Experimental
ONCOLOGY

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+ (Tregs) is the maintenance of immunologic homeostasis and prevention of autoimmune diseases. It should be mentioned that in peripheral blood of the almost healthy individuals, Tregs constitute only 10% in population of CD4+ T-lymphocytes. At the same time, the increase in the quantity of Tregs 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 Tregs 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 Tregs is transcription factor FOXP3+, expression of which has essential value for the differentiation of CD4+CD25+FOXP3+ lymphocytes and determines functions of Tregs 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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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 ⩽ 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).
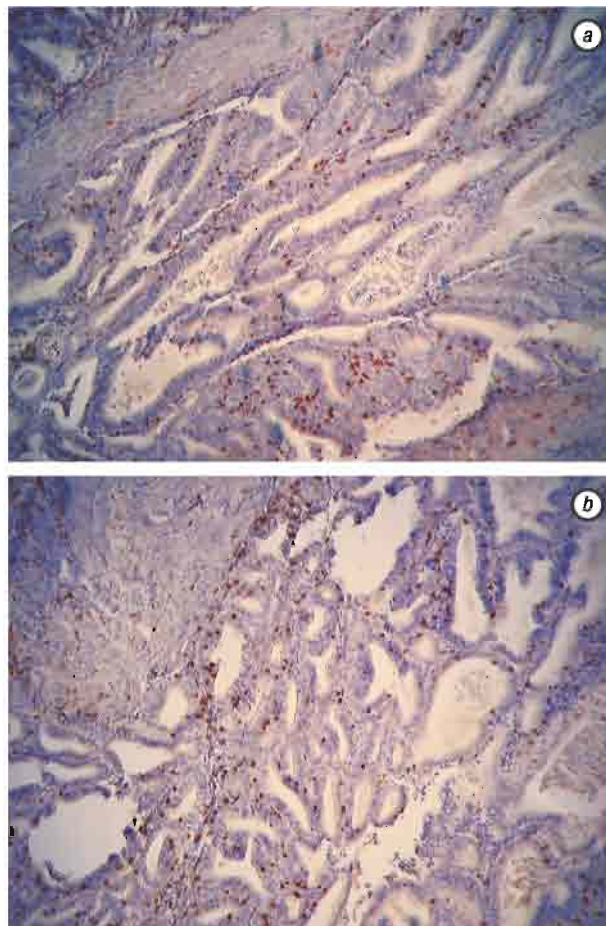
Fig. 1. CD4+ T-lymphocytes (a) and CD8+ T-lymphocytes (b) in the endometrioid adenocarcinoma, × 200

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 $42.3 \pm 0.7\%$, 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	$13,4 \pm 0.3$
G2	$35.1 \pm 5.1^*$	40.9 ± 5.6	$25.6 \pm 0.5^*$
G3	$21.2 \pm 5.1^*$	30.2 ± 4.2	$42.3 \pm 0.7^*$

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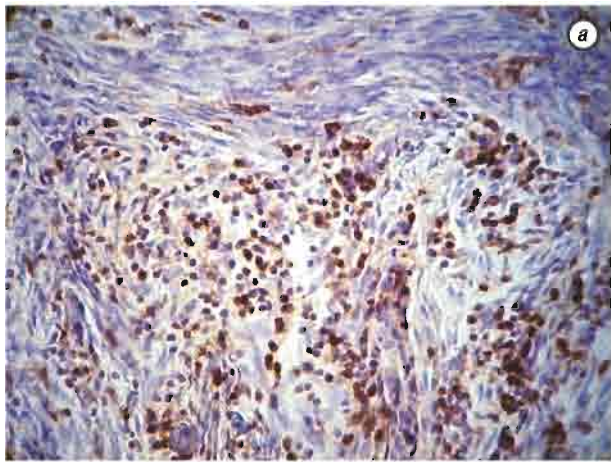
Fig. 2. FOXP3+ lymphocytes (*a*) and FOXP3 expression by tumor cells (*b*) in endometrial adenocarcinoma: *a* — × 400; *b* — × 200

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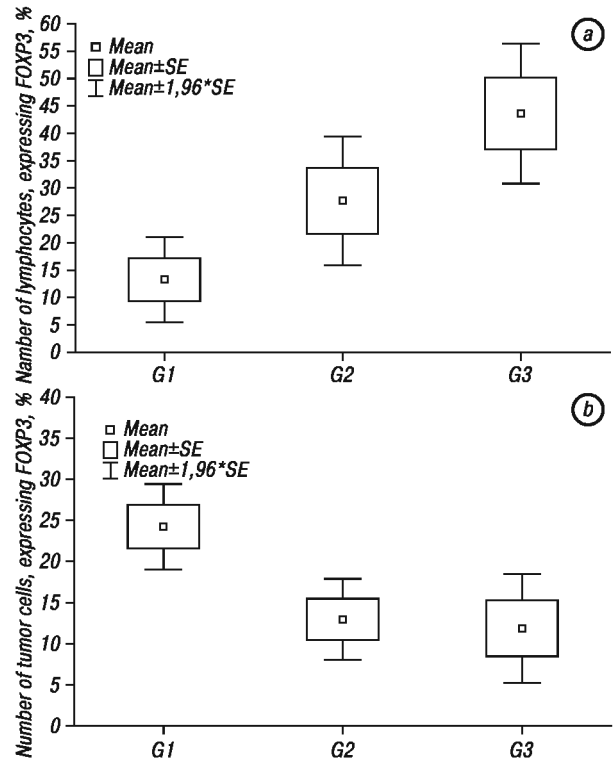
Fig. 3. Comparison of the number of FOXP3+ lymphocytes (*a*) and FOXP3+ tumor cells (*b*) in endometrial adenocarcinomas of different differentiation grade: *a* — H = 6.487719, p = 0.04; *b* — H = 6.893700, p = 0.03

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 Tregs in tumor microenvironment [34–36]. It is known that Tregs 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 Tregs 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.
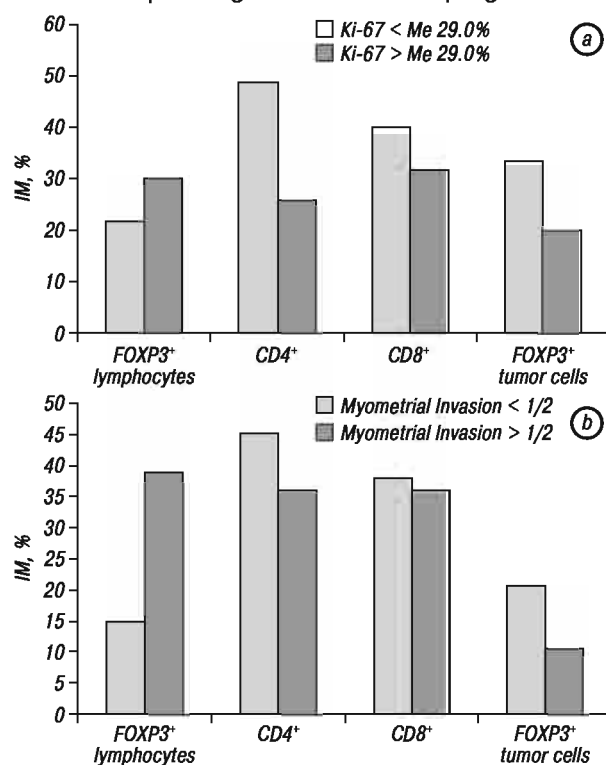
Fig. 4. Distribution of number of CD4+, CD8+ and FOXP3+ intratumoral lymphocytes, and FOXP3+ tumor cells depending on level of proliferative activity (*a*) and depth of invasion of EC (*b*)

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.

REFERENCES

1. **Germenis AE, Karanikas V.** Immunoepigenetics: the unseen side of cancer immunoediting. Immunol Cell Biol 2007; **85**: 55–9.

2. **Swann JB, Smyth MJ.** Immune surveillance of tumor. J Clin Invest 2007; **117**: 1137–46.

3. **Zitvogel L, Tesniere A, Kroemer G.** Cancer despite immunosurveillance: immunoselection and immunosurversion. Nat Rev Immunol 2006; 7: 1–13.

4. Immunology of malignant growth. Eds: NM Berezhnaya, VF Chekhun. K.: Naukova Dumka, 2005. 790 p.

5. **Berezhnaya NM.** Role of cells of immunity system in microenvironment of tumor. II. Interaction between cells of immunity system with other components of microenvironment. Onkologiya 2009; **11**: 86–93 (in Russian).

6. **Whiteside TL.** The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008; **27**: 5904–12.

7. **Zheng SG, Gray JD, Ohtsuka K, et al.** Generation *ex vivo* of TGF-beta-producing regulatory T cells from CD4$^+$CD25$^-$ precursors. J Immunol 2002; **16**: 336–44.

8. **Filaci G, Fravega M, Negrini S, et al.** Nonantigen specific CD8$^+$ T suppressor lymphocytes originate from CD8$^+$CD28$^-$ T cells and inhibit both T-cell proliferation and CTL function. Hum Immunol 2004; **65**: 142–56.

9. **Adurthi S, Mukherjee G, Krishnamurthy H, et al.** Functional tumor infiltrating TH1 and TH2 effectors in large early-stage cervical cancer are suppressed by regulatory T cells. Int J Gynecol Cancer 2012; **22**: 1130–7.

10. **Tanchot C, Terme M, Pere H, et al.** Tumor-infiltrating regulatory T cells: phenotype, role, mechanism of expansion *in situ* and clinical significance. Cancer Microenviron 2013; **6**: 147–57.

11. **Bates GJ, Fox SB, Han C, et al.** Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006; **24**: 5373–80.

12. **Yamagami W, Susumu N, Tanaka H, et al.** Immunofluorescence-detected infiltration of CD4$^+$FOXP3$^+$ regulatory T cells is relevant to the prognosis of patients with endometrial cancer. Int J Gynecol Cancer 2011; **21**: 1628–34.

13. **Berishvili AI, Tupitsyn NN, Laktionov KP.** Immunophenotypic characteristics of inflammatory breast cancer. Tumors Female Reprod System 2009; **3**: 15–9 (in Russian).

14. **Giatromanolaki A, Bates GJ, Koukourakis MI, et al.** The presence of tumor-infiltrating FOXP3$^+$ lymphocytes correlates with intratumoral angiogenesis in endometrial cancer. Gynecol Oncol 2008; **110**: 216–21.

15. **Wilke CM, Wu K, Zhao E, et al.** Prognostic significance of regulatory T cells in tumor. Int J Cancer 2010; **127**: 748–58.

16. **Yoshii M, Tanaka H, Ohira M, et al.** Expression of Forkhead box P3 in tumour cells causes immunoregulatory function of signet ring cell carcinoma of the stomach. Br J Cancer 2012; **106**: 1668–74.

17. **Ebert LM, Tan BS, Browning J, et al.** The regulatory T cell-associated transcription factor FoxP3 is expressed by tumor cells. Cancer Res 2008; **68**: 3001–9.

18. **Wang WH, Jiang CL, Yan W, et al.** FOXP3 expression and clinical characteristics of hepatocellular carcinoma. World J Gastroenterol 2010b; **16**: 5502–09.

19. **Hori S, Nomura T, Sakaguchi S.** Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; **299**: 1057–61.

20. **Sakaguchi S.** Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005; **6**: 345–52.

21. **Fontenot JD, Rasmussen JP, Williams LM, et al.** Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. Immunity 2005; **22**: 329–41.

22. **Liu W, Putnam AL, Xu-Yu Z, et al.** CD127expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006; **203**: 1701–11.

23. **Roncador G, Brown PJ, Maestre L, et al.** Analysis of FOXP3 protein expression in human CD4(+) CD25(+) regulatory T cells at the single-cell level. Eur J Immunol 2005; **35**: 1681–91.

24. **Takenaka M, Seki N, Toh U, et al.** FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. Mol Clin Oncol 2013; **1**: 625–32.

25. **Hinz S, Pagerols-Raluy L, Oberg HH, et al.** FoxP3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. Cancer Res 2007; **67**: 8344–50.

26. **Liu R, Wang L, Katoh H, et al.** FOXP3 up-regulates p21 expression by site-specific inhibition of histone deacetylase 2/histone deacetylase 4 association to the locus. Cancer Res 2009; **69**: 2252–9.

27. **Triulzi T, Tagliabue E, Balsari A, Casalini P.** FOXP3 expression in tumor cells and implications for cancer progression. J Cell Physiol 2013; **228**: 30–5.

28. **Kondratiev S, Sabo E, Yakirevich E, et al.** Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin Cancer Res 2004; **10**: 4450–6 (in Russian).

29. **de Jong RA, Leffers N, Boezen HM, et al.** Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. Gynecol Oncol 2009; **114**: 105–10. doi: 10.1016/j.ygyno.2009.03.022.

30. Cancer in Ukraine, 2011–2012. Incidence, mortality, indices of activity of cancer service. Bull Natl Cancer-Register of Ukraine, 2013; (14): 124 p.

31. **Nasman A, Romanitan M, Nordfors C, et al.** Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. PLoS One 2012; 7 (6): e38711. doi: 10.1371/journal.pone.0038711.

32. **Gooden MG, de Bock GH, Leffers N, et al.** The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Mol Diagnostics 2011; **105**: 93–103.

33. **Shen Z, Zhou S, Wang Y, et al.** Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. J Cancer Res Clin Oncol 2010; **136**: 1585–95.

34. **Solinas G, Germano G, Mantovani A, Allavena P.** Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 2009; **86**: 1065–73.

35. **Thomas DA, Massagué J.** TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 2005; **8**: 369–80.

36. **Toutirais O, Chartier P, Dubois D, et al.** Constitutive expression of TGF-beta 1, interleukin-6 and interleukin-8 by tumor cells as a major component of immune escape in human ovarian carcinoma. Eur Cytokine Network 2003; **14**: 246–55.

37. **Smith EL, Finney HM, Nesbitt AM, et al.** Splice variants of human FOXP3 are functional inhibitors of human CD4+ T-cell activation. Immunol 2006; **119**: 203–11.

38. **Babyshkina NN, Malinovska EA, Stakheeva MN, et al.** Role of transforming growth factor TGF-β1 in pathogenesis of breast cancer. Sib J Oncol 2010; **42**: 63–70 (in Russian).

39. **Zhan H-L, Gao X, Pu X-Y, Wang D-J.** Presence of tumour-infiltrating FOXP3$^+$ lymphocytes correlates with immature tumour angiogenesis in renal cell carcinomas. Asian Pacific J Cancer Prevention 2012; **13**: 867–72.

40. **Pere H, Tanchot C, Bayry J, et al.** Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer. Oncoimmunology 2012; **1**: 326–33.