Summary

Purpose: Hereditary breast/ovarian cancer (HBOC) occurs in families with several members affected. Most HBOC is caused by mutations in high penetrance BRCA genes accounting for about 5% of all breast cancers. There is a large number of genes with moderate or low penetrance, contributing to non-BRCA aggregation of breast and ovarian cancers. In the present study, we evaluated the presence and frequency of 1100delC and Del5395 mutations in CHEK2 gene in Serbian BRCA-negative HBOC cases.

Methods: We analyzed 57 BRCA-negative subjects from high risk breast/ovarian cancer families from Serbia for CHEK2 1100delC and Del5395 mutations. We used two multiplex allele specific PCR in order to detect them. All suspected positive cases were compared with controls and confirmed by DNA sequencing.

Results: 1100delC was not detected in the tested group. However, we detected one Del5395 mutation in a female diagnosed with breast cancer at the age of 32 and with apparent family history of breast cancer (her mother and sister were diagnosed with breast cancer at 42 and 39 years of age, respectively). The frequency of Del5395 mutation in our tested group was 1.7% (1/57).

Conclusions: 1100delC variant in CHEK2 gene was not present in the tested subjects from HBOC families in Serbia. However, the finding of Del5395 mutation does not allow us to discard a possible involvement of this gene in breast cancer susceptibility in Serbian population. It would be of great interest to assess the distribution of this large deletion in other countries from the Balkan region in order to assess its geographical distribution and possible founder effect.

Key words: CHEK2, HBOC, hereditary breast cancer, hereditary ovarian cancer, large deletion, moderate-penetrance variant

Introduction

Breast cancer is one of the most common malignancies and the major cause of cancer-related deaths among women worldwide with both environmental and genetic factors involved in its etiology [1]. The hereditary form of breast and ovarian cancer (HBOC) appears in families with several members affected, and is mainly caused by mutations in high penetrance breast cancer susceptibility genes such as BRCA1 and BRCA2 [1]. HBOC is also characterized by autosomal dominant transmission, earlier age of onset of cancers, multiple primary cancers in an individual and bilateral or multifocal cancer [2]. In just 5% of all breast cancer cases, disease will occur as a consequence of germline mutations in the high penetrance susceptibility genes while this percentage is higher among HBOC, being about 16% [1,2]. In the case of ovarian cancer, inherited mutations in BRCA genes account for up to 14% of all ovarian cancer cases [3]. These mutations are more common in families with clustering of early onset breast and ovarian cancers, male breast cancer and Ashkenazi Jewish ancestry [2]. Although mutations in BRCA genes are more frequent in HBOC, they still account for only a portion of these cases and there is still a significant number of families with multiple affected breast and ovarian cancer individuals with no BRCA mutation detected [4]. Although
the hereditary form of the disease accounts for a small proportion of patients with cancer of both anatomic localizations, it predominantly affects young population and screening for new genes responsible for development of disease is of great significance, especially from the aspect of disease prevention.

A large number of high, moderate or low penetrance alleles such as TP53, CDH1, ATM, CHEK2, NBS1, RAD51, BRIP1 and PALB 2 has been shown to best explain residual non-BRCA aggregation of breast and ovarian cancers [5-7]. It has been proposed that residual breast cancer risk might not be explained by mutations in high penetrance genes only but it probably involves defects in larger number of moderate or low penetrance genes [7].

The discovery of CHEK2 mutations in breast cancer families approximately one decade ago, revealed a new approach in studying the genetic background of familial breast cancer as well as other tumor types. The most extensively studied as possible third high breast cancer susceptibility gene is CHEK2 gene which is one of a few genes with rare alleles that are clearly associated with elevated breast cancer risk [8,9]. It has also been proposed that CHEK2 might act as multi-organ cancer susceptibility gene [10].

CHEK2 gene is located on the long arm of chromosome 22 and encodes the nuclear checkpoint serine/threonine protein kinase (Chk2) which maintains genomic stability through several fundamental processes in the cell. Chk2 protein structure shows three characteristic domains: N terminal SQ/TQ cluster, a fork head associated domain (FHA) responsible for protein-protein interactions and large C terminal serine/threonine kinase domain. In response to effects that cause DNA double strand breaks, Chk2 is activated by ATM and ATR kinases [10]. After the initial phosphorylation, activated Chk2 rapidly spreads the alert signal through phosphorylation of its substrates (Cdc25A, Cdc25C, Pik3 kinase, E2F1 transcriptional factor, BRCA1) activating the cell cycle checkpoints and increasing DNA repair efficiency [11]. Chk2 can prevent tumorigenesis through DNA damage repair or, if repair is not possible, through driving cell to enter apoptosis. Recently it has been shown that Chk2 is involved in the regulation of the proper mitotic spindle assembly and that even minor loss of protein activity can cause chromosomal instability [12].

Mutations in CHEK2 gene that cause inactivation of its protein product can lead to accumulation of DNA damage in the cells and neoplastic transformation. Several founder mutations in CHEK2 have been reported. Frameshift mutation 1100delC in exon 10 and the large deletion of 5395bp (Del5395), which removes both exons 9 and 10, are deleterious mutations and both cause protein truncation at codon 381 [13]. Carriers of CHEK2 truncating mutations have increased risk for breast cancer development of two to three-fold in women and ten-fold in men [14].

Mutation 1100delC is founder in Eastern and Northern Europe and its frequency ranges from 0.2 to 1.4%, but it’s less frequent in North America [15]. The frequency of this mutation is higher in familial cancer cases (2.1%) than in unselected breast cancer cases (0.4%) [15].

In 2006, a large deletion of exons 9 and 10 in CHEK2 gene was characterized in the USA in two families of Czech and Slovak origin with about 1% frequency [15]. Because Poland is contiguous with both Czech and Slovak Republics and they are all of Slavic origin, Cybulski et al. [16] conducted a study to determine the frequency of this mutation in Polish population and reported it as founder mutation in Poland. Considering that this mutation has only recently been discovered there is still no detailed information about its geographical distribution. Since BRCA mutations are population-specific, it is also reasonable to assume that CHEK2 mutations display the same characteristic. That is why it is important to search for this mutation in every population, especially those of Slavic origin.

Our study is a part of an ongoing project which deals with detection of mutations and establishment of fundamental genetic components in HBOC cases in Serbia. The aim of the study was to determine the presence and frequency of 1100delC and Del5395 mutations in CHEK2 gene in BRCA-negative individuals with positive family history of breast/ovarian cancer and to determine a more complex and detailed strategy for genetic screening.

Methods

Tested subjects

This study included 57 subjects (53 female and 4 male) from high risk breast/ovarian cancer families from Serbia. All of them were previously screened by direct sequencing of the entire coding regions for mutations in BRCA1 and BRCA2 genes in our laboratory (BRCAPRO calculated carrier probability CP>10%). They were all negative for sequence alterations in
CHEK2 1100delC and Del5395bp in BRCA-negative individuals

BRCA genes. All tested individuals provided written informed consent approved by the Ethics Committee of the Institute for Oncology and Radiology of Serbia and attended genetic counseling before and after the testing. All individuals included in the present study were of Caucasian descent and were selected from a larger group according to positive family history of breast and ovarian cancer, early age of breast cancer onset and absence of BRCA1 or BRCA2 mutations. Tested individuals were divided into six groups (Table 1).

### DNA isolation

DNA was isolated from peripheral blood on ABI PrismTM 6100 Nucleic Acid PrepStation using Blood-Prep Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

### Mutation detection

CHEK2 1100delC genotyping was performed by allele specific PCR amplification of the 120bp long DNA region. Specific primers used in the analysis were: WT- 5’TTGGAG TGCCAAAAATCAGT3’ (specific for the wild type allele), MUT- 5’CTTGGAGTG CCCAAAATCAT3’ (specific for the mutated allele) and C- 5’CTGATCTAGGCTACG TGGCT3’ (common primer). PCR was carried out using the Applied Biosystems 2720 Thermal Cycler, and performed in a final volume of 20μl. The reaction mixture contained 10μl AmpliTaqGold®PCR Master Mix, 200ng genomic DNA, 5pmol of CHLdel2F and CHLc2R, and 8pmol of CHLdelIR and CHLcF primers. The initial denaturation step at 94ºC for 10 min was followed by touch-down PCR with the following program: 9 cycles of 94ºC 25s, 68ºC 25s, 72ºC 35s lowering the temperature by 1.4ºC in each cycle, and 31 cycles of 94ºC 25s, 55ºC 30s, 72ºC 35s. A heterozygote mutation-positive control for CHEK2 Del5395 was included to ensure detection of specific mutation. The success of PCR reaction and presence of mutation was confirmed on 2% agarose gel electrophoresis (50min, 25mA). If the sample was wild type homozygote amplification this would occur only in the tube which contained WT+C primers producing a single band on the gel electrophoresis, while if the sample was heterozygote for 1100delC mutation, amplification would occur in both tubes producing two bands on gel (one for each PCR reaction).

Two primer pairs were used for allele specific multiplex PCR detection of large deletion of both exons 9 and 10 in CHEK2 gene, Del5395. The first pair (CHLdel2F- 5’TGTAAT GAGCTGAGATTGTGCG3’ and CHLc2R- 5’CAGAAATGAGACAGGAAAGTT3’) flanked breakpoint site in intron 8. The second pair (CHLdelIR- 5’GTCTAAAACCTTGCGTGCG3’ and CHLcF- 5’CTCTGGTGTAACAGTGAC3’) flanked breakpoint site in intron 10. PCR was carried out using the Applied Biosystems 2720 Thermal Cycler, and performed in a final volume of 20μl. The reaction mixture contained 10μl AmpliTaqGold®PCR Master Mix, 200ng genomic DNA, 5pmol of CHLdel2F and CHLc2R, and 8pmol of CHLdelIR and CHLcF primers. The initial denaturation step at 94ºC for 10 min was followed by touch-down PCR with the following program: 9 cycles of 94ºC 25s, 68ºC 25s, 72ºC 35s lowering the temperature by 1.4ºC in each cycle, and 31 cycles of 94ºC 25s, 55ºC 30s, 72ºC 35s. A heterozygote mutation-positive control for CHEK2 Del5395 was included to ensure detection of specific mutation. The success of PCR reaction and presence of mutation was confirmed on 3% agarose gel electrophoresis (50min, 25mA). In mutation-negative cases only two PCR fragments of 579bp (CHLdel2F+CHLc2R) and 522bp (CHLcF+CHLdelIR) were amplified. In mutation-positive cases the forward primer of the first pair and the reverse primer of the second pair (CHLdel2F+CHLdelIR) amplified additional 450bp PCR product. The presence of mutation was confirmed by sequencing.

### Results

Detection of two truncating CHEK2 mutations 1100delC and Del5395 was successfully performed in all 57 high risk breast/ovarian cancer probands, using two separate multiplex allele-specific PCR reactions (Figures 1 and 2). None of the samples analyzed carried the 1100delC variant in CHEK2 gene. However, we detected Del5395 mutation (Figure 3) in a female patient diagnosed with breast cancer at the age of 52 (Group B) and with apparent family history of breast cancer (her

<table>
<thead>
<tr>
<th>Tested individuals</th>
<th>N (%)</th>
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<tbody>
<tr>
<td><strong>Group A</strong>&lt;br&gt;Female breast cancer patients with at least one breast or ovarian cancer in first or second-degree relatives</td>
<td>25 (43.9)</td>
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<tr>
<td><strong>Group B</strong>&lt;br&gt;Patients with early age of breast cancer onset diagnosed before age of 35</td>
<td>14 (24.6)</td>
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<tr>
<td><strong>Group C</strong>&lt;br&gt;Male breast cancer patients</td>
<td>4 (7)</td>
</tr>
<tr>
<td><strong>Group D</strong>&lt;br&gt;Ovarian cancer patients with at least one case of breast or ovarian cancer in first-degree relatives</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td><strong>Group E</strong>&lt;br&gt;Patients with both breast and ovarian cancers</td>
<td>3 (5.3)</td>
</tr>
<tr>
<td><strong>Group F</strong>&lt;br&gt;Healthy subjects that had two or more cases of breast or ovarian cancer among first-degree relatives including at least one case with disease onset under the age of 50</td>
<td>10 (17.5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>57 (100)</td>
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Figure 1. Detection of CHEK2 1100delC mutation by allele specific PCR DNA ladder (O’GeneRuler™ Fermentas): column 1; heterozygote 1100delC: control sample (columns 2 and 3) - amplification products of both mutated and wild-type alleles. Homozygote wild-type samples: sample 1 (columns 4 and 5), sample 2 (columns 6 and 7), sample 3 (columns 8 and 9) – amplification of product only in tube which contains primers specific for wild-type allele.

Figure 2. Detection of CHEK2 Del5395 mutation by multiplex allele specific PCR DNA Ladder (O’GeneRuler™ Fermentas): column 1; heterozygote Del5395: control sample (columns 2 and 3) - beside two bands (379bp and 522bp) corresponding to wild-type allele, additional band of 450bp was amplified from mutated allele. Homozygote wild-type samples: sample 1 (columns 4 and 5), sample 2 (columns 6 and 7), sample 3 (columns 8 and 9) - only two amplified bands from wild-type alleles (379bp and 522bp) without 450bp mutation product.

Figure 3. A 5395bp deletion detected in Serbian population. Sequencing electropherogram of PCR product containing deletion. Dashed line represents site of deletion.
CHEK2 1100delC and Del5395bp in BRCA-negative individuals

mother and sister were diagnosed with breast cancer at 42 and 39 years of age, respectively; BRCA1 CP: 18.1%, BRCA2 CP: 11%) The sister of the affected proband was also tested and was negative for Del5395 mutation. The frequency of Del5395 mutation in our tested group was 1.7% (1/57).

Discussion

Mutations in BRCA1 and BRCA2 genes account for approximately 20-25% of the familial risk of breast/ovarian cancer [17]. The model that explains residual risk includes intermediate-risk variants, such as protein truncating mutations in ATM [18], BRIP1 [19], CHEK2 and PALB2 [20,21] genes. There are many reports of CHEK2 acting as moderate penetrant tumor suppressor gene which significantly contributes to breast cancer development [22]. It is also reported that mutations in this gene can be involved in prostate [23], gastric [24], colorectal [25], lung cancers [26] and lymphomas [27].

CHEK2 1100delC is a frameshift mutation occurring at the beginning of the 10th protein coding exon, resulting in stop codon formation at the position 381. In 2001, CHEK2 1100delC founder mutation became recognized as first low-penetrant allele associated to breast cancer development [28]. The frequency of 1100delC varies according to ethnicity and is mostly very rare (<1%) [29,30]. The highest population frequencies were found in north European countries like Finland (1.3%), the lowest in Australia (0.14%), with intermediate frequency in the UK (0.52%) [14]. The frequency of 1100delC mutation in Poland is around 0.25% [31], 0.3% in Czech Republic [32], 0.3-0.4% in USA [29], whereas this mutation was not found in Spanish population [33]. Founder allele 1100delC contributes to two-fold increased risk of breast cancer, but in individuals with family history of disease further increases the already elevated risk. It has been estimated that risk for breast cancer development before the age of 70 in 1100delC carriers is 13%, comparing to 5.7% in persons without mutation [34]. Contribution of 1100delC in male breast cancer is 9% and for male carriers the risk for breast cancer is 10 times higher than in the general population [35].

Our results considering 1100delC are in agreement with previously reported results. Considering that this mutation is founder in north European populations which are geographically distant from Serbia, as well as the fact that this mutation is not reported with high frequency in surrounding countries, we did not expect its high frequency in our population. We did not find any 1100delC mutation in the tested group, which might lead us to conclude that this mutation is very rare in the Serbian population, although we have to expand testing on larger groups of subjects in order to be certain.

In 2006 a large deletion of exons 9 and 10 in CHEK2 gene was discovered in two high-risk families in USA both of Czech and Slovakian ancestry. Further analysis revealed that this mutation appeared at the same haplotype, indicating that the mutation had a single source, somewhere in the Carpathian Mountains on the border of the present Czech and Slovak Republics. As 1100delC, this large deletion of 5395bp leads to stop codon formation at position 381, but contrary to 1100delC, Del5395 eliminates both exons 9 and 10. Considering that Del5395 is founder in Slavic countries like Poland, Czech and Slovak Republic, it is expected to be detected in other Slavic populations, as well as countries from the Baltic and Balkan regions. Therefore, we were interested to examine if this mutation is present in our group of high risk BRCA-negative breast/ovarian cancer individuals. The frequency of Del5395 in our tested group was 1.7%, which seems high comparing to other previous reports. The main reason for this may be the relatively small sample size of the group which was available for genetic testing. Further increase of the number of the tested subjects is going to provide us a clearer picture about the frequency of this mutation, and whether it can be considered as founder in the Serbian population.

The significance of 1100delC and Del5395 mutations in CHEK2 gene for disease development is difficult to determine because of low cancer penetrance, uneven geographical distribution and low frequency. Considering the low penetrance of CHEK2 variants, testing for CHEK2 mutations alone, would likely have little predictive value for an individual patient, however, CHEK2 testing could be included as part of a broader panel of variants suitable for genetic screening.

Conclusions

We found that 1100delC variant in the CHEK2 gene, previously described as founder allele in North European populations is not present in BRCA-negative subjects selected from hereditary breast and ovarian cancer families in Serbia. However, the finding of Del5395 mutation, previously described as founder mutation in Poland, does not allow us to discard a possible involvement of this gene in breast cancer susceptibility in our popu-
lation. Additional studies in countries from Balkan region are needed to more closely examine geographical distribution of these mutations and to help identification of genetic susceptibility factors as an important step in definition of individual risk to this malignancy.

Acknowledgement

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