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INDIVIDUAL PROGNOSIS OF SEROUS OVARIAN CANCER SURVIVAL PATIENTS BASED ON ADHESION AND PROLIFERATION OF TUMOR CELLS

Summary. ***Aim*** *was to study expression of (CD44s) and (Ki-67) markers serous ovarian cancer cells by immunohistochemistry and to evaluate their clinical significance.* ***Material and methods:*** *102 surgical specimens of stage I–III serous ovarian cancer (OC), mean age 59,3 ± 3,7. Methods: clinical, morphological, immunohistochemical, statistical.* ***Results:*** *All tumors had serous adenocarcinoma morphology with different quantity of papillary component and solid structures. We allocated high-, moderate- and low-differentiated forms of OC, as well as tumors with low and high degree of morphological malignancy. We found the heterogeneity between OC tumors by expression of Ki-67 and CD44 proteins. Number of tumors with high expression of Ki-67 (> 10%) and CD44 (> 10%) was significantly higher in patients with poorly differentiated OC and high degree of morphological malignancy. We allocated the molecular phenotypes of OC tumor cells: $CD44^+/Ki\text{-}67^+$, $CD44^+/Ki\text{-}67^-$, $CD44^-/Ki\text{-}67^+$ and $CD44^-/Ki\text{-}67^-$ and defined the dependence of patients' survival from the molecular phenotype of tumor cells.* ***Conclusions:*** *We defined intertumoral heterogeneity of OC by expression of CD44 and Ki-67 and variability of OC patients' overall survival periods, which depended from the tumor molecular phenotype. Stratification of tumors not only by morphology, degree of differentiation and malignancy, but also according to molecular phenotype is needed for individualized treatment and predictive prognosis of patients with serous OC, because it reflected the underlying potency of tumor to grow and form metastasis.*

INTRODUCTION

Among female reproductive system neoplasms, serous ovarian cancer (OC) is characterized by the most aggressive clinical course with frequent relapses in the area of small pelvis and abdominal cavity. Clinical and morphological factors, which influence the OC course, are stage of disease, volume of cytoreduction, grade of differentiation of tumor and age of patient. Clinical division of OC by stage only not always allows to evaluate clearly the prognosis of course of disease and prescribe the adequate treatment. It can be explained by the fact that tumors with different morphology, unequal grade of differentiation and malignancy are being united in one stage. These very features of tumors determine different growth potential and diffusion of neoplastic cells outside the limits of ovary with further development of implantation metastasis [1, 2].

Numerous studies of tumors of different genesis have determined that variability of clinical course is connected both with etiopathogenesis of cancer and with later alteration of gene expression and molecular phenotype of tumor cells [3–7], which are considered to be connected with inter- and intratumor heterogeneity of the last ones [8]. For instance, inter- and intratumor heterogeneity of such markers as CD44 and CD24 has been showed on cell lines of breast cancer (BC) (MDA-MB-468), kidneys (CAKI2), large intestine (HCT116), lungs (COR-L23), ovary (OVCAR3, SKOV3, CAOV3, A2780) cancers [9]. Heterogeneity of expression of VEGFR-2 has been determined not only in cells of tumors of the same genesis (gliomas), but also in different zones of the same tumor (central, periphery, near lesions of necrosis) [10]. Study of molecular phenotype in invasive focuses of multifocal carcinomas of mammary gland dependently on their size, morphology, and differentiation grade has showed that heterogeneity may appear in the similar by morphology tumor focuses [11]. However, clinical significance of inter- and intratumor heterogeneity and its role in progression of tumor growth has not been fully studied. Thus, it conditions the necessity of conduction of the further studies in this direction. The relevance of the problem in full measure concerns OC, for which frequent relapses are typical and are the sign of tumor progression [2].

Cardinal role in progression of tumor disease of different genesis, including OC, belongs to such biological processes as proliferation and adhesion, alteration of which causes the increase of the number of tumor cells, disorder of their junction and their invasion in surrounding tissues. Nuclear antigen Ki-67 is molecular marker of proliferation, which affects the speed of growth and aggressiveness of clinical course of tumors of different genesis [12, 13]. Molecule CD44s, which is multifunctional transmembrane protein and receptor of hyaluronic acid, also plays

significant role in processes of invasion and metastasis [9]. CD44s is involved in intercellular connections, modification of cellular-matrix interactions and participates in many signal cascades, which are connected with differentiation, proliferation, migration, and apoptosis of cells [14, 15]. One of the papers [16] has emphasized that disorder of intercellular adhesion lies in the basis of development of metastases and is biological marker of tumor growth.

Molecule CD44 has such peculiarity as expression in cancer stem cells (CSC) and tumor-initiating cells (TIC), which are being detected in neoplasms of different genesis. According to the data of studies, CSC/TIC are characterized by higher potential to proliferation, myofibroblast-like and invasive properties, capacity of dissociation, formation of spheroids in ascitic solution and being for a long time in rest upon the conditions of hypoxia [17, 18]. CSC/TIC in population of OC cells are being determined by series of markers (CD44, CD117, CD133, CD24) or their combinations [19]. The fact attracts attention that prevalence of CSC in tumors is associated with invasive phenotype of cells and relapses of tumor process even in the early stages of OC [20].

Due to the intensification of molecular-biological studies in oncology, more and more attention is given to the study of not only single molecular markers of tumor growth, but also their combinations. Such approach is used, for instance, in prognosis of course of BC. Several molecular phenotypes of BC have been allocated dependently on expression of receptors of estrogens, progesterone and epidermal growth factor and their significance for the individual prognosis of tumor disease and personalization of treatment has been studied [21]. Allocation of certain molecular phenotypes in OC patients is also relevant, but undeveloped issue.

The aim of the paper was to conduct immunohistochemical study of expression of adhesion (CD44s) and proliferation (Ki-67) markers in cells of serous OC and evaluate its clinical significance.

OBJECT AND METHODS

One hundred and two patients with serous OC of I–III stage, who have not undergone neoadjuvant polychemotherapy before surgical treatment, have been included in study. After surgical treatment, patients have been prescribed adjuvant polychemotherapy by CAP and CP schemes. All patients have been informed and have given their consent for the use of samples of their tumors with research goals. The dissemination of tumor process has been evaluated according to the FIGO classification. At morphological study of surgical material, the histological structure of tumors, differentiation grade, as well as stage of morphological malignancy according to the structural and cytomorphological criteria [21], has been evaluated. Immunohistochemical study (streptavidin-biotin-peroxidase method) of expression of markers Ki-67, CD44s has been carried out on histological sections (4 mkm) of paraffinic blocks of surgical material fixed in 10% solution of neutral formalin. Monoclonal Ki-67-specific (clone MIB-1, "Dako Cytomation", Denmark) and CD44s-specific (clone DF 1485, "Dako Cytomation", Denmark) antibodies (McAB) have been used as initial. For the visualization of the results of reaction, kit of reagents EnVision+ and 3,3-diaminobenzadine ("Dako LSAB2 system", Denmark) has been used according to the manufacturer's recommendations; sections have been stained with Mayer's hematoxylin. For the evaluation of expression of Ki-67, semi-quantitative method has been used: number of Ki-67$^+$ (Ki-67-positive) cells per 1000 analyzed cells has been considered proliferation index (PI, %). Also stage of staining of nuclei has been determined — high (+++), moderate (++), low (+). Being guided by data of literature [23] that even in the early stages of OC the number of Ki-67-positive cells (on the assumption of use of the McAB MIB-1) is a predictor of relapse, tumors with PI > 10% and high/moderate stage of staining of nuclei have been considered tumors with high proliferation. Expression of CD44s (further — CD44) has been evaluated, analyzing the whole histological section as fraction of positively stained cells (%). Expression of CD44 > 10% has been considered high, also taking into account only high and moderate stage of staining of cells. In negative control primary McAB were omitted. Lymphocytes in histological sections have been used as positive control for detection of CD44 expression. Expression of markers has been analyzed under magnification ×200–400.

Statistical processing of the results has been conducted using standard software Statistika 6.0. For the determination of significance of changes of frequency of tumors with different expression of markers dependently on stage of OC, differentiation and malignancy grade of tumors, χ^2 test, level of significance and Pearson correlation coefficient have been calculated. Survival of patients has been evaluated by Kaplan — Meier test using log-rank test. Difference has been considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Analysis of the general clinical characteristics of 102 patients with OC of I–III stage has showed that their mean age varied from 28 to 75 and has constituted in average 59.3 ± 3.7; 61.8% of patients were in menopause. In 81.4% of patients, the OC of II and III stages has been diagnosed; all tumors had structure of serous adenocarcinoma with varying part of papillary component and solid structures, variability of atypism and polymorphism of tumor cells. Taking into account these criteria, in analyzed material high-, moderate- and low-differentiated OC forms have been allocated, among which moderately differentiated forms prevailed (52.0%). Quantity of patients with low-differentiated OC has constituted 30.4%, with high-differentiated — 17.6%. For the evaluation of OC malignancy grade, assessment of tumors by the following parameters has been conducted: interrelation between glandular and solid structures, heterogeneity of morphology of tumor in its different zones, atypism and polymorphism of tumor cells, features of growth (solid/glandular focuses, clusters, accumulations of cells, single cells), presence of mitoses, lesions of necrosis, calcifications. Basing on this, tumors with low and high malignancy grades have been detected: they have constituted 38.2 and 61.8% correspondingly (Table 1). The mentioned data show that serous OC is characterized by intertumor heterogeneity not only by differentiation grade, but also by malignancy grade, which evaluation was based on cytomorphological data.

Table 1

General clinical features of OC patients

Indexes	Number of patients	
	n	%
Total	102	100.0
Menstrual cycle		
Preserved	39	38.2
Menopause	63	61.8
Stage by FIGO		
I	19	18.6
II	35	34.3
III	48	47.1
Differentiation grade		
High G1	18	17.6
Moderate G2	53	52.0
Low G3	31	30.4
Morphological malignancy grade		
Low	39	38.2
High	63	61.8

Results of immunohistochemical study have showed that individual indexes of expression of Ki-67 in tumors varied from 2 to 76% (mean PI — 45.0 ± 4.7%), CD44 — from 0.0 to 67.5% (mean index — 52.0 ± 3.1%). The mentioned data are the evidence of intertumor heterogeneity of expression of these markers that has given the ground for their analysis dependently on dissemination of tumor process, differentiation grade and OC morphological malignancy. As data in Table 2 show, expression of Ki-67 > 10% has been determined at different dissemination of OC: quantity of Ki-67$^+$ tumors in patients with OC of I stage has constituted 47.4%, and it has been increasing at II and III stages to 54.3 and 54.2% correspondingly, but significant difference has not been determined ($p > 0.05$). At the same time, occurrence of tumors with expression of this marker depended on their differentiation grade: χ^2 compared with quantity of Ki-67 tumors of high and low differentiation grade has constituted 7.51 ($p = 0.00617$, $r = 0.36$). Significant difference has been determined also when analyzing occurrence of tumors with expression of Ki-67 dependently on grade of their morphological malignancy ($\chi^2 = 8.7$; $p = 0.00318$; $r = 0.30$).

Similar data on increasing of Ki-67 expression with increasing of stage of tumor process are represented in paper [24], which authors consider high expression of Ki-67 the sign of aggressiveness and unfavorable prognosis of OC. In one of the last studies [13] on large sampling material (808 patients with oncologic pathology of ovary) also has been determined essential clinical significance of Ki-67 expression. Using criterion of evaluation of Ki-67 expression > 10% (the same as in our study), authors have determined that frequency of expression of this marker in OC significantly increases with dissemination of tumor process and decrease of differentiation grade. Also it is connected with decrease of lifespan of patients.

Analysis of quantity of tumors with CD44 expression has not determined changes of the last one dependently on stage of tumor process ($p > 0.05$). At the same time, despite significant heterogeneity of indexes, it has been determined that patients with low differentiation and high malignancy grade had significantly higher number of tumors with CD44 expression: $\chi^2 = 14.5$ ($p < 0.00157$; $r = 0.56$), $\chi^2 = 19.4$ ($p < 0.00157$; $r = 0.42$) correspondingly (see Table 2).

Table 2

Distribution of tumors (n = 102) with positive expression of Ki-67 and CD44 dependently on clinical and morphological features

Clinical and morphological indexes	Number of patients, n[1]	Number of tumors with expression of markers, n/%			
		Ki-67		CD44	
		Ki-67(+)	Ki-67(-)	CD44(+)	CD44(-)
Stage of tumor process					
I	19	9/47.4	10/52.6	10/52.6	9/47.4
II	35	19/54.3	16/45.7	20/57.6	15/42.4
III	48	26/54.2	22/45.8	28/58.3	20/41.7
Differentiation grade					
High	18	11/61.1*	7/38.9	10/55.5*	8/44.5
Moderate	53	30/56.6	23/43.4	29/54.7	24/45.3
Low	31	20/64.5	11/35.5	21/67.7	10/32.3
Morphological malignancy grade					
Low	39	30/58.9**	9/41.1	30/76.9**	9/23.1
High	63	39/61.9	24/30.1	56/88.8	7/11.2

Note: [1] taking into acounting the frequency of tumors with different expression of the investigated markers, 100% take the number of patients with each of the stages of tumor process, the degree of differentiation or morphological malignancy;* significant difference between high and low differentiation grade, ** significant difference between low and high morphological malignancy grade.

The obtained results show that there is significant intertumor heterogeneity of expression of studied molecular markers. It may be conditioned by complicated mechanisms of adhesion of cells and their disorders at tumor growth of different differentiation grade as well as peculiarities of microenvironment of tumors. It is also confirmed by the fact that CD44 interacts with receptors of hyaluronic acid and proteins of extracellular matrix (collagen, fibronectin, etc.) and acts as bioactive signaling transmitter [25], which takes part not only in intercellular adhesion, but also in migration and invasion of tumor cells. Formation of single clusters and spheroid structures in ascitic solution, which assist dissemination of OC in abdominal cavity with further proliferation of cells, is connected with disorder of adhesion of tumor cells [26]. Formation of such clusters and single accumulations of tumor cells with expression of CD44 has been determined in particular focuses of primary OC [22].

As mentioned above, at present time in oncologic clinical practice in order to determine the prognosis of aggressiveness of tumor process and survival of BC patients, the molecular phenotype of cells is being determined, which includes several molecular markers, to be exact receptors of estrogens, progesterone, EGFR (marker HER2/neu) [27–29]. In particular, it has been showed [27] that 5-year survival of BC patients depends on molecular phenotype and constitutes 96; 88; 81; 89 and 85% correspondingly at luminal A, luminal B, HER2+, basal-cellular and unclassified phenotypes. In a similar, taking into account the results of own study and data of literature concerning high significance of immunohistochemical markers for diagnosis of grade of malignancy and aggressiveness of ovarian tumors, it is quite possible to presume the presence of molecular phenotypes also in serous OC, which would include proliferation and intercellular adhesion markers. Basing on analysis of expression of CD44 and Ki-67 in patients with serous OC, we have determined its following molecular phenotypes: CD44$^+$ Ki-67$^+$ (n = 30; 29.5%), CD44$^+$ Ki-67$^-$ (n = 28; 27.4%), CD44$^-$ Ki-67$^+$ (n = 24; 23.5%), CD44$^-$Ki-67$^-$ (n = 20; 19.6%), which underlay the basis of

calculation of overall survival of patients (Figure). As seen from mentioned data, there is a difference between survival of patients with different molecular phenotypes, which turned out to be significant ($p < 0.05$) when comparing curves of survival of patients with molecular phenotype of tumors $CD44^+$ $Ki\text{-}67^+$ and $CD44^-$ $Ki\text{-}67^-$. Obtained results demonstrate that each molecular phenotype, obviously, reflects unequal biological peculiarities of tumor.

It should be mentioned that survival of patients with serous OC depends on series of factors. To these factors may be referred not only molecular phenotype of tumor, but also other factors, exactly resistance of OC to cytostatics, features of microenvironment of tumor, immune status of organism of patient. Despite prognosis is influenced by so many factors, at present time more and more authors abandon themselves to idea concerning the role of molecular inter- and intratumor heterogeneity in course of OC. Each tumor is unpredictable phenomenon with pleiotropism of molecular pathways [30], structural, phenotypic and functional heterogeneity of both tumor cells and the whole tumor [31, 32].

Certain contribution in inter- and intratumor heterogeneity was made also by molecule of intercellular adhesion CD44 as marker of stem cells, which are connected with progression of tumor growth. For instance, in serous OC, ovarian cells with increased expression of CD44 have been recognized as tumorigenic (ovarian cancer-initiating cells — OCIC), in contrast to the cells without expression of this marker [33]. Tumorigenic ovarian cells with phenotype $CD44^+/CD117^+$ are characterized by high proliferation, low differentiation grade and resistance to the chemodrugs [34]. When studying BC, it has been demonstrated that different tumorigenicity of tumor cells, which is conditioned by their phenotypic and functional polymorphism, may be the cause of different expression of basic markers of this tumor (receptors of estrogens, progesterone, receptor HER2/neu) in primary neoplasm and metastases in the same patients [35].

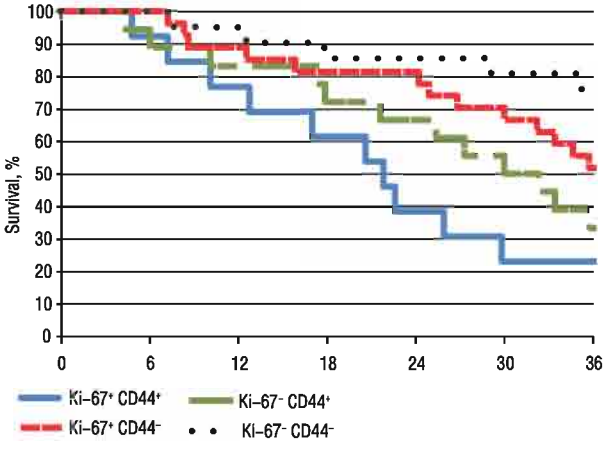

Figure. Overall survival of OC patients (Kaplan — Meier test) dependently on molecular phenotype of tumors ($p < 0.05$ between curves of survival of patients with phenotypes $CD44^+$ $Ki\text{-}67^+$ and $CD44^-$ $Ki\text{-}67^-$)

Obtained data also indicate that molecule of adhesion CD44 may become perspective target for the development of new target drugs of antitumor therapy. It is also confirmed by the study, which has showed that miR-199a regulates in specific way the expression of CD44 in $CD44^+/CD117^+$ ovarian tumors (TIC), suppressing the proliferation, migration and invasion of these cells, and can prevent further development of OC [34]. All mentioned above emphasizes the essential significance of heterogeneity of molecular phenotype of serous OC. Awareness about molecular phenotype of serous OC may help to develop new approaches to the individualized therapy of patients with OC and evaluate individual prognosis of the disease.

CONCLUSIONS

1. Results of immunohistochemical study of expression of markers of proliferation Ki-67 and intercellular adhesion CD44 in serous OC demonstrate their significant intertumor heterogeneity.

2. It has been showed that number of tumors with higher expression of Ki-67 and CD44 at low differentiation grade and high OC morphological malignancy grade significantly increases.

3. In analyzed material, molecular phenotypes of tumor cells $CD44^+$ $Ki\text{-}67^+$, $CD44^+$ $Ki\text{-}67^-$, $CD44^-$ $Ki\text{-}67^+$, $CD44^-$ $Ki\text{-}67^-$ have been allocated and the dependence of overall survival of patients with serous OC on molecular phenotype has been established.

4. For the predictive evaluation of individual prognosis for the patients with serous OC, is needed stratification of tumors not only by morphology, differentiation grade and morphological malignancy of tumor, but also by molecular phenotype, which represents the main potencies of tumor to grow and form metastasis.

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ІНДИВІДУАЛЬНИЙ ПРОГНОЗ ВИЖИВАНОСТІ ХВОРИХ З УРАХУВАННЯМ ПРОЛІФЕРАЦІЇ ТА АДГЕЗІЇ ПУХЛИННИХ КЛІТИН У ХВОРИХ НА СЕРОЗНИЙ РАК ЯЄЧНИКА

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Резюме. ***Мета**: провести імуногістохімічне дослідження експресії маркерів CD44s і Ki-67 у клітинах серозного раку яєчника (РЯ) та оцінити їх клінічне значення.* ***Об'єкт і методи**: операційний матеріал 102 хворих (середній вік 59,3 ± 3,7 року) на серозний РЯ I–III стадії. Використано клінічні, морфологічний, імуногістохімічний, статистичні методи дослідження.* ***Результати**: усі пухлини мали будову серозної аденокарциноми з різною часткою папілярного компонента і солідних структур. Виділено високо-, помірно- і низькодиференційовані форми РЯ і пухлини з низьким і високим ступенем морфологічної злоякісності. Встановлено міжпухлинну гетерогенність РЯ за експресією маркерів Ki-67 і CD44. Кількість пухлин Із високою експресією Ki-67 (> 10%) і CD44 (> 10%) була достовірно більшою у хворих на РЯ низького ступеня диференціювання і високого — морфологічної злоякісності. Виділено молекулярні фенотипи пухлинних клітин РЯ: $CD44^+$ $Ki\text{-}67^+$, $CD44^+$ $Ki\text{-}67^-$, $CD44^-$ $Ki\text{-}67^+$, $CD44^-$ $Ki\text{-}67^-$ та показана варіабельність загальної виживаності хворих залежно від молекулярного фенотипу пухлинних клітин.* ***Висновки**: для індивідуалізованого лікування і предиктивної оцінки у хворих на серозний РЯ необхідна стратифікація пухлин не тільки за морфологією, ступенем диференціювання і злоякісності, але й за молекулярним фенотипом, який відображає основні потенції пухлини до росту і метастазування.*

Ключові слова: рак яєчника, цитоморфологія, гетерогенність, CD44, Ki-67, молекулярний фенотип, виживаність.

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