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COMPREHENSIVE ANALYSIS OF INTRATUMORAL LYMPHOCYTES AND FOXP3 EXPRESSION IN TUMOR CELLS OF ENDOMETRIAL CANCER

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Aim: **To study the tumor microenvironment ($CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes) and FOXP3 expression by tumor cells and correlation of studied parameters with clinical and morphological characteristics of endometrial adenocarcinomas.** ***Materials and Methods:*** **Tumor samples from 40 patients (mean age 56.9 ± 2.8) with endometrial cancer (EC), who did not receive special treatment before surgery (chemotherapy, radiation therapy and hormontherapy), were investigated. Morphological, immunohistochemical methods as well as methods of mathematical statistics were applied in the study.** ***Results:*** **It has been determined that high quantity of $FOXP3^+$ tumor cells and intratumoral $CD4^+$ and $CD8^+$ T-lymphocytes along with the low content of $FOXP3^+$-lymphocytes is typical for the endometrial adenocarcinomas of high differentiation grade (G1). In poorly differentiated (G3) EC an increase of number of $FOXP3^+$-lymphocytes and decrease of $CD4^+$ and $CD8^+$ lymphocytes in lymphocytic infiltrate have been observed. Moreover, decrease of the content of $FOXP3^+$ tumor cells has been determined. In EC patients correlation between the following parameters has been detected: proliferative activity and deep invasion of tumor in myometrium ($R = 0.74$); depth of invasion correlated with the number of the $FOXP3^+$ tumor cells ($R = -0.63$) and number of $CD4^+$ and $CD8^+$ lymphocytes ($R = 0.68$ and $R = -0.55$ respectively) in lymphocytic infiltrate. Thus, results of this study are the evidence of significance of the lymphocytic components of tumor microenvironment and content of FOXP3 expressing tumor cells in EC progression.** ***Conclusion:*** **Quantitative changes of tumor microenvironment, such as number of $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes and content of $FOXP3^+$ tumor cells correlate with biological characteristics of EC.**

Key Words: **endometrial cancer, $FOXP3^+$ tumor cells, intratumoral $FOXP3^+$ lymphocytes, intratumoral $CD4^+$, $CD8^+$ T-lymphocytes, depth of invasion, proliferative potential.**

According with the "immune-editing" or "three E" theory (elimination, equilibrium, escape), immunocompetent cells being important component of the tumor microenvironment can both show antitumor activity and contribute to the progression of malignant growth that occurs through selection and survival of the most aggressive clones of tumor cells resistant to the impact of cytotoxic T-lymphocytes as well as due to the changes in functioning of immunocompetent cells in tumor microenvironment [1–3]. Tumor pathophysiological features affect the structural and functional changes of certain components of tumor microenvironment, in particular decrease or lack the expression of antigen-recognizing receptors of lymphocytes, antigens of I and II classes of HLA, increase the quantity of macrophages of type 2 and T-lymphocytes with regulatory and suppressive activity. These macrophages synthesize appropriate range of immune suppressing cytokines, which cause inhibition of effector functions of immunocompetent cells, in particular $CD8^+$ and $CD4^+$ T-lymphocytes [4–9].

The main function of regulatory T-lymphocytes $CD4^+CD25^+FOXP3^+$ ($Treg_s$) is the maintenance of immunologic homeostasis and prevention of autoimmune diseases. It should be mentioned that in peripheral blood of the almost healthy individuals, $Treg_s$ constitute only 10% in population of $CD4^+$ T-lymphocytes. At the same time, the increase in the quantity of $Treg_s$ up to 30–50% both in peripheral blood and in tumor microenvironment of patients with cancer of the different genesis correlates with unfavorable clinical course. According to the data of several studies, particular functional features of $Treg_s$ may play key role in the mechanism of tumor cell escape from the immune response that contributes to the progression of malignant process [10–20]. Marker of $Treg_s$ is transcription factor $FOXP3^+$, expression of which has essential value for the differentiation of $CD4^+CD25^+FOXP3^+$ lymphocytes and determines functions of $Treg_s$ regardless of the level of expression of $CD25^+$ [14–22].

In the most of cases, FOXP3 is mainly expressed in subpopulation of $CD4^+CD25^+$ lymphocytes, but small quantity of $CD8^+CD25^-$ cells also express FOXP3 antigen [23].

Until recently, FOXP3 was considered to be expressed by the population of immunocompetent cells. However, over recent years, some studies have shown that FOXP3 is expressed not only in cells of immune system, but also in tumor cells of different tissues (epithelial and not epithelial) and is the main factor, which determines inhibition of proliferative activity of these cells [24, 25].

Besides mentioned above, in tumor cells of many solid neoplasms, $FOXP3^+$ regulates expression of such oncogenes and tumor suppressors as C-Myc, ERBB2, SKP2 and $p21^{WAF1/CIP1}$ [26, 27].

At the present time, there are only few data about the role of $FOXP3^+$ lymphocytes in progression of endometrial adenocarcinoma [12, 14, 28, 29] and there is no information concerning significance of the FOXP3 expression in the endometrial tumor cells. The question remains

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Abbreviations used: EC – endometrial cancer; FOXP3 – Forkhead box P3.

essential concerning the significance of antitumor immunity in the development and clinical course of endometrial cancer (EC). Also, mechanisms of interaction between tumor and immunocompetent cells were not clarified.

Relevance of the study is substantiated by the fact that EC is one of the most widespread malignant tumors of the female reproductive system. Study of epidemiological situation in Ukraine has showed that in female population, EC incidence has significantly increased over the last 5 years from 15.9 to 17.9% per 100,000 of the female population and takes third place (8.6%) after breast cancer and non-melanoma skin cancer [30].

Considering the mentioned above, the aim of the study was to investigate the characteristics of the tumor microenvironment ($CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes) and FOXP3 expression by tumor cells and correlation of studied parameters with clinical and morphological characteristics of endometrial adenocarcinoma.

MATERIALS AND METHODS

Samples of surgical material from 40 patients (mean age 56.9 ± 2.8 years) with EC of FIGO stage I, who did not receive special treatment before surgery (chemotherapy, radiation therapy and hormonal therapy) were taken for a study. All patients underwent treatment in oncological gynecology department of the National Cancer Institute of Ministry of Health of Ukraine. All patients have given informed consent for the use of their surgical material for the research purpose.

Assessment of the population of lymphocytes infiltrating tumor was carried out by immunohistochemistry using primary monoclonal antibodies: CD4 (clone 4B12, "Millipore", USA) and CD8 (clone RIV — 11, "Millipore", USA). Expression of FOXP3 was determined both in tumor cells and in intratumoral lymphocytes using primary monoclonal antibody to antigen FOXP3 (clone 5H5L12, "Invitrogen", USA). Proliferative potential of endometrial tumor cells was determined by the number of cells expressing Ki-67 (clone MIB1, "DakoCytomation", Denmark). Results of the immunohistochemical reaction were assessed by semi-quantitative method via calculation of the number of positively stained cells in percentage — labeling index (LI, %). Proliferative potential was evaluated by determination of the number of cells expressing protein Ki-67 (proliferation index — PI). Expression of markers was evaluated in 800–1000 tumor cells. For corrected interpretation of the results statistical method of median (Me) determination was applied: if values of IM and PI were lower than Me, expression of the corresponding marker was low, and if values of IM and PI were higher than Me — high. Statistical analysis was carried out using the software Statistica 8.0 (StatSoft, Inc.) with use of Kruskal — Wallis nonparametric criterion. A p value of $\leqslant 0.05$ was considered to be statistically significant.

RESULTS

Morphological analysis has determined that all studied tumors were endometrioid adenocarcinomas of different histological differentiations: 11 (27.5%) tumors were high (G1), 15 (37.5%) — moderately (G2) and 14 (35.0%) — poorly differentiated (G3). The distribution of tumors with respect to the depth of invasion in myometrium was as follows: 16 (40.0%) tumors invaded less than 1/2 of myometrium, 24 (60.0%) neoplasms — more than 1/2.

Immunohistochemical study of the population of intratumoral T-lymphocytes has revealed that CD4-lymphocytes were detected in 88.9% and CD8-lymphocytes — in 95.0% of studied endometrial tumors (Fig. 1).

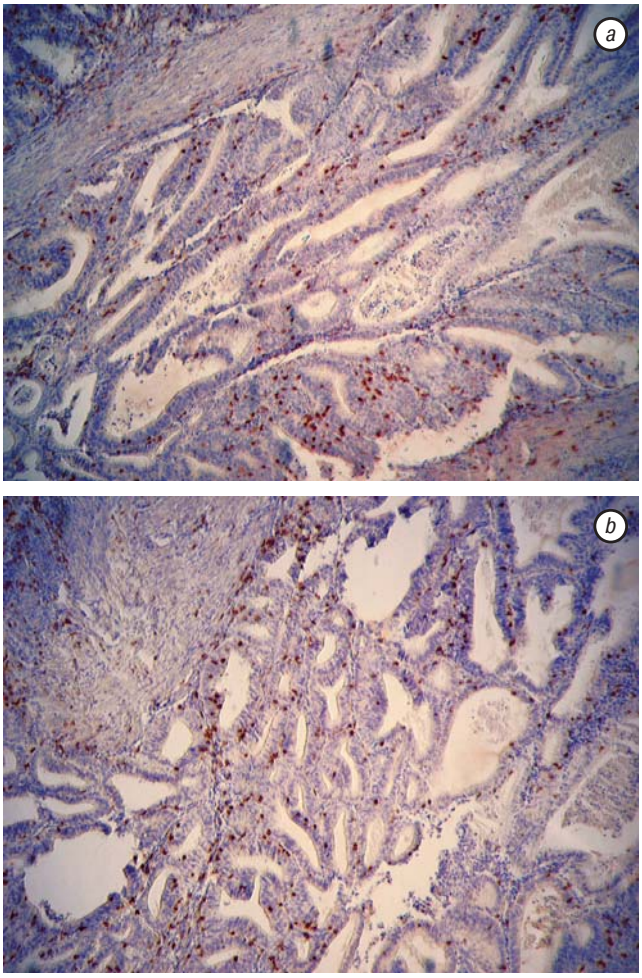

Fig. 1. $CD4^+$ T-lymphocytes (*a*) and $CD8^+$ T-lymphocytes (*b*) in the endometrioid adenocarcinoma, × 200

Average number of $CD4^+$ T-lymphocytes in the lymphocytic infiltrate of EC was 35.3 ± 4.1%, and $CD8^+$ T-lymphocytes — 39.5 ± 4.8%. At the same time, individual values of quantity of $CD4^+$ and $CD8^+$ T-lymphocytes in lymphocytic infiltrate of endometrial adenocarcinoma significantly varied and constituted 3.0–48.9% and 5.0–65.0%, respectively. Differences in number of $CD4^+$ and $CD8^+$ T-lymphocytes in the endometrial tumors of different histological differentiation grade were determined. In G2- and G3-tumors, the number of intratumoral $CD4^+$ and $CD8^+$ T-lymphocytes decreased as compared to G1 tumors. It should be mentioned that the number of EC cells with Ki-67 expression also depended on histological differentiation of endometrial adenocarcinoma. As differentiation grade of EC was decreasing, quantity of endometrial tumor cells expressing Ki-67 was progressively increasing. In cells of G2 and G3 tumors, the number of Ki-67 positive tumor cells was higher

($p < 0.05$) as compared to well differentiated tumors and constituted 25.6 ± 0.5 and 42.3 ± 0.7%, respectively (Table).

Table. The number of T-lymphocytes in lymphocytic infiltrate and proliferative potential of endometrial adenocarcinomas of different differentiation grade

Tumor differentiation	Number of T-lymphocytes, M±m, %		The number of cells expressing Ki-67, M±m, %
	$CD4^+$	$CD8^+$	
G1	52.0 ± 2.7	46.4 ± 5.6	13,4 ± 0.3
G2	35.1 ± 5.1*	40.9 ± 5.6	25.6 ± 0.5*
G3	21.2 ± 5.1*	30.2 ± 4.2	42.3 ± 0.7*

Note: *p < 0.05 as compared to G1.

Expression of FOXP3 has been found in cells of 75.0% endometrial adenocarcinomas whereas $FOXP3^+$ intratumoral lymphocytes were detected in 83.3% tumors (Fig. 2).

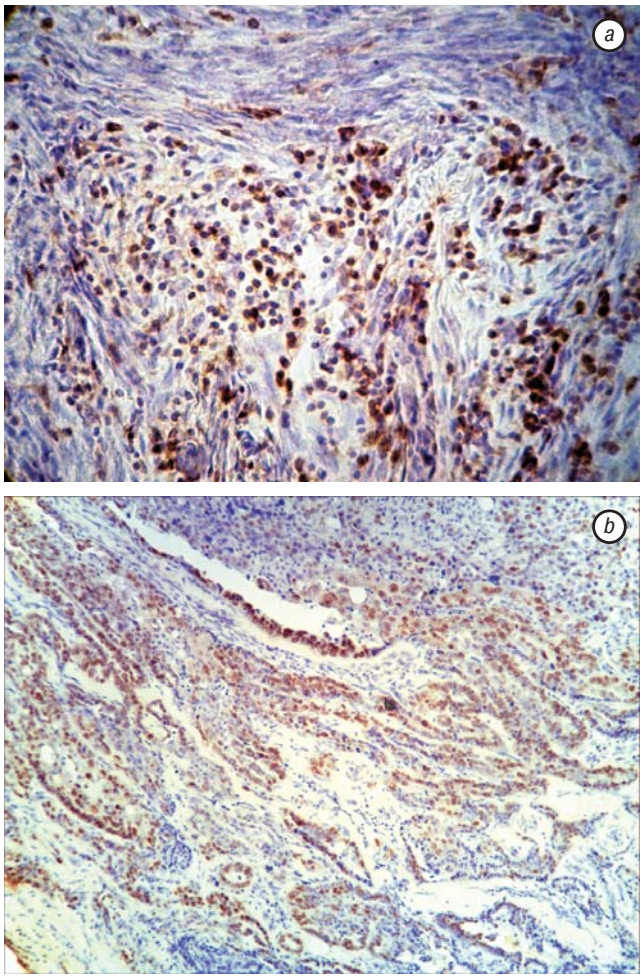

Fig. 2. $FOXP3^+$ lymphocytes (*a*) and FOXP3 expression by tumor cells (*b*) in endometrial adenocarcinoma: *a* — × 400; *b* — × 200

The number of cases with positive expression of FOXP3 by tumor cells decreased from G1 to G3 tumors and reached 100% for G1, 60.0% — for G2 and 50.0% — for G3 endometrial adenocarcinomas whereas the number of cases with $FOXP3^+$ intratumoral lymphocytes in lymphocytic infiltrate conversely increased from G1 to G3 (G1–62.5%; G2–90.0%; G3–100%) tumors.

At the same time, the number of tumor cells with FOXP3 expression in G3 was lower ($p < 0.03$) as compared with G1 adenocarcinomas. In lymphocytic infiltrate of these tumors, increase ($p < 0.04$) of the number of $FOXP3^+$ lymphocytes was observed. These results show that changes in expression of FOXP3 both in tumor cells and lymphocytes are associated with morphological features of endometrial tumors, but oppositely directed (Fig. 3).

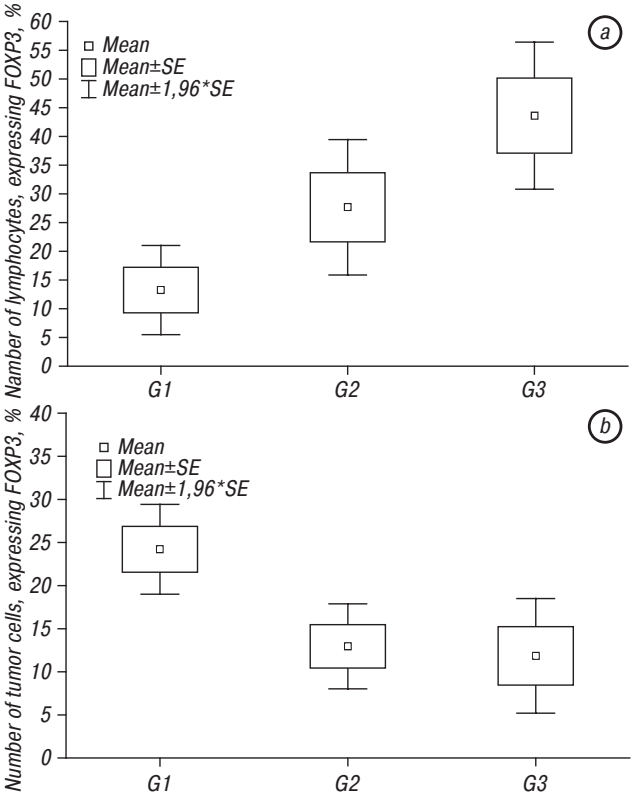

Fig. 3. Comparison of the number of $FOXP3^+$ lymphocytes (*a*) and $FOXP3^+$ tumor cells (*b*) in endometrial adenocarcinomas of different differentiation grade: *a* — H = 6.487719, p = 0.04; *b* — H = 6.893700, p = 0.03

Detailed analysis of cell population in tumor microenvironment has determined that at decrease of differentiation grade in endometrial adenocarcinomas, significant decrease of ratio of $CD4^+/FOXP3^+$ lymphocytes and $CD8^+/FOXP3^+$ lymphocytes was observed. In G1 tumors, this ratio equaled to 2.3 and 2.1, respectively, in G2 tumors — 1.3 and 1.6, respectively, in G3 tumors — 0.5 and 0.9, respectively. Comprehensive assessment of the content of intratumoral T-lymphocytes, especially quantitative indices of ratio of subpopulations of $CD8^+$ to $FOXP3^+$ lymphocytes, gives opportunity to determine their balance that can be important factor for the determination of the role of tumor microenvironment in the progression of malignant growth [31–33].

Thus, at decrease of differentiation grade of endometrial adenocarcinoma, quantitative redistribution of subpopulation of $FOXP3^+$ lymphocytes towards increase of the number of intratumoral $FOXP3^+$ cells and decrease of the number of $CD4^+$, $CD8^+$ T-lymphocytes occurs. The causes of detected changes can be conditioned, on the one hand, by the impact of such cytokine as TGF-β1 expressed by most of the tumor cells and tumor-associated macrophages of the II type (M2), which in turn cause the increased number of $Treg_s$ in tumor microenvironment [34–36]. It is known that $Treg_s$ also possess high secretion of inhibitory cytokines TGF-β, IL-10, IL-35 and IL-6, which contribute to the decrease or inhibition of functional activity of effector cells, in particular $CD4^+$ and $CD8^+$ lymphocytes. Intratumoral $Treg_s$ produce

high level of VEGF that can contribute to the angiogenesis and growth of tumors. Also, conversion of effector $CD4^+$ and $CD8^+$ T-lymphocytes in regulatory and suppressive $FOXP3^+$ T-lymphocytes is possible [7–10, 13, 22, 37–39]. Regulatory T-lymphocytes can suppress immune reaction not only due to the secretion of cytokines, but also due to their direct cytotoxic impact [40].

One of the indices associated with progression of EC is invasive potential. Therefore undoubtedly important was comparison of the number of cells with expression of $FOXP3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes with proliferative potential and depth of invasion of endometrial tumor in myometrium. It has been determined that in the most of well differentiated endometrial adenocarcinomas (81.8%), IP was lower than Me and 91.0% of these tumors were characterized by not deep (< 1/2) invasion in myometrium. At the same time, in G2 and G3 tumors compared to G1 tumors, significant increase of the number of cases with high proliferative potential and deep invasion (> 1/2) in myometrium was observed — (60.0%) and (54.3%), and (78.6%) and (85.7%), respectively.

It has been determined that in highly proliferating cells (Ki-67 > 29.0%), number of $FOXP3^+$ tumor cells (11.4 ± 2.8%; p = 0.01) and $CD4^+$ (25.8 ± 5.9%; p = 0.008) and $CD8^+$ intratumoral T-lymphocytes (31.8 ± 4.3%; p = 0.3) was lower, and number of $FOXP3^+$ intratumoral lymphocytes (41.0 ± 6.7%; p = 0.04) was higher as compared with tumors with low proliferative activity (Ki-67 < 29.0%), in which these indices constituted: 20.8 ± 2.0%; 48.7 ± 4.7%; 41.4 ± 4.3% and 22.0 ± 4.9%, respectively. Obtained data show significance of expression of transcription factor FOXP3 in modulation of proliferative potential in endometrial adenocarcinomas. For instance, increase of number of immunocompetent cells with expression of FOXP3 contributes to the inhibition of functions of effector T-lymphocytes, increase of immunity suppression and proliferative activity. At the same time, in tumor cells, impact of FOXP3 protein realized through the activation of expression of inhibitor of cyclin-dependent kinases $p21^{WAF1/CIP1}$ and causes arrest of cellular cycle in S-phase that contributes to the decrease of proliferative activity of EC cells [26, 27]. It should be mentioned that the same correlation was detected in endometrial adenocarcinomas with different invasive potential of tumor in myometrium (Fig. 4).

For instance, in EC with deep (>1/2) invasion, significantly lower number of $FOXP3^+$ tumor cells (10.5 ± 2.0%, p = 0.004), $CD4^+$ lymphocytes (36.5 ± 2.0%, p = 0.03), decreasing tendency of intratumoral $CD8^+$ lymphocytes (36.0 ± 2.0%, p = 0.3) was observed and number of $FOXP3^+$ lymphocytes (39.1 ± 4.9%, p = 0.01) increased as compared with tumors, which invaded myometrium less than 1/2 (20.6 ± 2.4; 45.3±5.1; 38.0±4.3 and 15.0±2.9%, correspondingly).

Correlation analysis (Spearman's rank correlation, p < 0.05) showed strong positive correlation (R = 0.74) between proliferative activity of cancer cells and depth of invasion of tumor in myometrium. Moreover, correlation between depth of invasion of endometrial adenocarcinoma and number of $FOXP3^+$ tumor cells (R = −0.63) and components of lymphocytic infiltrate, such as $FOXP3^+$ and $CD4^+$ lymphocytes, was determined (R = 0.68 and R = −0.55, correspondingly). Determined correlations are the evidence of significance of components of tumor microenvironment and content of FOXP3 expressing tumor cells in EC progression.

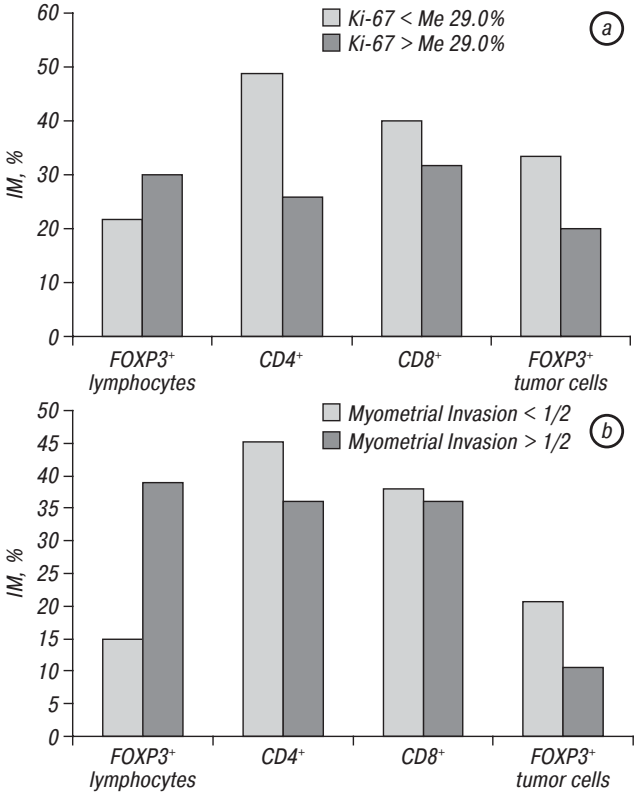

Fig. 4. Distribution of number of $CD4^+$, $CD8^+$ and $FOXP3^+$ intratumoral lymphocytes, and $FOXP3^+$ tumor cells depending on level of proliferative activity (*a*) and depth of invasion of EC (*b*)

Thus, this study determined the changes in the number of $CD4^+$, $CD8^+$, $FOXP3^+$ lymphocytes and $FOXP3^+$ tumor cells in patients with EC depending on clinical and morphological features of tumor, as histological differentiation grade, proliferative potential and depth of myometrial invasion. It has been demonstrated that high percentage of intratumoral $CD4^+$ and $CD8^+$ T-lymphocytes and tumor cells with expression of FOXP3 as well as decrease of the content of $FOXP3^+$ lymphocytes are associated with high differentiation grade of endometrioid adenocarcinoma, low expression of marker of proliferating cells Ki-67 and low invasion of tumor in myometrium. When differentiation grade decreases, the increase of population of $FOXP3^+$ lymphocytes in tumor microenvironment is observed that correlates with decrease of the number of intratumoral $CD4^+$ and $CD8^+$ T-lymphocytes and FOXP3 expressing tumor cells, high proliferative activity and deep invasion of tumor in myometrium.

These findings show that quantitative changes of components of tumor microenvironment, such as $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes and the content of $FOXP3^+$ expressing tumor cells objectively reflect the biological characteristics of EC and may represent a promising tool toward the identification of EC patients with poorer prognosis.

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