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# SIGNIFICANCE OF ADHESION MOLECULES EXPRESSION FOR ESTIMATION OF SEROUS OVARIAN CANCER PROGNOSIS

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***Aim*: To study the adhesion molecules CD44 and E-cadherin expression in serous ovarian cancer (OC) and their relationship with clinicopathological peculiarities of tumor process and prognosis. *Materials and Methods:* The study was performed on OC samples from 72 patients with serous OC of stages I-III. Expression of CD44 and E-cadherin in tumor samples was evaluated with the use of immunohistochemical analysis. *Results*: Immunohistochemical analysis has revealed that in 58.3% of OC patients CD44 expression was detected in more than 10% of cells with average level of 30.0±5.6%. E-cadherin expression was present in 47.2% of tumors, and 12.2±3.6% cells were immunopositive. CD44 and E-cadherin expression depends on degree of cytomorphological malignancy and cell differentiation. A reverse correlation between CD44 and E-cadherin expression in primary serous OC ($r=-0.38$, $p<0.05$) has been found. Increased CD44 expression (mean index) was not observed in peritoneal metastases compared to primary tumors, but analysis of individual indexes showed increase of CD44 expression in 46.6% OC patients with metastases. The dependence between overall survival of OC patients and the molecular phenotype of tumor cells, in particular, poor prognosis for OC patients with CD44(+)/E-cadherin(-) and CD44(+)/budding(+) tumor cells phenotype, has been revealed. *Conclusion:* The results of morphological and immunohistochemical analysis has shown the increase of adhesive features of serous ovarian cancer cells what may be significant for estimation of ovarian cancer aggressiveness and development of metastatic process.**

***Key Words*: serous ovarian cancer, CD44, E-cadherin, immunohistochemical analysis, morphology, budding, malignancy, prognosis.**

It is known that ovarian cancer (OC) is characterized by late diagnostics and results in the high morbidity of patients in many countries in spite of cytoreductive operations and repeated courses of chemotherapy [1]. Histological structure, degree of tumor differentiation and stage (FIGO) are the main criteria for estimation of OC prognosis. Such criteria do not always represent the real biological characteristics of tumors, especially their invasive and metastatic features. It was shown that biological characteristics of OC are associated with altered patterns of proliferation, angiogenesis and expression of cell adhesion molecules that play the critical role in mechanisms of tumor growth and metastasis [2–5]. The violation of cell adhesion leads to escape of tumor cells from tumor node and their spreading by blood and lymphatic vessels or per continuitatem [6].

Adhesive peculiarities of normal and malignant cells are related to cadherin–catenin complex and CD44 molecule [7, 8]. Cadherins (E-, N-, P-cadherin) are known as basic and crucial factors of homotypic intercellular adhesion [9, 10]. Epithelial cadherin (E-cadherin) is type I transmembrane glycoprotein. In humans this protein is encoded by *CDH1* gene, which is a tumor suppressor gene. E-cadherin is a key determinant of cell recognition, tissue morphogenesis, and E-cadherin ligation alone reduces the frequency of cells entering the S-phase of the cell cycle, demonstrating the direct transduction of growth or inhibitory signals [11, 12]. Recent findings indicated that classical cadherins are adhesion-activated signalling receptors in cancer cells. Loss of E-cadherin activity after epidermal growth factor induction is a major determinant of tumor progression and invasion and is observed in many physiological and pathological processes [13]. Cadherin molecules mediate intercellular contacts (zona adhaerence), and complexes via their cytoplasmic domains with α-, β- and γ-catenin receptors participating in WNT signalling pathway [14]. Cadherin switching may have a profound effect on cell phenotype and behaviour and is one of the aspects of epithelial-mesenchymal transition (EMT) [15] and development of OC metastases [16, 17].

CD44 was firstly characterized as multistructural and multifunctional cell surface adhesion molecule. It is involved in cell-cell and cell-matrix interactions, lymphocyte homing and has hyaluronan receptor functions. CD44 is the transmembrane glycoprotein that is encoded by a single gene on chromosome 11p13, but represents a polymorphic group of transmembrane glycoproteins owing to extensive alternative splicing and posttranslational modifications. The human CD44 gene is composed of 19 exons, 10 of which (exons 1–5 and 15–19) are included in the standard form of CD44 (CD44) [8, 18, 19]. It was shown that CD44 is related to cell proliferation and adhesion to form epithelial groups owing to adherent peculiarity and take the participation in VEGFR-2 signalling and angiogenesis.

Adhesion processes are involved in all levels of the metastatic cascades, and CD44 expression in invaded tumor areas were evaluated as prognostic indicators of lymph node metastases [20–23]. CD44-positive tumor cells were found in both primary and metastatic sites, as well as in ascites, a higher percentage

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*Abbreviations used*: CSCs – cancer stem cells; OC – ovarian cancer.

of CD44-positive cells were observed in metastases and ascites of OC patients. Also percentage of CD44-positive cells was maintained stable for over 20 passages suggesting that they were able to self-renew compared to CD44-negative cells [24]. The decrease of level of CD44-positive cells correlates with tumor response to chemotherapy in patients with gastrointestinal cancer [25]. Complex molecular investigations demonstrated also the involvement of CD44 expression in mechanisms of carboplatin resistance [26].

Recently an interest to adhesion proteins has increased because CD44 was identified as a marker of cancer stem cells (CSCs) and as prerequisite for tumor cell migration [27–29]. Subpopulation of ovarian cancer-initiating cells ($CD44^+CD117^+$) from primary OC tissues is fully capable for a serial propagation of their original tumor phenotype in animals. Prevalence of epithelial ovarian CSCs correlates with recurrence in patients with early stage OC [30]. It was suggested that $CD44^+$ cells might be involved in OC cell proliferation and progression. But in some studies it was shown that CD44 molecules performs a double role — as a growth and invasiveness-promoting molecule and as a tumor suppressing cofactor, and also as a molecule involved in reparation mechanisms and tumor cells chemoresistance [24, 31, 32].

One of the main features of tumor cell proliferation are peculiarities of invasive front of carcinomas allowing them to invade (by single cells or small clusters of up to five tumor cells) in stroma. This morphological phenomenon was named "budding", and is most studied in colorectal cancer [33, 34]. The presence of tumor buds has been considered to be a feature of aggressive tumor. It was shown that budding of colorectal carcinoma along the tumor invasive margin has been associated with increased malignancy potential. In tongue squamous cell carcinoma tumor budding was observed in 71.7% cases with high-intensity in 48.3% patients. In these patients the presence of budding was associated with tumor size, differentiation, clinical stage, lymph node metastases, and correlated with EMT and overall survival of patients. It should be mentioned that budding results from such features of malignant growth as cellular discohesion and active invasion, which is typical for tumor growth dynamics [35].

Tumor budding is characterized by decreased cadherin expression associated with EMT, and is thought to be a target for treatment of aggressive colorectal cancer. Tumor budding is a predictor of lymph node metastasis, metastatic disease, local recurrence, worse overall and disease-free survival and as an independent prognostic factor [35, 36]. Thus, adhesive alteration and budding as morphological phenomenon may have direct relation to OC progression and prognosis of the disease, molecular phenotype and spreading of tumor cells. The results of such investigations are widely discussed in literature.

The aim of this investigation was to study the CD44 and E-cadherin expression in serous OC and their relation to clinicopathological peculiarities of tumor process and prognosis of the disease.

## MATERIALS AND METHODS

***OC patients***. 72 patients with stage I–III serous OC without neoadjuvant chemotherapy were included in the study. Definition of stage was performed according to the International Federation of Gynecology and Obstetrics classification (FIGO). The studied clinicopathological parameters included age and menopausal status of patients, dissemination of OC, histological degree of differentiation (well-differentiated — G1, moderately differentiated — G2, or poorly differentiated — G3) according to the WHO histological classification and criteria of cytomorphological malignancy (high/low) [2]. All patients received adjuvant chemotherapy (CAP/CEP, 3–7 cycles) after primary cytoreductive operation. The study protocol of investigation was approved by the local bioethic committee.

***Immunohistochemical analysis***. Formalin-fixed, paraffin-embedded tissue blocks and sections (thickness 5 μm) were stained by haematoxylin and eosin for evaluation of histological structure, degree of differentiation and cytomorphological malignancy of serous OC. Immunohistochemical reaction was performed on parallel sections of formalin-fixed paraffin-embedded tissues by streptavidin-biotin-peroxidase method with monoclonal antibodies (clone DF 1485, DakoCytomation, Denmark) which were able to detect CD44 (all isoforms of CD44), and E-cadherin (clone NCH-38), with visualization of results of immunohistochemical assay by EnVision system (Dako LSAB2 system, Denmark). Internal lymphocytes in tumor sections were used as positive control for detection of expression of CD44. In negative control primary antibodies were omitted. Results of immunohistochemical reactions were scored semiquantitatively as a percent of positively stained cancer cells in the whole tumor area. Tumor tissue as isolated single cells or their complexes and clusters were considered CD44-positive if positive staining was detected. The intensity of CD44 was categorized into three grades: 0 — negative, 1 — weak to moderate, or 2 — strong. For further statistical analysis, CD44 expression was categorized into two groups according to the median percentage of the tumor cells with positive expression of studied protein: low expression — $\leqslant$10% of CD44 expressing tumor cells; high expression — >10% of CD44 expressing tumor cells. The percentage of tumor cells showing E-cadherin staining was analysed from the total cells of tumor area and further categorised into two groups — reduced expression (<5% of tumor cells with positive expression), and preserved expression (>5% of tumour cells with positive expression). The immunostained sections were observed under high-power magnification (×200–400).

***Statistical analysis.*** Significance of differences between expression of biological markers and other clinicopathologic parameters were measured using $\chi^2$ test. Survival of patients calculated on the basis

of period after the primary operation, was evaluated using the Kaplan — Meier method, and multivariate analysis was completed using the Cox proportional regression model and Log-rank test. Differences were considered significant at $p < 0.05$.

## RESULTS AND DISCUSSION

General characteristics of OC patients are presented in Table 1. The age of patients ranged from 28 to 73 years and the average age was equal to 58.6±4.3 years, median age was 57 years. The majority of the studied patients were older than 50 years, and also 76.5% of patients had OC stage II and III. All studied tumors had histological structure of serous adenocarcinomas or serous papillary cancer of different degree of differentiation. Half of the studied tumors had moderate degree of differentiation, while percent of tumors with low and high degrees of differentiation was equal to 30.6 and 19.4% respectively. OC showed various cytomorhologic degrees of malignancy: high degree was registered in 62.5% of patients, low — in 37.5%. Patients with stage II and III OC had implantational metastases, in particular in omentum (56.9%) and peritoneum (44.4%).

**Table 1.** Clinicopathological characteristics of OC patients

| Criteria | | Number of patients | |
|---|---|---|---|
| | | n | % |
| Total | | 72 | 100.0 |
| Age (years) | Average | 58.6±4.3 | |
| | Range | 28–73 | |
| Menstrual cycle | | | |
| Preserved | | 30 | 41.6 |
| Menopause | | 42 | 58.4 |
| Stage (according to FIGO) | | | |
| IA – IC | | 17 | 23.5 |
| IIA – IIC | | 23 | 32.0 |
| IIIA – IIIC | | 32 | 44.5 |
| Histological tumor structure | | | |
| Serous cancer (papillar or adenocarcinoma) | | 72 | 100 |
| Degree of OC differentiation | | | |
| G1 | | 14 | 19.4 |
| G2 | | 36 | 50.0 |
| G3 | | 22 | 30.6 |
| Degree of cytomorphological malignancy | | | |
| Low | | 27 | 37.5 |
| High | | 45 | 62.5 |
| Metastases localization* | | | |
| Omentum | | 41 | 56.9 |
| Implantation metastases in peritoneum | | 32 | 44.4 |
| Lymphatic nodes (paraortal, sacral) | | 9 | 12.5 |
| Pelvis, uterus, urinary bladder | | 7 | 9.7 |
| Implantation metastases in intestine | | 6 | 8.3 |

Note: *general number of metastases is higher than number of patients because some patients had several metastases with different localization.

Immunohistochemical assay has shown that in epithelium of serous, mucinous, follicular cysts, which were diagnosed in contralateral ovaries of stage I OC patients and served as a control, CD44 expression was absent. In epithelial hyperplasia of some non-malignant ovarian cysts we have found single cells expressing this adhesion marker. CD44 expression widely varied even in tumors with the same histological structure and in different fields of the same histological sample. CD44 expression was also variable in OC with different degree of differentiation. These results correspond to the data [37] — the significant correlation between the expression of CD44, which was higher in cancer tissue than in benign cystadenomas and in borderline ovarian tumor.

Qualitative analysis of CD44 adhesion molecule expression in serous OC has revealed some features. First of all, we found a focality of CD44 expression in most part of tumors. Expression of this marker had heterogenic localization in different tumor sites, and also in solid zones of tumors. It should be mentioned that CD44-positive cells were observed in central zones, as well as in some layers of cells or in individual cells adjacent to stroma. CD44-positive immunohistochemical reaction was detected not only in tumor cells, but also in stroma near tumor buds as hyaluronan receptor. In 30 (41.7%) from 72 OC cases CD44 expression was low (not higher than 10% of immunopositive cells) or absent. In 42 (58.3%) of 72 OC cases the number of immunopositive CD44s cells was higher than 10% and average level of CD44s expression was 30.0±5.6%, median expression — 27.5%.

E-cadherin also demonstrated a focal character of expression especially in solid zones, but its expression in contrast to CD44 was not detected in proliferative zones and in zones with spindle-like tumor cells and in distant from primary tumor buds. E-cadherin expression was predominantly absent (38/52.8% patients). In 34 (47.2%) cases immunopositive tumors had membranous and/or cytoplasmatic (aberrant) E-cadherin expression. E-cadherin expression index in serous OC was lower than CD44 expression and was equal to 12.2±6.6%. In tumors of other genesis [35] significant associations were observed among deregulation of E-cadherin and tumor budding. This points on significance of E-cadherin expression in tumor proliferation and budding as a morphological criterion of this process.

CD44 expression was frequently observed in individual groups of cells and on separate papillas in the cavity of glands, and even in individual cells in these cavities, and, also, in stroma cells in form of separate clusters of several cells (buds) with lymphocyte infiltration around. CD44 is the main hyaluronan receptor and its expression is observed in stroma particularly on the border between tumor and surrounding stroma, around vessels and in stromal component around glandular structures, and also in lymphocytes, macrophages, and endothelium. These features were mainly observed in individual cells or in their groups (budding) in zones of cell invasion into stroma. In these regions tumor cells had spindle-like shape and were detected in regions with expression of CD44 in both tumor cells and adjacent stroma, or in tumor cells/stroma alone (Fig. 1, 2).

It is known, that CD44 function depends on hyaluronan state: CD44 is expressed in tumor epithelium, where hyaluronan synthetase expression is restricted to stroma-associated cells. This distinct CD44 and hyaluronan pattern of distribution suggests an important role of epithelial-stromal interaction in CD44 functioning. On the one hand, nuclear signalling induced

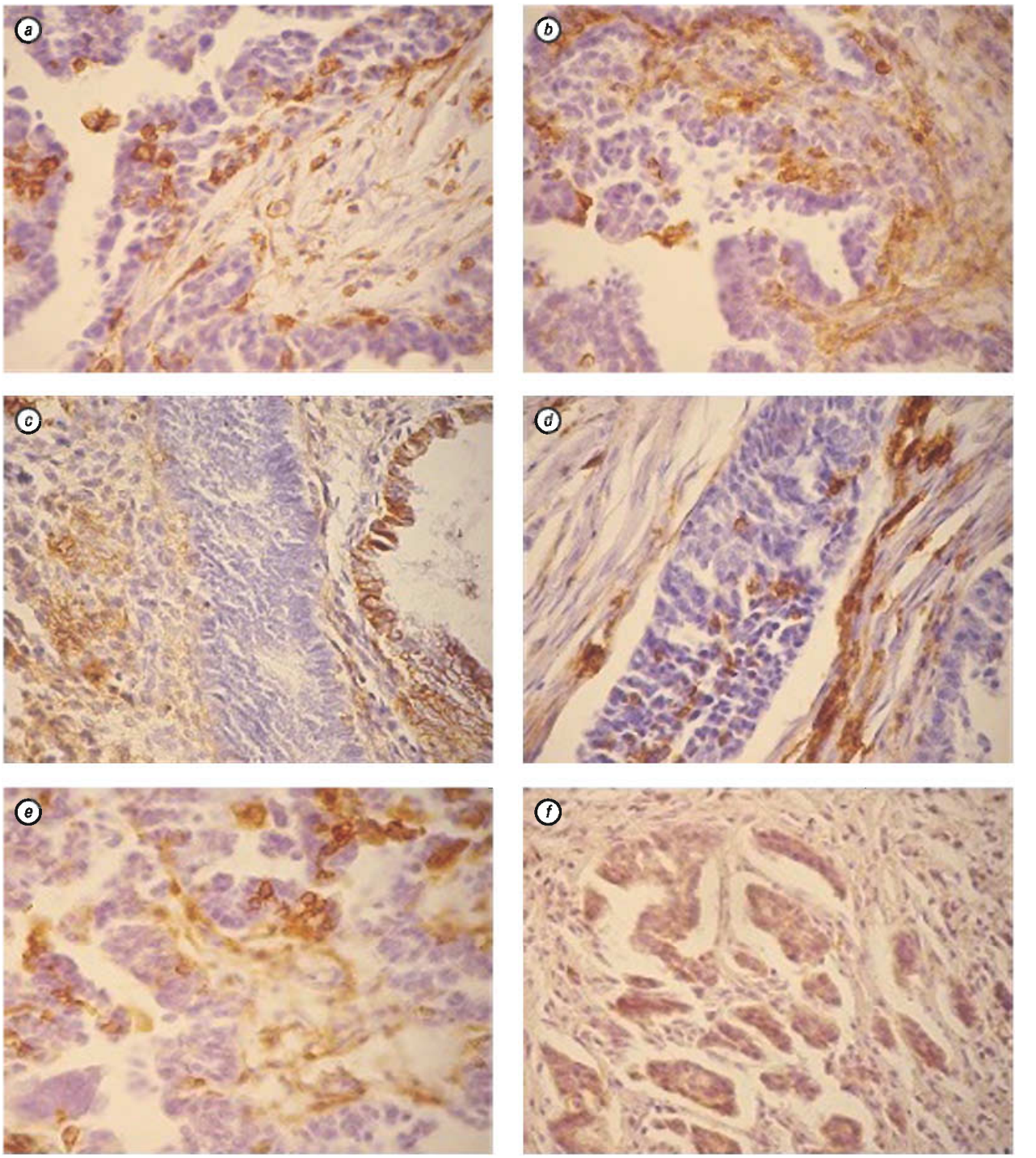

**Fig. 1.** Immunohistochemical analysis of adhesion molecules expression in serous ovarian cancer. *a*) CD44 expression in tumor cells mostly on the board with stromal component and in lymphocytes. *b*) CD44 expression in tumor cells and stromal component and single budding. *c*) E-cadherin expression in differentiated tumor cells including glandular structure, and absence of E-cadherin expression in less differentiated cells. *d*) CD44 expression in cells of low differentiated zone tumors and in stromal component, absence CD44 expression in cells of glandular structure. X 200. *e*) CD44 expression in tumor cells and single budding. *f*) CD44 expression in cells of OC metastasis

by the loss of cell/matrix adhesion stimulates anchorage-independent growth of OC cells. On the other hand, CD44 expression in tumor cells on the border with surrounding stroma might point on elevation of tumor cells adhesive properties, what is essential for their further invasion. It is important that hyaluronan-CD44 interactions can activate stem cell marker Nanog, Stat-3-mediated *MDR1* gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells [38].

In our investigation CD44 and E-cadherin expression varied widely and depended on clinical factors of tumor processes (Table 2). Number of tumors with high CD44 expression varied from 52.9% (stage I) to 59.4 and 60.2% tumors (stage III and II, respectively), what reflects the tendency for elevation of CD44 expression along with tumor cell dissemination ($p>0.05$). The alterations of number of tumors with high E-cadherin expression also varied — most E-cadherin-positive tumors (70.5%) were detected

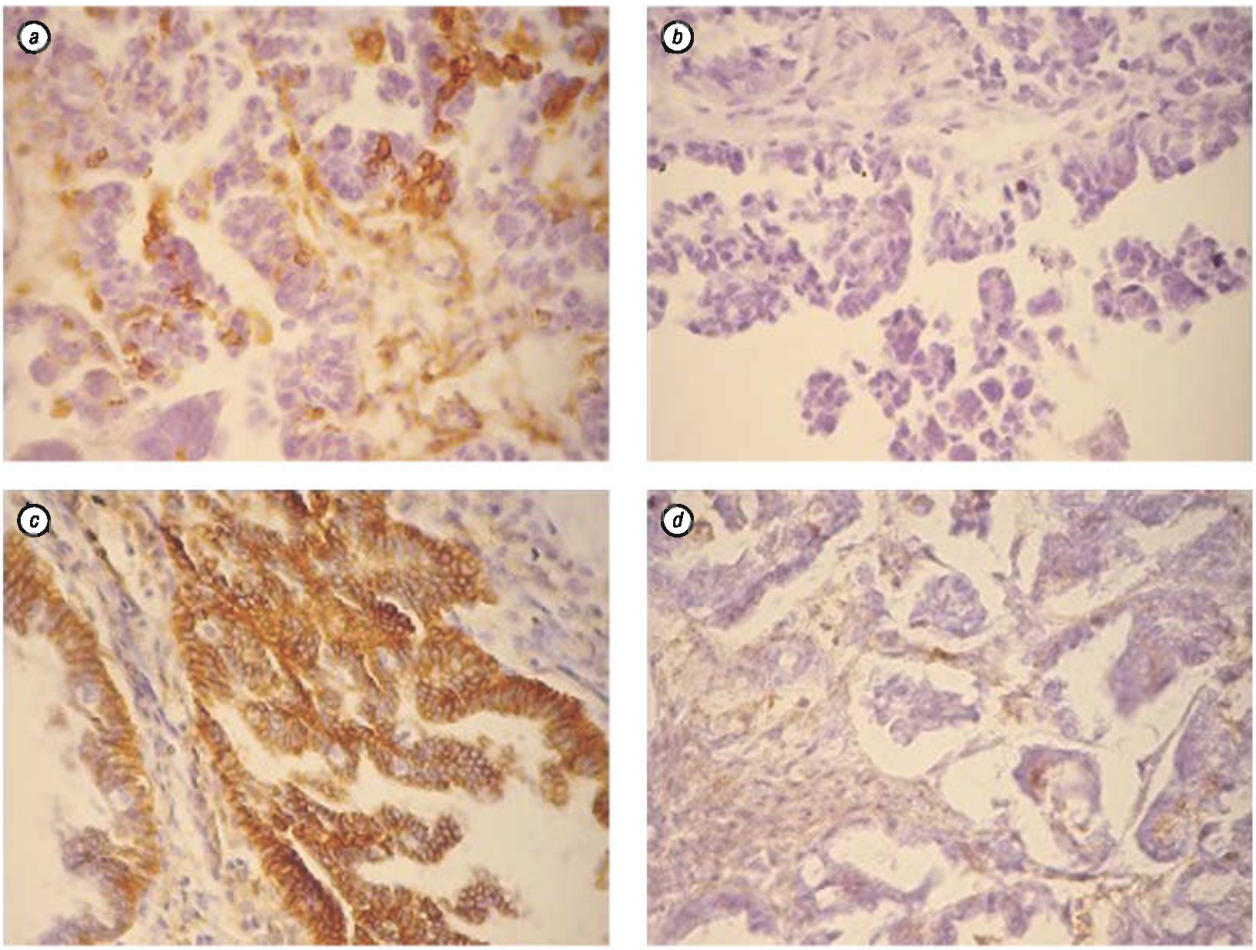

**Fig. 2.** Immunohistochemical analysis of adhesion molecules expression in serous ovarian cancer. *a*) Adhesion violation of tumor cells and forming the buddings with/without CD44 expression. *b*) Absence of E-cadherin expression. *c*) High E-cadherin expression in differentiated carcinoma. *d*) E-cadherin expression in single tumor cells. ×200

in patients with stage I OC. In OC patients with stage II/III number of tumors with high E-cadherin expression decreased ($p<0.05$) to 43.4% and 37.5%, respectively.

**Table 2.** Clinicomorphological indexes and immunohistochemical evaluation of CD44 and E-cadherin expression in OC

| Clinicomorphological indexes | Patients (n=72) | Tumors with high expression of markers (n/%) | |
|---|---|---|---|
| | | CD44(+) (n=42) | E-cadherin+ (n=34) |
| Stage of tumor process | | | |
| IA–IC | 17/100 | 9/52.9 | 12/70.5 |
| IIA–IIC | 23/100 | 14/60.9 | 10/43.4[1] |
| IIIA–IIIC | 32/100 | 19/59.4 | 12/37.5[1] |
| Degree of cytomorphological malignancy | | | |
| Low | 27/100 | 16/38.1[2] | 23/85.2[2] |
| High | 45/100 | 26/61.9[2] | 11/24.4[2] |
| Degree of tumor differentiation | | | |
| G1 | 14/100 | 3/21.4 | 11/78.5 |
| G2 | 36/100 | 21/58.3[3] | 19/52.7[3] |
| G3 | 22/100 | 18/81.8[3] | 4/18.2[3] |

Notes: [1] $p<0.05$ compared to group of patients with OC IA–IC stage; [2] $p<0.05$ between groups of patients with high and low cytomorphological malignancy; [3] $p<0.05$ compared to group of patients with G1 OC

Comparison of adhesion molecules expression with degree of cytomorphological malignancy OC has shown that CD44 was expressed more often in OC with high degree of malignancy (61.9% cases) than in OC with low degree (38.1% cases) ($p<0.05$). On the contrary, E-cadherin expression was observed more often in ovarian tumors with low rather than with high degree of malignancy (85.2 and 24.4% cases, respectively, $p<0.05$). The same tendency of CD44 expression in serous OC tumors was observed: this protein was more frequently expressed in G2 and G3 tumors, while E-cadherin — in G1 tumors ($p<0.05$). Using Pearson's correlation coefficient we have found a dependence between expression of the studied markers: reverse correlation between CD44 and E-cadherin expression ($r=-0.38$, $p<0.05$) in serous OC. Obtained results about correlation between nuclear β-catenin and CD44 corresponded to the data from literature [41], and pointed on the role of E-cadherin expression in CD44 expression regulation.

The heterogeneity of markers expression in different tumors can be explained by different mechanisms that might depend on each other: genomic instability, both genetic and epigenetic, which might be related with them in cell selection with the highest disturbance of genome and clonal tumor growth; molecular phenotype of tumor cells with different markers of differentiation including stem cell markers; adhesive and invasive phenotype and plasticity of tumor cells [39, 40].

We have compared CD44 expression in primary tumors and implantation metastasis from the same patients with OC stage III (15 patients). It has been shown that mean number of CD44-positive cells in primary tumors and metastases did not significantly differ and was equal to 25.6±3.4 and 27.5±2.8%, respectively ($p>0.05$) (Fig. 3). Analysis of individual expression

indexes has revealed significant variations of this adhesion molecule expression (Fig. 4). In 7 (46.6%) from 15 patients the increase of expression index has been registered, in 4 (26.7%) — the decrease, and in 4 (26.7%) — no changes (Fig. 5).

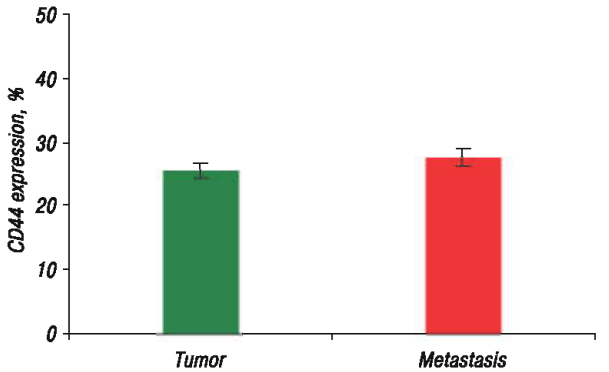


**Fig. 3.** Mean number of CD44-positive tumor cells in primary OC and implantation metastasis

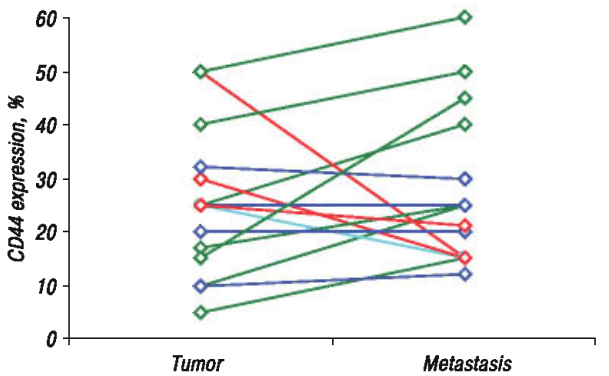


**Fig. 4.** Individual changes of CD44 expression in primary serous OC and implantation metastasis

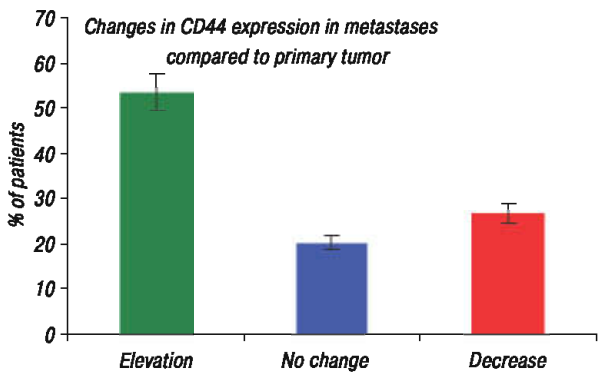


**Fig. 5.** Changes in CD44 expression in implantation metastasis cells in comparison with primary serous OC

We have used Cox proportional hazards model to detect the possibility of use of studied adhesion proteins as prognostic markers of OC patients survival. It has been shown that significant changes of CD44s and E-cadherin expression could be used as independent prognostic markers for OC patients (Table 3).

**Table 3.** Results of multifactor analysis (Cox proportional hazards model)

| Marker | β | P |
|---|---|---|
| CD44 | -0.28 | <0.05 |
| E-cadherin | 0.34 | <0.05 |

Prognostic significance of adhesion molecules expression was acquired by analysis of OC patients overall survival dependent on the molecular phenotype of tumor cells. Kaplan — Meier overall survival curves for patients with serous OC with CD44(+)/E-cadherin(-) and CD44(-)/E-cadherin(+) cell phenotype defined better prognosis for OC patients with CD44(-)/E-cadherin(+) OC phenotype (Fig. 6, 7). So, CD44(+)/E-cadherin(-) cell phenotype is biological feature of more aggressive serous OC and is associated with poor survival.

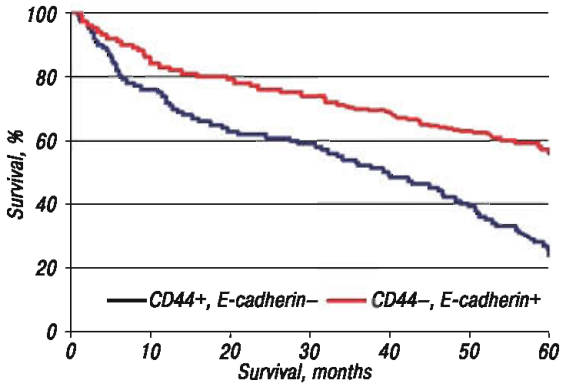


**Fig. 6.** Kaplan — Meier curves of overall survival in patients with serous OC with CD44s(+)/E-cadherin (-) and CD44s(-)/E-cadherin (+) cell phenotype

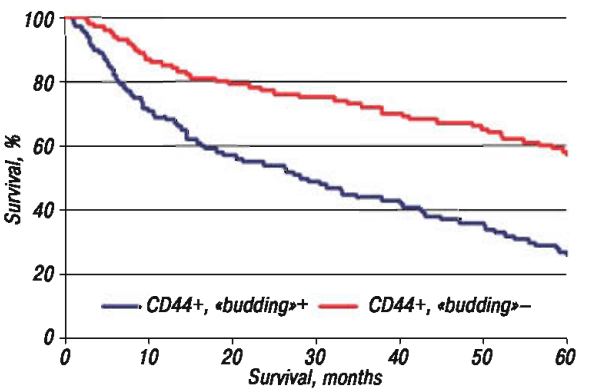


**Fig. 7.** Kaplan — Meier curves of overall survival in patients with serous OC with CD44s(+)/budding(+) and CD44s(+)/budding(-) cell phenotype

The data present in literature about prognostic value of CD44 expression in tumors remain controversial because influence of CD44s(-)/E-cadherin(+)-mediated tumor adhesion properties on OC prognosis may be related to various factors. For example, epidermal growth factor receptor expression is frequently elevated in OC, and this often causes disruption of adherent junctions and reduces E-cadherin protein levels in tumor tissue [42]. It was shown that CD44 expression is related to well-differentiated, early-stage OC and long survival of the patients, thus indicating a favourable prognosis [43, 44]. According to the data [45] CD44v6 expression significantly correlated with histological type, FIGO stage and histological grade of ovarian carcinomas but 5-year survival of patients with CD44v6(+) was shorter than that of patients with CD44v6(-) (36.6% *versus* 66.7%,). Evidently, survival of OC patients depended on correlation between CD44-positive and CD44-negative tumor cell populations characterized by variable gene expression. Analysis of the global gene expression pattern of OC cells has shown that 10% of 24000 studied

genes were differentially expressed between the two cell populations — CD44-positive and CD44-negative isolated from three OC samples. These differentially expressed genes were predominantly associated with control of apoptosis, signal transduction, transcription regulation and cell differentiation [24].

The literature data describe complex mechanisms of E-cadherin gene (*CDH1*) inactivation and CD44 activation in human cancer cells: mutations (inherited/somatic), aberrant protein processing, methylation, induction of transcriptional repressors (SNAIL, ZEB1, ZEB2), epithelial mesenchymal transition, which is also associated with induction of "mesenchymal" cadherins [46, 47]. Decrease of E-cadherin expression is associated with clinical stage, lymph node metastasis, and degree of differentiation [48], but it was found that E-cadherin expression alone shows no statistically prognostic significance [49]. The data on function of E-cadherin in OC cells remains controversial and further and deeper studies for detection of correlations with traditional clinicopathological factors including budding are required.

Functions of CD44 and its isoforms also depend on hyaluronan status. CD44 is expressed in tumor cells, while hyaluronan synthase expression is restricted to stromal-associated cells. This distinct hyaluronan pattern of distribution and extremely sensitive changes of CD44 expression in tumor microenvironment suggests the role of epithelial-stromal interaction in function of adhesion molecules. The loss of cell/matrix adhesion stimulates anchorage-independent growth of OC cells, including budding. There is a hypothesis that CD44 function varies during different stages of tumor growth — from initiation to formation of metastases [50] that may be an actual question for future studies.

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