and 1 patient with breast cancer diagnosed at the age of 55 and positive family history (breast cancer diagnosed in the sister aged 53). All 6 of the healthy positive individuals declared a positive family history, however, based on the Claus model scoring (using published tables) their estimated life-time risk for breast cancer was below the indication threshold 25% (in the range 16–20%).

All 45 positive individuals from group 1 were patients with diagnosed cancer: breast cancer in 33, ovarian cancer in 3, bilateral breast cancer in 3, bilateral ovarian cancer in 1, duplex of breast and ovarian cancer in 4 and duplex of

**Figure 1. Examples of the results of SNaPshot and fragment analysis.** A.) Selected results of SNaPshot multiplex analysis of *BRCA1*, *BRCA2* genes. Sample 1 – Presence of deletion c.4243delG in exon 13 of *BRCA1* gene is manifested by double peak A/G. Sample 2 – Presence of duplication c.9098dupA in exon 23 of *BRCA2* gene is manifested by double peak A/G. B.) Selected results of fluorescent multiplex PCR and fragment analysis of exon 11 of *BRCA1* gene. Sample 1 – Sample without variant. Sample 2 – Presence of 4-base deletion. Sample 3 – Presence of 5-base deletion.

**Table 2. Variants detected using our diagnostic approach.**

c. DNA level	Protein level	Gene	Exon	Samples	Indicated	Not indicated	Method
c.181T>G	p.Cys61Gly		5	7	6	1	SNP
c.1881_1884del4	p.Val627Serfs*2			1	1	0	FA
c.1938_1947del10	p.Ser646Argfs*2			1	1	0	FA
c.2068delA	p.Lys690Lysfs*10	<i>BRCA1</i>	11	2	2	0	FA
c.3018_3021del4	p.His1006Glnfs*16		8	7	1	FA	
c.3700_3704del5	p.Val1234Glnfs*7		3	1	2	FA	
c.4243delG	p.Glu1415Lysfs*3		13	2	2	0	SNP
c.5266dupC	p.Gln1756Profs*73		20	15	13	2	SNP
c.3076A>T	p.Lys1026*			3	2	1	SNP
c.5645C>A	p.Ser1882*	<i>BRCA2</i>	11	1	1	0	SNP
c.9098dupA	p.Thr3033Asnfs*9		23	4	4	0	SNP
c.4986+4A>T	exon 16 skipping	<i>BRCA1</i>	16	1	1	0	MPS
c.5511G>A	p.Trp1837*		20	1	1	0	MPS
c.4327C>T	p.Arg1443*		13	1	1	0	MPS
c.7069_7070delCT	p.Leu2357Valfs*1	<i>BRCA2</i>	14	1	1	0	MPS
c.7558C>T	p.Arg2520*		15	1	1	0	MPS

SNP – SNaPshot analysis, FA – fluorescent PCR analysis, MPS – massive parallel sequencing.

bilateral breast and unilateral ovarian cancer in 1 case. The average age in this subgroup was 41.7 years (SD=10.11, range 24–66).

Using the sequencing analysis for determining the variant status in group 1 allowed us to determine the extent to which the both readings would confirm. The proportion of correctly classified individuals was 88.9% (40/45) of positive and 100% (86/86) of negative results. The negative and positive predictive values (NPV and PPV, respectively) were calculated using a prevalence of 52.3% (95%CI: 41.3–63.2%) as found in the group 1. The following results for the screening test were obtained:

- PPV was 100% (95%CI: 91.2–100%),
- NPV was 89.1% (95%CI: 76.4–96.4%),
- post-test variant likelihood despite negative test was 10.1% (95%CI: 3.6–23.6%),
- sensitivity (true positive rate): 88.9% (95%CI: 76.0–96.3%),
- specificity (true negative rate): 100% (95%CI: 91.4–100%).

To adapt the estimated PPV and NPV to the prevalence of *BRCA1/2* pathogenic variant in the general population, we used a value of 0.4% reported by Maxwell et al. (10) To recall, our “prevalence” based on the frequency of variant detected by the screening test and confirmed by sequencing in 100%, amounted to 1.16%. Assuming the detected perfect level of specificity and acceptable level for sensitivity of the screening test, we could easily show that recalculated PPV and NPV yielded 100% and 99.96%, respectively.

Based on the results of the screening test which was conducted in both, group 1 and 2, the observed diagnostic odds ratio amounted to 72.6 (95%CI: 30.1–203.1). Assuming the fact that sensitivity and specificity can be considered

fixed properties of a diagnostic test, we could expect one person without indication classified as false negative. Thus, the odds ratio in our sample would change to 63.5 (95%CI: 27.3–166.5), which means that a person with indication had on average 63.5 times higher odds of having variant than a person without indication.

## Discussion

In our previous report, we have identified several variants in *BRCA1/2* genes frequently occurring in Slovak population and therefore we have decided to design a rapid screening test. Altogether, nine variants (c.68\_69delAG, c.181T>G, c.3700\_3704del5, c.4243delG, c.5266dupC in *BRCA1*, c.3076A>T, c.5645C>A, c.9098dupA, c.9403delC in *BRCA2*) accounted for 76% of the *BRCA1/2*-positive HBOC families (3). We designed our screening test as a combination of only two multiplex PCR reactions, variant-specific multiplex SNaPshot analysis and fluorescent multiplex PCR amplification followed by fragment analysis. Fragment analysis of possible ins/dels in exon 11 of *BRCA1* increased the theoretical detection limit of the test up to 80%. Previous analysis of *BRCA1/2* genes in Slovak HBOC families revealed variant detection frequency at the level of 17.1% [3]. The overall actual frequency of variants was only 6.8%, which is in contrast with expected rate of 13.7% (80% of expected 17.1% variant carriers). However, we previously tested patients indicated for a complex *BRCA1/2* analysis, while the current set included a considerable number of individuals who did not meet the criteria. Only 13% of tested samples came from patients indicated for the complex *BRCA1/2* analysis (group 1). Interestingly, variant detection rate in this set was at the high level of 46.5%. Brief comparison with other studies

showed detection rate at 19.8% (n=197) [11] and 29.1% (n=294) [12] in Czech, 27% in Polish (n=60) [13], 20% in Austrian (n=86) [14], 16% in Sicilian (n=106) [15], and 17% in Spain population (n=42) [16]. However, several factors, such as number of samples, composition of families, selection criteria or used screening techniques might contribute to this difference in the detection rate also between historically associated populations.

Similar screening approach was used by Filippini et al. [17] in Spanish HBOC population, however authors used only multiplex SNaPshot detection for ten recurrent and founder *BRCA1/2* mutations, what represents approximately 50% of all observed Spanish mutations. All 48 samples were previously analyzed by Sanger sequencing and authors identified no false positive result. Another screening approach was published by Khachibi et al. [18] using HRM analysis for identification of mutations only in exon 11 of single *BRCA1* gene, exactly in the group of 71 patients with diagnosed breast cancer. Altogether, they have identified causal mutations only in 2 samples with diagnosed breast cancer and positive family history. Cini et al. [19] published a study of the origin of most recurrent *BRCA1/2* mutations in north-east Italy. For the quick identification of the eight frequent mutations they also developed a pre-screening test based on the in-house primer extension SNaPshot assay. Generally, using the rapid screening tests of *BRCA1/2* mutations was previously mainly focused on the founder mutations specific for populations e.g. Ashkenazi Jewish [20], Iceland [21], Poland [22], Slovenia [23], Italy [24], North America [25] or Latin America [26] and were based as population specific genetic tests [27]. To our knowledge, this is the first publication which reports the rapid *BRCA1/2* screening test not targeted only at founder mutations and designed for such broad spectrum of *BRCA1/2* mutations.

Further analysis of samples from group 1 by massive parallel sequencing identified 5 more pathogenic variants [c.4986+4A>T, c.5511G>A (p.Trp1837\*), c.4327C>T (p.Arg1443\*), c.7069\_7070delCT (p.Leu2357Valfs\*1), c.7558C>T (p.Arg2520\*)]. However, all were localized in parts of genes that were not included in the screening test, which strongly supports the high estimated specificity (100%). Pathogenic variant carriers from group 1 were all diagnosed with some type of cancer, majority of carriers were diagnosed with breast cancer (73%), duplex of breast and ovarian cancer was diagnosed in 8%, ovarian cancer or bilateral breast cancer in 6.7%.

As mentioned before, for patients from group 2 the test for most frequent variants was recommended for various reasons, e.g. psychological indications, personal decision to undergo the test, positive family history or positive personal history below the threshold criteria. In this group of 601 samples, we have detected the presence of pathogenic variants only in 1.1% of samples. The common characteristic of these samples was positive family history, which appears to be the strongest criterion for the DNA analysis.

The most frequently detected variant in the analyzed group was c.5266dupC identified in 32%, followed by variant c.3018\_3021del5 detected in 17% and exon 5 variant c.181T>G detected in 15% of *BRCA1* variant positive families. These three variants were also most frequent ones in previous reports (3, 12). The most frequently detected *BRCA2* variant was c.5645C>A identified in 8.5% of positive families. As expected also in group 2, the most frequent variant c.5266dupC in *BRCA1* was identified in 2 individuals. Surprisingly, a relatively rare variant c.3700\_3704del5 in *BRCA1* gene was also detected in two individuals. Other three variants (c.181T>G, c.3018\_3021del4 in *BRCA1* and c.3076A>T in *BRCA2*) were determined each in one individual. It needs to be stated that based on the recent data about the prevalence of *BRCA2* variants in central European region, we need to widen the spectrum of variants.

Using statistical analysis, the observed odds ratio of the screening *BRCA1/2* test was estimated to 73.8 and the conditional maximum likelihood estimate was 72.6 (30.1 to 203.1). Sensitivity (true positive rate) of this approach was counted at 89% (range 75.9% to 96.3%) and specificity (true negative rate) was determined to 100% (range 91.4% to 100%).

We take into account the general population prevalence of *BRCA1/2* pathogenic mutation (0.4%) and detected levels of specificity and sensitivity to define positive and negative predictive value of the screening test. Lower positive predictive values of the test (4%) show that we can expect some results as false positive, however these samples should undergo approbation by Sanger sequencing. On the other side, negative predictive value of the screening test reaches 99.9%.

With rare conditions, the PPV is driven by specificity. If it were the specificity different from 100%, for example, if we use the lower 95% confidence limit for the specificity, the falling prevalence would lead to a rapid decrease in PPV (4%) and thus, in an increase in the false positive results. This should be kept in mind when estimating the real-life performance of the screening method, and positive samples should always undergo approbation by Sanger sequencing.

In the conclusion, we suppose that even in the bioinformatic NGS era this simple, rapid, and cost-effective test represents a reliable alternative, sufficiently sensitive to identify individuals in the targeted part of population who have an increased lifetime risk for developing cancer, but do not meet the standard selection criteria. Naturally, the test would not provide complex results, but it offers testing accessible to a broader spectrum of individuals, whose clinical history puts them just under the threshold of indication for complex *BRCA1/2* analysis.

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