Introduction

BRCA1 and BRCA2 tumor suppressor genes play an important role in DNA damage and repair pathways. Germline mutations in these genes are strongly associated with an increased risk of breast and ovarian cancer [1, 2]. Previous studies estimated that approximately 15% of the Polish patients diagnosed with ovarian cancer carry germline BRCA1/2 mutation [3–5]. This frequency is comparable to the overall prevalence of BRCA1/2 mutations among ovarian cancer patients worldwide [6, 7].

Although 20–70% of sporadic ovarian tumors display loss of heterozygosity (LOH) in the BRCA1/2 loci, indicating essential role of these genes in ovarian cancer pathogenesis, somatic mutations of these genes are relatively rare finding [8–10]. To date, somatic BRCA1 mutations were reported in 5–9% of sporadic ovarian cancer cases, whereas somatic genetic variants of BRCA2 were identified in 3–4% of tumors [11–15].

Detection of somatic BRCA1/2 mutations in ovarian cancer – next-generation sequencing analysis of 100 cases

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Keywords
BRCA1, BRCA2, next-generation sequencing, ovarian cancer, PARP inhibitors, somatic mutation

Abstract
The overall prevalence of germline BRCA1/2 mutations is estimated between 11% and 15% of all ovarian cancers. Individuals with germline BRCA1/2 alterations treated with the PARP1 inhibitors (iPARP1) tend to respond better than patients with wild-type BRCA1/2. Additionally, also somatic BRCA1/2 alterations induce the sensitivity to iPARP1. Therefore, the detection of both germline and somatic BRCA1/2 mutations is required for effective iPARP1 treatment. The aim of this study was to identify the frequency and spectrum of germline and somatic BRCA1/2 alterations in a group of Polish patients with ovarian serous carcinoma. In total, 100 formalin-fixed paraffin-embedded (FFPE) ovarian serous carcinoma tissues were enrolled to the study. Mutational analysis of BRCA1/2 genes was performed by using next-generation sequencing. The presence of pathogenic variants was confirmed by Sanger sequencing. In addition, to confirm the germline or somatic status of the mutation, the nonneoplastic tissue was analyzed by bidirectional Sanger sequencing. In total, 27 (28% of patient samples) mutations (20 in BRCA1 and 7 in BRCA2) were identified. For 22 of 27 patients, nonneoplastic cells were available and sequencing revealed the somatic character of two BRCA1 (2/16; 12.5%) and two BRCA2 (2/6; 33%) mutations. Notably, we identified six novel frameshift or nonsense BRCA1/2 mutations. The heterogeneity of the detected mutations confirms the necessity of simultaneous analysis of BRCA1/2 genes in all patients diagnosed with serous ovarian carcinoma. Moreover, the use of tumor tissue for mutational analysis allowed the detection of both somatic and germline BRCA1/2 mutations.

Introduction

BRCA1 and BRCA2 tumor suppressor genes play an important role in DNA damage and repair pathways. Germline mutations in these genes are strongly associated with an increased risk of breast and ovarian cancer [1, 2]. Previous studies estimated that approximately 15% of the Polish patients diagnosed with ovarian cancer carry germline BRCA1/2 mutation [3–5]. This frequency is comparable to the overall prevalence of BRCA1/2 mutations among ovarian cancer patients worldwide [6, 7].

Although 20–70% of sporadic ovarian tumors display loss of heterozygosity (LOH) in the BRCA1/2 loci, indicating essential role of these genes in ovarian cancer pathogenesis, somatic mutations of these genes are relatively rare finding [8–10]. To date, somatic BRCA1 mutations were reported in 5–9% of sporadic ovarian cancer cases, whereas somatic genetic variants of BRCA2 were identified in 3–4% of tumors [11–15].
Recently, many clinical trials for specific therapies targeting cells with defect BRCA signaling pathway are ongoing, that is, with poly (ADP-ribose) polymerase 1 (PARP1) inhibitors. PARP1 is a member of chromatin-associated polymerases involved in the single-strand breaks repair, a common form of DNA damage [16]. The clinical response rate to PARP inhibitor treatment among BRCA1/2 mutation carriers was higher than in wild-type patients [17–21]. The eligibility for iPARP1 treatment is thus determined by the BRCA1/2 mutation status, both germline and somatic. Therefore, complex mutational analysis of BRCA1/2 genes could increase the number of patients who might benefit from PARP1 inhibitors treatment.

In this study, we established the frequency and type of BRCA1/2 mutations. Mutational analysis of both genes was performed in 100 formalin-fixed paraffin-embedded tissues (FFPE) tissues from ovarian cancers.

Materials and Methods

Study material

In total, 100 FFPE serous ovarian carcinoma samples were enrolled to the study. All samples were obtained from the files of the Department of Pathomorphology, Medical University of Gdansk and were collected between 2008 and 2012. The histological diagnosis of ovarian serous carcinoma and the tumor tissue content (TTC%) of each sample were evaluated by pathologists. In order to obtain cancer cells from heterogeneous histological samples, tissue macrodissection was performed. The average patient age at diagnosis was 60 years (range: 36–81). Informed consent was obtained from all the patients and the research was approved by local ethics committee.

DNA extraction

Genomic DNA was extracted from the macrodissected FFPE tissues using Cobas DNA Sample Preparation Kit (Roche, Basel, Switzerland) according to manufacturer’s protocol. Quantity and quality of isolated DNA was determined with NanoDrop 1000 UV Spectrophotometer (Thermo Scientific, Canton, GA, USA) and Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). In a selected 22 samples, DNA from uterus or peripheral blood samples was isolated by using Cobas DNA Sample Preparation Kit (Roche, Basilea, Switzerland) or Blood Midi kit (A&A Biotechnology, Gdynia, Poland).

Mutational analysis

BRCA1 and BRCA2 mutation screening was performed using the BRCA Tumor MASTR Plus assay (Multiplicom, Niel, Belgium) according to the manufacturer’s protocol. MiSeq targeted resequencing 99x (Illumina, San Diego, CA, USA) was performed. The read length was pair-end and cut-off of 4% for the Variant Allele Frequency was applied. The median coverage for all samples was 1700.

The analysis was performed with Sophia DDM software (Sophia Genetics, Saint-Sulp). The presence of the BRCA1/2 mutation was confirmed by bidirectional Sanger sequencing (ABI PRISM 3130, Life Technologies, Carlsbad, CA, USA). Finally, in order to determine the somatic or germline status of detected alteration in 22 BRCA1/2 positive tumor samples, mutational analysis of DNA isolated from nonneoplastic cells was performed by PCR followed by Sanger sequencing.

Results

From the 100 samples three (#8, #34 and #95) were excluded from the analysis due to low quality of the results. In the remaining 97 patients diagnosed with serous ovarian carcinoma, pathogenic BRCA1/2 variants were identified in 27 (28%) samples. All genetic variants are presented at Figure 1 and Table 1. Among the BRCA1/2 positive cases, 20 (74%) BRCA1 and 7 (26%) BRCA2 mutations were identified. The most frequent alteration was c.5266dupC (rs80357906, p.Gln1756Profs*74) in BRCA1, detected in 6.2% (n = 6/97) of tumors.

For 22 patients with pathogenic BRCA1/2 mutations, DNA extracted from uterus or peripheral blood leukocytes was available for further molecular studies. Within this group, only four (4.1%) mutations were found to be somatic while 18 (18.6%) were also identified in a non-neoplastic cells and were classified as germline variants. In total, 14 (78%) and four (22%) germline mutations were found in BRCA1 and BRCA2, respectively.

To our knowledge, six of the 27 (22.2%) variants were not previously reported. These six novel mutations included three deletions, two substitutions, and one duplication. All these genetic variants resulted in a premature stop codon and protein truncation (Table 1).

Mutations in the BRCA1 gene were more frequently found in patients diagnosed before or at the age of 50 compared to older individuals – 40% (8/20) versus 16% (12/77), respectively. None of the BRCA2 alteration was identified in the group of patients younger than 50 years old. The mean age of ovarian cancer diagnosis was 54.5 (range: 36–76), 58.4 (range: 51–76) and 61.9 (range: 40–81) years for BRCA1, BRCA2 and wild-type patients, respectively.

In the study, three individuals were compound heterozygous for BRCA1 and BRCA2 mutations. One alteration was pathogenic and a second was previously classified as
unknown variant although resulting is premature STOP codon.

Detailed histopathological and molecular data, including all variants identified in the studied group, are shown in Table S1.

**Discussion**

Since personalized target therapy appears more common in modern oncology, evaluation of the highly sensitive and cost-effective analysis accessible for routine diagnostics
Table 1. Pathogenic mutations in the BRCA1/2 genes in 27 serous ovarian tumors.

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<th>Age at diagnosis (years)</th>
<th>FIGO stage</th>
<th>Tumor content (%)</th>
<th>Exon/intron</th>
<th>Mutation in corresponding cDNA</th>
<th>Predicted effect</th>
<th>Mutation type</th>
<th>RS Number</th>
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1Mutation type according to the Human Genome Variant Society (HGVS) nomenclature.
2A reference SNP number.
Nd, no data; F, frameshift mutation; N, nonsense mutation; S, splicing mutation.
has become a high priority. Currently, the gold standard for detecting somatic mutations is Sanger sequencing, but its diagnostic yield is relatively low. In addition, the analysis of FFPE tumor material is challenging, because the extracted DNA is typically of poor quality and highly fragmented. Moreover, tumor cells are histologically and genetically heterogeneous and tumor DNA may be contaminated with DNA from nonneoplastic cells. Therefore, implementation of novel, powerful tools enabling the detection of low-level mutations, such as next-generation sequencing should be considered as a standard diagnostic procedure. The application of next-generation sequencing allows to detect somatic mutations that may be present in a low proportion of the total DNA. In this report, we determined the frequency of somatic and germ line BRCA1/2 mutations in serous ovarian carcinomas, the entire coding sequence of these genes was analyzed by next-generation sequencing. To distinguish true alterations from artifacts, the cut-off of 4% for the Variant Allele Frequency was applied. The presence of pathogenic variants has been confirmed by Sanger sequencing.

Molecular analysis of BRCA1/2 revealed the presence of pathogenic mutations in 28% of 97 studied samples. Among the BRCA1/2 positive cases, the frequency of germ line and somatic mutations was 18.6% and 4.1%, respectively. The percentage of identified somatic changes is comparable to previous reports [11–15], whereas the BRCA1/2 germline mutation frequency is higher than the expected frequency in a constitutive ovarian cancer cases (~19% vs. 11–15.3%) [3–7]. However, the explanation of this phenomena could be due to the differences in the studied patient population. In this study, all cases were classified as serous cancers which is the most frequent histological subtype of ovarian carcinomas and has the highest incidence of BRCA1/2 mutations [7, 22] of all ovarian tumors. Not surprisingly, the majority of mutation-positive samples (n = 25/27; 93%) were classified as high-grade serous ovarian tumors. In contrast, only in two (~7%) low-grade tumors BRCA1/2 mutation was detected (Table 1).

According to the guidelines of the American Society of Clinical Oncology genetic testing for BRCA1/2 mutations should be performed in each patient with ovarian cancer (1996) [23]. The advantages of this approach were presented in our previous study, where more than one-third of BRCA1/2-positive patients had no family history of breast or ovarian cancer [5]. Unfortunately, in this study, no information about family history of the patients was available. Another important issue is the source of the biological material used for molecular studies. In contrast to peripheral blood samples, use of a tumor tissue for mutational analysis allow to identify somatic mutations which account for additional 3–9% of patients with ovarian cancer [11–15]. In this study, the frequency of somatic alterations was significantly higher, accounting for approximately 15% of all detected mutations.

The recommendations of the European Molecular Genetics Quality Network (EMQN) suggest to perform mutational analysis of the both BRCA1 and BRCA2 genes in each individual [24], which has important implications for genetic counseling, especially for cosegregation analysis. So far, the phenomena of double BRCA1 and BRCA2 mutation carriers was mainly, but not only, described in the Ashkenazi Jewish population [25–27]. In this study, we identified three individuals with two different germline alterations. One genetic variant was identified as pathogenic and a second was unknown variant, although resulting is premature STOP codon (Table S1). Two patients (#73 and #98) carried BRCA1 splicing alterations (c.4484+1G>A and c.4357+2T>G) and nonsense mutations in the BRCA2 gene (c.9976A>T; p.Lys3326*) whereas patient #47 carried BRCA1 founder mutation (c.5266dupC; Gln1756Profs*74) and a known frameshift change in the BRCA2 gene (c.10095delinsGAATTATATCT; p.Ser3366Asnfs*4). According to the BIC database [28], c.9976A>T change is designated as benign based on its location (the last exon of BRCA2) and a frequency (variant was present in 2.5% of patients and 2.1% of healthy controls). In contrast, Martin et al. (2005) described the higher frequency among patients with familial pancreatic cancer indicating its potential pathogenicity [29]. c.10095delinsGAATTATATCT variant of BRCA2 was previously detected in two Polish patients with familial aggregation of the disease [30]. In both individuals, the presence of other BRCA1/2 mutations was excluded. Finally, in silico analysis suggest a possible impact of c.10095delinsGAATTATATCT on mRNA splicing.

Finally, to our knowledge six presumably novel mutations in the BRCA1 and BRCA2 genes were identified (Table 1). These included two nonsense and four frameshift variants, all resulting in premature termination of translation.

In conclusion, the application of a targeted assay using next-generation sequencing of FFPE samples allowed the identification both somatic and germ line BRCA1/2 mutations in a group of patients with serous ovarian tumors. As we previously mentioned, the routine genetic screening for at least five recurrent BRCA1 mutations (c.5266dup, c.181T>G, c.3700_3704del, c.68_69delAG, and c.4034delA) in all ovarian cancer patients in the Polish population should be performed [30]. However, the use of a tumor sample for molecular analysis and further mutational analysis of the entire coding sequence of both BRCA1 and BRCA2 may significantly increase the total number of patients who may potentially benefit from targeted therapies with PARP inhibitors. In addition, such a genetic
testing would allow to identify more family members with BRCA1/2 mutations.

**Conflict of Interest**

L.J. did receive honoraria for advisory activities from AstraZeneca. W.B. did receive honoraria for advisory and consulting functions and educational activities from AstraZeneca.

**References**


Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. All identified variants in the BRCA1/2 genes in the serous ovarian tumors.