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MOLECULAR PHENOTYPE OF HIGH-GRADE ENDOMETRIOID CARCINOMA OF THE ENDOMETRIUM

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Background: **Prognosis of the course of tumor progression is one of urgent problems of clinical oncology. A relevant specificity of endometrial cancer is its clinical polymorphism within the same histological type of the disease. The search for molecular-biological features associated with the aggressive phenotype of endometrioid carcinomas is indisputably urgent.** ***Aim:*** **To study molecular-biological features of endometrioid carcinoma of the endometrium (ECE) and to identify the molecular subtype of tumors with high potential of malignancy.** ***Materials and Methods:*** **Surgical specimens of 127 patients with EC, stages I–II, aged 36–72 (the average age — 59.3 ± 3.2) were studied using morphological and immunohistochemical methods. The multivariant analysis with the Kullback's informative measure and PanelomiX were used to estimate the significance of the expression of specific biomarkers.** ***Results:*** **The expression of a complex of multifunctional markers was evaluated in ECE cells of different malignancy stage: p53, FOXP3, $p21^{WAF1/CIP1}$, $p16^{INK4a}$, E2F1, cyclins E and D1, Her2/neu, c-Myc, E-cadherin, β-catenin, vimentin, CD44, CD24. A triad of biomarkers with threshold expression levels was determined (p53 > 45%; FOXP3 < 14%; c-Myc > 10%). The high expression of oncogene c-Myc and oncosuppressor p53 along with the low level of FOXP3 in tumor cells of ECE was associated with high proliferative potential, low differentiation grade, and deep invasion of a tumor into the myometrium.** ***Conclusions:*** **The molecular phenotype of ECE, most informative in terms of specificity and sensitivity (95%) — $p53^{high}FOXP3^{low}c\text{-}Myc^{high}$, was first characterized, which would help identify a high-grade subtype of this cancer form.**

Key Words: **endometrioid carcinoma of the endometrium, molecular phenotype, expression of biomarkers, informative relevance.**

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The incidence rate of endometrial cancer (EC) is among the highest in the structure of malignant neoplasms of the female reproductive system both in Ukraine and globally [1, 2]. The predominant majority of EC cases (70–80%) are endometrioid carcinomas of the endometrium (ECE) by their histological type, which are characterized by a favorable clinical course. At the same time, tumor progression occurs in 15–25% of ECE cases. The different clinical course of ECE could be partially attributed to the biological polymorphism of this disease [3, 4–7].

The ambiguity in EC etiology was first noted by Ya.V. Bokhman, who formulated the concept of two pathogenic variants. The first one is a hormone-dependent variant, when tumors occur on the background of hyperplastic processes and have predominantly high and moderate grades of differentiation. As for the other pathogenic variant of EC, neoplasms evolve on the background of atrophic endometrium and have a low grade of differentiation [8].

Current model of EC pathogenesis is viewed from the position of state-of-the-art data, obtained from the studies of molecular-biological specificities of tumors. These studies helped to determine the biological heterogeneity of ECE defining the clinical course and being responsible for the differences in the aggressiveness of the tumor process [9–16].

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Abbreviations used: EC – endometrial cancer; ECE – endometrioid carcinoma of the endometrium; EMT – epithelial-mesenchymal transition; IQR – interquartile range; Me – median; LI – labeling index; PI – proliferation index.

The results of a complex genomic and transcriptomic analysis of ECE, conducted within the project The Cancer Genome Atlas Research Network have demonstrated that these neoplasms are characterized by specific mutational changes, defining the molecular-biological features of EC. It was demonstrated that some neoplasms diagnosed as endometrioid G3-carcinomas of the endometrium, have a molecular profile characteristic of serous tumors justifying an assumption about the "serous-like" subtype of ECE [9, 10, 17].

The existing data of scientific literature and the results of our research have demonstrated that the tumor progression in ECE is associated with unfavorable clinical course and the impaired expression of some suppressor genes, oncogenes, cellular cycle regulators, and markers of epithelial-mesenchymal transition (EMT) [6, 14–16, 18]. However, it has not yet been clear how to discern ECE with high potential of malignancy.

The abovementioned arguments substantiate the need of searching for informative molecular markers that would be useful to characterize the most aggressive subtype of cancer within one morphological form — ECE, which, according to the concept of 3P systemic medicine, could ensure personalized diagnostics of the pathological process and further efficient treatment [19].

Therefore, our research was aimed at studying molecular-biological features of ECE and identifying the molecular subtype of tumors with high potential of malignancy.

MATERIALS AND METHODS

The surgical specimens of 127 patients with EC, stage I–II, who had not received any special treatment

prior to surgical intervention were studied. The average age of the patients was 59.3 ± 3.2. All patients treated at the Department of Oncogynecology (Head Prof. V.S. Svintsitskiy) of the National Cancer Institute of the Ministry of Health of Ukraine in 2014–2018 gave the informed consent to the use of their biological material for the research. According to the conclusion of the commission on bioethics of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, all the necessary ethical standards were observed during the study in accordance with the requirements of generally accepted international rules in the framework of the Declaration of Helsinki 2008.

The specimens stained with hematoxylin and eosin were used for the morphological verification of the pathological process in the endometrium and differentiation grade of the tumors according to the WHO criteria [20].

The immunohistochemical determination of the protein expression was conducted on the deparaffinized sections of endometrial tumors using the relevant monoclonal antibodies to the markers, specified below: cyclins D1 and E1 (AB2070433 Santa Cruz Biotechnology, USA; 13A3, Novocastra, UK, respectively), E2F1 (2E10, Sigma, USA), Her2/neu (polyclonal variant, Diagnostic BioSystems, USA), c-Myc (9E10, Diagnostic BioSystems, USA), CD44 (196-3C11 Thermo Fisher Scientific, USA), CD24 (Ab-1 Thermo Fisher Scientific, USA), vimentin (V9 Diagnostic BioSystems, Netherlands), to E-cadherin (NCH-38 Dako, Denmark), β-catenin (β-catenin-1, Dako, Denmark), to FOXP3 (5H5L12 Invitrogen, USA), p53 (DO-7, Dako, Denmark), $p21^{WAF1/CIP1}$ (HZ52 Dako, Denmark), $p16^{INK4a}$ (JC8, Dako, Denmark), Ki-67 (MIB-1 Dako, Denmark) and PolyVue HRP/DAB Detection System (Diagnostic BioSystems, USA).

The expression of markers was estimated by counting the number of positively stained cells — the labeling index (LI, %). The proliferative potential was determined by counting the number of Ki-67-positive cells (the proliferation index — PI, %). 800–1000 tumor cells were analyzed in each case. If the values of LI and PI were under the median (Me), the expression of the relevant marker was considered to be low, and if the values were above Me, it was considered to be high.

The statistical processing of the data was conducted in Statistica 8.0 (StatSoft Inc., USA). The following statistical methods were used: standard descriptive method, calculation of the Me and interquartile range (IQR), non-parametric Mann — Whitney test. The differences were considered significant at $p < 0.05$.

The multivariant analysis with the Kullback's informative measure [21] and a web-based tool PanelomiX http://www.panelomix.net) were used to estimate the significance of the expression of specific biomarkers and determine the optimal panel of features for the identification of the aggressive molecular subtype of ECE. In PanelomiX analysis, the optimal cut-off point was accepted as a level of expression of the markers, which provided for the highest accuracy of identifying the phenotypes, related to high-grade malignancy of tumor process in the endometrium. The prognostic significance of the study results was estimated at the pre-set 95% sensitivity and specificity.

RESULTS

All the investigated tumors were verified as ECE of moderate (G2) — (46.5% cases) and low (G3) — (53.5% cases) differentiation grade. G2-tumors with low (< ½) invasion into the myometrium were present in 64.4% of cases. Most (72.2%) G3-tumors deeply infiltrated the myometrium.

We analyzed the specificities of the expression of biomarkers associated with such traits of EC malignancy as the differentiation grade, the depth of tumor invasion into the myometrium and the proliferative potential. G2-tumors with deep invasion into the myometrium demonstrated a reliably higher expression of p53 (Me = 32.0%) and low expression of FOXP3 (Me = 13.0%) as compared with similar indices for G2-neoplasms, which infiltrated less than ½ of the myometrium (Me = 23.4% and Me = 19.8%, respectively). It was shown that the expression of proteins — cell cycle regulators, $p21^{WAF1/CIP1}$, $p16^{INK4a}$ and cyclin D1 in G2-tumors, practically did not depend on the depth of the tumor invasion into the myometrium. The transcription factor E2F1 and cyclin E1 demonstrated a tendency to higher expression in tumors, which deeply infiltrated the myometrium (Me = 11.2% and Me = 14.6%, respectively) as compared with the same indices for ECE, which infiltrated less than ½ of the myometrium (Me = 8.2% and Me = 10.2%, respectively). In addition, in G2-tumors, oncoproteins Her-2/neu and c-Myc were expressed at the same level regardless of the depth of ECE invasion into the myometrium. The presented specificities of the expression of biomarkers for moderately differentiated endometrial rumors with deep invasion into the myometrium were associated with high indices of the expression of the proliferation marker Ki-67, whose expression Me was 30.0%, and in case of invasion below ½ decreased down to 21.0% ($p < 0.05$) (Figure, *a, b*).

Since the tumor progression, including invasive growth is related both to the changes in the expression of suppressor genes and oncogenes, and to the molecules defining the density of intercellular contacts [22–26], we further analyzed the expression of several markers, associated with EMT in ECE cells.

A reliably lower expression of E-cadherin, the decrease in the expression of β-catenin and CD24, and the increase in CD44 and vimentin in G2-tumors of the endometrium with the invasion of < ½ was determined as compared with the indices for G2-tumors with the invasion of > ½ of the myometrium (Figure, *a, b*).

ECE of low differentiation grade, which deeply infiltrated the myometrium, were characterized by higher expression of p53 (Me = 55.0%), and low — of FOXP3 (Me = 10.3%) as compared with similar indices for tumors with the invasion of less

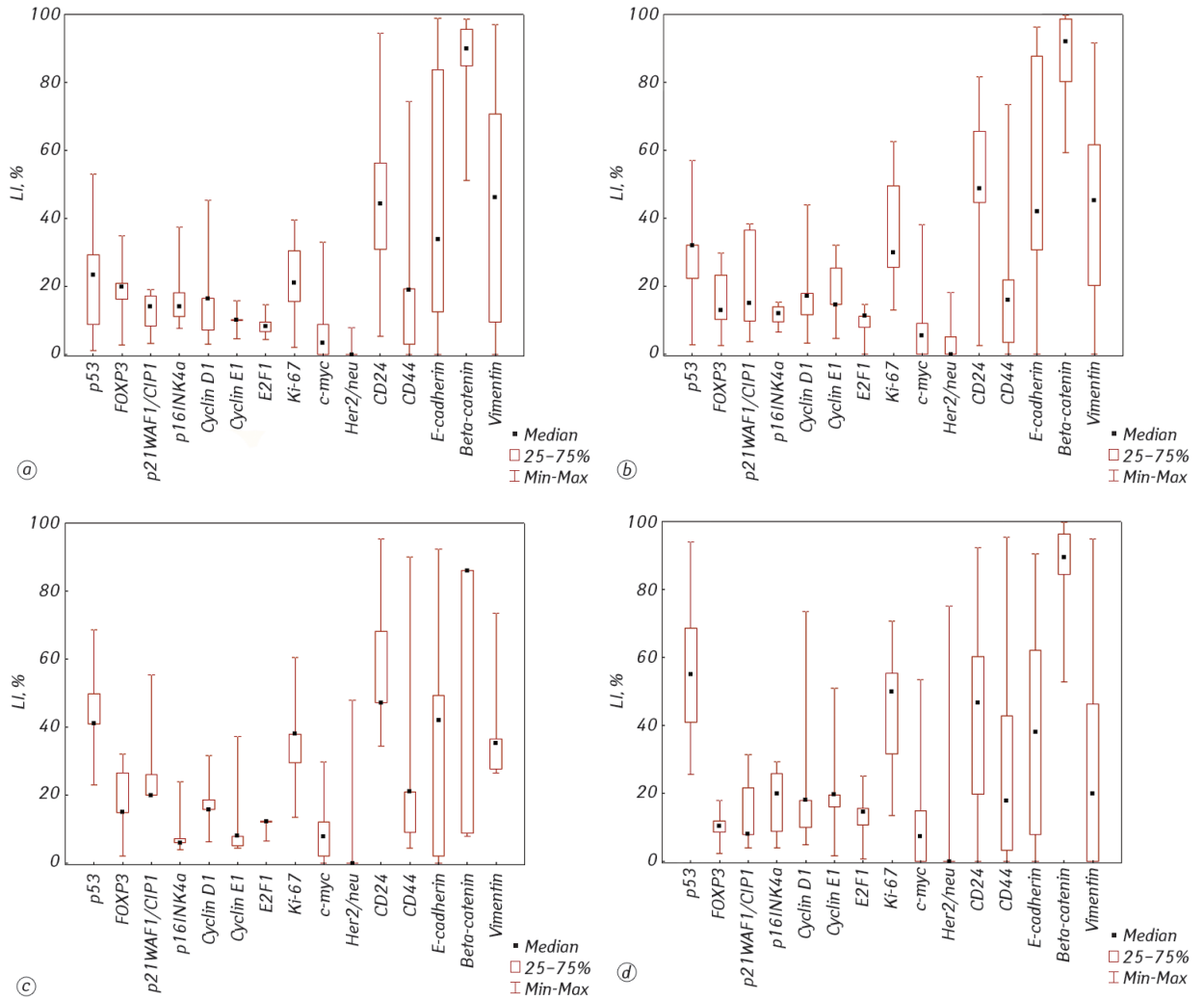

Figure. The expression of biomarkers in ECE cells of different malignancy grade: *a* — G2-tumors with the invasion of < ½ of the myometrium; *b* — G2 — tumors with the invasion of > ½ of the myometrium; *c* — G3-tumors with the invasion of < ½ of the myometrium; *d* — G3-tumors with the invasion of > ½ of the myometrium

than ½ of the myometrium (Me = 41.0% $p < 0.05$, Me = 15.0%, $p > 0.05$, respectively). G3-tumors with the invasion of less than ½ of the myometrium, had higher expression of $p21^{WAF1/CIP1}$ (Me = 20.0%) and lower expression of $p16^{INK4a}$ (Me = 6.0%) as compared with the neoplasms with the invasion of > ½ of the myometrium (Me = 8.0%, $p < 0.05$ and Me = 20.0%, $p < 0.05$, respectively). G3-tumors deeply infiltrating the myometrium demonstrated a tendency to the increase in the expression of E2F1 and cyclin D1, and a reliable increase in the expression of cyclin E1 contrary to these indices in the neoplasms of the endometrium with the invasion of < ½ of the myometrium. G3-tumors, deeply infiltrating the myometrium, demonstrated a reliable increase in the expression of c-Myc as compared with the tumors, infiltrating less than ½ of the myometrium. In addition, these tumors showed a tendency towards a decrease in the expression of CD24, CD44, and E-cadherin, the increase in the expression of β-catenin and a reliable decrease in the expression of vimentin (Me = 20.0%, $p < 0.05$) as compared with the expression of vimentin (Me = 35.2%, $p < 0.05$) and other markers of EMT in the tumors, infiltrating < ½ of the myometrium (Figure, *c, d*).

The variability of ECE determined within one histological type and differentiation grade and invasion depth of the tumor into the myometrium, substantiated the reasonability of further search for potential molecular markers defining the malignancy grade for ECE.

According to our results as well as the findings of other authors, G2-tumors of the endometrium with low invasion into the myometrium are associated with low grade of malignancy, while G3-tumors, deeply infiltrating the myometrium, — with high grade of malignancy [6, 16, 27, 28]. We have further compare the indices of the expression for the markers under investigation and PI in ECE of different grade of malignancy. It was determined that the neoplasms with low grade of malignancy had reliably lower expression of p53 (Me = 23.4%, IQR = 20.5), cyclin E1 (Me = 10.2%, IQR = 0.3), E2F1 (Me = 8.2%, IQR = 2.7), c-Myc (Me = 3.4%, IQR = 8.9) and higher expression of FOXP3 (Me = 19.8%, IQR = 4.7) as compared with the tumors with high grade of malignancy (Me = 55.0%, IQR = 27.8; Me = 19.6%, IQR = 3.6; Me = 14.6%, IQR = 5.0; Me = 7.3%, IQR = 15.0; Me = 10.3%, IQR = 3.2, respectively). The expression of other markers did not differ in ECE with high and low grade of malignancy. The analysis of proliferative

potential demonstrated that the tumors of high grade of malignancy had a reliably higher PI (Me = 42.1%, IQR = 25.1) as compared with neoplasms of low grade of malignancy (Me = 21.9%, IQR = 14.9) (Figure, *a, d*).

Thus, the results obtained demonstrate that the molecular-genetic polymorphism of the ECE, is a significant factor, modulating the morphofunctional characteristics of neoplasms, whose integral impact defines the variable nature of tumor progression. Therefore, the determination of significant prognostic biomarkers is extremely urgent since it would help identify the most aggressive tumor variant within one morphological form of ECE.

The data on the specificities of the expression of genes with suppressing and oncogenic functions, became the foundation for determining the molecular phenotype of the most aggressive forms of ECE via estimating the informative value of the specified factors.

Up to date, several approaches have been developed, based on the analysis of the data of experimental research using multifactor regression models, Bayesian and artificial neural networks, etc. One of the methods, ensuring high probability analysis of the indices is the Kullback's informative measure [21].

The determination of the most informative markers of the aggressiveness of the tumor process in the endometrium required the estimation of the significance of the expression for each biomolecular marker under investigation (p53, FOXP3, $p21^{WAF1/CIP1}$, $p16^{INK4a}$, E2F1, cyclins E and D1, Her2/neu, c-Myc, E-cadherin, β-catenin, vimentin, CD24, CD44) in patients with ECE of different grade. The Kullback's method was applied to determine the informative relevance (threshold value I = 0.5) of the expression indices for some biomolecular markers in accordance with their ranking in the order, presented in Table, in the groups of patients with high-grade (G3 tumors, invasion > ½ of the myometrium) and low-grade (G2 tumors, invasion < ½ of the myometrium) tumors.

These informative relevance coefficients demonstrate that the expression of such biomarkers as p53, FOXP3 and c-Myc is of the highest significance for the determination of high-grade forms of ECE.

Our estimation of the validity of the defined triad of biomarkers involved testing (web-based tool PanelomiX) their expression in high-grade and low-grade tumors. The threshold values for the expression of p53, FOXP3 and c-Myc were used to determine the optimal informative combination of the expression of genes with specificity and sensitivity (95%): p53 > 45.0; FOXP3 < 14.0; c-Myc > 10.0, which are associated with the aggressiveness of ECE.

Table. The estimation of the informative relevance of the markers for the identification of the features of aggressive ECE subtype

№	Expression of the biomarker	Informative relevance coefficient (I)
1	**p53 > Me**	**5.91**
2	**FOXP3 < Me**	**4.48**
3	**C-Myc > Me**	**1.08**
4	Vimentin < Me	0.54
5	E2F1 > Me	0.26
6	E-cadherin > Me	0.17
7	CD44 < Me	0.17
8	Her2/neu > Me	0.17
9	Cyclin D1 < Me	0.08
10	β-catenin < Me	0.05
11	$p21^{WAF1/CIP1}$ > Me	0.03
12	CD24 > Me	0
13	Cyclin E < Me	0

For the first time, we determined the molecular phenotype of the aggressive variant of ECE, namely, $p53^{high}FOXP3^{low}c\text{-}Myc^{high}$, which presents the most objective potential capacity of the tumor progression and ensures timely diagnostics of this form of cancer.

DISCUSSION

Recent studies have demonstrated that ECE is characterized by considerable biological heterogeneity, which determines the different clinical course complicating the selection of treatment strategy [6, 11–13]. Therefore, at present there is an active search for optimal approaches to the identification of the most aggressive molecular subtype of EC, which would be applicable for clinical pathologists [5, 9, 13, 29].

The results of our study have demonstrated that the increased expression of p53, c-Myc and low FOXP3 in endometrioid G3-tumors of the endometrium with deep invasion into the myometrium is associated with high proliferative potential of a neoplasm, i.e. is characterized by the indices, defining the aggressive clinical course.

Our results are in line with the data stating that among the cases of ECE of moderate and low differentiation grade some tumors are similar to serous carcinomas of the endometrium in terms of their molecular features [28, 30]. For instance, according to the data presented in the Cancer Genome Atlas, the unfavorable prognosis is observed in 25% of patients with endometrioid G3-tumors defined as "serous-like"-subtype. These tumors were characterized by the amplification of genes c-*MYC*, *ERBB2 (HER-2/neu)*, *CCNE1, FGFR3, SOX17* and *TP53* mutation [9, 31]. It was shown that functional impairment of *TP53* gene in ECE is related to the infiltrative and metastatic potential of the tumor [32–34].

The results of our study demonstrated that hyperexpression of p53 is found in 18% of low differentiated tumors of the endometrium,deeply infiltrating the myometrium. It is possible that such cases of endometrial carcinoma may be referred to as "serous-like" endometrioid tumors.

FOXP3 is a direct target of p53. Both proteins activate the expression of genes from *CIP/KIP* family, which facilitates the suspension of the cell cycle in S-phase and the decrease in the proliferation of tumor cells [35, 36]. In addition, FOXP3 inhibits the expression of such oncogenes as *HER2/neu, c-MYC* and chemokine receptor *CXCR4* [37, 38].

In our previous study, we have demonstrated the increase in the level of methylation for *FOXP3* in endometrial carcinomas of low differentiation grade and tumors with the invasion of over ½ of the myometrium [39]. These data are in good agreement with the results of the research on breast tumors demonstrating that low expression of FOXP3 was related to the tumor

expansion, the presence of lymph node metastasis and low indices of overall survival of patients [40, 41].

The invasiveness of a malignant neoplasm correlates with considerable changes in DNA content (aneuploidy) in tumor cells, which are often combined with high expression of c-Myc, defining low survival rate of cancer patients [42–44]. In ECE with high-grade potential, we found out the amplification of c-*MYC*, related to the overexpression of its protein product, in 25% of patients [15].

It is noteworthy that the activation of transcription for some genes, whose functioning controls DNA replication and the transition from G1- into S-phase of the cell cycle, occurs during the interaction of c-Myc and E2F1 [45]. It was shown that high expression of c-Myc and E2F1 was observed in endometrial tumors with high proliferative activity, which is one of the factors of tumor progression. In addition, the increase in the proliferative potential may be caused by the impairment of p53 and FOXP3 expression. This is confirmed by our results demonstrating reliably higher proliferative activity in ECE with higher expression of p53 and low expression of FOXP3. Therefore, the results of our studies and the scientific literature demonstrate the association between high expression of p53, c-Myc, low expression of FOXP3, and potential high-grade malignancy of the tumors.

To sum up, we have shown that the molecular phenotype $p53^{high}FOXP3^{low}c\text{-}Myc^{high}$ is an indicator of aggressive tumor growth in the endometrium and may be applied for the diagnostics of the pathological process, as well as the objective prognosis of the clinical course.

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