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Dissimilar tumor cell populations in ascitic fluid of ovarian cancer patients

Kaigorodova E.V.[1, 2], Fedulova N.V.[2], Ochirov M.O.[1], Molchanov S.V.[1], Dyakov D.A.[2], Chasovskikh N.Yu.[2], Rogachev R.R.[2]

[1] *Cancer Research Institute, Tomsk National Research Medical Center (NRMC) of the Russian Academy of Sciences*
5, Kooperativny Str., Tomsk, 634009, Russian Federation

[2] *Siberian State Medical University (SSMU)*
2, Moscow Trakt, Tomsk, 634050, Russian Federation

ABSTRACT

Ovarian cancer is one of the most aggressive and hard-to-treat cancers. About 75% of ovarian cancer cases are detected at later stages of the disease. Ascitic fluid is promising biological material to get information about the tumor nature in ovarian cancer. Peritoneal dissemination is one of the most unfavorable factors of malignant tumor progression. However, prognostic factors associated with malignant ascites are not well understood.

The aim of the study was to evaluate various tumor cell populations in ascitic fluid of ovarian cancer patients by laser multicolor flow cytometry using a molecular panel of EpCam, CD45, CD44, CD24, CD133, and N-cadherin markers. The prospective study included 16 patients aged 36 to 76 years with newly diagnosed FIGO stage Ic–IV ovarian cancer, who were admitted for treatment to the Cancer Research Institute of Tomsk National Research Medical Center. The study material included EDTA-stabilized ascitic fluid sampled during laparoscopy. Various populations of ascitic tumor cells (with stemness features, with epithelial mesenchymal transition (EMT) features, without stemness and EMT features, with a combination of these features, as well as atypical/hybrid cell populations) were identified by multicolor flow cytometry on a BDFACSCanto apparatus (USA) using fluorochrome-labeled EpCam, CD45, CD44, CD24, CD133, and N-cadherin monoclonal antibodies and the BD FACSDiva software. The study revealed twelve populations of Epcam-positive cells in ascitic fluid of ovarian cancer patients. The cell composition of ascitic fluid in ovarian cancer patients is represented by a heterogeneous population. A large fraction of ascitic tumor cells are atypical/hybrid tumor cells with stemness features as well as Epcam+CD45–CD44+CD24+CD133+/– cancer stem cells, both with and without EMT features.

Key words: ovarian cancer, ascitic tumor cells, cancer stem cells, multicolor flow cytometry, liquid biopsy.

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✉ *Kaigorodova Evgeniya V.*, e-mail: zlobinae@mail.ru.

Различные популяции опухолевых клеток в асцитической жидкости больных раком яичников

Кайгородова Е.В.[1, 2], Федулова Н.В.[2], Очиров М.О.[1], Дьяков Д.А.[2], Молчанов С.В.[1], Часовских Н.Ю.[1]

[1] *Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук*
Россия, 634009, г. Томск, пер. Кооперативный, 5

[2] *Сибирский государственный медицинский университет (СибГМУ)*
Россия, 634050, г. Томск, Московский тракт, 2

РЕЗЮМЕ

Введение. Рак яичников (РЯ) является одним из самых агрессивных и тяжело поддающихся лечению онкологических заболеваний. Около 75% случаев РЯ выявляется на поздних стадиях заболевания. Асцитическая жидкость является перспективным биологическим материалом для получения информации о характере опухолевого процесса при РЯ. Перитонеальная диссеминация считается одним из наиболее неблагоприятных факторов прогрессирования злокачественных опухолей. Однако прогностические факторы, связанные со злокачественным асцитом, изучены недостаточно.

Целью данного исследования явилась оценка различных популяций опухолевых клеток в асцитической жидкости больных РЯ методом многоцветной проточной лазерной цитометрии на основе молекулярной панели маркеров EpCam, CD45, CD44, CD24, CD133 и N-cadherin. В проспективное исследование включены 16 больных с впервые диагностированным РЯ, стадии Ic–IV по системе FIGO, возраст 36–76 лет, поступившие на лечение в НИИ онкологии Томского НИМЦ. Материалом для исследования служила асцитическая жидкость, стабилизированная ЭДТА, взятая во время лапароскопии. Различные популяции асцитических опухолевых клеток (с признаками стволовости, с признаком EMT (epithelial-mesenchymal transition), без признаков стволовости и EMT, с сочетанием этих признаков, а также атипичные / гибридные популяции клеток определяли методом многоцветной проточной лазерной цитометрии на аппарате BDFACSCanto (США) с помощью меченных различными флуорохромами моноклональных антител к EpCam, CD45, CD44, CD24, CD133 и N-cadherin и программного обеспечения BD FACSDiva. В результате исследования в асцитической жидкости больных РЯ было выявлено 12 популяций Epcam-положительных клеток. Клеточный состав асцитической жидкости больных РЯ представляет собой гетерогенную популяцию. Большую концентрацию асцитических опухолевых клеток представляют собой атипичные / гибридные формы опухолевых клеток с признаком стволовости, а также стволовые опухолевые клетки Epcam+CD45-CD44+CD24+CD133+/- как с признаком EMT, так и без него.

Ключевые слова: рак яичников, асцитические опухолевые клетки, стволовые опухолевые клетки, многоцветная проточная цитометрия, жидкостная биопсия.

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INTRODUCTION

Ovarian cancer is one of the most aggressive and hard-to-treat cancers. More than 225,000 new cases of ovarian cancer are annually detected across the world, and more than 140,000 females die from this disease. Despite success achieved in the diagnosis, about 75% of ovarian cancer cases are detected at later stages of the disease. Treatment of patients with advanced cancer is difficult and not always provides a positive outcome. The five-year survival rate in patients with stage III or stage IV cancer is 23.8% and 11.6%, respectively. The asymptomatic course of the disease results in late diagnosis and a five-year mortality rate of 60%.

Unlike other tumors, ovarian cancer is characterized by a unique metastatic process. The earliest and most common way of metastasis is through implantation. It is often accompanied by accumulation of fluid in the abdominal cavity, which is called ascites. Ovarian cancer accounts for up to 38% of ascites cases associated with malignancies in females. Ascitic fluid is promising biological material to get information about the tumor nature. Unlike serum, ascitic fluid is more informative, especially at an early stage of the malignant process. In general, ascites is a multicomponent, dynamic system where all elements combined facilitate the formation of a pro-inflammatory and immunosuppressive environment. Ascites consists of a complex mixture of cell populations and a rich cytokine profile. The variety of cells is related to several factors. Firstly, it is phenotypic plasticity arising from the influence of soluble factors and microenvironment signals from immune and stromal cells. Secondly, the heterogeneity is associated with hydrodynamic forces that significantly change cell morphology [1]. Thirdly, the cause of tumor cells in ascitic fluid is the tumor, in particular, ovarian cancer, that is a heterogeneous cell population itself.

Currently, the heterogeneity of tumor cells is evaluated based on their antigenic properties, spectrum of various cell surface markers, and activity of the key signaling pathways. If circulating tumor cells (CTCs) are detected, the epithelial cell adhesion molecule (EpCAM) is widely used as a specific biomarker because it is overexpressed in more than 70% of ovarian cancer cases, and its level is closely associated with malignant ascites, chemoresistance, and decreased survival rate in patients. The role of EpCAM is not limited to cell adhesion; there is abundant evidence of its involvement in the epithelial mesenchymal transition (EMT). The EMT is known to enable cells to separate, lose their apical-basal polarity typical of epithelial cells, demonstrate enhanced resistance to apoptosis, and return to a more mobile mesenchymal phenotype that promotes peritoneal dissemination. This molecule is also used as a marker for cancer stem cells (CSC) [2].

Along with EpCAM, the CD44 receptor, widely present on the surface of tumor cells, is used to identify CTCs. It mediates attachment of ovarian cancer cells to peritoneal mesothelium by binding to hyaluronic acid (HA). CD44, as a biomarker, has several advantages. Firstly, normal cells have a low level of CD44 expression and poor adhesion to hyaluronic acid. Secondly, HA is one of the main components of the tumor extracellular matrix that, along with binding to the CTCs, supports cell integrity [3].

Another CTCs marker in ascitic fluid is CD24 that is expressed in 70.1% of ovarian cancer cases and is an independent predictor of survival. CD24 increases the metastatic potential of tumor cells because it is a ligand of P-selectin, an adhesion receptor on activated endothelial cells. In addition, CD24 induces EMT, which leads to the formation of a highly proliferative phenotype and resistance to chemotherapy via activation of PI3K/Akt, NF-κB, and ERK signaling cascades [4].

Morphologically, leucocytes in ascitic fluid resemble circulating tumor cells; therefore, it is advisable to use the differentiation antigen CD45 for affinity binding to leucocytes.

A common EMT feature is reduced expression of epithelial cadherin (E-cadherin) and a concomitant increase or *de novo* expression of neural cadherin (N-cadherin). This so-called "cadherin switch" is associated with increased migratory and invasive behavior of tumor cells. Increased expression of N-cadherin in solid tumors promotes "collective" cell migration, enhances transmission of fibroblast growth factor signals, and modulates the canonical Wnt pathway, which leads to the formation of an aggressive phenotype [5].

CD133 is the most commonly used cell surface antigen for detection and isolation of CSCs in various malignant diseases, including ovarian cancer. High expression of CD133 in tumors is considered

a prognostic marker of disease progression. Despite the fact that the functional role of CD133 in malignancies is not fully understood, most studies suggest that CD133 has a significant prognostic value for assessing overall and progression-free survival in various cancers [6].

A complete picture of different tumor cell populations in ovarian cancer may enable to predict the disease course, overall and relapse-free survival, and response to chemotherapy. Peritoneal dissemination caused by ascites is one of the most unfavorable factors for progression of malignancies. However, prognostic factors associated with malignant ascites are not well studied. In this regard, investigation of different tumor cell populations in ascitic fluid is a topical issue. The aim of this study was to evaluate tumor cell populations in ascitic fluid of ovarian cancer patients by laser multicolor flow cytometry using a molecular panel of EpCam, CD45, CD44, CD24, CD133, and N-cadherin markers.

MATERIALS AND METHODS

The prospective study included 16 patients aged 36 to 76 years with newly diagnosed FIGO stage Ic–IV ovarian cancer, who were admitted for treatment to Cancer Research Institute of Tomsk National Research Medical Center.

The study material included EDTA-stabilized ascitic fluid sampled during laparoscopy. Different populations of ascitic tumor cells (with stemness features, with epithelial mesenchymal transition (EMT) features, without stemness and EMT features, with a combination of these traits, as well as atypical/hybrid cell populations) were identified by multicolor flow cytometry on a BDFACSCanto apparatus (USA) using a molecular panel of EpCam, CD45, CD44, CD24, CD133, and N-cadherin markers and the BD FACSDiva software.

For this purpose, ascitic fluid was incubated with fluorochrome-labeled monoclonal antibodies to CD45 clone HI30 (APC/Cy7) (Biolegend, USA), EpCAM clone 9C4 (PE) (Biolegend, USA), CD44 clone BJ18 (FITC) (Biolegend, USA), CD24 clone ML5 (PE/Cy7) (Biolegend, USA), N-cadherin clone 8C11 (PerCP/Cy5.5) (Biolegend, USA), and CD133 clone AC-133 (APC) (Miltenyi Biotec, USA). Then, erythrocytes in the sample were lysed with a BD Facs lysing solution and washed twice with CellWash buffer; next, 1 mL of BD Flow was added to the cell pellet. All samples were stored in the dark at 4 °C and were analyzed on a flow cytometer within an hour. The cell level was expressed as the amount of cells per 1 μL of ascitic fluid.

The obtained data were processed using variation statistics methods. The statistical significance of differences was evaluated by a nonparametric Wilcoxon test for dependent samples with the Bonferroni correction using the Statistica 12.0 statistical software (StatSoft). The data are presented as a median (Me) and upper and lower quartiles [Q_1–Q_3]. The differences were considered statistically significant at a significance level of $p < 0.05$.

RESULTS

Multicolor flow cytometry of ascitic fluid revealed twelve populations of Epcam-positive cells. These included ascitic tumor cells with stemness features, without EMT features, and with phenotypes Epcam+CD45–CD44+CD24–CD133+Ncadherin–; Epcam+CD45–CD44+CD24–CD133–Ncadherin–; Epcam+CD45–CD44+CD24+CD133+Ncadherin–; ascitic tumor cells without stemness and EMT features: Epcam+CD45–CD44–CD24–CD133–Ncadherin–; ascitic tumor cells without stemness features and with EMT features: Epcam+CD45–CD44–CD24–CD133–Ncadherin+; ascitic tumor cells with stemness features and EMT features: Epcam+CD45–CD44+CD24–CD133+Ncadherin+; Epcam+CD45–CD44+CD24–CD133–Ncadherin+; Epcam+CD45–CD44+CD24+CD133+/–Ncadherin+/–; Epcam+CD45–CD44–CD24+CD133+/–Ncadherin+/–; Epcam+CD45–CD44+CD24+CD133+Ncadherin+; atypical/hybrid cells without stemness features: Epcam+CD45+CD44–CD24–CD133–Ncadh+/–; atypical/hybrid cells with stemness features: Epcam+CD45+CD44+CD24+/–CD133+/–Ncadh+/–.

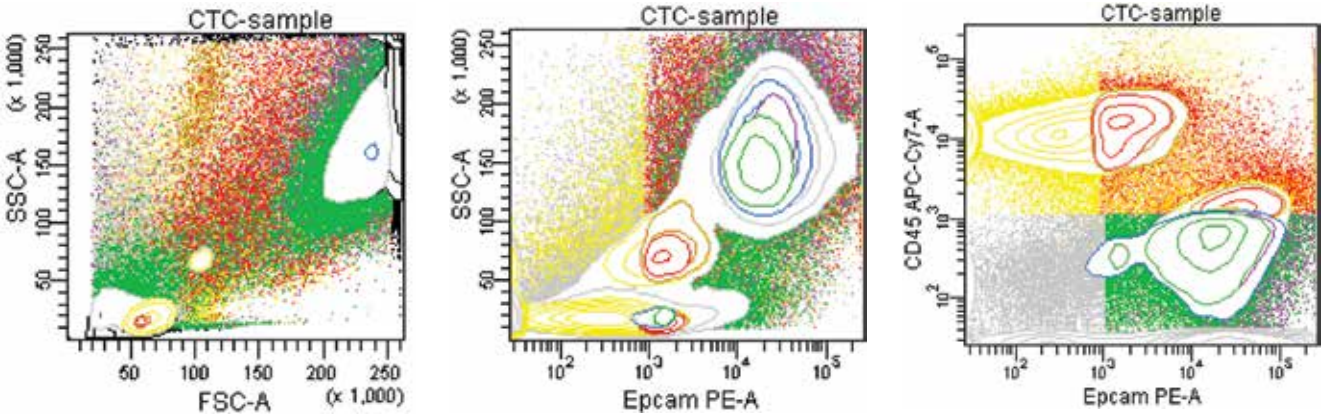
Figure 1 presents the results of multicolor flow cytometry in assessing different populations of Epcam+ cells in ascitic fluid.

Statistical analysis of the data using a nonparametric Friedman-Kendall test and pairwise Wilcoxon comparison revealed statistically significant differences between levels of these populations. The highest concentration of ascitic tumor cells was observed in atypical/hybrid forms with stemness traits. For example, the level of Epcam+CD45+CD44+CD24+/–CD133+/–Ncadh+/– cells

was 240.97 (80.54–383.5) cells/μL, while the levels of ascitic tumor cells without stemness and EMT traits (Epcam+CD45–CD44–CD24–CD133–Ncadh–) and atypical/hybrid tumor cells without stemness traits (Epcam+CD45+CD44–CD24–CD133–Ncadh+/–) amounted to 0.28 (0.11–4.27) cells/μL ($p = 0.0009$) and 2.07 (0.29–7.16) cells/μL ($p = 0.00098$), respectively.

The amount of tumor stem cells with positive expression of CD24, both with and without EMT features, (Epcam+CD45–CD44+CD24+CD133+/–Ncadherin+/–) did not significantly differ from the amount of tumor cells without stemness and EMT features (Epcam+CD45–CD44–CD24–CD133–Ncadherin–) and exceeded the level of cells without stemness traits in the EMT state (Epcam+CD45–CD44–CD24–CD133–Ncadherin+) at the trend level ($p = 0.073$) (Table 1).

In this case, the amount of tumor stem cells with positive expression of CD24 (Epcam+CD45–CD44+CD24+CD133+/–Ncadherin+/–) was significantly higher than that of tumor stem cells with negative expression of CD24, both with and without EMT features (Epcam+CD45–CD44+CD24–CD133+/–Ncadh+ and Epcam+CD45–CD44+CD24–CD133+/–Ncadh–, respectively), and amounted to 2.07 (0.53–11.42) cells/μL vs. 0.29 (0.03–0 72) cells/μL and 0.02 (0.00–0.52) cells/μL, respectively. The level of tumor stem cells with positive expression of CD24 with the Epcam+CD45–CD44–CD24+CD133+/–Ncadherin+/– phenotype amounted to 1.58 (0.22–3.87) cells/μL and was significantly higher than that of tumor stem cells with the Epcam+CD45–CD44+CD24–CD133+/–Ncadherin+ phenotype (Table 1).



a *b* *c*

Fig. 1 Different cell populations in ascitic fluid of an ovary cancer patient, which were identified by multicolor flow cytometry: *a*) ascitic cell populations in forward (FSC) and side (SSC) scattered light modes; *b*) populations of EpCam-positive and EpCam-negative cells in acidic fluid; *c*) populations of CD45+EpCam–, CD45+Epcam+, CD45–Epcam–, and CD45–Epcam+ cells

Table

Levels of different cancer cell populations in ascitic fluid of ovarian cancer patients, *Me* [Q_1–Q_3]

ACC	Phenotype of ascitic cancer cells	Cells / μL
ACC-1	Epcam+CD45-CD44-CD24-CD133-Ncadherin-	0.28 [0.11–4.27]
ACC-2	Epcam+CD45-CD44-CD24-CD133-Ncadherin+	0.09 [0.00–1.22] $p_{1-2} = 0.054$
ACC-3	Epcam+CD45-CD44+CD24-CD133+/-Ncadherin-	0.02 [0.00–0.52] $p_{1-3} = 0.021$. $p_{2-2} = 0.91$
ACC-4	Epcam+CD45-CD44+CD24-CD133+/-Ncadherin+	0.29 [0.03–0.72] $p_{1-4} = 0.035$. $p_{2-4} = 0.27$. $p_{3-4} = 1.00$
ACC-5	Epcam+CD45-CD44+CD24+CD133+/-Ncadherin+	0.34 [0.21–0.53] $p_{1-5} = 0.085$. $p_{2-5} = 0.31$. $p_{3-5} = 0.77$. $p_{4-5} = 0.37$

Table (continued)

ACC	Phenotype of ascitic cancer cells	Cells / μL
ACC-6	Epcam+CD45-CD44+CD24+CD133+/-Ncadherin-	0.09 [0.00–1.45] $p_{1-6} = 0.21$ $p_{2-6} = 0.76$ $p_{3-6} = 0.91$ $p_{4-6} = 0.76$ $p_{5-6} = 0.88$
ACC-7	Epcam+CD45-CD44-CD24+CD133+/-Ncadherin+	0.09 [0.04–0.78] $p_{1-7} = 0.26$ $p_{2-7} = 0.32$ $p_{3-7} = 0.95$ $p_{4-7} = 0.85$ $p_{5-7} = 0.76$ $p_{6-7} = 0.85$
ACC-8	Epcam+CD45-CD44-CD24+CD133+/-Ncadherin-	0.17 [0.00–0.74] $p_{1-8} = 0.068$ $p_{2-8} = 0.39$ $p_{3-8} = 0.77$ $p_{4-8} = 0.85$ $p_{5-8} = 0.85$ $p_{6-8} = 0.95$ $p_{7-8} = 0.39$
ACC-9	Epcam+CD45-CD44+CD24+CD133+/-Ncadherin+/-	2.07 [0.53–11.42] $p_{1-9} = 0.36$ $p_{2-9} = 0.073$ $p_{3-9} = 0.039$ $p_{4-9} = 0.030$ $p_{5-9} = 0.043$ $p_{6-9} = 0.011$ $p_{7-9} = 0.31$ $p_{8-9} = 0.21$
ACC-10	Epcam+CD45-CD44-CD24+CD133+/-Ncadherin+/-	1.58 [0.22–3.87] $p_{1-10} = 0.36$ $p_{2-10} = 0.086$ $p_{3-10} = 0.084$ $p_{4-10} = 0.047$ $p_{5-10} = 0.514$ $p_{6-10} = 0.374$ $p_{7-10} = 0.027$ $p_{8-10} = 0.017$ $p_{9-10} = 0.27$
Atipical / hybrid cell without stemness features	Epcam+CD45+CD44-CD24-CD133-Ncadherin+/-	2.07 [0.29–7.16] $p_{1-11} = 0.55$ $p_{2-11} = 0.0029$ $p_{3-11} = 0.177$ $p_{4-11} = 0.013$ $p_{5-11} = 0.015$ $p_{6-11} = 0.0506$ $p_{7-11} = 0.011$ $p_{8-11} = 0.011$ $p_{9-11} = 0.68$ $p_{10-11} = 0.506$
Atipical/ hybrid cell with stemness features	Epcam+CD45+CD44+CD24+/-CD133+/-Ncadherin+/-	240.97 [80.54–383.50] $p_{1-12} = 0.0009$ $p_{2-12} = 0.0009$ $p_{3-12} = 0.0009$ $p_{4-12} = 0.0009$ $p_{7-12} = 0.0076$ $p_{8-12} = 0.0076$ $p_{9-12} = 0.0076$ $p_{10-12} = 0.0009$ $p_{11-12} = 0.0009$

Note: ACC- ascitic cancer cells; Wilcoxon Matched Pairs Test. Marked tests are significant at $p < 0.05$

DISCUSSION

Metastatic spread of ovarian cancer occurs mainly due to detachment of cells from the primary tumor and invasion of the abdominal cavity filled with malignant ascites. The cells spread widely with fluid flow and cause secondary tumor growth. At all stages of this unique process, tumor cells change their phenotype and co-evolve together with surrounding fibroblasts, macrophages, adipocytes, endothelial, and other cells.

During metastasis, ovarian cancer cells demonstrate tropism for the omentum. Their interaction leads to the formation of a highly active cancer-associated adipocyte (CAA) phenotype that produces most of the tumor metabolic microenvironment [7]. In addition, adipose tissue is a source of multipotent mesenchymal stem cells (MSCs) capable of self-renewing, differentiating into other pro-oncogenic cells (cancer-associated fibroblasts (CAFs) and CAA), and maintaining the cancer stem cell (CSC) population [8].

This study revealed different tumor cell populations in ascitic fluid: atypical forms/hybrid cells, both with and without stemness traits, with EMT traits, and with a combination of these traits; stromal and immune cell populations, identification and characterization of which may be a useful tool in predicting the disease course and response to chemotherapy.

The largest group of cells in ascitic fluid of ovarian cancer patients has an atypical phenotype and is represented by hybrid cells with stemness features (Epcam+CD45+CD44+CD24+/−CD133+/−Ncadherin+/−). It may be suggested that the formation of hybrid cells promotes carcinomatosis of ovarian cancer and prevents initiation of apoptosis and anoikis of tumor cells in ascites. This suggestion and the identified cells require further research.

According to the literature data, out of 150 different marker combinations, the most common panel includes three markers: CD44, CD24, and Epcam. Expression of these molecules in OVCAR-5, SKOV-3, and IGROV-1 lines corresponded to cells with greater colony-forming ability. These cells demonstrated a short *in vivo* relapse-free interval after xenotransplantation and a greater migratory capacity in an *in vitro* invasion study, compared to CD44-CD24-Epcam cells. In addition, doxorubicin, cisplatin, and paclitaxel promoted an increase in this population, which indicates drug resistance, but Мьllerian inhibiting substance (MIS) effectively suppressed its growth [9].

It may be supposed that the presence of tumor cells with the Epcam+CD45−CD44+CD24+CD133+/−Ncadherin+/− phenotype in ascitic fluid is associated with the aggressive course of ovarian cancer. Numerous studies have shown that the Epcam+CD44+CD24+CD133+CD117+ population has increased ability to initiate cancer and/or stimulate metastasis *in vivo* [10]. In a mouse model (NOD/Shi-scid/IL-2Rγ null mice), it was demonstrated that CD24+ and CD133+ cells were more capable of forming spheres, spreading, and initiating tumors *in vivo*. In addition, CD24+ cells showed a more “mesenchymal” phenotype with higher expression of Twist1, Snail, and Vimentin, which connects the CD24 marker to the EMT phenotype. Interestingly, CD24 cells were also capable of initiating tumor growth, albeit to a lesser extent than CD24+. This probably occurs due to the fact that a subset of CD24 cells with stem properties has a lower proliferation rate than CD24+ cells [11].

According to E. Meng et al., ovarian cancer cells with the CD44+/CD24− phenotype have a high potential for intraperitoneal dissemination due to the properties similar to those of CSCs. Compared with other phenotypes, they demonstrate a 60-fold increase in the Matrigel invasion ability of the SKOV3 cell line ($p < 0.001$) and are characterized by an aggressive clinical course of the disease [12].

The presence of CD44 indicates a great potential for the formation of cell spheroids that are known to promote metastasis and chemotherapy resistance. This is confirmed by a study that demonstrated the formation of large hollow spheroids from the CD44high/CD166high population derived from an ovarian cancer line (SKOV3, OV90) in comparison with CD44low/CD166low analogs [13].

Therefore, our findings demonstrate a large heterogeneity of tumor cells in ascitic fluid of ovarian cancer patients. The presence of atypical/hybrid forms of Epcam-positive cells and different populations of tumor cells with stemness features in ascitic fluid is of great interest for further research in the field of personalized medicine.

CONCLUSION

The cell composition of ascitic fluid in ovarian cancer patients is heterogeneous. A large fraction of ascitic tumor cells are represented by atypical/hybrid forms of cells with stemness features and cancer stem cells Epcam+CD45−CD44+CD24+CD133+/−, both with and without EMT. Further investigation of different tumor cell populations in ascitic fluid and their relationship with the clinical course and efficacy of chemotherapy of ovarian cancer patients is very important and opens up great opportunities for practical developments in the field of liquid biopsy.

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Authors contribution

Kaigorodova E.V., Fedulova N.V., Dyakov D.A. – carrying out of research and interpretation of data. Ochirov M.O., Molchanov S.V – diagnostics and treatment of patients with ovarian cancer. Rogachev R.R., Chasovskikh N.Y. – statistical data processing, data retrieval from different bioinformatics resources. Kaigorodova E.V. – conception and design, drafting of the manuscript.

Authors information

Kaigorodova Evgeniya V., Dr. Sci. (Med.), Leading Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk; Professor, Division of Biochemistry and Molecular Biology with a Course in Clinical Laboratory Diagnostics, Siberian State Medical University, Tomsk. ORCID ID 0000-0003-4378-6915.

Fedulova Natalya V., 6th-Year Student, Siberian State Medical University.

Ochirov Maxim O., Post-Graduate Student, Department of Gynecology, Cancer Research Institute, Tomsk National Research Medical Center, Tomsk. ORCID ID 0000-0001-6628-2918.

Dyakov Denis A., Assistant, Division of Biochemistry and Molecular Biology with a Course in Clinical Laboratory Diagnostics, Siberian State Medical University, Tomsk.

Rogachev Roman R., 6th-Year Student, Siberian State Medical University.

Molchanov Sergey V., Cand. Sci. (Med.), Senior Researcher, Department of Gynecology, Cancer Research Institute, Tomsk National Research Medical Center, Tomsk. ORCID ID 0000-0002-0522-1772.

Chasovskikh Nataliya Yu., Dr. Sci. (Med.), Professor, Division of Medical and Biological Cybernetics, Siberian State Medical University, Tomsk. ORCID 0000-0001-6077-0347.

(✉) **Kaigorodova Evgeniya V.**, e-mail: zlobinae@mail.ru.

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