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GENETIC VARIATION OF THE *BRCA1* AND *BRCA2* GENES IN MACEDONIAN PATIENTS

Maleva I[1], Madjunkova S[1], Bozhinovski G[1], Smickova E[2], Kondov G[3], Spiroski Z[3], Arsovski A[4], Plaseska-Karanfilska D[1,*]

***Corresponding author:** Professor Dr. Dijana Plaseska-Karanfilska, Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov," Macedonian Academy of Sciences and Arts, Krste Misirkov 2, Skopje 1000, Republic of Macedonia; Tel: +389(0)2 3235410; Fax: +389 (0)2 3115434; E-mail: dijana@manu.edu.mk

ABSTRACT

The most significant and well characterized genetic risk factors for breast and/or ovarian cancer are germline mutations in the *BRCA1* and *BRCA2* genes. The *BRCA1* and *BRCA2* gene mutations strikingly increase breast cancer risk, suggesting that polymorphisms in these genes are logical candidates in seeking to identify low penetrance susceptibility alleles. The aim of this study was to initiate a screen for *BRCA1/2* gene mutations in order to identify the genetic variants in the Republic of Macedonia, and to evaluate the association of several single nucleotide polymorphisms (SNPs) in these genes with breast cancer risk. In this study, we included 100 patients with invasive breast cancer from the Republic of Macedonia, classified according to their family history and 100 controls. The methodology included direct sequencing, single nucleotide primer extension method and multiplex ligation probe amplification (MLPA) analysis, all followed by capillary electrophoresis (CE) on an ABI PRISM™ 3130 Genetic Analyzer. We identified a total of seven carriers of mutations in the *BRCA1/2* genes. None of the tested polymorphisms was associated with sporadic breast cancer risk, however, polymorphism rs8176267 in *BRCA1* and N372H in *BRCA2* showed an association with breast cancer risk in patients with at least one family member with breast cancer.

Keywords: *BRCA1* and *BRCA2* genes, Breast cancer, Macedonian patients, Polymorphisms.

INTRODUCTION

The most significant and well characterized genetic risk factors for breast and/or ovarian cancer are germline mutations in the *BRCA1* (17q chromosome) [1] and *BRCA2* (13q chromosome) [2] genes. Other relevant genes, such as *CHEK2, NBS1, PALB2, BRIP1*, *etc.,* also contribute to hereditary breast cancer, although their impact appears to be more population-specific [3]. It has been estimated that 5.0-10.0% of all breast cancer and 10.0-15.0% of ovarian cancer patients carry mutations on one of the *BRCA* genes [4]. The prevalence of the *BRCA1/2* gene mutation carriers in the general population is approximately 0.2% (1/500), however, it can vary significantly in different countries and ethnic groups due to founder effects [5]. The mutations in these high-penetrance genes confer a high lifetime risk of breast and ovarian cancer. Women with an inherited

1 Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov," Macedonian Academy of Science and Arts, Skopje, Republic of Macedonia
2 Clinic for Oncology and Radiotherapy, Skopje, Republic of Macedonia
3 Clinic for Thoracic Surgery, Skopje, Republic of Macedonia
4 Re-Medika General Hospital, Skopje, Republic of Macedonia

BRCA1 gene mutation have a 65.0-80.0% risk of developing breast cancer and 37.0-62.0% of developing ovarian cancer over their lifetime, while *BRCA2* gene mutation carriers have a 45.0-85.0% risk for breast cancer and 11.0-23.0% for ovarian cancer [6]. The identification of *BRCA1* and *BRCA2* gene mutation carriers is therefore a critical step in individualized risk assessment [7]. Once a mutation is identified in a given family, a very informative predictive (or presymptomatic) oncogenetic test can be offered to all adult family members. Moreover, oncogenetic testing is becoming the powerful therapeutic predictive tool, as new targeted therapeutic opportunities, such as poly(ADP-ribose) (PARP) inhibitors emerge [8] and chemosensitivity to platinum-based therapy is constantly reported [9]. It is now evident that in the near future the demand for rapid *BRCA1/2* gene mutation testing will increase. However, a full *BRCA1* and *BRCA2* gene screening still remains a labor- and time-consuming challenge due to the large size of the genes and the high diversity of mutations and variants of unknown significance. On the other hand, the distribution of known *BRCA1* and *BRCA2* gene mutations is well documented worldwide. Several recent reviews have summarized the evidence that the *BRCA1/2* gene mutation spectrum in given countries and ethnic communities is limited to a few founder mutations [4,5,10]. To date, no systematic study has assessed the distribution of *BRCA1/2* gene mutations in the Macedonian population. We aimed to initiate screening for *BRCA1/2* gene mutations in order to identify the genetic variants common in the Republic of Macedonia.

The fact that *BRCA1* and *BRCA2* gene mutations drastically increase breast cancer risk suggests that polymorphisms in these genes could represent low penetrance susceptibility alleles [11]. Whether common polymorphisms contribute to disease risk has not yet been thoroughly evaluated. The importance of these common variants is still conflicting and more data on large cohorts are needed to better understand their significance. We present data on several single nucleotide polymorphisms (SNPs) including allele frequencies and association with breast cancer risk.

MATERIALS AND METHODS

We included 100 patients with invasive breast cancer from the Republic of Macedonia in this study. The patients were referred to us from the Clinic for Oncology, Skopje and the Re-Medika General Hospital, Skopje, Republic of Macedonia. Patients were classified into three main groups, according to their family history: ***group 1)*** patients with two or more close relatives with breast cancer (n = 19); ***group 2)*** patients with only one affected relative with breast cancer (n = 31); and ***group 3)*** patients with no family history (sporadic cases) (n = 50). The control group consisted of healthy women from the general population (n = 100).

The DNA was isolated from peripheral EDTA blood samples using standard proteinase K/SDS digestion followed by phenol chloroform extraction. All patients were screened for six mutations in the *BRCA1* gene (185delAG, C61G, E368X, 4154delA, 4184del4 and 5382insC) and four in the *BRCA2* gene (D2723G, 3034del4, 5950delCT and 9326insA) by a single nucleotide primer extension assay utilizing the ABI PRISM™ SNaPshot Multiplex Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions (manuscript in preparation). Patients from the first group and patients younger than 40 years from the second group (n = 30) were screened for mutations in all coding sequences of the *BRCA1* and *BRCA2* genes by direct sequencing using the ABI PRISM™ Big Dye Terminator v.1.1 Kit (Life Technologies). Multiplex ligation probe amplification (MLPA) was used for the detection of large rearrangements in these genes using commercially available kits from MRC Holland, Amsterdam, The Netherlands. For the case-control association study of seven common variants in *BRCA1* [rs1799949 (S694S), rs799917 (P871L), rs16941 (E1038G), rs16942 (E1138G), rs8176267, rs8176166 and rs3737559) and one in *BRCA2* (rs144848 (H372N)] with breast cancer risk, we also used single nucleotide primer extension.

RESULTS AND DISCUSSION

Point mutations in the *BRCA* genes are the most common deleterious mutations in familial breast cancer patients. Complete sequencing remains the gold standard for initial mutation identification. However, large rearrangements in these genes have been described in a significant proportion of breast cancer families and are responsible for up to one-third of the identifiable BRCA mutations in a cer-

Table 1. Mutations in the *BRCA1* and *BRCA2* genes detected by sequencing analysis.

Gene	BIC[a] MutationName	HGVS Nomenclature	Exon	Amino Acid Change	Location in Gene	Gender
BRCA1	**C61G**	c.181T>G	5	Cys→Gly	RING domain	F
BRCA1	**E368X**	c.1102G>T	11	Glu→Stop	RING domain	F
BRCA1	**del ex5-8**		5-8		ex5,6-RING domain	F
BRCA1	**5382insC**	c.5266dupC	20	Glu→Stop	BRCT domain	F
BRCA1	**del ex23**		23		BRCT domain	F
BRCA2	**D2723G**	c.8167G>C	18	Asp→His	DNA binding domain	M

[a] BIC: Breast Cancer Information Core.

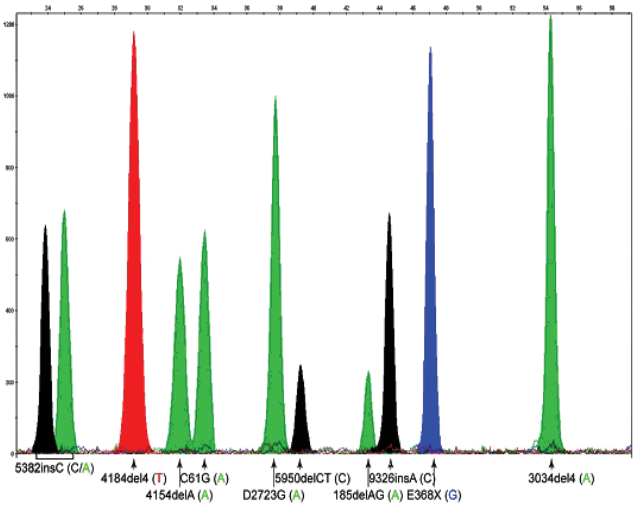

Figure 1. Single nucleotide primer extension assay. Analysis of a patient carrying the 5382insC mutation in the *BRCA1* gene: the fluorescent peaks formed by specific primer extension products are labeled below the electropherogram; labels correspond to mutation names (see Table 2).

Table 2. Specific primer extension products data for most common mutations in the *BRCA* genes.

No.	Mutation	Nucleotide Change	SNaPshot Result (N/M)	SNaPshot Fragment Size N (bp)	SNaPshot Fragment Size M (bp)
1	**5382insC**	A/C	A/C	25	24
2	**4184del4**	T/G	T/G	29	n.d.
3	**4154delA**	A/G	A/G	32	n.d.
4	**C61G**	T/G	T/G	33.5	32
5	**D2723G**	A/G	A/G	38	37
6	**5950delC**	C/A	C/A	39	n.d.
7	**185delAG**	A/T	A/T	43	n.d.
8	**9326insA**	C/A	C/A	44.5	n.d.
9	**E368X**	G/T	G/T	47	48
10	**3034del4**	A/G	A/G	54	n.d.

n.d.: not done.

tain population [12]. In our group of familial breast cancer patients (group 1 as defined in Materials and Methods), we identified a total of seven carriers of mutations: four point mutations and two large deletions in the *BRCA1* gene and a point mutation in the *BRCA2* gene (Table 1). Mutations appear to be evenly distributed across the coding sequence of the genes. Bearing in mind that certain mutations have been observed to be common to specific populations, we designed an assay for detection of the most common mutations in the Slavic populations. Our aim was to expand the mutation screen to breast cancer patients regardless of their family history. To this end, we developed a single nucleotide primer extension as a rapid and economical one-tube test for genetic testing of hereditary breast cancer that can be applied to a wider population setting (Figure 1, Table 2). We screened all sporadic patients and did not identify any mutations until now. More analyses including direct sequencing are needed in order to assess the distribution of mutations in the Macedonian population. This is important because it will allow the development of effective mutation-specific tests for the common mutations in the future. In patients with a strong familial history of breast cancer (n = 6), we performed mutational screening in all coding exons of the *PALB2* gene using the high resolution melting (HRM) method. These analyses were performed at the Gynecology Unit, Hannover

Table 3. Specific primer extension products data for most analyzed SNPs in the *BRCA* genes.

No.	Mutation	Nucleotide Change	SNaPshot Result (N/M)	SNaPshot Fragment Size N (bp)	SNaPshot Fragment Size M (bp)
1	**rs16942**	A/G	T/C	27	25
2	**rs8176267**	A/G	A/G	30	28
3	**rs16941**	A/G	T/C	31	28
4	**rs3737559**	A/G	A/G	31	33
5	**rs799917**	C/T	C/T	34	35.5
6	**rs8176166**	A/G	T/C	39	38
7	**rs1799949**	C/T	C/T	44	45
8	**rs144848**	T/G	A/C	50	49

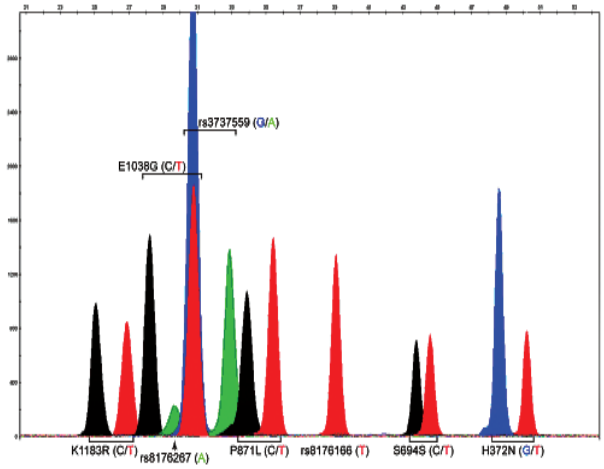

Figure 2. Single nucleotide primer extension assay for detection of selected SNPs in the *BRCA* genes: the fluorescent peaks formed by specific primer extension products are labeled below the electropherogram; labels correspond to SNP names (see Table 3).

Medical School, Hannover, Germany. We found three already published polymorphisms and one potentially damaging variant.

Many studies are focused on rare, highly penetrant germline mutations in *BRCA* genes that strongly predispose women to a familial form of breast cancer. However, there is a possibility that common germline variation in coding and non coding regions may also contribute to predisposition to breast cancer. In the present study, we tested seven common variations in the *BRCA* genes (Figure 2, Table 3) in all our patients in comparison to the controls. Our results showed that none of the polymorphisms tested were associated with the risk of sporadic breast cancer (group 3) suggesting that the variations *per se* do not play a significant role in the development of sporadic breast cancer. However, polymorphism rs8176267 in the *BRCA1* gene showed an association with breast cancer risk when we analyzed the results for patients with at least one family member with breast cancer (groups 1 and 2 combined) *vs.* controls [$p = 0.0151$; OR (odds ratio) (95% CI) (95% confidence interval) = 2.31 (1.16-4.61)]. These results are in concordance with published data [13]. Recent meta-analysis suggests that the *BRCA2* N372H allele may be a low-penetrant risk factor for developing breast cancer [14], however, there is conflicting evidence regarding the role of this variant as a modifier of breast cancer risk. We observed that N372H is associated with slightly increased risk in patients with a family member with breast cancer [$p = 0.0081$; OR (95%CI) =2.37 (1.24-4.56)] [14].

Further analyses on larger cohorts of patients and controls are needed in order to build a high-quality database of genetic *BRCA1/2* gene variants in the Macedonian population, and to obtain accurate estimates as to the association of various polymorphisms with breast cancer risk.

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