ASSOCIATION OF 22 POTENTIAL PATHOGENIC VARIANTS OF NEW CANDIDATE GENES AND THE RISK OF OVARIAN CANCER

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Abstract. The high risk of ovarian cancer is primarily associated with mutations in *BRCA1* and *BRCA2* genes. However, mutations in these explain only a small proportion of cases. Mutations in other genes are also involved in the disease. As a result of previous exome sequencing of DNA samples from breast cancer Germany patients with clinical signs of a hereditary form of the disease without major mutations in the *BRCA1, BRCA2, CHEK2* and *NBN* genes, potentially pathogenic genetic variants in new breast and ovarian cancer candidate genes were selected. Selected as a result of bioinformatics analysis genes are involved in vital cell signaling pathways such as repair, apoptosis, cell cycle regulation, cell proliferation, migration, differentiation, as well as immune response and inflammation. Recently, biological microarray technologies have been widely used to study the general genetic variability throughout the human genome in order to determine genetic associations with the disease and search for genes involved in the pathogenesis of multifactorial pathologies. The use of such approaches can be very useful for identifying risk markers for the development and severity of diseases. Our case-control study is aimed at researching potentially pathogenic variants selected as a result of exome sequencing of DNA samples from Caucasian patients using microarray technology Fluidigm to assess their contribution to ovarian cancer pathogenesis in Bashkortostan. Most of the researched alleles were found with different frequencies among cases and controls; however, our data indicate that the researched potentially pathogenic variants do not contribute to ovarian cancer pathogenesis in Bashkortostan populations.

Keywords: ovarian cancer, candidate genes, exome sequencing, potential pathogenic variants, risk of disease, Fluidigm BioMark™ HD.

List of Abbreviations

OC – ovarian cancer
OCAC – cancer association consortium
WES whole – exome sequencing
DNA – deoxyribonucleic acid
HWE –Hardy–Weinberg equilibrium
PCR – polymerase chain reaction
LSP – locus-specific primer
STA – specific target amplification

Introduction

Ovarian cancer (OC) is one of the most common gynecological malignancy that has the highest mortality rate. Worldwide, approximately 313,000 women are diagnosed with ovarian cancer each year, and 207, 000 dies. In 2020, in Russia has estimated 13,192 new cases of ovarian cancer, and more than half of the cases were fatal (Kaprin *et al.*, 2021). The high mortality rate is primarily due to the detection of ovarian cancer in the late stages of development (III-IV). In the first year after diagnosis, every third patient dies. There has been a tendency over the last years towards the rejuvenation of this cancer type, so the disease is more often diagnosed in a group of women under the age of 30 (Cress *et al.*, 2015; Torre *et al.*, 2018).

Ovarian cancer is a complex, multifactorial, heterogeneous disease that includes a number of different histological type. The high risk of

this pathology is primarily associated with mutations in the tumor suppressor genes *BRCA1* and *BRCA2* (Antoniou *et al.*, 2003; Bateneva *et al.*, 2013; Bermisheva *et al.*, 2018; Lyubchenko, 2009; Shubin & Karpukhin, 2011). Other genes with moderate and low penetrance are also involved in OC development, which are part in maintaining the integrity of the genome, the processes of cell proliferation and migration, and repair (*NBN, RAD50, MRE11, CHEK2, BLM, PALB2, ATM, BRIP1, BARD1, MDC1, STK11, TP53, CDK12* and others) (Bateneva *et al.*, 2013; Bermisheva *et al.*, 2018; Bogdanova *et al.*, 2019; Gordiev *et al.*, 2018; Koczkowska, *et al.*, 2018; Prokofieva, 2013).

Despite the fact that to date some progress has been achieved in the study of genetic predisposition to ovarian cancer, there are still many unclear aspects. International Ovarian Cancer Association Consortium (OCAC) has been established for a comprehensive study of the OC. These researchers have identified new genetic risk factors and targets for treatment of patients diagnosed with ovarian cancer (Johnatty *et al.*, 2015; Knijnenburg *et al.*, 2018).

Recently, biological microarray technologies have been widely used to study the general genetic variability throughout the human genome in order to determine genetic associations with the disease and search for genes involved in the pathogenesis of multifactorial pathologies, which allow simultaneous testing of thousands of samples in a short time. The use of such approaches can be very useful for identifying risk markers for the development and severity of diseases, as well as for creating a person's "genetic passport" (Chan *et al.*, 2017).

We have screened and analyzed the association of 22 potential pathogenic variants of new OC candidate genes. The options under consideration were selected as a result of bioinformatic analysis of data from exome sequencing of DNA samples from patients with hereditary breast cancer and OC, carried out earlier by colleagues from Germany (School of Medicine, Hannover).

Materials and Methods

Study populations

The material for this research was DNA samples from women diagnosed with ovarian cancer (n = 212) and women without cancer at the time of blood sampling (n = 212) at the age of 17-87 years from the Republic of Bashkortostan. All OC patients and healthy women originated from the Volga-Ural region but belonged to different ethnic groups, including Russians (47.9%), Tatars (30.6%), Bashkirs (12.3%), Ukrainians (2.9%), and patients of other (3.9%) or mixed ancestry (2.3%). In terms of ethnic composition, the control group corresponded to the group of patients.

Peripheral venous blood was taken by employees of the State Autonomous Institution of Health Republican Clinical Oncology Center of the Health Ministry of the Bashkortostan Republic (Ufa) and the Oncology Department of the City Clinical Hospital No. 1 (Sterlitamak). All participants of this research signed voluntary informed consent for molecular genetic studies. The work was approved by the Bioethical Committee of the Institute of Biochemistry and Genetics, Ufa Federal Research Center of the Russian Academy of Sciences.

In this study were included DNA samples from patients with epithelial OC. Of these, 76,4% of women had poorly differentiated serous tumors, 2% had highly differentiated serous tumors, 5% had mucinous tumors, 1,5% had clear cell tumors, and 1% had endometrioid tumors. Tumors were predominantly of a high grade (G1-G2) – 31.2%. A low-grade tumor (G3-G4) was detected in 15.2% cases, and grading of cancer cells was not histologically determined in 52.9% patients. Bilateral OC was present in 57% women with OC. Stage I of disease was established in 16.8% of patients; II – in 33.2%; III – in 43.4% and stage IV – in 6.6% of cases. Family history of OC and/or breast cancer was found in 9.5% of patients.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional re-

search bioethical committee of the Institute of Biochemistry and Genetics, Ufa Federal Research Center of the Russian Academy of Sciences and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Each participant gave written informed consent.

Methods

Genomic DNA was isolated from peripheral blood samples from ovarian cancer patients and controls by routine phenol-chloroform extraction. The DNA concentration was measured using NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

As a result of previous whole exome sequencing (WES), 24 potentially pathogenic genetic variants were selected, which became the object of study in this project. Genetic variants were selected by them role in protein function: truncating variants (essential splice-site, frameshift, stop gained and one genetic variant that results in the substitution of the first amino acid). We genotyped using standard protocol of Fluidigm 192.24 SNPtype Genotyping Technology (Fluidigm, South San Francisco, CA, USA) (Olwagen *et al.*, 2019). Investigated genetic variants of ovarian cancer candidate genes presented in the study are shown in Table 1.

The genotypes of the polymorphisms listed above were determined by polymerase chain reaction (PCR). First, for the polymerase chain reaction analysis, the amount of DNA was quantified to 50 ng and the DNA fragment was amplified using two preamplification primers (locus-specific primer (LSP) and specific target amplification (STA) primer to amplify the target region containing the genetic variants. Multiplex PCR was performed on an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), with the following conditions: hold at 95 °C for 15 min, 14 cycles at 95 °C for 15 s, and 60 °C for 4 min. The ready STA-product was diluted 100-fold in suspension DNA buffer.

A second amplification was performed on the Fluidigm 96.96 Dynamic Array. Assay mixes was prepared by mixing 3 μL of each allele-specific primer (ASP), 8 μL of each locus-specific primer (LSP), and 29 μL DNA hydration buffer. 0.8 μl of each assay mix was combined with 2.0 μL of 2 × Assay Loading Reagent and 1.2 μL of nuclease-free water. To prepare a pre-mix for samples, 2.25 μl Biotium 2X Fast Probe Master Mix, 0.225 μl 20X SNP Type Sample Loading Reagent, 0.075 μl 60X SNP Type Reagent, 0.027 μl ROX, 0.048 μl nuclease-free water were mixed. Sample mixture with a volume of 4.5 μL was prepared by combining 2.6 μL of the pre-mix with 1.9 μL of 1: 100 diluted STA amplifier. The mixture with samples was vortexed for 20 seconds and then centrifuged for 30 seconds. The BioMark HD dynamic array was first primed with control line fluid, and then loaded with the samples and assay mixtures via the appropriate inlets using an IFC (integrated fluidic circuit) controller. Samples were applied to the chip according to the manufacturer's instructions. The array chip was placed in the BioMark HD Instrument. PCR was carried out using the following cycling conditions: 95 °C for 5 min, followed by four touchdown cycles (95 °C for 15 s, from 64 °C to 61 °C for 45 s, 72 °C for 15 s) and 34 additional cycles (95 °C for 15 s, 60 °C for 45 s, 72 °C for 15 s). Each PCR reaction used distilled water instead of DNA as negative control. For variants rs201755391 and rs763189487 in genes *PARP14* and *TSR1*, respectively, low fluorescence signals were obtained and subsequently their studies were excluded. Results were plotted on a two-dimensional scatter plot of the major versus the minor allele using the BioMark SNP Genotyping Analysis software version 2.1.1. Genotyping calls were assessed based on the allele discrimination plots and manually reviewed by looking at the single amplification plots. Genotyping calls were exported as a CSV file and processed for secondary analysis.

Chi-square was used to test association and Hardy–Weinberg equilibrium (HWE) for each variant. Variants that did not pass the HWE criteria have been discarded from further analysis. All statistical assessments were two-sided and considered to be significant when p-value was < 0.05.

Table 1

The potential pathogenic variants of ovarian cancer candidate genes presented in the research

№	Gene	Variant	Type of variant	dbSNP	Chr (GRCh37)	Ref Allele	Alt Allele
1	*USP39*	g.85876139G > C, c.*208G > C	violation of the acceptor site	*rs*112653307	Chr.2:85876139	*G*	*C*
2	*EGF*	g.110932393dupC, c.3406dupC, p.Gln1136fs	Frameshift	*rs*11569144	Chr.4:110932393	*CCCC*	*CCCCC*
3	*SMARCAL1*	*g.217342939G > T, c.2542G > T, p.Glu848Ter*	Nonsense	*rs*119473033	Chr.2:217342939	*G*	*T*
4	*BCLAF1*	g.136599127G > A, c.892C > T, p.Arg298Ter	Nonsense	*rs*138333275	Chr.6:136599127	*G*	*A*
5	*ANKRD36*	g.97779488delC,c.12delC, p.Lys5fs	Frameshift	*rs*141447363	Chr.2:97779488	*C*	*delC*
6	*PHKB*	*g.47495300G > A, c.39G > A, p.Trp13Ter*	Nonsense	*rs*141733590	Chr.16:47495300	*G*	*A*
7	*TP53I3*	g.24302375G > C, c.755C > G, p.Ser252Ter	Nonsense	*rs*145078765	Chr.2:24302375	*G*	*C*
8	*PZP*	*g.9321534G > A, c.2038C > T, p.Arg680Ter*	Nonsense	*rs*145240281	Chr.12:9321534	*G*	*A*
9	*APLF*	g.68805146delA, c.1528delA, p.Arg510fs	Frameshift	*rs*149897324	Chr.2:68805146	*AAAA*	*AAA*
10	*EXO5*	g.40981245_40981246insG, c.1029_1030insG, p.Arg344fs	Frameshift	*rs*150018949	Chr.1:40981245	*D*	*insG (I)*
11	*BABAM2*	g.28532947A > C, c.1088+11589A > C	violation of the acceptor site	*rs*150302537	Chr.2:28532947	*A*	*C*
12	*HERC6*	g.89318021T > A, c.906T > A, p.Tyr302Ter	Nonsense	*rs*192005184	Chr.4:89318021	*T*	*A*
13	*DCLRE1A*	g.115610226C > T, c.638G > A, p.Trp213Ter	Nonsense	*rs*200026311	Chr.10:115610226	*C*	*T*
14	*SLX1B*	g.29469248G > C, c.711-1G > C	violation of the acceptor site / occurrence new acceptor site	*rs*200435542	Chr.16:29469248	*G*	*C*

The end of the table 1

№	Gene	Variant	Type of variant	dbSNP	Chr (GRCh37)	Ref Allele	Alt Allele
15	*PARP14*	g.122404166G > A	violation of the donor site	*rs*201755391	Chr.3:122404166	*G*	*A*
16	*APOBEC1*	g.7805414C > T, c.62G > A, p.Trp21Ter	Nonsense	*rs*34275479	Chr.12:7805414	*C*	*T*
17	*H4C2*	g.26027457dupA, c.24dupA, p.Lys9Ter	Nonsense	*rs*535221714	Chr.6:26027457	*A*	*dupA*
18	*H4C12*	g.27799162_27799163insA, c.143_144insT, p.Gly49fs	Frameshift	*rs*544620282	Chr.6:27799162	*D*	*insA (I)*
19	*AUNIP*	g.26161770_26161779del, c.780_789delTTCACTGATT, p.Glu260fs	Frameshift	*rs*564635111	Chr.1:26161770	*TTTCACTGATT*	*delTTTCACTGATT*
20	*MYCT1*	g.153019103_153019106delTAGA, c.66_69delTAGA, p.Asp22fs	frameshift	*rs*3841162	Chr.6:153019103	*AGATAGA*	*delTAGA*
21	*NANOG*	g.7947687_7947688delTG, c.914_915delTG,p.Ter306LysextTer	Nonsense	*rs*762642172	Chr.12:7947687	*GTGTG*	*delTG*
22	*TSR1*	g.2238179G > A, c.568C > T, p.Gln190Ter	Nonsense	*rs*763189487	Chr.17:2238179	*G*	*A*
23	*ATP23*	g.58335486T > A, c.2T > A, p.Met1Lys	Missense	*rs*768622289	Chr.12:58335486	*T*	*A*
24	*FANCL*	g.58386930_58386933dupAATT, c.1111_1114dupAATT, p.Thr372fs	Frameshift	*rs*759217526	Chr.2:58386930	*TAATT*	*dupAATT*

Results

As a result of previous exome sequencing of DNA samples from breast cancer patients with clinical signs of a hereditary form of the disease, in whom major mutations in the *BRCA1*, *BRCA2*, *CHEK2* and *NBN* genes were not detected, potentially pathogenic genetic variants in new breast and ovarian cancer candidate genes were selected. All variants are truncating. The genes included in the study are involved in vital cell signaling pathways such as repair, apoptosis, cell cycle regulation, cell proliferation, migration, differentiation, as well as immune response and inflammation.

The role predisposing to OC was studied for 22 alleles. Three alleles *SMARCAL1* p.Glu848Ter, *DCLRE1A* p.Trp213Ter and *ATP23* p.Met1Lys were not detected. These alleles are also extremely rare in population databases. Especially the *ATP23* p.Met1Lys allele, which was found with a frequency of 0.0004% to 0.002% in the ALFA and gnomAD-Exomes projects. Missense variant *ATP23* p.Met1Lys leads to the replacement of the start codon with lysine, which identifies it as pathogenic and is confirmed by predictive algorithms. Such a low frequency of occurrence of a rare allele also testifies in favor of the high pathogenicity of the considered genetic variant. Minor allele of variants *USP39* c.*208G>C (0.72%), *BCLAF1* p.Arg298Ter (1.21%), *TP53I3* p.Ser252Ter (1.67%), *PHKB* p.Trp13Ter (1.90%) and *HERC6* p.Tyr302Ter (1.44%) with different low frequencies were found among controls only. These alleles occurred at a much higher frequency than the 1000 Genome Project, ALFA and gnomAD-Exomes populations (Table 2). For 14 potentially pathogenic variants, rare alleles were identified both among patients and control and we could association research (Table 3).

Minor allele of variants *APLF* p.Arg510fs (1.65% vs 0.72%), *EXO5* p.Arg344fs (1.66% vs 0.72%) and *H4C12* p.Gly49fs (0.98% vs 0.79%) were more common in cases than controls, respectively. Potentially pathogenic genetic variants *MYCT1* p.Asp22fs (4.95%) and *NANOG* p.Ter306LysextTer (3.10%) were detected at a markedly higher frequency in case and control, respectively. The highest frequency of the minor allele was found for *ANKRD36* p.Lys5fs (12.44% in patients with OC and 9.38% in controls). The allele frequencies of the studied genetic variants assessed are reported in Table 3.

Discussion

As mentioned earlier, the selected truncating variants were found by exome sequencing and most of them have not been described in the literature. However, several alleles have been described in relation to various diseases.

The *EXO5* c.1029_1030insG (p.Arg344fs) variant, which leads to a frameshift and synthesis of the defective protein, was also identified by full exome sequencing in Spanish patients with testicular cancer with a family history. Using algorithms that predict the effect of this genetic variant on the structure and function of the protein, the authors of the study concluded that it is highly pathogenic (Phred = 28.3). Subsequently, this variant was searched for in an expanded sample of patients and healthy individuals, which revealed an association of the p.Arg344fs polymorphic locus with a moderate risk of developing testicular cancer (Paumard-Hernández *et al.,* 2018). In our study, the frequency of the minor allele in the group of patients was more than two times higher (3.32%) than in the control group (1.44%) for this reason, we do not exclude the possibility of an association with OC with an increase in the statistical power of the study.

The variant *USP39* (c.*208G>C) leads to disruption of mRNA splicing acceptor site in the 3′-untranslated region of the gene (Fujiwara *et al.,* 2014). This variant was associated with triple-negative breast cancer in Russians, as well as with breast cancer in a combined analysis of three populations (Russians, Germans, and Belarusians) (Kuligina *et al.,* 2020). Interestingly, carriers of this variant were found only in the control group (0.72%) in our research.

The *PZP* p.Arg680Ter variant, which leads to the loss of protein function, was previously identified by whole exome sequencing in female patients with clinical signs of hereditary breast cancer from Brazil (Thompson *et al.,*

Table 2

Frequency investigated alleles of ovarian cancer candidate genes in this case-control study and few projects of allele frequency in different populations

№	Gen	Variant	Cases	Controls	1000 Genomes	ALFA	gnomAD - Exomes
1	*USP39*	c.*208G>C	0	0.72	0.1	0.78	0.5
2	*EGF*	c.3406dupC, p.Gln1136fs	0.71	2.13	8.7	0.50	3.97
3	*SMARCAL1*	c.2542G>T, p.Glu848Ter	0	0	–	0.02	0.008
4	*BCLAF1*	c.892C>T, p.Arg298Ter	0	1.21	0.10	0.073	0.2
5	*ANKRD36*	c.12delC, p.Lys5fs	12.44	9.38	2.42	5.91	7.40
6	*PHKB*	c.39G>A, p.Trp13Ter	0	1.90	0.14	0.11	0.22
7	*TP53I3*	c.755C>G, p.Ser252Ter	0	1.67	–	0.13	0.19
8	*PZP*	c.2038C>T, p.Arg680Ter	0.24	0.47	0.14	0.44	0.43
9	*APLF*	c.1528delA, p.Arg510fs	1.65	0.72	0.46	2.08	1.1
10	*EXO5*	c.1029_1030insG, p.Arg344fs	1.66	0.72	0.84	1.71	1.3
11	*BABAM2*	c.1088+11589A>C	0.94	1.19	0.04	0.25	0.41
12	*HERC6*	c.906T>A, p.Tyr302Ter	0	1.44	0.16	0.45	0.6
13	*DCLRE1A*	c.638G>A,p.Trp213Ter	0	0	–	0.004	0.02
14	*SLX1B*	c.711-1G>C	0.71	0.95	0.02	–	0.1
15	*APOBEC1*	c.62G>A, p.Trp21Ter	0.24	0.48	0.22	0.9	0.6
16	*H4C2*	c.24dupA, p.Lys9Ter	0.47	0.24	0.06	0.02	0.14
17	*H4C12*	c.143_144insT, p.Gly49fs	0.98	0.79	0.06	0	0.2
18	*AUNIP*	c.780_789delTTCACTGAT, p.Glu260fs	0.50	1.77	0.22	0.4	0.41
19	*MYCT1*	c.66_69delTAGA, p.Asp22fs	4.95	3.15	8.43	3.45	5.48
20	*NANOG*	c.914_915delTG, p.Ter306LysextTer	3.10	4.76	1.74	1.2	1.1
21	*ATP23*	c.2T>A, p.Met1Lys	0	0	0	0.002	0.0004
22	*FANCL*	c.1111_1114dupAATT, p.Thr372fs	0.47	0.48	–	0.43	0.3

Table 3

Analysis of Case-Control Association Study for 22 po Frequency investigated potential pathogenic variants of ovarian cancer candidate genes in patients and controls

№	Gen	Variant	Alleles(A1)*/A2	Frequency A1 (cases)	Frequency A1 (control)	$\chi 2$	p
1	*EGF*	c.3406dupC	*dupC/C*	0.71	2.13	2.14	0.14
2	*ANKRD36*	c.12delC	*delC/C*	12.44	9.38	1.71	0.19
3	*PZP*	c.2038C>T	*T/C*	0.24	0.47	0.00002	1
4	*APLF*	c.1528delA	*delA/A*	1.65	0.72	0.85	0.36
5	*EXO5*	c.1029_1030insG	*D/I*	1.66	0.72	0.88	0.35
6	*BABAM2*	c.1088+11589A>C	*C/A*	0.94	1.19	0.0002	0.99
7	*SLX1B*	c.711-1G>C	*C/G*	0.71	0.95	0.00004	0.99
8	*APOBEC1*	c.62G>A	*T/C*	0.24	0.48	0.0003	0.99
9	*H4C2*	c.24dupA	*dupA/A*	0.47	0.24	0.00007	0.99
10	*H4C12*	c.143_144insT	*I/D*	0.98	0.79	0.01	0.92
11	*AUNIP*	c.780_789delTTCACTGATT	*D/I*	0.50	1.77	1.87	0.17
12	*MYCT1*	c.66_69delTAGA	*D/I*	4.95	3.15	1.30	0.25
13	*NANOG*	c.914_915delTG	*D/I*	3.10	4.76	1.14	0.29
14	*FANCL*	c.1111_1114dupAATT	*Dup AATT/ AATT*	0.47	0.48	0.24	0.63

* Allele1 (A1)-minor allele

2012; Torrezan *et al.*, 2018). However, a replicative study by Kuligina et al. among breast cancer patients of Russian, Belarusian and German ethnicity did not reveal a significant association of this variant with the disease (Kuligina *et al.*, 2020). In our research, this variant occurred at a low frequency among cases and controls, more common in health women (0.24% vs 0.47%, respectively). The frequency of this allele among controls corresponded to the prevalence of the genetic variant in population databases the 1000 Genome Project, ALFA and gnomAD-Exomes (0.14–0.44%). So, we do not to draw unambiguous conclusions about its role in the development of OC.

We research of the role of new ovarian cancer candidate genes. Many of alleles occur at a low frequency in populations and were not identified in this work. We do not exclude the possibility of finding associations with the ovarian cancer risk for these alleles with an increase in the number of samples (≥ 1 000).

Thus, our data indicate that the researched potentially pathogenic genetic variants do not contribute to the ovarian cancer pathogenesis in Bashkortostan populations.

Conflicts of interest: the authors declare no conflict of interest.

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