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FOXP3 GENE PROMOTER METHYLATION IN ENDOMETRIAL CANCER CELLS

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Aim: **To determine the methylation level of promoter region of the *FOXP3* gene promoter depending on the heterogeneity of intracellular localization of its protein product in endometrial cancer (EC) cells and assess its relation to the clinical and morphological features of tumor.** ***Materials and Methods***: **Samples of surgical material of 40 EC patients who have not received any specific treatment before the surgery, were studied. Real time methylation-specific PCR (MSP) as well as morphological and immunohistochemical methods were used in the study.** ***Results***: **Methylation of promoter region of the *FOXP3* gene was determined in all EC cases, but variability of the methylation level in EC cells from 45.0% to 85.0% was observed. With tumor progression and in tumors with deep ($\geqslant 1/2$) invasion in myometrium, an increase of the methylation level of the *FOXP3* and of cell number with cytoplasmic FOXP3 localization was observed. In EC patients the correlation between of methylation level of the *FOXP3* gene and the number of FOXP3$^+$ tumor cells with cytoplasmic expression ($r = 0.41$) was determined.** ***Conclusion***: **The methylation level of *FOXP3* gene promoter region and intracellular localization of its protein product are associated with tumor differentiation grade and the depth of myometrial invasion.**

Key Words: **endometrial cancer, methylation of promoter region, FOXP3.**

Data accumulated over the last decades show that epigenetic changes are the interface between genes and environment, and methylation of suppressor genes in epithelial cells is observed in patients with cancer of different genesis [1, 2]. The forkhead box protein 3 (FOXP3) is a transcription factor that regulates functional activity of large amount of genes (almost 2000) [3]. FOXP3 was initially identified as a specific marker of immunosuppressive CD4$^+$CD25$^+$ regulatory T cells (Tregs) [4]. Although the role of FOXP3 in the generation of Tregs is clearly defined, the issue concerning the importance of this marker in tumor cells remains open. According to the data of literature, changes of *FOXP3* expression in different forms of cancer are ambiguous. In hormone-sensitive tissues (breast, prostate gland), FOXP3 protein is detected in morphologically unchanged epithelial cells, but its expression decreases in 70% of cases at these forms of cancer. In contrast, in melanoma and pancreatic cancer, expression of FOXP3 is restrained by the tumor cells [5]. FOXP3 is insignificantly expressed in cells of serous ovarian cancer, but their transfection with the *FOXP3* gene causes the inhibition of proliferation, decrease of cell migration and invasion [6]. There are also observed certain peculiarities in FOXP3 localization in different tumor types. For instance, FOXP3 cytoplasmic expression prevails in melanoma, colon cancer, breast and pancreatic cancer [7]. Moreover, FOXP3 expression in the cytoplasm is determined in all HER2-positive breast tumors [8, 9]. Similar regularities were detected in lung cancer cells and T-cell leukemia [10]. Nuclear localization of the FOXP3 is found in less than 30% of cells of these tumor types [7, 10]. Our previous studies have showed that low number of FOXP3$^+$ tumor cells and intratumoral CD4$^+$ and CD8$^+$ T cells, along with the high content of the FOXP3$^+$ lymphocytes in lymphocytic infiltrate, significantly correlate with such indexes of endometrial cancer (EC) progression as low differentiation grade, high proliferative activity and deep invasion of tumor in myometrium [11]. However, molecular mechanisms, which cause changes of intracellular localization of FOXP3 and stipulate its role in tumor progression, are not fully studied.

Despite well known risk factors for EC (obesity, hypertonic disease, hormonal imbalance expressed in functional domination of estrogens over progesterone) as well as antiproliferative impact of progesterone on endometrial epithelium mediated by receptors of stromal but not epithelial cells [12, 13], the exact molecular mechanisms of this pathology remain uncertain. Among them one could mention epigenetic changes of tumor suppressor genes, in particular methylation of their promoter regions detected in tumors of different localizations [14]. In has been recognized that aberrant methylation of DNA is an early and common event in EC causing the loss of expression of many critical genes. For instance, the study of the DNA methylation profile in endometrial tumors has showed that among 24 tumor suppressor genes, a number of methylated promoters increases in the following direction: normal endometrium — simple endometrial hyperplasia — atypical endometrial hyperplasia — EC [15]. Methylation of *PTEN* gene promoter was found in about 20% of sporadic EC of I pathogenetic variant that was associated with microsatellite instability and metastases [16, 17]. The study [18] has demonstrated an aberrant hypermethylation of $p16^{INK4a}$ gene in 11–75% of sporadic EC cases. Interesting results were obtained by the

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Abbreviation used: EC – endometrial cancer; FOXP3 – forkhead box protein 3; MSP – methylation-specific PCR; Tregs – regulatory T cells.

group of authors who studied the methylation of tumor suppressor gene *RASSF1A* [19, 20]. Hypermethylation of promoter of this gene was determined in 33–85% of EC patients. At the same time, in 36% of EC cases, hypermethylation of the *RASSF1A* gene was detected in the regions of morphologically unchanged cells proximate to endometrial tumor cells what allowed to assume that epigenetic changes may be an early event in development of EC. The latter can be confirmed by aberrant DNA methylation of many tumor suppressor genes (CDH13, GSTP1, etc.), revealed in complex glandular endometrial hyperplasia with mutations in DNA mismatch repair genes [15]. The results of these studies demonstrated the significance of epigenetic changes in endometrial carcinogenesis. Epigenetic modifications of promoter region of the *FOXP3* gene in EC remain unstudied yet.

The aim of this study was to analyze the methylation level of promoter region of the *FOXP3* gene and intracellular localization of its protein product in tumor cells of EC depending on clinical and morphological features of tumors.

MATERIALS AND METHODS

The study was performed on the samples of surgical material from 40 patients (mean age 56.9 ± 2.8) with EC of stage I (FIGO), who have not received any specific treatment before surgery. All patients were cured in the Research Department of Cancer Gynecology of the National Cancer Institute MH of Ukraine and have given an informed consent for the use of their surgical material for the research.

To study the methylation level of regulatory region of the *FOXP3* gene, we have used samples of genome DNA, isolated from tumor tissue via phenol-chloroform method as well as sample of methylated DNA (control DNA), which underwent bisulfite processing. For bisulfite conversion of DNA, EpiTect Plus DNA Bisulfite Kit (Qiagen, USA) was used. Modified DNA was analyzed using real time methylation-specific PCR (MSP) with methylation-specific and non-methylation-specific primers to the *FOXP3* gene (FOXP3MF-GGTCGTTATGACGTTAATGGC, FOXP3MR-TAAAAAACCGAACTAAACAACCG; FOXP3UF-GGTTGTTATGATGTTAATGGTGG, FOXP3UR-CTAAAAAACCAAACTAAACAACCAA). Each MSP reaction (volume 20 μL) contained 10 μL of the maxima SYBR Green qPCR master mix (2X) (Thermoscientific, USA), 1.5 μL of each primer (10 pM/μL), 4 μL of RNAse-free water and 3 μL bisulfite-modified DNA. Quantitative analysis of PCR-products was carried out using Applide Biosystems 7500 fast real-time PCR system under the following conditions — initial denaturation — 95 °C, 10 min, 1 cycle; 40 cycles: denaturation — 95 °C, 15 sec, annealing — 60 °C, 30 sec, extension — 72 °C, 30 sec. Methylation level of the *FOXP3* promoter region was determined by percentage (%) of methylated molecules in the *FOXP3* locus in tumor tissue regarding the level of such molecules in the *FOXP3* gene in control DNA taken as 100%. At immunohistochemical assessment of the FOXP3 expression in tumor endometrial cells, we have used primary monoclonal antibody (Invitrogen, USA; clone 5H5L12) with further detection of protein with the help of visualization system PolyVue HRP/DAB Detection System (Diagnostic BioSystems, USA). Results of immunohistochemical reaction were assessed by semi-quantitative method via calculation of the number of positively stained cells determined in percentage — labeling index (LI, %). Obtained data were statistically processed using software Statistica 8.0 (StatSoft Inc, USA). For detection of statistically significant differences between samples, Mann — Whitney criteria were used. Correlation analysis was carried out using Spearman method. A p value of $\leqslant 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Morphological analysis has demonstrated that all studied tumors were endometrial adenocarcinomas of various differentiation grade: G1 (n = 11, 27.5%), G2 (n = 15, 37.5%), and G3 (n = 14, 35.0%). By the depth of myometrial invasion, the studied tumors were divided as follows: 16 (40.0%) tumors invaded less than ½ of myometrium, 24 (60.0%) tumors — over than ½.

The results of the molecular-genetic study of the EC samples have showed that effective amplification with methylation-specified primers for the *FOXP3* gene occurred in all cases. Values of the methylation level of promoter region of the *FOXP3* gene in DNA of EC cells varied from 45.0 to 85.0%.

When carrying out comparative analysis of the methylation level of promoter region of the *FOXP3* gene depending on the EC differentiation grade, we have determined that methylation level of the gene was higher in G3 tumors (76.2 ± 3.4%) as compared to the highly and moderately differentiated tumors (56.4 ± 2.1% and 67.0 ± 5.3%, respectively). Intracellular localization of the FOXP3 protein was associated with level of methylation of promoter of this gene and tumor differentiation grade (Table, Figure). For instance, when differentiation grade decreased and methylation level of the *FOXP3* gene significantly increased, the number of cells with $FOXP3^+$ nuclei significantly decreased (from 25.04 ± 2.4% in G1 to 11.9 ± 3.4% in G3, $p < 0.03$) and cytoplasmic FOXP3 expression tended to increase.

Table. Methylation level of promoter region of the *FOXP3* gene and FOXP3 cellular localization in EC cells depending on clinical and morphological features of tumor

Features of tumor	Methylation of the *FOXP3* gene, mean ± SE, %	Number of tumor cells, which express FOXP3, mean ± SE, %	
		Nucleus	Cytoplasm
Differentiation grade			
G1	56.4 ± 2.1	25.0 ± 2.4	17.9 ± 6.4
G2	67.0 ± 5.3	13.5 ± 2.2*	31.6 ± 5.02
G3	76.2 ± 3.4*	11.9 ± 3.4*	31.1 ± 5.9
Invasion			
< ½	60.0 ± 3.4	20.6 ± 2.5	19.2 ± 4.2
> ½	77.3 ± 5.2	10.5 ± 2.1**	37.1 ± 4.0**

Note: *$p < 0.05$ as compared to G1; ** $p < 0.05$ as compared to group of tumors with < ½ invasion in myometrium.

According to the data of correlation analysis, significant and moderate positive correlation was established between methylation level of the *FOXP3* gene

and the number of FOXP3$^+$ tumor cells with cytoplasmic expression ($r = 0.41$, $p < 0.05$). Negative correlation was established between this index and the number of cells with FOXP3 nuclear expression ($r = 0.37$). We have to mention that methylation level of the studied gene in G1-neoplasms constituted about a half (56.4 ± 2.1%) of maximal possible level of methylation of its alleles as compared to moderately and poorly differentiated tumors.

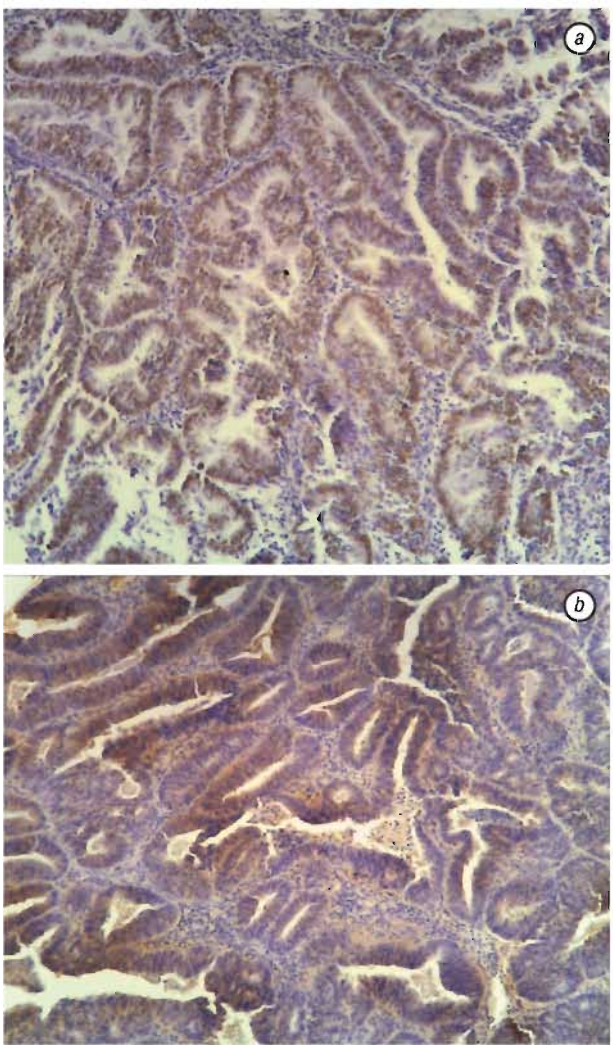

Figure. FOXP3 expression in endometrial adenocarcinoma: nuclear localization (*a*), cytoplasmic localization (*b*). Immunohistochemistry with anti-FOXP3 antibody, counterstained with Mayer's hematoxylin, × 100

Obtained results probably demonstrate the lack of methylation in the second allele of promoter of the *FOXP3* gene in highly differentiated tumors, since this gene is coupled with X-chromosome, one of which is initially inactivated via epigenetic modification [3].

The study of the methylation level of *FOXP3* gene promoter and intracellular localization of its protein product has shown that in 15.0% of EC cases low nuclear (4.2–6.8%) and high cytoplasmic (27.1–32.1%) expression of FOXP3 was observed at the background of low *FOXP3* methylation level (55.0–60.0%).

According to the data of literature, translocation of FOXP3 protein to cytoplasm mostly occurs due to the somatic mutations in *FOXP3* gene. Some tumors, including breast cancer, mostly develop from cells with inactive *FOXP3* allele of the wild type and with activated mutant locus [3, 14]. We suppose that accumulation of FOXP3 protein in cytoplasm along with decrease of *FOXP3* methylation level in EC of lower differentiation grade, may reflect tumor genome instability.

When analyzing methylation level of FOXP3 promoter region and heterogeneity of FOXP3 intracellular localization depending on myometrial invasion depth (Table), it was determined that in a case of invasion less than ½, methylation level of the studied gene constituted 60.0 ± 3.4%. When invasion increases (more than ½), this index increases up to 77.3 ± 5.2%. However, no significant differences between the levels of FOXP3 promoter methylation depending on the depth of myometrial invasion were detected. But, along with increase of the depth of myometrial invasion, FOXP3 intracellular localization changed as follows: cytoplasmic expression increased and nuclear expression decreased. In tumors with invasion over ½ of myometrium, the number of cells with FOXP3 cytoplasmic expression was reliably ($p < 0.05$) higher as compared to the one in tumors with invasion ⩽ ½.

In 10% of cases with deep tumor invasion, low nuclear (6.8–10.4%) and cytoplasmic (8.7–14.0%) expression of the FOXP3 protein in EC cells was observed. *FOXP3* methylation level in these cases was lower than 50.0%. According to the data of literature, DNA methylation patterns is age-related and under strict genetic control [14, 17]. Usually the highest number of methylated cytosine bases is observed in DNA of embryos or newborns, and its number decreases with years [18]. The decrease of the DNA methylation level is known not only age-dependent but also tissue- and gene-specific [18, 19], and is higher in cancer and immune diseases [20].

In tumor cells epigenetic processes occur via total demethylation with simultaneous hypermethylation of promoters of certain tumor suppressor genes [21]. Our comparative analysis of the methylation level of the *FOXP3* gene in tumor cells dependent on EC patients age has showed that in a group of patients younger than 60 years old, the methylation level of promoter region of gene constituted 62.5 ± 6.4%, while in patients older than 60, a value of the same index was 78.3 ± 5.3%.

Thus, an analysis of the methylation level of promoter region of the *FOXP3* gene depending on intracellular localization of its protein product and its association with clinical and morphological features of tumor has showed that promoter region of the gene is significantly methylated in poorly differentiated tumors with deep invasion in myometrium. At the background of relatively high methylation level of the gene, low nuclear expression and high cytoplasmic expression of the FOXP3 in EC cells was observed. Taking into account that FOXP3 is a transcriptional factor, functional activity of which is stipulated by its nuclear localization, we may assume that FOXP3 performs suppressor function in highly differentiated EC. In our study, large amount of FOXP3$^+$ tumor cells with cytoplasmic expression was associated with poor

differentiation and deep myometrial invasion. These data are in accordance with the results of other studies [22, 23], where suppressor function of FOXP3 and prognostic significance of its expression in breast cancer have been shown, in particular, cytoplasmic localization was associated with unfavorable prognosis and low survival of breast cancer patients.

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