8. Rashidova, D.A. Hasanova, M.T. Onkomery v onkologii: biokhimicheskie aspekty kancerogeneza, opredeleniya strategij lecheniya, razrabotki innovacionnykh metodov diagnostiki [Oncomers in oncology: biochemical aspects of carcinogenesis, definition of treatment strategies, development of innovative diagnostic methods. Oriental renaissance: Innovative, educational, natural and social sciences. 2024; 4(4): 186-194. (In Russ.).]

9. Ca 19-9 https://helix.ru/kb/item/08-006

10. Solenova L.G. Sovremennye podkhody k ocenke vliyaniya zagryazneniya okruzhayushchej sredy na onkologicheskij risk [Modern approaches to assessing the impact of environmental pollution on cancer risk]. Uspekhi molekulyarnoj onkologii. [Advances in Molecular Oncology. 2020; 7 (1): 17-22. (In Russ.)].

11. Solomatina T.V. Onkomarkery kak faktor riska v rajonakh ehkologicheskogo neblagopoluchiya [Cancer markers as a risk factor in areas of ecological distress]. Byulleten' sibirskoj mediciny. [Bulletin of Siberian medicine. 2002; 1: 114-117. (In Russ.)].

12. Nikolaev V.M., Efremova S.D., Gol'derova A.S., i dr. Sravnitel'naya kharakteristika urovnya onkomarkerov u zhitelej Yakutii v rajonakh ehkologicheskogo neblagopoluchiya [Comparative characteristics of the level of tumor markers in residents of Yakutia in areas of ecological trouble]. Byulleten' VSNC SO RAMN [Bulletin of the SB RAMS. 2011; 3(79), part 1: 86-88 (In Russ.).]

13. Veremchuk L.V., Kikul P.F., Zhernovoj M.V., Ehkologicheskaya zavisimost' rasprostraneniya onkologicheskikh zabolevanij v Primorskom krae [Ecological dependence of the spread of oncological diseases in Primorsky Krai]. Sibirskij onkologicheskij zhurnal. [Siberian oncological journal. 2012; 16: 19-25 (In Russ.).]

14. Yagudina L.A. Novozhilova A.A. Immunodiagnostika opukholej [Immunodiagnostics of tumors]. Medicinskij al'manakh [Medical Almanac 2016; 2(42): 48-52 (In Russ.).]

15. Kaur G., Kumar R., Mittal S., Sahoo P.K., & Vaid U. Ground/drinking water contaminants and cancer incidence: A case study of rural areas of South West Punjab, India / Human and Ecological Risk Assessment: An International Journal. 2021; 27(1): 205–226. https://doi.org/10.1080/10807039.2019.1705145

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PROSPECTS FOR THE DETECTION OF OVARIAN CANCER BY GENETIC TESTING OF ASPIRATE FROM THE UTERINE CAVITY

In the article, a genetic study of biological samples from uterine cavity aspirate was performed in patients with serous ovarian carcinoma in order to identify gene mutations characteristic of ovarian tumor lesions. It has been proven that the aspiration from the uterine cavity contains diagnostically significant numbers of cells or fragments of ovarian cancer cells necessary for molecular genetic analysis and detection of mutations in the *TP53, FAT3, CSMD3, BRAF*, and *KRAS* genes. Mutations of the *BRCA1/2* genes are rare. The detection of *TP53, FAT3, CSMD3, BRAF,* and *KRAS* gene mutations in uterine aspirate cells requires an active diagnostic search for the detection of ovarian serous carcinoma.

Keywords: ovarian cancer, serous carcinoma, morbid obesity, oncogenic mutations, aspiration biopsy.

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Introduction. Ovarian cancer is the ninth most common type of cancer in women in Russia, accounting for approximately 3,7% of all cancers and 4,7% of deaths among patients with oncological pathology [2]. Malignant ovarian tumors occupy the first place among the causes of death of women with oncogynecological pathology [16]. Most cases of ovarian cancer are first diagnosed at late stages, which is due to the lack of effective methods of early diagnosis and the almost asymptomatic course of the disease [11]. It is extremely difficult to identify clear clinical signs of ovarian tumors, which explains the interest of oncologists in the risk factors for the development of this pathology and the development of effective methodological approaches for its early detection [14].

The use of ultrasound, tomographic (X-ray and magnetic resonance) diagnostic methods helps to identify malignant ovarian pathology in many ways, but these approaches cannot be considered as generally available to a wide range of women, especially at the screening stage [8]. Unfortunately, the determination of the concentration of adenogenic cancer antigen CA-125, the secretory protein of human epididymis 4 (HE4) in the blood, is of little informative value for detecting ovarian cancer at the initial stages [13]. In clinical practice, multiple determination of markers in the blood is used primarily to monitor treatment results and timely detect relapses [15].

Recently, there has been a growing number of proponents that screening should be performed only for primary

epithelial serous ovarian tumors, as they are the main cause of mortality from this disease [16]. Currently, it has been established that the majority of highly differentiated serous ovarian carcinomas occur in the fimbriae of the fallopian tube funnels [12]. It has been proven that circulating tumor cells in ovarian cancer, and especially when the tumor process spreads to the fallopian tubes, enter the uterine cavity through the fallopian tubes and concentrate in the uterine cavity [10]. For this reason, aspirate from the uterine cavity obtained by Pipel biopsy, which is regularly performed in women of pre- and postmenopausal age, especially in the presence of risk factors for oncogynecological diseases, becomes a valuable biological medium for the detection of ovarian cancer. Biological samples taken near the tumor process are always more informative for detecting diseases than venous blood.

Scientific papers have proved that DNA of ovarian cancer cells is detected in the biological medium obtained for the detection of cervical cancer during liquid cytology and uterine body cancer during aspiration biopsy [1,6]. The use of uterine aspirate, which contains the contents of the fallopian tubes and peritoneal fluid in contact with the ovaries, as well as circulating tumor cells, can be considered as an alternative medium to blood serum. Consideration of the risk stratification of ovarian cancer is also aimed at improving the effectiveness of screening by identifying those most at risk of the disease [8]. In this regard, the aim of the work was to evaluate the frequency of mutations of genes characteristic of ovarian tumor lesions in uterine aspirate cells in patients with serous ovarian carcinoma, taking into account the degree of malignancy of tumor cells and comorbid pathology.

Materials and methods. Two groups were formed for the study: the main group and the control group. The main group included 274 patients with newly diagnosed ovarian cancer. The control group consisted of 226 women of the same age as the main group, but without oncogynecological pathology, who attended preventive gynecological examinations with a Pipel aspiration biopsy. Depending on the presence or absence of morbid obesity in the main and control groups, 1 and 2 subgroups were distinguished. The criterion for morbid obesity was an increase in BMI of more than 40 kg/m2. Morbid obesity is a known risk factor for oncogynecological pathology [7]. In the patients of the main group, morbid obesity was found in 67 people (1 main subgroup), and in 207 women it was absent (2 main subgroup). In the control group, morbid obesity was detected in 47 (1 control subgroup), and in 179 women it was absent (2 control subgroup).

When including patients in the main group, the following criteria were followed: primary diagnosis of ovarian cancer; serous ovarian carcinoma by histological type; absence of cancer of the body and cervix; collection of a biological sample before the start of antitumor treatment.

The exclusion criteria were as follows: oncological diseases of other localization; decompensation of somatic diseases with the development of functional insufficiency of the main life-supporting systems (cardiac, respiratory, renal, liver failure).

The study was approved by the Ethics Committee of the Federal State Budgetary Institution "NMIC of Oncology" of the

Table 1

Initial general characteristics of patients in clinical groups

Indicator	The main group (n=274)		Control group (n=226)		p
	1 subgroup (n=67)	2 subgroup (n=207)	1 subgroup (n=47)	2 subgroup (n=179)	
Age, years (M±m)	55.3±1.54	58.4±1.49	58.6±0.66	59.4±1.12	p1=0.81 p2=0.92 p3=0.84 p4=0.95
BMI, kg/m² (M±m) (M±m)	44.9±1.92	23.1±1.54	40.9 ±1.67	22.3±1.38	p1<0.001 p2<0.001 p3=0.67 p4=0.85
Insulin resistance, abs. (%)	67 (100)	4 (1.9)	47 (100)	2 (1.1)	p1<0.001 p2<0.001 p3=1.0 p4=0.98
Diabetes mellitus, abs. (%)	31 (46.3)	3 (1.4)	16 (34)	1 (0.6)	p1<0.001 p2<0.001 p3=0.19 p4=0.98
Grade of malignancy, abs.(%): low grade high grade	 9 (13.4) 58 (86.6)	 44 (21.3) 163 (78.7)	-	-	p1=0.16
Stage, abs.(%): IIA IIB IIIA1 IIIA2 IIIB IV	 8 (11.9) 13 (19.4) 19 (28.4) 15 (22.4) 11 (16.4) 1 (1.5)	 24 (11.6) 39 (18.8) 63 (30.4) 48 (23.2) 31 (15) 2 (1)	-	-	p1=0.99

Note: p1 is the p–value when comparing 1 and 2 main subgroups, p2 is the p–value when comparing 1 main and 1 control subgroups, p3 is the p-value when comparing 2 main and 2 control subgroups, p4 is the p-value when comparing the indicators of 2 main and 2 control subgroups.

Ministry of Health of the Russian Federation. All patients reviewed and gave written informed consent to be included in the study.

The patients of the corresponding main and control subgroups did not differ in terms of age, BMI, or the frequency of concomitant diseases. In the case of morbid obesity, the frequency of insulin resistance and diabetes mellitus was higher in both the main and control groups. All women with morbid obesity had insulin resistance syndrome.

In the main group, the frequency of high-grade malignancy (low differentiation of cancer cells) was higher than the frequency of low-grade malignancy (high and moderate differentiation), regardless of the presence or absence of morbid obesity. Advanced stages of ovarian cancer (III-IV) were more common than early stages (I-II) in both the 1st (88.1% vs. 11.9%) and 2nd (88.4% vs. 11.6%) main subgroups.

The study was to determine in cells from the uterine cavity aspirate somatic mutations specific for ovarian cancer.

From the uterine cavity aspirate obtained by the Pyle biopsy, DNA was isolated using the AllPrep kit (Qiagen) according to the manufacturer's instructions. Five volumes of RLTM buffer (Qiagen) were added to the uterine aspirate samples when the amount of liquid was ≤3 ml. When the volume of liquid exceeded 3 ml, cells and cell fragments were precipitated by centrifugation at 1000 rpm for 5 min, and the precipitate was dissolved in 3 ml of RLTM buffer (Qiagen).

Purified DNA was amplified in three multiplex PCR reactions using primers for the amplification of 110- to 142-nucleotide segments of the genes studied: BRAF, KRAS, TP53, CSMD3, FAT3, BRCA1, and BRCA2. Three multiplex reactions were performed for each sample from a single patient, each containing non-overlapping amplicons. Each sample was evaluated in two duplicate wells. To improve the detection of low-frequency mutations, we used the Safe-SeqS (Safe-Sequencing System) technology, which assigns a unique identifier to each template molecule. PCR fragments with the same unique identifier were considered mutant only if they contained an identical mutation in 95% or more of the cases. To characterize each gene mutation, we calculated the minor allele frequency (MAF).

The statistical analysis of the study results was performed using the STATISTICA 12.0 program (StatSoft, USA).

Results and discussion. A complex of genes with recurrent somatic mutations characteristic of serous ovarian adenocarcinomas was identified according to a meta-analysis of the results of genetic studies of tumor cells in 489 patients within the Cancer Genome Atlas project [9]. According to the summarized results, in serous ovarian cancer, especially in cases with a high degree of malignancy, mutations in the TP53 gene are detected in 100% of cases, and mutations in the BRCA1/BRCA2, BRAF, KRAS, CSMD3, and FAT3 genes are also detected with a certain frequency [9].

All patients in the main group did not have cervical or uterine body cancer, and the diagnosis of ovarian serous adenocarcinoma was verified by ultrasound and tomography. All patients in the main group underwent surgery. The histological examination of tumor samples revealed the histotype of the tumor (serous carcinoma) and the grade of malignancy of the tumor cells. Before starting specific antitumor treatment, the patients of the main group underwent a standard liquid cervical cytology examination and a Pyle aspiration biopsy. The women of the control group underwent a Pyle aspiration biopsy as part of their preventive gynecological examinations.

Table 2

Frequency of gene mutations in uterine cavity aspirate samples in patients with ovarian cancer and in the control group

The gene	The main group (n=274)				The control group (n=226)				p
	1 subgroup (n=67)		2 subgroup (n=207)		1 subgroup (n=47)		2 subgroup (n=179)		
	Abs.	%	Abs.	%	Abs.	%	Abs.	%	
TP53	63	94.0	202	97.6	1	2.1	2	1.1	p1=0.16 p2<0.001 p3<0.001 p4=0.95
KRAS	7	10.4	18	8.7	-	-	1	0.6	p1=0.67 p3<0.001
BRAF	7	10.4	20	9.7	-	-	-	-	p1=0.85
CSMD3	11	16.4	31	15.0	-	-	-	-	p1=0.78
FAT3	28	41.2	37	17.9	5	10.6	-	-	p1=0.49 p2=0.0001 p4<0.001
BRCA1	5	7.5	9	4.3	1	2.1	-	-	p1=0.32 p2=0.89
BRCA2	3	4.5	5	2.4	-	-	-	-	p1=0.38

Note. p1 – the value of p when comparing the 1st and 2nd main subgroups, p2 – the value of p when comparing the 1st main and 1st control subgroups, p3 – the value of p when comparing the 2nd main and 2nd control subgroups, p4 – the value of p when comparing the indicators of the 2nd main and 2nd control subgroups.

Table 3

The frequency of gene mutations in aspirate samples from the uterine cavity in patients in the main group, depending on the degree of malignancy

The gene	The degree of malignancy				p
	low (n=55)		high (n=219)		
	Abs.	%	Abs.	%	
TP53	46	83.6	219	100.0	<0.001
KRAS	21	38.2	4	1.8	<0.001
BRAF	24	43.6	3	1.4	<0.001
CSMD3	-	-	42	19.2	0.002
FAT3	1	1.8	64	29.2	<0.001
BRCA1	1	1.8	13	5.9	0.33
BRCA2	-	-	8	3.7	0.15

In the uterine cavity aspirate of patients with ovarian cancer, mutations of the TP53 gene were found in the majority of cases (265/274, 96.7%). In one-fifth of the cases (65/274, 23.7%), patients in the main group had mutations in the FAT3 gene, in 15.3% (42/274), in the CSMD3 gene, in one-tenth of the cases of the BRAF genes (27/274, 9.9%) and KRAS (25/274, 9.1%). Mutations of the BRCA1/2 genes were rare (22/274, 8%).

Morbid obesity did not affect the frequency of gene mutations in patients with ovarian cancer, except for the FAT3 gene (Table 2). Mutations of the FAT3 gene were more common in patients with morbid obesity in the main group. In the control group, there were only a few cases of mutations in the studied genes. In the 1st control subgroup with morbid obesity, mutations of the FAT3 gene were found in one-tenth of the cases (10.6%). Consequently, mutations in the FAT3 gene were associated with both ovarian cancer and morbid obesity.

Among the patients of the main group, a low degree of malignancy of tumor cells (low grade) occurred in 55 (20,1%), and a high degree of malignancy of cancer cells prevailed in frequency (n=219, 79,9%). The characteristics of the effect of the degree of malignancy on the distribution of patients with gene mutations are presented in Table 3.

In patients with low-grade serous ovarian carcinoma, mutations were found in the *TP53* (83,6%), *BRAF* (43,6%), and *KRAS* (38,2%) genes in uterine aspiration cells. In patients with high-grade serous ovarian carcinoma, the number of genes and the frequency of their mutations were higher: *TP53* gene mutations were detected in all patients, *FAT3* in 29,2%, *CSMD3* in 19,2% and *BRCA1/2* in 9,6%.

According to literature data, mutations in the *BRAF, KRAS,* and *CSMD3* genes activate uncontrolled growth of tumor cells, which leads to an increase in tumor size and rapid metastasis [5]. Point mutations of the *FAT3* gene are simultaneously associated with both the development of ovarian cancer and impaired carbohydrate metabolism [4]. The *TP53* gene acts as an oncosuppressor. The mutant P53 protein can activate genes that stimulate tumor growth, metastasis, and resistance to chemotherapy of cancer cells [5]. Mutations in the *BRCA1* and *BRCA2* genes lead to an increased risk of certain malignancies. The main function of the proteins encoded by these genes is to maintain DNA integrity. With mutations of the *BRCA1* and *BRCA2* genes, the cell's ability to repair DNA decreases, which leads to the accumulation of genetic errors [3].

Thus, we performed targeted sequencing of certain genes that are often mutated in ovarian cancer. The most important fact of the study was the proof that in the early and late stages of ovarian cancer, diagnostically significant numbers of cells or fragments of tumor cells necessary for molecular genetic analysis are present in the aspirate from the uterine cavity. This circumstance indicates the effectiveness of a genetic test in the examination of uterine cavity aspirate for early detection of ovarian tumor lesions. If ovarian tumors are detected at a late stage with a positive genetic test for mutations in the *BRAF, KRAS, TP53, CSMD3, FAT3, BRCA1,* and *BRCA2* genes, then this circumstance may also be beneficial. One of the most important prognostic indicators for ovarian cancer is the size of the residual tumor after surgical removal. The earlier ovarian cancer is diagnosed at a late stage, the smaller the total tumor volume and the higher the probability of optimal tumor removal. In addition, it is possible that a small volume of the tumor will be more sensitive to cytotoxic chemotherapy than a large, voluminous tumor characteristic of symptomatic serous carcinoma of high malignancy.

Conclusions

1. In biological samples, aspiration biopsy by Pipel in patients with ovarian serous carcinoma revealed mutations in 96,7% of the *TP53* gene, 23,7% of the *FAT3* gene, 15,3% of the *CSMD3* gene, 9,9% of the *BRAF* gene and 9,1% of the *KRAS* gene. *BRCA1/2* gene mutations are rare (8%).

2. In patients with high-grade serous ovarian carcinoma, the frequency of mutations in the *TP53, FAT3, CSMD3,* and *BRCA1/2* genes is higher than in low-grade tumors.

3. In patients with serous ovarian carcinoma with morbid obesity, the frequency of *FAT3* gene mutations increases.

4. The detection of mutations of *TP53, FAT3, CSMD3, BRAF,* and *KRAS* genes in uterine aspirate cells requires further active diagnostic search for the detection of ovarian serous carcinoma.

The authors declare no conflict of interest.

References

1. Kit. O.I., et al. Vozmozhnosti skrininga raka tela matki po soderzhaniju molekuljarnyh markerov v moche i vaginal'no-cervikal'nom secrete [Possibilities of screening for cancer of the uterine body by the content of molecular markers in urine and vaginal-cervical secretion]. Klinicheskaja laboratornaja diagnostika [Russian Clinical Laboratory Diagnostics]. 2023; 68 (3): 141-145 (In Russ.).] doi: 10.51620/0869-2084-2023-68-3-141-145.

2. Savinova A.R., Gataullin I.G. Rannjaja diagnostika i skrining raka jaichnikov [Early diagnostics and screening for ovarian cancer]. Kazanskij medicinskij zhurnal [Kazan medical journal]. 2022; 3 (103): 476-483. doi: 10.17816/KMJ2022-476.

3. Al-Moghrabi N., et al. Constitutional BRCA1 and MGMT Methylation Are Significant Risk Factors for Triple-Negative Breast Cancer and High-Grade Serous Ovarian Cancer in Saudi Women. Int J Mol Sci. 2024 Mar 7; 25(6): 3108 (In Russ.).] doi: 10.3390/ijms25063108.

4. Talhouk A., et al. Development and validation of the gene expression predictor of high-grade serous ovarian carcinoma molecular SubTYPE (PrOTYPE). Clin. Cancer Res. 2020; 26: 5411–5423. doi: 10.1158/1078-0432.CCR-20-0103.

5. Heitz F.; et al. Dilution of molecular-pathologic gene signatures by medically associated factors might prevent prediction of resection status after debulking surgery in patients with advanced ovarian cancer. Clin. Cancer Res. 2020; 26: 213–219. doi: 10.1158/1078-0432.CCR-19-1741. doi: 10.1016/j.bpobgyn.2020.02.010

6. Kinde I., et al. Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. Sci. Transl. Med. 2013; 5. 167ra4.

7. Friedenreich C.M., Ryder-Burbidge C., McNeil J. Physical activity, obesity and sedentary behavior in cancer etiology: epidemiologic evidence and biologic mechanisms. Mol. Oncol. 2021; 15: 790–800 (2021). doi: 10.1002/1878-0261.12772.

8. Gaona-Luviano P., Medina-Gaona L.A., Magaña-Pérez K. Epidemiology of ovarian cancer. Chin Clin Oncol. 2020; 9(4): 47. doi: 10.21037/cco-20-34.

9. Ramachandran D., et al. Genome-wide association analyses of ovarian cancer patients undergoing primary debulking surgery identify candidate genes for residual disease. NPJ Genom. Med. 2024; 9 (19). doi: 10.1038/s41525-024-00395-y

10. Cafforio P., et al. Liquid biopsy in cervical cancer: hopes and pitfalls. Cancers. 2021; 13: 3968. DOI: 10.3390/cancers13163968.

11. Nash Z., Menon U. Ovarian cancer screening: current status and future directions. Best Pract. Res. Clin. Obstet. Gynaecol. 2020; 65: 32–45.

12. Hanley G.E., et al. Outcomes from opportunistic salpingectomy for ovarian cancer prevention. JAMA Netw Open. 2022 Feb1; 5(2). e2147343. doi: 10.1001/jamanetworkopen.2021.47343.

13. Potenza E., et al. Prognostic and predictive value of combined HE-4 and CA-125 biomarkers during chemotherapy in patients with epithelial ovarian cancer. Int J Biol Markers. 2020; 35(4): 20–27. doi: 10.1177/1724600820955195.

14. Gersekowski K., et al. Risk Factors for Ovarian Cancer by BRCA Status: A Collaborative Case-Only Analysis. Cancer Epidemiol Biomarkers Prev. 2024 Apr 3; 33(4): 586-592. doi: 10.1158/1055-9965.

15. Funston G., et al. The diagnostic performance of CA-125 for the detection of ovarian and non-ovarian cancer in primary care: A population-based cohort study. PLoS Med. 2020; 17(10): e1003295. doi: 10.1371/journal.pmed.1003295.

16. Webb P.M., Jordan S.J. Global epidemiology of epithelial ovarian cancer. Nat Rev Clin Oncol. 2024 May; No 21(5); 389-400. doi: 10.1038/s41571-024-00881-3.