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Population-based study of *BRCA1/2* mutations: Family history based criteria identify minority of mutation carriers

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The two major susceptibility genes, *BRCA1* and *BRCA2*, are involved in hereditary breast and ovarian cancer syndrome. Early detection of mutation carriers has crucial clinical importance, as it allows identification of women who may benefit from intensive clinical follow-up or prophylactic surgery. Generally accepted inclusion criteria for *BRCA1/2* mutation testing are based either upon family history of breast or ovarian cancer or young age at cancer diagnosis. In order to analyze the impact of *BRCA1/2* mutations on breast cancer development in the Czech population and to confront the clinical and histopathological data of mutation carriers with current criteria for mutation testing we examined the frequency of mutations in unselected breast cancer cases. Mutational analysis of *BRCA1/2* genes performed in 679 unselected female breast cancer patients included all recurrent deleterious alterations previously identified in the Prague area and truncating mutations in the whole exon 11 of *BRCA1.* Within analyzed gene sequences more than 80% of mutations were identified previously in high-risk patients. A total of 16 breast cancer patients (2.4%) carried a mutation. *BRCA1* mutations were identified in 14 (2.1%) whereas *BRCA2* in 2 (0.3%) women. Family history of ovarian cancer was a strong predictor of a *BRCA1/2* mutation (OR = 8.3; p = 0.01), however, family history of breast cancer was not indicative of carrier status. A significant association between medullary breast cancer and mutation status was observed. Current criteria for *BRCA1/2* mutation testing would distinguish only 6 out of 16 (37.5%) carriers identified in our study. Ten breast cancer patients with confirmed *BRCA1/2* germ-line mutation exhibited no clinical characteristics that would predict their carrier status. Therefore, we believe that the testing for *BRCA1/2* mutations in the Czech Republic may not be restricted only to high-risk patients. Our results indicate that analysis of locally prevalent *BRCA1/2* mutations in all breast cancer patients might extend substantially the percentage of identified mutation carriers.

Keywords: *hereditary breast cancer, BRCA/2 mutation analysis, population-based study*

Breast cancer (BC) is the most common malignancy affecting women in developed countries. Genetic susceptibility resulting from germ-line inactivation in cancer predisposition genes accounts for approximately 5-10% of BC cases [1]. Hereditary BC is characterized by an early onset [2], high bilateral incidence and association with ovarian cancer (OC) [3]. Two highly penetrant cancer susceptibility genes, *BRCA1* (MIM *113705) and *BRCA2* (MIM *600185), have been identified [4, 5]. Mutations in these genes are responsible for more than 60% of diseases in families with BC only and for more than 90% of cases in families with both BC and OC [6]. Mutations in *BRCA1/2* confer a lifetime risk of BC that ranges between 60 and 85% and a risk of OC between 15 and 40% [7, 8]. Detection of mutation carriers has an important clinical relevance, as it allows identification of women who may benefit from intensive clinical follow-up [9] or prophylactic surgery [10].

Analyses that have been performed in various geographic regions revealed significant differences in frequencies and types of mutations in *BRCA1/2* genes. Elucidation of recurrent population-specific mutations facilitates the analysis of high-risk patients in specific ethnic groups. Testing for three common mutations in *BRCA1* in Poland identified 198 (3.9%) carriers of 5024 women who had a first- or second-degree relative with BC before 50 years of age or OC at any age or women who themselves had such history of BC or OC [11]. In Iceland, the single *BRCA2* mutation 999del5 was responsible for a substantial fraction (8.5%, 39/459) of BC patients [12]. The three frequently identified *BRCA1* and two *BRCA2* mutations accounted for 3.6% (18/500) of female BC patients in Hungary [13].

The *BRCA1* mutations 5382insC and 300T>G predominate in populations of Central, Eastern and Southeastern Europe [14], including Austria [15], Hungary [13], Slovenia [16], Greece [17], Poland [18], Slovakia [19], Russia [20], and the Czech Republic [21, 22]. Previous analyses of entire coding sequences of *BRCA1/2* genes performed in 500 and 1010 high-risk BC or OC patients from the two centers in the Czech Republic [21–23 and unpublished results] consistently demonstrated that mutations in *BRCA1* account for more than 70% of identified gene alterations and that more than 90% of alterations in this gene represent most frequent recurrent mutations 5382insC and 300T>G and truncating mutations in exon 11 (25 different mutations). In *BRCA2,* the three repeatedly occurring mutations (5873C>A, 5910C>G and 5991insT) represented almost 40% (9/23) of deleterious defects [22].

We performed analysis of population-specific mutations in *BRCA1/2* genes in a cohort of unselected BC patients from the Prague area to estimate the frequency of mutation carriers among BC patients. Association of *BRCA1/2* -related carcinomas with specific clinical and histopathological characteristics was analyzed to evaluate the relevance of currently used criteria of mutation testing.

Patients and methods

Subjects. Genetic testing was performed in a series of consecutive female patients 27 to 86 years of age with a histologically confirmed diagnosis of primary invasive BC who were treated at the Department of Oncology of the General Teaching Hospital and the 1st Medical Faculty, Charles University in Prague or at the Department of Clinical Oncology, Central Military Hospital, Prague from September 2004 to December 2006. All patients had Czech ancestries and were living in Central Bohemia. Participants were included into the study without regard to age at diagnosis and BC or OC family history. Testing was performed immediately after confirmation of the pathologic diagnosis. Medical history and other clinical data on patients were obtained from medical records. Age at diagnosis, family history concerning the first- and second-degree relatives, tumor histology, receptor status and TNM stage were recorded. A total of 730 control blood samples were obtained regardless of age or sex from random blood donors originating from the same geographical region as the tested patients. The group comprised randomly chosen healthy individuals enrolled between April 2006 and November 2006 at the Department of Blood Transfusion of the Thomayer Faculty Hospital in Prague. The study was approved by the Ethical Committee of the First Faculty of Medicine and the General Teaching Hospital and all participants gave their written informed consent prior to genetic testing.

DNA isolation. Genomic DNA was isolated from EDTA blood samples using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the supplier's instructions.

Analysis of population-specific alterations in BRCA1/2 genes. The test panel was designed to harbor all previously identified recurrent mutations in *BRCA1/2* genes [22]. In addition, analysis also included truncating mutations in the whole exon 11 of *BRCA1.* 83% (83/100) of mutations previously found in high-risk families were identified within analyzed gene sequences. Mutations in exon 11 of *BRCA1* leading to the premature termination of protein translation were pre-screened by the protein truncation test (PTT) as described previously [24]. The most common pathogenic *BRCA1* mutation 5382insC was identified by a mismatch polymerase chain reaction (PCR) assay and restriction enzyme analysis as described in detail by Backe et al. [25]. The second most frequently occurring *BRCA1* mutation 300T>G was detected by the use of restriction fragment length polymorphism (RFLP) analysis. PCR-fragments containing the site of mutation were obtained with primers (5´-CTCTTAAGGGCAGTTGTGAG-3´ - forward and 5´-TTGGAAATAATTTACTGTGTGC-3´ - reverse) flanking exon 5 of the gene. 10 μl reaction mixtures contained 1 μl of 10 x PCR buffer (Roche, Mannheim, Germany), 0.2 mM of each dNTP, 0.4 μM of each primer, 0.5 U of Fast Start Taq DNA polymerase (Roche) and 50-100 ng of genomic DNA. After an initial denaturation (at 95°C for 3 min), 35 cycles (at 95°C for 30 sec, 58°C for 30 sec and 72°C for 2 min) and final extension (at 72°C for 7 min) were performed. Wild type PCR products carrying a single site for the restriction endonuclease *Cfr13I* (Fermentas, St. Leon Rot, Germany) are digested into a 144-bp and a 186-bp fragment. The 300T>G mutation introduces the second restriction site. Therefore, the aberrant allele is digested by the *Cfr13I* into a 144-bp, a 174-bp and a 12-bp fragment. PCR products digested by *Cfr131* under conditions described by the supplier were run on precast Spreadex EL300 gels [26] using SEA 2000 electrophoretic apparatus (Elchrom Scientific AG, Cham, Switzerland). Separated fragments were visualized by SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Screening for *BRCA2* mutations included analysis of a 230 bp fragment of exon 11 by denaturing high-performance liquid chromatography (DHPLC). Amplifications were performed in 15 μl reaction mixtures containing 1.5 μl of 10 x PCR buffer, 0.2 mM of each dNTP, 0.4 μM of each primer (5´-CGTTTGTGTTTCACATGAAAC-3´ - forward and 5´-CTTCACTCTGAATGTCAGCA -3´ - reverse), 0.6 U Gold Taq DNA polymerase (Applied Biosystems, Foster City, CA) and 100 ng of genomic DNA. After an initial denaturation (at 95°C for 10 min), 10 cycles (at 95°C for 30 sec, 65°C -1°C/cycle for 30 sec and 72°C for 2 min) followed by 25 cycles (at 95°C for 30 sec, 55°C for 30 sec and 72°C for 2 min) and final extension (at 72°C for 7 min) were performed. 5 μl aliquots of the PCR reactions were resolved using DHPLC (WAVE 3500 System; Transgenomic, Omaha, NE) on the DNASep cartridge at 55°C in a gradient of 58–67% acetonitrile-containing Buffer B.

DNA sequencing. Alterations detected by PTT, RFLP or DHPLC-analyses were confirmed and characterized by direct sequencing from independently amplified PCR fragments using the BigDye terminator cycle sequencing kit version 3.1 in a ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Statistical analysis. The mean, median, SD, variance, quartiles and other basic statistical measurements were computed in given groups and subgroups. Proportions, frequencies, odds ratios and 95% confidence intervals were computed using CRAN 2.4.0. statistical analysis software release 8.02. Statistical significance was assessed using logistic regression analysis and non-parametric Wilcoxon test.

Results

Analysis of pathogenic mutations in BRCA1/2 genes. A total of 679 incident BC patients were examined for mutations in *BRCA1/2* genes. Sixteen deleterious germ-line mutations were identified (Table 1), accounting for 2.4% (95% CI 1.2-3.5) of invasive BC cases associated with alterations in *BRCA1/2* genes. *BRCA1* mutations were found in 14 (2.1%; 95% CI 0.9–3.1) patients whereas *BRCA2* mutations in 2 (0.3%; 95% CI 0.0–0.7) patients. The most frequent mutation was the *BRCA1* 5382insC detected in 7 (1.0%) participants. The *BRCA1* 300T>G was identified in 3 (0.4%) patients and other previously identified locally prevalent mutations – *BRCA1* 1806C>T and 3819del5 and *BRCA2* 5991insT and 5873C>T – were found each in one patient (0.15%). Two *BRCA1* mutations –2607dup10 and 1135insA – were not previously detected in our series of high-risk families [22]. Among 730 control blood samples analyzed for *BRCA1* 5382insC one positively tested individual was present. Population frequency of 0.14% (95% CI 0.0–0.4) was estimated for this mutation. None of the control samples carried recurrent mutations 3819del5 and 3875delGTCT identified in exon 11 of *BRCA1*.

Clinical and histopathological characteristics of BC patients carrying alteration in BRCA1/2 genes. Table 2 summarizes clinical and histopathological data of analyzed BC patients in relation to their carrier status. The mean age at diagnosis of BC was lower in *BRCA1/2* mutation carriers (50.8 years) than in non-carriers (56.0 years) nevertheless the difference did not reach statistical significance (p = 0.06). However, BC patients diagnosed at the age ≤50 had a more than two-fold increased chance of being a mutation carrier than patients diagnosed after 50. Of 16 identified mutation carriers, two patients (12.5%) with a family history of OC and one (6.25%) with a history of BC were registered. In non-carriers, family history was available in 590 cases and BC and OC were reported in 80 (13.6%) and 10 (1.7%) families, respectively. In our series of BC patients, family history of OC strongly predicted the presence of a *BRCA1/2* mutation (OR = 8.3; 95% CI 1.7-41.4; p = 0.01). On the contrary, family history of BC did not increase the chance of being a mutation carrier. No family history of BC or OC was registered in 13 out of 16 (81.3%) mutation carriers.The incidence of bilateral BC was significantly higher in *BRCA1/2* mutation carriers (2/16; 12.5%) than that in non-carriers (11/663; 1.7%; OR = 7.5; 95% CI 1.5–37.3; p = 0.01). The histological data were available for all mutation carriers and 558 non-carriers. Invasive ductal carcinomas predominated both in women with *BRCA1/2*-related BC and in mutation negative patients. A significant association was found between a mutation status and medullary carcinoma. A total of 4 (25.0%) medullary carcinomas were identified among 16 *BRCA1/2*-related breast tumors whereas 15 (2.7%) tumors of this histological subtype were found in a group of 558 mutation negative women (OR = 12.1; 95% CI 3.5–41.8; p = 0.0001). We did not reveal any significant difference in tumor grade or stage comparing *BRCA1/2* positive and negative individuals (data not shown). The *BRCA1/2*-associated tumors were more often progesterone receptor negative than tumors in non-carriers [68.8% vs. 34.6% (OR = 4.2; 95% CI 1.4–12.2; p = 0.01)]. Further, we registered increased frequency of triple-negativity (a lack of expression of estrogen, progesterone, and ErbB-2 receptors) in *BRCA1/2*-related tumors [12.5% vs. 3.6% (OR = 3.8; 95% CI 0.8–18.3; p = 0.09)]. On the contrary, no association between mutation status and estrogen receptor expression was found.

Discussion

BRCA1/2 mutations were intensively studied in families at high-risk of BC. However, in unselected BC cases, the role of these mutations in BC development remains unclear in many populations. We have searched for population-specific mutations of *BRCA1/2* genes in a cohort of BC patients selected regardless of age at diagnosis or family history and found 2.4% of mutation carriers. Assuming 83% mutation detection sensitivity (this percentage of gene alterations was previously found within analyzed gene sequences in high-risk patients from the Prague area), the approximate frequency of pathogenic *BRCA1/2* mutations in BC patients may be estimated at 2.8%. Nevertheless, this calculation may be underestimated since analysis was focused only on identified point mutations and short deletions or insertions and screening for large intragenic deletions and rearrangements, which may represent more than 10% of all deleterious mutations in the Czech Republic [27, 28], was omitted. The spectrum of cancer-predisposing mutations detected in this study was similar to that obtained previously in high-risk patients and all identified gene alterations were already described in the Czech Republic [21, 22]. The most common *BRCA1* alterations, 5382insC and 300T>G, were identified in 50% (7/14) and 21.4% (3/14) of *BRCA1* mutation carriers whereas frequencies of these mutations previously identified in high-risk families were 51.4% (18/35) and 8.6% (3/35), respectively [22]. Further, we revealed other locally prevalent mutations; 1806C>T and 3819del5 in *BRCA1* and 5991insT and 5873C>T in *BRCA2* [22]. *BRCA1* mutations – 1135insA and 2607dup10 – were previously disclosed at the Masaryk Memorial Cancer Institute, Brno, Czech Republic [21]. Different spectra but similar frequencies of *BRCA1/2*

Table 1 Clinical Characteristics of *BRCA1* and *BRCA2* Mutation Carriers

Patient No.	Exon	Mutation	Effect	Histology of breast cancer	Age at dg. years	Family history of cancer [1] (age at diagnosis)
BRCA 1						
196	20	5382insC	Gln1756fsX1829	Lobular	58	-
197	20	5382insC	Gln1756fsX1829	Ductal	44	Ovarian – M (50); ovarian – MGM (50)
302	20	5382insC	Gln1756fsX1829	Medullary	55	-
304	20	5382insC	Gln1756fsX1829	Medullary	39	-
592	20	5382insC	Gln1756fsX1829	Ductal	46	Endometrial – M (?); ovarian – S (?)
639	20	5382insC	Gln1756fsX1829	Ductal	61	Lung – F (?)
689	20	5382insC	Gln1756fsX1829	Ductal in situ	50	-
278	5	300T>G	Cys61Gly	Ductal	61	Breast – M (57)
364	5	300T>G	Cys61Gly	Mucoid	47	-
365	5	300T>G	Cys61Gly	Medullary	28	-
214	11	1135insA	Lys339fsX345	Medullary/Ductal	43/51	Brain – F (?); prostatic – MGF (?)
708	11	1806C>T	Gln563X	Ductal	53	-
660	11	2607_2616dup10	Lys833fsX	Ductal	50	-
331	11	3819del 5	Val1234fsX1241	Ductal/Ductal	51/53	-
BRCA2						
691	11	5873C>A	p.Ser1882X	Ductal	67	Endometrial – M (?)
542	11	5991insT	p.Ala1922fsX1923	Lobular	61	Stomach – M (62); lung – S (67)

[1] Abbreviations: M, mother; MGM, maternal grandmother; S, sister; F, father; MGF, maternal grandfather.

Table 2 Clinical and Histopathological Characteristics of Breast Cancer Cases

	BRCA 1/2 Carriers (%)	Non-carriers (%)	OR[1]	CI 95%	p value
Age at Diagnosis (n=651)[2]					
≤ 50 *years*	8 (50.0)	199 (31.3)	2.2	0.8 - 5.9	0.1
> 50 *years*	8 (50.0)	436 (68.7)	0.5	0.2 - 1.2	0.1
Family History of (n= 606)					
Breast cancer	1 (6.3)	80 (13.6)	0.4	0.1 - 3.3	0.4
Ovarian cancer	2 (12.5)	10 (1.7)	8.3	1.7 - 41.4	0.01
Bilateral BC (n=679)	2 (12.5)	11 (1.7)	7.5	1.5 – 37.3	0.01
Histological Type (n= 574)					
Invasive ductal	8 (50.0)	403 (72.2)	0.4	0.1 - 1.0	0.06
Invasive lobular	2 (12.5)	79 (14.2)	0.9	0.2 - 3.9	0.8
Medullary	4 (25.0)	15 (2.7)	12.1	3.5 – 41.8	0.0001
Other types	2 (12.5)	61 (10.9)	1.2	0.3 - 5.2	0.8
ER Status (n= 487)					
ER positive	12 (75.0)	371 (78.8)	0.8	0.3 - 2.6	0.7
ER negative	4 (25.0)	100 (21.2)	1.2	0.4 - 3.9	0.7
PR Status (n= 400)					
PR positive	5 (31.25)	251 (65.4)-	0.2	0.1 - 0.7	0.01
PR negative	11 (68.75)	133 (34.6)	4.2	1.4 - 12.2	0.01
Triple Negative Tumors (n=462)	2 (12.5)	16 (3.6)	3.8	0.8 - 18.3	0.09

[1] Value corresponds to the difference between mutation carriers and non-carriers.
[2] Number of patients with data available.

mutations were reported in other population-based series of BC cases tested for locally common mutations. Prevalence of mutations in these genes was 1.8% (19/1035) in the Finnish study [29], 2.5% in the Norwegian study [30], and 3.6% (18/500) in the Hungarian study [13]. Testing for the three most frequently occurring founder mutations of *BRCA1* in

3472 unselected incident cases of early-onset BC in Poland identified 198 gene alterations (5.7%) [31]. Screening of the entire coding sequence of both genes was performed in the UK in a series of 1220 BC patients diagnosed before the age of 55 and in a group of 1628 American BC cases of age range 35-64 years. In the UK, *BRCA1* mutation carriers were identified in 0.7% (8/1220) of cases whereas *BRCA2* carriers were found in 1.3% (16/1220) of cases [32]. Among American women, 2.4% and 2.3% carried deleterious mutations in *BRCA1* and *BRCA2*, respectively [33].

As expected, carriers of *BRCA1/2* mutations were diagnosed earlier compared to BC patients without a mutation. Frequency of 3.9% (8/207) was found for cases diagnosed under or at the age of 50 whereas among individuals diagnosed after 50 the mutation frequency was 1.8% (8/444). Two deleterious *BRCA1* mutations were found in a group of 12 patients (16.7%) with invasive BC who reported a family history of OC. Thus, in analyzed BC patients a family history of OC seems to be a strong indicator of mutation in *BRCA1*. This result is in agreement with the findings of the Finnish [29] and Spanish [34] studies. On the other hand, only one pathogenic *BRCA1* alteration was detected in 80 patients (1.3%) from families with a history of BC. Medullary carcinoma is referred to account for about 2-3% of BC cases [35]. In our series, the incidence of medullary carcinoma was 3.3% (19/574). Remarkably, a total of 4 (21.1%) mutation carriers in *BRCA1* were found among women with this histological tumor subtype. Family history of BC or OC was not reported by these *BRCA1*-related BC patients. One of these women was diagnosed at the age of 28; the other exhibited the triple-negative phenotype, which is reported to be common among *BRCA1* germ-line mutation carriers [36]. The other two women did not fulfill currently used criteria for genetic testing [22] (Table 1). Therefore, it seems that indication of patients with medullary carcinoma for *BRCA1* testing, regardless of the family history and age at diagnosis, may be helpful in mutation screening. Our results are in agreement with the study of Eisinger et al. [37] who found mutations in *BRCA1* in 11% of patients with medullary carcinoma. Another French study found medullary histology in 14.3% *BRCA1/2* mutation carriers [38].

Among 16 *BRCA1/2* mutation carriers identified in our study, family history based criteria would have distinguished only two patients. Indication of patients with an early age of onset and with medullary carcinoma for genetic testing would identify additional four carriers. Unexpectedly, 10 out of 16 patients (62.5%) did not meet any of currently used criteria for mutation screening [22] (Table 1).

In conclusion, no clinical characteristics that would indicate alterations in predisposition genes were found in the majority of carriers identified in our population-based study. Accordingly, testing for *BRCA1/2* mutations in the Czech population may not be restricted only to patients with a family history of BC and/or OC or to other high-risk patients who meet currently applied criteria for genetic testing. Our results indicate that rapid and inexpensive testing of all BC patients for the most frequent recurrent mutations - prior to analysis of entire coding sequences in high-risk patients - might extend substantially the percentage of identified mutation carriers.

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