Original paper

Somatic mutations in *BRCA1* and *2* in 201 unselected ovarian carcinoma samples – single institution study

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Ovarian cancer (OC) is the most lethal among gynecologic malignancies worldwide. Unfortunately, in around 70% of cases cancer is diagnosed in late stages (III-IV) which decreases the 5-year survival rate to 25%. The standard of care in ovarian cancer is debulking surgery followed by chemotherapy regimens based on platinum salts. Since 2014 PARP inhibitors became available for OC patients with germline or/and somatic mutations in *BRCA1/2*, including maintenance therapy. *BRCA1/2* Next Generation Sequencing (NGS)-based analysis of formalin-fixed paraffin-embedded (FFPE) ovarian cancer samples becomes the standard of care. The aim of the present study was to evaluate the frequency of mutations in 201 unselected ovarian cancer tissues using the NGS method. In total, pathogenic mutations in both genes were detected in 24% (49/201) of the ovarian cancer cases tested. For 41 patients the results of testing of DNA isolated from blood sample revealed that 17% (35/201) mutations were germline origin, whereas 3% (6/201) mutations were somatic. In 4% (8/201) cases blood sample was inaccessible. The presence of pathogenic mutations was correlated with younger age at diagnosis and serous subtype. Close cooperation between many specialists (gynecologist, pathologist, oncologist, clinical genetics and molecular biologist) is indispensable for efficient and on-time *BRCA1/2* ovarian tumor tissue testing.

Key words: covarian cancer, NGS, BRCA1, BRCA2, somatic mutation.

Introduction

Ovarian cancer is the most lethal among gynecologic malignancies with 238700 new cases and 151900 deaths reported annually worldwide [1]. In Europe alone the statistics points towards 65500 new cases and 42700 deaths each year [2]. As the cancer initially develops without any specific symptoms, only 20-30% of women are diagnosed at early stages (I-II) which translates into 90% 5-year survival rates. Unfortunately, in around 70% of cases cancer is diagnosed at late stages (III-IV) which decreases the 5-year survival rate to 25%. Despite numerous research efforts, the combination of low specificity symptoms and lack of reliable biochemical markers makes the early detection of ovarian cancer one of the main challenges for health care systems [3].

The standard of care in ovarian cancer is debulking surgery followed by chemotherapy regimens based on platinum salts [3]. In 2014 this standard was enriched by first registration of poli-ADP-ribose Polymerase inhibitors. Since 2014 several other indications of PARP inhibitors became available for OC patients with germline or/and somatic mutations in *BRCA1* & *BRCA2* genes, including maintenance therapy in the 1st line setting translating to spectacular progression-free survival (PFS) difference [4].

The prevalence of mutations in *BRCA1* & *BRCA2* genes in OC patients population is approximately 14-18% and 7-8% for germline and somatic setting, respectively [5, 6]. In Poland, until recently the dominant constitutive mutation detection system was based on founder mutations screening, focusing on 3-5 most frequent loci [7, 8]. Dramatic cost reduction of Next Generation Sequencing (NGS) and advent of PARP inhibitors have been the main driving forces for the founder screening system to be gradually replaced by full genes sequencing of DNA isolated either from leukocytes (germline mutation) or tumour specimen (germline and somatic mutation detection). Initial studies clearly showed that founder mutations cover as little as 48-65% of the germline mutation spectrum further reinforcing the testing paradigm shift towards NGS-based approach [9, 10, 11]. In Poland, germline mutations are now detected in 15% of unselected ovarian cancer cases and as many as 28% for testing from tumour specimen [10, 12, 13]. Introduction of NGS-based tumour testing has the advantage of detection both germline and somatic mutations yet poses several challenges in the patients pathway and lab pipeline [14]. Here we present the results of analysis of 201 unselected ovarian cancer cases specimens that were tested for *BRCA1*/*BRCA2* mutations using Next Generation Sequencing of tumour-derived DNA.

Table I. Clinical characteristic of the studied 201 ovarian carcinoma cases

	Study group No. 201
Age at diagnosis, years; median (Q1-Q3)	59.0 (52.0-67.0)
Age at diagnosis, years; mean ±SD	58.5 ±10.6
Histological subtype	
Serous	155 (77.1%)
Endometrioid	21 (10.4%)
Clear cell	9 (4.5%)
Mucinous	6 (3.0%)
Undifferentiated	6 (3.0%)
Mixed (serous and endometrioid)	4 (2.0%)
Histological grade of serous carcinomas (n = 155)	
high	122 (78,7%)
low	33 (21,3%)
FIGO stage	
I, II	40 (19.9%)
III, IV	152 (75.6%)
unknown	9 (4.5%)

Material and methods

The material for the study derives from 201 unselected patients with ovarian carcinomas operated in 2015-2017 at the Department of Gynecologic Oncology Clinic Holycross Cancer Center in Kielce (Table I). Mean and median age of patients at diagnosis were 58.5 and 59 years, respectively. Formalin-fixed paraffin-embedded tissue blocks stored at the Department of Pathology were used for the study. In terms of histopathology, the majority of cases (78%) in the study group were serous and 11% were endometrioid. The other histopathological types were clear cell, mucosal, undifferentiated and mixed. Among serous carcinomas, the high grade (HG) subtype constituted 78,7% and the low grade (LG) –21.3 (6/33; 38%). According to FIGO (International Federation of Gynecology and Obstetrics) classification, 76% of patients were in stage III/IV, 20% in I/II and for 4% no data were available.

All of the study procedures were approved by the Institutional Review Board at the Holycross Chamber of Physicians in Kielce (10/2016) and performed according to the Declaration of Helsinki. All patients gave their informed consent and all methods were performed in accordance with the relevant guidelines and regulations.

DNA isolation

The pathologist marked the area containing OC tumor cells on a hematoxylin and eosin-stained slide. In all studied cases in the marked area tumor cell content was more than 70%, range (10-100%). Then, the tumor tissue on matched unstained slides was deparaffinized and the selected area was transferred to a tube for DNA isolation using the Maxwell 16 and Maxwell® 16 FFPE Tissue LEV DNA Purification Kit, according to the manufacturer's instructions (Promega, USA). The isolated DNA concentration was measured by using NanoDrop (TkBiotech, Warsaw, Poland). Mean concentration of isolated DNA was 100ng/μl and purity for 260/280 ratio ranged between 1.8-2.0.

Next Generation Sequencing Library preparation

The DNA was diluted to 10 ng/μl. The libraries were prepared using the Oncomine™ BRCA Research Assay, Manual Library Preparation and the Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific), according to the manufacturer's instructions (Thermo Fisher Scientific). Oncomine™ BRCA Research Assay is two pool Ion AmpliSeq™ design containing 265 amplicons. Two separate polymerase chain reaction (PCR)-multiplex reactions were performed for each of the samples tested, and the samples were combined into one. The resulting multiplex PCR products were subjected to partial enzymatic digestion to remove primer sequences. Next, adapters for multiplex PCR products were enzymatically attached using the Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific). One of the adapters contains barcodes that allow identification of sequences from a given patient among a mixture of libraries. The prepared libraries were cleaned using Agencourt AMPure XP (Beckman Coulter Genomics) according to the manufacturer's instructions (Ion AmpliSeq Library Kit 2.0 – Thermo Fisher Scientific).

Preparation of clonally amplified template for sequencing – emulsion PCR (emPCR) for S5 using IonChef

The concentration of libraries was measured by quantitative PCR with real-time detection (qRT-PCR) using the Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) on a Rotor-Gene Q instrument (Qiagen). Based on the values obtained with qRT-PCR, all prepared libraries were diluted to a concentration of 100pM. Then, with Ion Chef (Thermo Fisher Scientific) and Ion 520 & Ion 530 Kit-Chef and Ion 530 ™ Chip Kit (Thermo Fisher Scientific), emPCR was performed, enrichment and two 530 chips were loaded (16-24 samples per chip, cov x1000).

Sequencing

Sequencing was performed on an Ion S5 sequencer (Thermo Fisher Scientific) according to the manufacturer's instructions.

Bioinformatic analysis

The raw data generated during sequencing was processed using the Torrent Server Suite 5.6(TSS) (Thermo Scientific, USA). The obtained sequences were matched (mapped) to the reference sequence of the human genome (hg19). Searching for different variants (single nucleotide polymorphism [SNP], deletions, insertions) was carried out using the Variant Caller 5.6 program which is a part of Torrent Server Suite 5.6. The following basic parameters of the variants were used: minimum allele frequency – SNP = 0.01 / INDEL = 0.05, minimal quality – 10, minimal sequencing depth – 10. Variant Caller is compatible with the IGV genomic browser – Integrative Genomics Viewer (Broad Institute), which enables fast visualization of sophisticated variants. To annotate the detected variants with the TSS, the wANNOVAR software (www.wannovar.usc.edu) was used. Additionally, Torrent Server Suite 5.6 generated FASTQ files that were used for analysis using the CLC Biomedical Workbench 5.0 (Qiagen). The basic parameters used in the analysis using CLC software were: minimum allele frequency – 0.01, minimal quality – 10, minimal sequencing depth – 100. Detected mutations, SNP, insertions and deletions of the coding regions of the analyzed genes were filtered to detect pathogenic mutations by COSMIC base, dbSNP database (to discard hereditary polymorphisms) and population base of the 1000GENOMES project. Only variants with minimal 5% allelic frequency were reported.

Classification of pathogenicity of mutations.

Detected mutations were classified based on the information deposited in the ClinVar database and according to the American the College of Medical Genetics and Genomics (ACMG) recommendations [15]. In addition, in the case of variant of unknown significance or conflicting results we have performed *in silico* analysis using Varsome (https://varsome.com/) which integrates useful algorithms and databases, frequency in the populations etc. and literature search [16, 17].

Sanger Sequencing

Thanks to the close cooperation with our Genetic Clinic, mutations detected in tumor tissue were verified (germline *vs.* somatic) using DNA isolated from blood sample when it was available from the same patient. The Sanger Sequencing reaction

used PCR amplification products that were purified using 10 U of exonuclease I (EN 0582) and 1 U of phosphatase Fast-AP (EF 0651) (both from ThermoFisher Scientific, Waltham, MA). The reaction was incubated for 15 min at 37°C, followed by 20 min at 80°C. Sequencing reactions were performed using forward and reverse sequence-specific primers and the ABI PRISM Big Dye Terminator Kit, version 3.1 (catalogue number 4337450, Applied Biosystems/ThermoFisher Scientific), according to the manufacturer's instructions. The sequencing results were analyzed using the 3130 Capillary Sequencer (Applied Biosystems/ThermoFisher Scientific). The generated sequences were compared to the reference sequence using the NCBI Blast Nucleotide program.

Statistical analysis

Categorical data were summarized by frequencies and percentages and compared by Fisher's exact test. Numerical variables were presented as mean and standard deviations as well as median and quartiles (Q1-Q3); differences between groups were analyzed (due to non-normality) by Mann-Whitney U-test. A two-tailed p-value < 0.05 was considered as statistically significant. All statistical analyses were performed using R software (version 3.1.2; The R Foundation for Statistical Computing, Vienna, Austria).

Results

NGS sequencing of *BRCA1* and *BRCA2* was successful in 98% (197/201) of the cases studied, while in four cases (2%) good quality DNA sequencing results were not obtained (Fig. 1).

In 36 (18%) tissue samples, pathogenic mutations were detected in the *BRCA1* gene and in 13 (6%) – in the *BRCA2* gene. In total, pathogenic mutations in both genes were detected in 24% (49/201) of the ovarian cancer cases tested (Fig. 1, Table II). In 11 (11/49; 22%) cases, pathogenic mutations in *BRCA1*: 4x p.Cys61Gly, 7x p.Gln1756ProfsTer74, which are well known as a founder for the Polish population, were detected (Table II). In addition, in 4 (4/49; 8%) cases we detected a pathogenic mutation (*BRCA1*: p.Arg1751Ter) which was recognized as recurrent in the Polish population. The above-mentioned mutations were most often detected mutations in our cohort. Due to close cooperation with our Genetic Clinic, for 41 patients results of testing of DNA isolated from blood sample were available. Thirty five (17%; 35/201) mutation were germline origin, whereas 6 (3%; 6/201) mutations were somatic. We were unable to reveal germline vs. somatic status in 8 (4%; 8/201) patients (Fig. 2). However, in this group of unknown status two (1%; 2/201) mutations (*BRCA1*: one p.Cys61Gly and one p.Gln1756ProfsTer74) are founder for Polish population.

Variants of unknown significance (VUS) were detected in 12 (6%) tissue samples (Table III). In the remaining 136 (68%) cancer tissue samples no mutations in the *BRCA1/2* genes were detected.

Considering the histopathological type of ovarian cancer, pathogenic mutations in *BRCA1/2* were detected in 29% (45/155) cases of serous ovarian cancer. In the serous HG subtype, 32% (39/121) of pathogenic mutations were detected and in the serous LG ovarian cancer, pathogenic mutations were detected in 18% (6/33) of cases. In endometroid ovarian cancer, in 10% (2/21) of cases pathogenic mutations in *BRCA1/2* were detected. In clear cell ovarian carci-

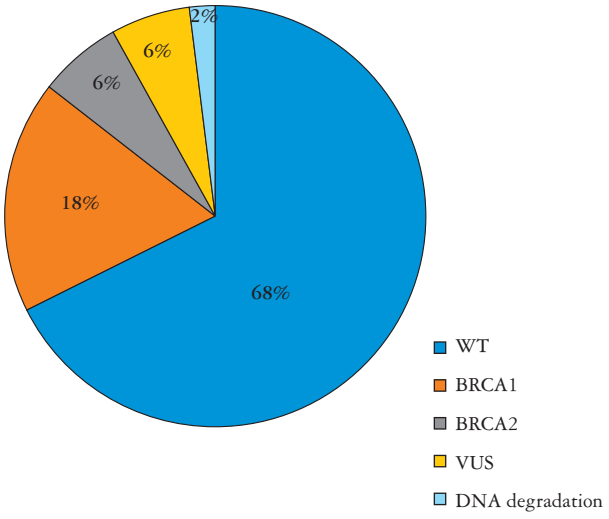

Fig. 1. Percentage share of *BRCA1/2* NGS testing results in 201 studied ovarian carcinoma tissues

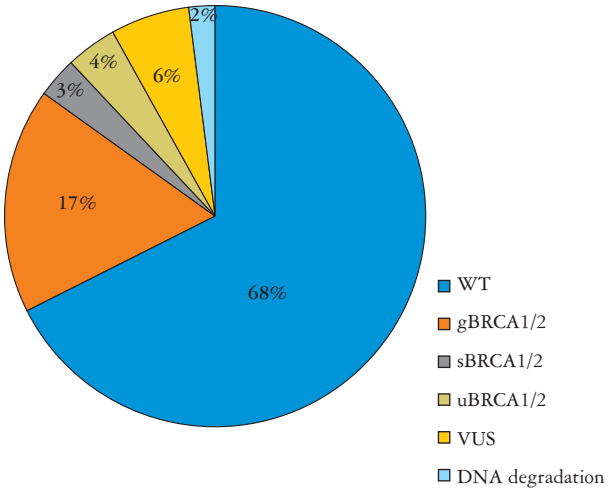

Fig. 2. Percentage share of germline *vs.* somatic status of *BRCA1/2* NGS testing results in 201 studied ovarian carcinoma tissues

Table II. Clinical and pathological characteristics of the pathogenic mutation carriers detected with NGS

No.	Age at diagnosis	Histology	FIGO	% of tumor cells	Gene	P- Pathogenic, PF- Pathogenic founder, PR- Pathogenic recurrent	cDNA *, **	Predicted effect	Exon	Type of mutation: F – frameshift variant; N – nonsense variant; M – missense variant; S – splicing variant	% of mutated allel frequency NGS	Mutation category according to ACMG Classification #	Database	G-germiline S-somatic U-unknown
1	57	Serous HG	IIIC	90%	*BRCA1*	PF	c.181T>G	p.Cys61Gly	5	M	71%	Pathogenic	ClinVar	G
2	46	Serous HG	III	95%	*BRCA1*	P	c.191G>A	p.Cys64Tyr	4	M	50%	pathogenic	ClinVar	G
3	42	Serous HG	IIIC	95%	*BRCA1*	PF	c.181T>G	p.Cys61Gly	5	M	50%	Pathogenic	ClinVar	G
4	54	Serous HG	IV	40%	*BRCA1*	PF	c.181T>G	p.Cys61Gly	5	M	64%	Pathogenic	ClinVar	U
5	45	Serous HG	IIIC	90%	*BRCA1*	PF	c.181T>G	p.Cys61Gly	5	M	77%	Pathogenic	ClinVar	G
6	42	Serous HG	IIIC	80%	*BRCA1*	P	c.1612_1616delCAAAC	p.Gln538GlyfsTer11	10	F	50%	Pathogenic	ClinVar	G
7	63	Serous HG	IIIC	90%	*BRCA1*	P	c.2089_2090insTG	p.Phe697LeufsTer5	10	F	70%	Pathogenic	Novel	G
8	62	Serous HG	IIIC	90%	*BRCA1*	P	c.1612_1616delCAAAC	p.Gln538GlyfsTer11	10	F	50%	Pathogenic	ClinVar	U
9	64	Serous HG	IIIC	95%	*BRCA1*	P	c.2209delA	p.Thr737GlnfsTer16	10	F	79%	Pathogenic	ClinVar	S
10	57	Serous HG	IIIC	95%	*BRCA1*	P	c.3449delC	p.Pro1150LeufsTer5	10	F	71%	Pathogenic	Novel	S
11	69	Serous HG	IIIC	80%	*BRCA1*	P	c.2679_2682delGAAA	p.Lys893AsnfsTer106	10	F	84%	Pathogenic	ClinVar	U
12	45	Serous HG	III	90%	*BRCA1*	P	c.4185+1delG	Alteration of the WT donor site, most probably affecting splicing.	12	S	74%	Pathogenic	Novel	G
13	54	Serous HG	IIIC	90%	*BRCA1*	P	c.4186C>T	p.Gln1396Ter	12	N	87%	Pathogenic	ClinVar	G
14	73	Serous HG	IIIC	80%	*BRCA1*	P	c.4689C>G	p.Tyr1563Ter	15	N	47%	Pathogenic	ClinVar	G
15	52	Serous HG	IIIC	95%	*BRCA1*	P	c.4689C>G	p.Tyr1563Ter	15	N	50%	Pathogenic	ClinVar	U
16	57	Serous HG	IIIC	90%	*BRCA1*	PR	c.5251C>T	p.Arg1751Ter	19	N	68%	Pathogenic	ClinVar	G
17	40	Serous HG	IIIC	90%	*BRCA1*	PR	c.5251C>T	p.Arg1751Ter	19	N	89%	Pathogenic	ClinVar	G
18	42	Serous HG	III	90%	*BRCA1*	PR	c.5251C>T	p.Arg1751Ter	19	N	60%	Pathogenic	ClinVar	G
19	58	Serous HG	III	80%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	70%	Pathogenic	ClinVar	G

Table II. Cont.

No.	Age at diagnosis	Histology	FIGO	% of tumor cells	Gene	P- Pathogenic, PF- Pathogenic founder, PR- Pathogenic recurrent	cDNA *, **	Predicted effect	Exon	Type of mutation: F – frameshift variant; N – nonsense variant; M – missense variant; S – splicing variant	% of mutated allel frequency NGS	Mutation category according to ACMG Classification #	Database	G-germiline S-somatic U-unknown
20	35	Serous HG	IC	80%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	70%	Pathogenic	ClinVar	G
21	67	Serous HG	IIIC	80%	*BRCA1*	PR	c.5251C>T	p.Arg1751Ter	19	N	75%	Pathogenic	ClinVar	G
22	90	Serous HG	IIA	95%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	58%	Pathogenic	ClinVar	U
23	40	Serous HG	IIIB	40%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	53%	Pathogenic	ClinVar	G
24	52	Serous HG	IC	40%	*BRCA1*	PR	c.5251C>T	p.Arg1751Ter	19	N	66%	Pathogenic	ClinVar	G
25	39	Serous HG	IIA	75%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	92%	Pathogenic	ClinVar	G
26	49	Serous HG	IIIC	95%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	55%	Pathogenic	ClinVar	G
27	68	Serous HG	IIIC	95%	*BRCA1*	PF	c.5266dupC	p.Gln1756ProfsTer74	19	F	78%	Pathogenic	ClinVar	G
28	46	Serous HG	IIIC	80%	*BRCA1*	P	c.5291delA	p.Gln1764ArgfsTer23	20	F	38%	Pathogenic	Novel	S
29	70	Serous HG	IIIC	90%	*BRCA2*	P	c.1800T>G,	p.Tyr600Ter	10	N	68%	Pathogenic	ClinVar	G, G
30	59	Serous HG	IIIC	90%	*BRCA2*	P	c.5946delT	p.Ser1982ArgfsTer22	11	F	20%	Pathogenic	ClinVar	G
31	60	Serous HG	IIIC	40%	*BRCA2*	P	c.6405_6409delCTTAA	p.Asn2135LysfsTer3	11	F	35%	Pathogenic	ClinVar	G
32	66	Serous HG	IIIB	70%	*BRCA2*	P	c.5778_5779delTG	p.Ser1926ArgfsTer7	11	F	10%	Pathogenic	ClinVar	S
33	61	Serous HG	IVB	95%	*BRCA2*	P	c.5722_5723delCT	p.Leu1908ArgfsTer2	11	F	70%	Pathogenic	ClinVar	G
34	65	Serous HG	IIIC	80%	*BRCA2*	P	c.4483_4484delGT	p.Val1495ProfsTer18	11	F	70%	Pathogenic	ClinVar	G
35	53	Serous HG	IIIC	95%	*BRCA2*	P	c.5946delT	p.Ser1982ArgfsTer22	11	F	60%	Pathogenic	ClinVar	G
36	65	Serous HG	IA	85%	*BRCA2*	P	c.3847_3848delGT	p.Val1283Lysfs	11	F	84%	Pathogenic	ClinVar	G
37	55	Serous HG	IIIC	90%	*BRCA2*	P	c.7211_7212delAA	p.Lys2404Serfs	14	F	60%	Pathogenic	ClinVar	U
38	62	Serous HG	IVA	90%	*BRCA2*	P	c.7512delT	p.Pro2505HisfsTer19	15	F	44%	Pathogenic	Novel	S
39	61	Serous LG	IIC	70%	*BRCA1*	P	c.1612_1616delCAAAC	p.Gln538GlyfsTer11	10	F	60%	Pathogenic	ClinVar	G
40	61	Serous LG	IIC	95%	*BRCA1*	P	c.1612_1616delCAAAC	p.Gln538GlyfsTer11	10	F	74%	Pathogenic	ClinVar	G
41	59	Serous LG	BD	60%	*BRCA1*	P	c.4185G>A	p.Gln1395=	11	N	50%	Pathogenic	ClinVar	G

Table II. Cont.

No.	Age at diagnosis	Histology	FIGO	% of tumor cells	Gene	P- Pathogenic, PF- Pathogenic founder, PR- Pathogenic recurrent	cDNA *, **	Predicted effect	Exon	Type of mutation: F – frameshift variant; N – nonsense variant; M – missense variant; S – splicing variant	% of mutated allel frequency NGS	Mutation category according to ACMG Classification #	Database	G-germiline S-somatic U-unknown
42	40	Serous LG	IIIC	70%	*BRCA1*	P	c.4186C>T	p.Gln1396Ter	12	N	60%	Pathogenic	ClinVar	G
43	46	Serous LG	III	100%	*BRCA1*	P	c.4689C>G	p.Tyr1563Ter	15	N	68%	Pathogenic	ClinVar	U
44	69	Serous LG	IIIC	90%	*BRCA2*	P	c.6295delA	p.Arg2099AspfsTer20	11	F	33	Pathogenic	Novel	G
45	61	Serous HG	IIIC	80%	*BRCA1*	P	c.1612_1616delCAAAC	p.Gln538GlyfsTer11	11	F	60%	Pathogenic	ClinVar	G
46	39	Endometroid G1	IIB	90%	*BRCA2*	P	c.289G>T	p.Glu97Ter	3	N	22%	Pathogenic	ClinVar	S
47	57	Endometrioid	BD	95%	*BRCA1*	P	c.3756_3759delGTCT	p.Ser1253ArgfsTer10	11	F	82%	Pathogenic	ClinVar	G
48	59	Clear cell	IA	70%	*BRCA1*	P	c.68_69delAG	p.Glu23ValfsTer17	2	F	55%	Pathogenic	ClinVar	G
49	68	Mixtum (Serous HG, Endometroid G2)	IIIC	75%	*BRCA2*	P	c.G7007A	p.Arg2336His (p.Gly2313AlafsX31)]	13	M	75%	Pathogenic	ClinVar	U

** BRCA1 Refrence sequence: NM_007294.3*

*** BRCA2 Refrence sequence: NM_000059.3*

 # based on the American the College of Medical Genetics and Genomics (ACMG) recommendations

HG – high grade, LG – low grade

Table III. Clinical and pathological characteristics of the VUS carriers identified with NGS

No	Age at diagnosis	Histology	FIGO	tumor cell content %	Gene	cDNA *, **	Predicted effect	Exon	Type of mutation	Mutation category according to ACMG Classification #	% of mutated allel frequency NGS	Database	G-germiline S-somatic U-unknown
1	56	Serous HG	III	80%	*BRCA1*	c.518C>G	p.P173R	6	missense	VUS	23%	Varsome	S
2	56	Serous LG	IIIC	60%	*BRCA1*	c.648C>G	p.I216M	10	missense	VUS	7%	Novel	S
3	61	Endometrioid	IC	70%	*BRCA1*	c.3347C>T	p.A1116V	11	missense	VUS	24%	Novel	U
4	69	Serous HG	BD	70%	*BRCA1*	c.1768A>G	p.S590G	11	missense	VUS	22%	ClinVar	U
5	48	Serous LG	IIIC	95%	*BRCA2*	c.938C>T	p.S313F	10	missense	VUS	16%	ClinVar	U
6	71	Serous LG	IIIC	70%	*BRCA2*	c.3070A>G	p.I1024V	11	missense	VUS	50%	ClinVar	G
7	75	Undifferentiated carcinoma	BD	90%	*BRCA2*	c. 4951C>G	p.Pro1651Ala	11	missense	VUS	33%	Varsome	U
8	70	Serous HG	IA	90%	*BRCA2*	c.7768T>C	p.Ser2590Pro	16	missense	VUS	77%	ClinVar	U
9	74	Serous HG	IIA	80%	*BRCA2*	c.7768T>C	p.Ser2590Pro	16	missense	VUS	81%	ClinVar	U
10	50	Clear cell carcinoma	IIC	95%	*BRCA2*	c.9542T>G	p.M3181R	26	missense	VUS	49%	ClinVar	G
11	47	Endometrioid	IA	60%	*BRCA2*	c.9443C>T	p.A3148V	27	missense	VUS	36%	ClinVar	U
12	53	Serous HG	IIB	90%	*BRCA2*	c.9869T>C	p.V3290A	27	missense	VUS	7%	Novel	S

** BRCA1 Refrence sequence: NM_007294.3*

*** BRCA2 Refrence sequence: NM_000059.3*

 # based on the American the College of Medical Genetics and Genomics (ACMG) recommendations

HG – high grade, LG – low grade

Table IV. Frequency of detected pathogenic mutation in *BRCA1/2* in studied histopathological subtypes of ovarian carcinoma

Histological subtype of ovarian cancer	**Cases**	**BRCA1/2 mut**	*%*
Serous	155	45	29
Serous HG	122	39	32
Serous LG	33	6	18
Endometrioid	21	2	10
Clear cell	9	1	11
Mucinous	6	0	0
Undifferentiated	6	0	0
Mixted (serous+endometrioid)	4	1	25

LG – low grade

HG – high grade

noma, in 1 out of 9 cases pathogenic mutation was detected and in 1 out of 4 cases of mixed ovarian (serous and endometroid) cancer. In the mucinous and undifferentiated subtypes mutations were not detected (Table IV).

The analysis indicated that patients with pathogenic mutations in ovarian cancer were on average 3 years younger compared to patients without mutations ($p = 0.041$). The presence of pathogenic mutation in *BRCA1/2* correlated with the onset of serous histological type ($p = 0.003$). There is no relationship between the presence of pathogenic *BRCA1/2* mutation and FIGO stage ($p = 0.95$; Table V).

In the subgroup with only serous histology ($n = 151$), pathogenic mutations in *BRCA1/2* were detected in patients on average 4 years younger than patients without mutation. In the case of serous subtypes and FIGO stage no statistically significant correlation was found (Table VI).

Discussion

As there are no reliable markers that would support early detection of ovarian cancer, around 70% of patients are diagnosed when the disease has already spread (FIGO stage III/IV). A well established and optimal therapeutic approach usually consists of extensive cytoreductive surgery followed by chemotherapy regimen based on platinum salts [3]. In 2014 this armamentarium was enriched by the advent of PARP inhibitors with indication for use in patients with somatic or germline mutation in *BRCA1 & BRCA2* genes [18]. Recently published phase III study of Olaparib as maintenance therapy in newly diagnosed ovarian cancer patients with *BRCA* mutation showed a 70% reduction in the risk of disease progression or death [19]. Therefore, it has become of utmost importance to utilize the most efficient testing tools in order to maximize the pool of patients who could benefit from this treatment. As sequencing of DNA derived from tumor specimen allows to detect both germline and somatic mutations, NGS-based analysis of FFPE ovarian cancer samples becomes the standard of care [14].

NGS-based analysis of large genes such as *BRCA1* and *BRCA2* from FFPE sample has proved to be more challenging than similar testing performed on blood-derived DNA.

First of all, formalin fixation modifies bases in the DNA which are the source of artefacts that can blur identification of pathogenic variants. Additionally, formalin-based fixation should be performed strictly to the protocol as DNA is prone to break and shear in spots of formaldehyde-introduce cross-links. Overfixation of sample is a major reason of excessive fragmentation of DNA which is not compatible with downstream analysis steps, such as library generation [20, 21].

In the current study, with in-house derived and processed material, only 2% of samples gave inconclusive test result due to insufficient quality of DNA which is lower than published from other centers [22]. We attribute this low testing failure rate for FFPE samples to an excellent crossdisciplinary cooperation model between surgery unit, pathology and molecular diagnostics laboratory we have set up in our center to ensure optimal pipeline of sample acquisition, sample fixation & processing and downstream molecular analysis.

Secondly, the availability of PARP inhibitors (PARPi), now also in newly diagnosed ovarian cancer patients, has remodelled *BRCA* testing referral pattern. In order to ensure that *BRCA1, BRCA2* genes status is known in time for first line treatment decisions, patients should be referred for molecular testing at the time of cytoreductive surgery by the operating surgeon. In our center, a vast majority of patients are automatically referred for testing upon debulking surgery and confirmation of the diagnosis by the pathologist. This ensures on-time identification of patients qualifying for PARPi treatment and allows to minimize the number of tests performed on archival FFPE samples that could be subject to degradation [23].

In this study we identified pathogenic *BRCA* mutation in 24% of unselected ovarian cancer cases. Among patients with serous subtype, mutation was found in 29% of samples. These results are in line with similar analyses of smaller groups performed in the Polish population (28%) by other investigators, as well as studies performed by Italian or Chinese groups, 28% and 26%, respectively [13, 24, 25].

Table V. Comparison of clinical characteristics of cases with detected pathogenic mutation in *BRCA1/2* and the cases without mutations

	BRCA1/2 pathogenic mutation	no *BRCA1/2* mutation (VUS included)	P-value
	No. 49	No. 148	
Age at diagnosis, years; median (Q1-Q3)	57.0 (46.0-63.0)	60.5 (52.0-67.0)	0.041
Age at diagnosis, years; mean ±SD	56.0 ±11.2	59.3 ±10.4	
Histological subtype of cancer			0.099
Serous	45 (91.8%)	106 (71.6%)	
Endometrioid	2 (4.1%)	19 (12.8%)	
Clear cell	1 (2.0%)	8 (5.4%)	
Mucinous	0 (0.0%)	6 (4.1%)	
Undifferentiated	0 (0.0%)	6 (4.1%)	
Mixed (serous + endometrioid)	1 (2.0%)	3 (2.0%)	
Histology			0.003
non-serous	4 (8.2%)	42 (28.4%)	
serous HG	39 (79.5%)	79 (53.4%)	
serous LG	6 (12.2%)	27 (18.2%)	
Histological type of cancer			0.003
non-serous	4 (8.2%)	42 (28.4%)	
serous	45 (91.8%)	106 (71.6%)	
FIGO stage			0.95
I, II	9 (18.4%)	31 (20.9%)	
III, IV	38 (77.6%)	111 (75.0%)	
unknown	2 (4.1%)	(4.1%)	

HG – high grade, LG – low grade

Table VI. Comparison of clinical characteristics of cases with detected pathogenic mutation in *BRCA1/2* and the cases without mutations in the subgroup of 151 patients with serous ovarian carcinoma

	BRCA1/2 pathogenic mutation	no *BRCA1/2* pathogenic mutation (VUS included)	P-value
	No. 45	No. 106	
Age at diagnosis, years; median (Q1-Q3)	57.0 (46.0 - 63.0)	62.0 (53.0 - 68.0)	0.018
Age at diagnosis, years; mean (±SD)	56.0 (±11.2)	60.0 (±10.5)	
Histological subtype			0.13
serous HG	39 (86.2%)	79 (74.5%)	
serous LG	6 (13.3%)	27 (25.5%)	
FIGO stage			1.0
I, II	7 (15.6%)	16 (15.1%)	
III, IV	37 (82.2%)	88 (83.0%)	
unknown	1 (2.2%)	2 (1.9%)	

HG – high grade, LG – low grade

Many countries with strong founder effect in *BRCA1* & *BRCA2* mutation epidemiology such as Poland have originally set up their testing system on a panel of most frequent founder loci analysis to optimize cost effectiveness. In this study we show that in our region only 30% (16/49) of identified mutations belong to the founder/recurrent spectrum, further reinforcing the need to shift from founder to NGS-based approach (Table II) [9]. Thanks to cooperation with the Genetic Counselling Unit, for 41 (84%) of identified patients it was possible to determine somatic *vs.* germline mutation status and provide extensive genetic counselling. The analysis showed that 17% of the mutations were germinal and only 3% were somatic. The frequency of germline mutations (17%) detected in this work is similar to those reported in the literature (14-23%) and could be higher if we verified the status (germinal *vs.* somatic) in 8 (4%) cases (Fig. 2) [6, 10, 12]. According to published data, prevalence of somatic *BRCA1* and *BRCA2* mutations varies between 3% and 10%. Our results (3%) fall almost below this range most likely due to the sample size and inability to verify somatic *vs.* germline status for 8 (4%) patients in the cohort. Additionally, in our bioinformatic pipeline we apply a minimum 5% allelic frequency cut-off which might have contributed to lower detection of somatic variants.

In subtypes other than serous pathogenic *BRCA1* and *BRCA2* variants were also detected but with significantly lower frequencies, this observation is in line with available data that points towards mutations in other genes (*PIK3CA, PTEN, CTNNB1, ARIDA1, KRAS*) as more frequent aberrations in non-serous ovarian cancer [26].

Age analysis revealed that ovarian cancer patients with *BRCA1*, *BRCA2* mutations are on average 3 years younger than patients without pathogenic variant and mutations more often occur in the serous type in comparison to other histological types. As previously observed, we also did not observe any effect of FIGO stage between the groups [5]. However, in the group of serous carcinomas (155) only age at diagnosis remained a parameter that differentiated cancers with pathogenic mutation in *BRCA1/2* in comparison to non-mutant cancers ($p = 0.018$).

To further optimize identification of ovarian cancer patients with *BRCA1/2* mutations we plan to extend the molecular analysis of large rearrangements that may be found in up to 5% of Polish population [27]. Additionally, we also consider to introduce a broader gene panel for ovarian cancer patients that would include, apart from *BRCA1* and *BRCA2*, a number of other genes involved in homologous recombination-mediated repair of DNA double strand breaks that have been linked with familial ovarian cancer, with which we may detect additional 6% of carriers [12].

Conclusion

The availability of efficient, newly developed PARP inhibitors in *BRCA*-mutated OC patients and NGS technologies becoming an affordable testing tool, have driven a dynamic shift in the way *BRCA1/BRCA2* mutation testing is performed. This paradigm change from test-to-prevent to test to-to-treat implied some important challenges in the inter-disciplinary OC patients care. From the experience of our center, we identified the following key touch points: 1) need of efficient communication between the pathologist and the surgeon performing debulking surgery that ensures correct acquisition and fixation of adequate, representative cancer specimen for histopathology report and that is compatible with downstream molecular analysis; 2) robust cooperation between molecular laboratory and pathologists securing on-time and quality sample processing; 3) working out format of test result report that meets expectations of oncologists to support informed therapeutic decisions and, lastly; 4) strict cooperation between molecular laboratory and Genetic Counselling Unit that would ensure all OC patients to receive genetic counselling and, if needed, further molecular analysis to determine germline or somatic background of detected variants.

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