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ASSESSMENT OF *HER-2/neu, c-MYC* AND *CCNE1* GENE COPY NUMBER VARIATIONS AND PROTEIN EXPRESSION IN ENDOMETRIAL CARCINOMAS

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Aim: **To analyze copy number variations of *HER-2/neu*, *c-MYC* and *CCNE1* oncogenes and their protein expression in endometrioid endometrial carcinomas in relation to the degree of tumor progression and presence of a family history of cancer in cancer patients.** ***Materials and Methods:*** **The study was conducted on endometrial cancer (EC) samples from 68 patients with I–II FIGO stages of disease. Copy number analysis of *HER-2/neu*, *c-MYC* and *CCNE1* genes was performed by quantitative PCR. Protein expression was analyzed using immunohistochemistry.** ***Results:*** **Assessment of copy number variations of *HER-2/neu*, *c-MYC* and *CCNE1* genes revealed their amplification in the tumors of 18.8, 25.0 and 14.3% of EC patients, respectively. High expression of corresponding proteins was detected in 14.6, 23.5 and 65.6% of patients, respectively. It was established that *HER-2/neu* gene amplification is more common in the group of tumors of low differentiation grade than in moderate grade EC (35.7 and 5.5% of cases, respectively, $p < 0.05$). Also, high expression of c-Myc protein was more frequently observed in low differentiated tumors compared to the moderately differentiated EC (36.6 and 13.2% of cases, respectively, $p < 0.05$). Expression of HER-2/neu and cyclin E proteins was found to be dependent on the depth of tumor invasion into the myometrium. High expression of HER-2/neu protein was observed in 25.0 and 4.1% of EC patients with tumor invasion > ½ and < ½ of the myometrium, respectively, and cyclin E — in 86.7 and 46.6% of cases, respectively, $p < 0.05$. It was shown that among patients with a family history of cancer, a larger proportion of cases with high expression of c-Myc protein was observed compared to the group of patients with sporadic tumors (43.8 and 17.3%, respectively; $p < 0.05$).** ***Conclusions:*** **Amplification of *HER-2/neu* gene, along with high expression of c-Myc, HER-2/neu and cyclin E proteins, are associated with such indices of tumor progression as a low differentiation grade and deep myometrial invasion, suggesting the potential possibility of including these markers in the panel for determining the molecular EC subtype associated with an aggressive course of the disease. In a certain category of EC patients, there is a relationship between a family history of cancer and high expression of c-Myc protein.**

***Key Words*: endometrial cancer, copy number high subtype, *HER-2/neu*, *c-MYC*, *CCNE1*.**

Endometrial cancer (EC) is characterized by significant heterogeneity both of morphological structure and of clinical course, which greatly complicates the diagnosis and makes a choosing of treatment strategy much more difficult [1]. As a result of a comprehensive study conducted by The Cancer Genome Atlas (TCGA) Research Network on samples of endometrioid, serous and mixed endometrial carcinomas, four molecular subtypes of EC were found, which differed in their molecular genetic characteristics and aggressiveness of the disease [2]. The most unfavorable prognosis was revealed in patients with tumors of the serous-like (high copy number) subtype, which included the majority of serous and mixed endometrial carcinomas and approximately 25% of endometrioid grade 3 tumors with a high frequency of copy number variations of *c-MYC*, *HER-2/neu*, *CCNE1*, *FGFR3*, *SOX17* genes, mutations in *TP53* gene and low mutation rate.

Currently the search for optimal approaches to the identification of serous-like carcinomas has been actively pursued [3–5]. It is assumed that copy number changes of the genes established in the TCGA study may be the determining criteria for this category of endometrial tumors. From this point of view, the *c-MYC*, *HER-2/neu* and CCNE1 genes look promising as their proteins are involved in numerous processes, violation of which may initiate transformation of normal cells to malignancy. It is known that the pathological activation of the *c-MYC* protooncogene is accompanied by impaired cell division, apoptosis, stimulation of cell proliferation and deregulation of cell metabolism [6]. The *HER-2/neu* gene encodes the receptor for epidermal growth factor 2, which is a tyrosine kinase, whose excessive activation leads to a perturbation of signaling pathways involved in the regulation of cell growth and proliferation, in particular PI3K/AKT and Raf/MAPK, PKC, etc. [7]. The product of the *CCNE1* gene is cyclin E1 protein, which via binding to cyclin-dependent kinase 2 (CDK2), regulates the transition from G1 to the S phase of the cell cycle [8].

Separately, it is important to note that it is well known about the significant contribution of the hereditary factor to the development of EC [9–14]. However, to date the role of various types of genetic disorders in determining the hereditary susceptibility to EC is not fully understood [10, 11]. In addition, the search continues for typical molecular biological features that distinguish tumors of EC patients with a family history of cancer from sporadic neoplasms.

Considering the above, the analysis of copy number variations of *c-MYC*, *HER-2/neu* and *CCNE1* genes and expression of the corresponding proteins in endo-

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Abbreviations used: EC – endometrial cancer; G2 – grade 2; G3 – grade 3; LI – labelling index; TCGA – The Cancer Genome Atlas.

metrial carcinomas depending on the indices of tumor progression process and a family history of cancer is required.

MATERIALS AND METHODS

68 EC patients with I–II stage by FIGO were included in the study (mean age 60.3 ± 2.7 years). All patients were informed about the study and provided written consent for participation. Morphological analysis was performed on histological specimens stained with hematoxylin and eosin. The histological type of all examined tumors was diagnosed as endometrioid endometrial carcinoma of moderate (grade 2, G2) and low (grade 3, G3) differentiation grades (38 and 30 samples, respectively) [15].

Copy number variations of *HER-2/neu*, *c-MYC* and *CCNE1* genes were analyzed using 38 paired DNA samples obtained from tumor tissue and peripheral blood of the same patient. Genomic DNA was isolated using the InnuPREP mini kit (Analytik Jena AG, Germany) following the manufacturer's protocol. Estimation of gene copy number variations was performed by qPCR using the *HBB* gene as a reference control. Copy number variations of the mentioned genes in the tumor DNA samples were determined relative to normal DNA isolated from the blood of the same patient.

The nucleotide sequences of the primers were as follows:

HER-2/neu:
Forward: 5'-AACTGGTGTATGCAGATTGC-3'
Reverse: 5'-AGCAAGAGTCCCCATCCTA-3'
c-MYC:
Forward: 5'GGACGACGAGACCTTCATCAA-3'
Reverse: 5'CCAGCTTCTCTGAGACGAGCTT-3'
CCNE1:
Forward: 5'-CTGGGCAAATAGAGAGGAAGTC-3'
Reverse: 5'-CATGAAGCGAACAGGAAGACTC-3'
HBB:
Forward: 5'CACCAACTTCATCCACGTTCA3'
Reverse: 5'GTGCATCTGACTCCTGAGGAGAA3'

PCR was performed in a volume of 20 µl containing SYBR Green MasterMix M02 reagents (UkrGenTech, Ukraine), 100 ng total DNA, 0.3 µM forward and reverse primers with an ABI 7500 real-time PCR System. After 12 min preheating at 95 °C, 40 amplification cycles were carried out as follows: denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Quantification of the PCR results was calculated according to the formula $2^{-\Delta\Delta Ct}$. The values in the range of 0.85–1.5 were considered as indicators of normal copy number samples. Samples with a value more than 1.5 were regarded to characterize by amplification of the particular gene and less than 0.85 by gene deletion [16, 17].

Evaluation of protein expression was performed by immunohistochemistry using primary antibodies to HER-2/neu (polyclonal, Diagnostic BioSystems, USA), c-Myc (clone 9E10, Diagnostic BioSystems, USA) and cyclin E (clone 13A3, Novocastra, UK). For the visualization, a Mouse/Rabbit PolyVue Plus™ HRP/DAB Detection System (Diagnostic BioSystems, USA) was used. Immunohistochemical reactions were evaluated by assessment of percentage of stained cells (labelling index — LI). Membrane (HER-2/neu) and nuclear (c-Myc та cyclin E) staining in more than 10% of tumor cells was interpreted as high expression.

To collect a family history of patients, a special questionnaire was used, which included information about occurrence of malignancy in relatives, patients' living conditions and concomitant diseases. The criteria for assigning EC patients to a group of participants with a family history of cancer was the presence of malignant tumors of the female reproductive system and the gastrointestinal tract in first and second-degree relatives [13].

All statistical analyses were performed with the Statistica 8.0 software package (StatSoft, Inc., USA). Fisher's exact test was used to compare groups with a p-value of < 0.05 being considered significant. To identify the relationship between the data, the Spearman's rank correlation coefficient (r) was calculated.

RESULTS

Copy number analysis revealed *HER-2/neu* gene amplification in 18.8% of EC patients (Fig. 1, *a*). According to immunohistochemical staining, high expression of the HER-2/neu protein was observed in 14.6% of tumors (Fig. 2, *a*). A tendency towards a positive correlation between the *HER-2/neu* gene copy number and the corresponding protein expression was found ($r = 0.51$, $p > 0.05$). It was determined that the proportion of tumors with amplification of *HER-2/neu* gene among G3 carcinomas was significantly higher compared to the group of G2 tumors (35.7 and 5.5%, respectively) ($p < 0.05$) (Table 1). In addition, high expression of HER-2/neu protein was more frequently observed in the group of G3 tumors compared to the group of G2 tumors (22.7 and 7.7%, respectively) (Table 1). The *HER-2/neu* gene copy number analysis considering the invasive potential of the tumor showed that there was a smaller proportion of cases with *HER-2/neu* amplification among carcinomas with an invasion of < ½ myometrium than among tumors that invade > ½ of the myometrium (7.1 and 27.8%, respectively). In addition, among the latter, a significantly higher number of tumors with high expression of HER-2/neu was determined (25.0 compared to 4.1% among carcinomas with an invasion of < ½ myometrium) ($p < 0.05$). No significant differences were found in the number of patients whose tumors were characterized by amplification of *HER-2/neu* gene (16.7 and 19.2%, respectively) and high expression of the corresponding protein in the group of EC patients with a family history of cancer relative to group with sporadic EC (14.3 and 15.4%, respectively).

According to the obtained data, amplification of the *c-MYC* gene and high expression of its protein was observed in 25.0 and 23.5% of the EC pa-

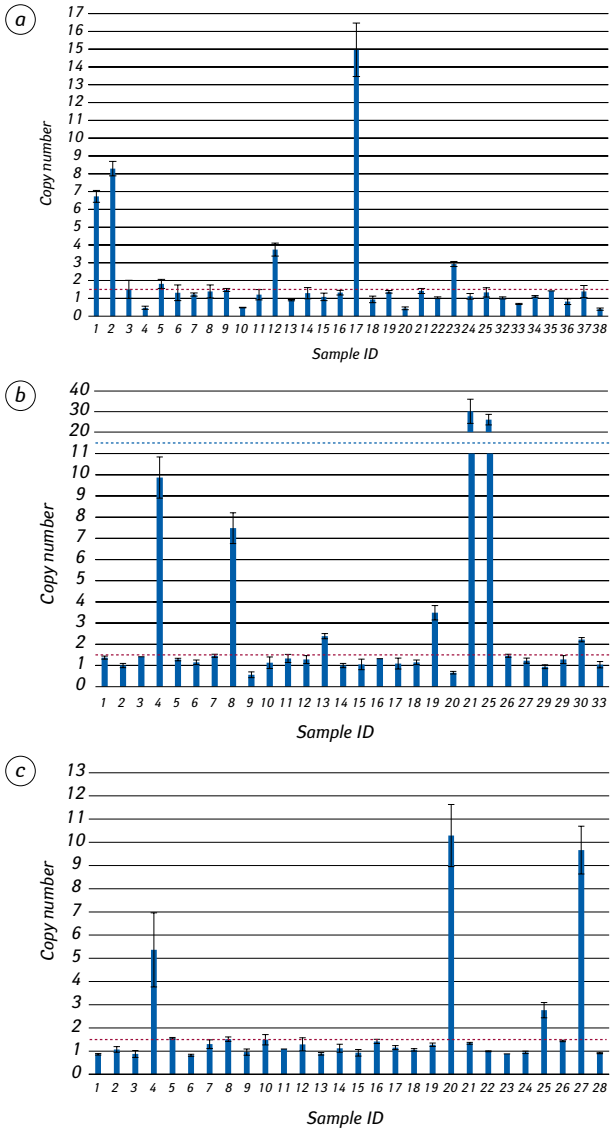

Fig. 1. Copy number analysis of *HER-2/neu* (*a*), c-*MYC* (*b*) and *CCNE1* (*c*) genes in EC samples

tients, respectively (Fig. 1, *b*; Fig. 2, *b*). The analysis of the relation between copy number and expression of this marker showed a tendency for positive correlation between these parameters ($r = 0.60$, $p > 0.05$). It was established that the increased copy number of *c-MYC* gene was more common in G3 tumors than in G2 (36.4 and 17.6% of cases with amplification, respectively), and among G3 tumors a significantly higher number of cases with high expression of this protein was observed (36.6 and 13.2%, respectively, $p < 0.05$) (Table 2). Carcinomas with increased copy number of *c-MYC* gene and high protein expression were more often found among tumors that deeply invade into the myometrium. Thus, gene amplification was revealed among 33.3% of cases with invasion > ½ of myometrium and among 15.4% with invasion < ½ of myometrium. High protein expression was observed in 26.8 and 18.5% of cases, respectively. An increased number of cases with amplification of *c-MYC* gene was also found in the group of EC patients with a family history of cancer compared to the women with sporadic tumors (42.9 and 19.0%, respectively). Furthermore,

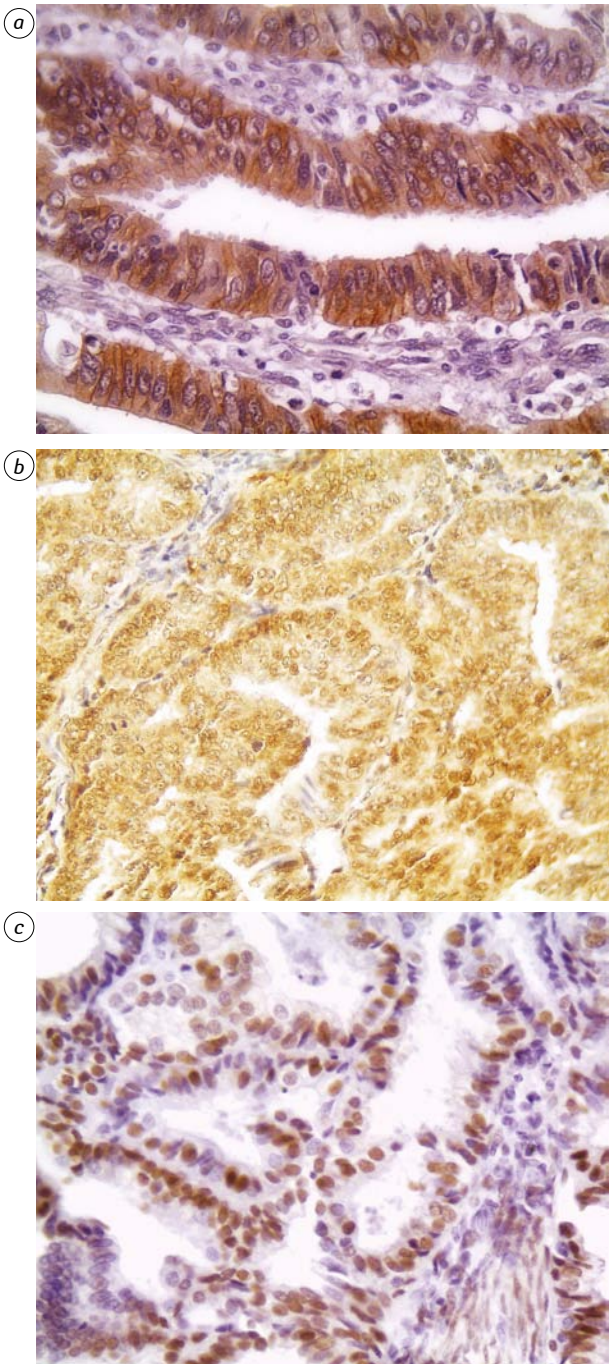

Fig. 2. Immunohistochemical expression of HER-2/neu (*a*), c-Myc (*b*) and cyclin E1 (*c*) proteins in EC (magnification × 400)

there was significantly higher number of cases with high c-Myc protein expression in the first group of EC patients than in the second (43.8 and 17.3%, respectively) ($p < 0.05$).

Copy number analysis of the *CCNE1* gene showed its amplification in 14.3% of EC patients (Fig. 1, *c*). High expression of cyclin E1 protein was observed in 65.6% of cases (Fig. 2, *c*). There was no correlation between the copy number of this gene and the expression of its protein ($r = 0.16$, $p > 0.05$). Amplification of *CCNE1* gene was found to be more common among G3 than among G2 carcinomas (21.4 and 7.1% of cases, respectively) (Table 3). There were no significant differences in the expression level

Table 1. Comparative analysis of *HER-2/neu* gene copy number and expression of its protein in EC regarding clinical and pathological characteristics of patients

Characteristics		Gene copy number		Protein expression	
		No changes	Amplification	LI < 10.0% or absence of expression	LI > 10.0% (high expression)
Differentiation grade	G2 (n, %)	17 (94.5%)	1 (5.5%)	24 (92.3%)	2 (7.7%)
	G3 (n, %)	9 (64.3%)	5 (35.7%)	17 (77.3%)	5 (35.7%)
p (F test)		$p < 0.05$		$p > 0.05$	
Depth of tumor invasion into the myometrium	< ½ (n, %)	13 (92.9%)	1 (7.1%)	23 (95.9%)	1 (4.1%)
	> ½ (n, %)	13 (72.2%)	5 (27.8%)	18 (75.0%)	6 (25.0%)
p (F test)		$p > 0.05$		$p < 0.05$	
Family history	Absent (n, %)	21 (80.8%)	5 (19.2%)	29 (85.7%)	5 (14.3%)
	Present (n, %)	5 (83.3%)	1 (16.7%)	11 (84.6%)	2 (15.4%)
p (F test)		$p > 0.05$		$p > 0.05$	

Table 2. Comparative analysis of c-*MYC* gene copy number and expression of its protein in EC regarding clinical and pathological characteristics of patients

Characteristics		Gene copy number		Protein expression	
		No changes	Amplification	LI < 10.0% or absence of expression	LI > 10.0% (high expression)
Differentiation grade	G2 (n, %)	14 (82.4%)	3 (17.6%)	33 (86.8%)	5 (13.2%)
	G3 (n, %)	7 (63.6%)	4 (36.4%)	19 (63.4%)	11 (36.6%)
p (F test)		$p > 0.05$		$p < 0.05$	
Depth of tumor invasion into the myometrium	< ½ (n, %)	11 (84.6%)	2 (15.4%)	22 (81.5%)	5 (18.5%)
	> ½ (n, %)	10 (66.7%)	5 (33.3%)	30 (73.2%)	11 (26.8%)
p (F test)		$p > 0.05$		$p > 0.05$	
Family history	Absent (n, %)	17 (81.0%)	4 (19.0%)	43 (82.7%)	9 (17.3%)
	Present (n, %)	4 (57.1%)	3 (42.9%)	9 (56.2%)	7 (43.8%)
p (F test)		$p > 0.05$		$p < 0.05$	

Table 3. Comparative analysis of *CCNE1* gene copy number and expression of its protein in EC regarding clinical and pathological characteristics of patients

Characteristics		Gene copy number		Protein expression	
		No changes	Amplification	LI < 10.0% or absence of expression	LI > 10.0% (high expression)
Differentiation grade	G2 (n, %)	13 (92.9%)	1 (7.1%)	6 (33.3%)	12 (66.7%)
	G3 (n, %)	11 (78.6%)	3 (21.4%)	5 (35.7%)	9 (64.3%)
p (F test)		$p > 0.05$		$p > 0.05$	
Depth of tumor invasion into the myometrium	< ½ (n, %)	11 (84.6%)	2 (15.4%)	8 (53.3%)	7 (46.6%)
	> ½ (n, %)	13 (86.7%)	2 (13.3%)	2 (13.3%)	13 (86.7%)
p (F test)		$p > 0.05$		$p < 0.05$	
Family history	Absent (n, %)	17 (81.0%)	4 (19.0%)	7 (28.0%)	18 (72.0%)
	Present (n, %)	7 (100%)	0 (0%)	4 (57.1%)	3 (42.9%)
p (F test)		$p > 0.05$		$p > 0.05$	

of cyclin E protein in tumors of different differentiation grade (66.7 and 64.3% cases with high expression among G2 and G3 tumors, respectively). However, a larger proportion of cases with high expression of cyclin E1 were determined among tumors with invasion > ½ of the myometrium than among tumors with invasion < ½ of the myometrium (86.7 and 46.6%, respectively, $p < 0.05$). The obtained results indicated that amplification *CCNE1* gene was common for 19.0% of patients with sporadic EC, while it was not observed in tumors of patients with a family history of cancer. In addition, the high expression of cyclin E1 protein was more often detected in the group of patients with sporadic EC than with a family history of cancer (72.0 and 42.9%, respectively).

DISCUSSION

The use of modern technologies allowed to characterize in detail the molecular profile of endometrial tumors. It has been established that among endometrioid tumors there is a category with a molecular profile that is more characteristic of serous carcinomas. This became the basis for revising the existing views on the classification and treatment of EC [2, 18, 19]. In particular, it was suggested that the use of polychemotherapy, which is common in the treatment of serous EC, is advisable for patients with serous-like endometrioid carcinomas that are characterized by the most unfavorable outcome [2]. Given the urgency of this issue, there is an active search for approaches to identifying the serous-like subtype of EC using routine methods. Several methods have already been proposed that are based on a comprehensive analysis of certain molecular genetic changes and clinical and morphological characteristics typical for each of the TCGA subtypes [3–5]. A distinctive feature of the serous-like EC subtype is a high level of changes in the copy number, such as deletions and amplifications, of genes with oncogenic and suppressor properties. The latter suggests a possible expediency of gene copy number analysis for the identification of certain more malignant EC cases [4, 20].

The present study showed the amplification of *HER-2/neu* gene and high protein expression in 18.8 and 14.6% of EC patients, respectively. We determined that these characteristics were more pronounced in EC patients whose tumors were characterized by a low differentiation grade and deep tumor invasion into the myometrium. It is noteworthy that according to various data, the amplification of this gene is observed in 2.5–38.0% of endometrioid endometrial carcinomas [21–23] and protein overexpression in 1.0–47.0% of EC cases [24]. It was shown that increased copy number of *HER-2/neu* gene and protein overexpression positively correlated with the degree of EC malignancy [22, 25]. In addition, it was established that amplification and/or overexpression of *HER-2/neu* in endometrial carcinomas is associated with a low survival rate of these patients [24]. Consequently, the data we obtained along with the results of other researchers point to the possibility of assigning of changes in *HER-2/neu* gene copy number and its expression to markers of the aggressive course of EC.

In the present study, amplification of *c-MYC* gene was detected in 25.0% of the EC patients and high

protein expression in 23.5% of cases. We observed that high expression of this protein was associated with a low differentiation grade. It should be noted that, according to various estimates, the increase in copy number of 8q24 chromosomal locus, where the *c-MYC* gene is localized, is observed in 5–26% of EC cases [26–28]. A number of authors have determined an association between amplification or high expression of this marker and the unfavorable course of the disease [26–29], that, together with our results, allows to consider it as an indicator of the EC progression.

According to the obtained data, *CCNE1* gene amplification occurs in 14.3% of endometrioid tumors. Other researchers found the amplification of this gene in 8.3 and 10.4% of EC cases [30, 31]. It should be noted that the increase in the copy number of CCNE1 is more often observed among serous carcinomas (in 45% of cases), including 41% of intraepithelial serous carcinomas of the endometrium [32]. High expression of this marker was detected in 65.6% of the examined endometrial carcinomas. We revealed a tendency to increase the copy number of *CCNE1* gene with a decrease in the degree of differentiation. Moreover, the association of high expression of this marker with a deep tumor invasion into the myometrium was established. The obtained results and literature data, according to which *CCNE1* amplification is observed in endometrial carcinomas with a high degree of malignancy [30–32], may be regarded as the basis to consider copy number variations of *CCNE1* gene as a marker of an unfavorable course of EC.

Our study revealed no association between amplification of *HER-2/neu* and *CCNE1* genes and high expression of their proteins with the presence of a family history in EC patients. Interestingly, a comparative analysis of the frequency of *HER-2/neu* amplification and protein expression conducted by El-Gerzawy *et al*. didn't reveal any differences between patients with familial *vs* sporadic breast cancer by these parameters [33]. In addition, no such dependence was found among patients with hereditary (in *BRCA1/2* mutation carriers) and sporadic ovarian cancer [34]. However, in a study by Chappuis *et al.* a significant increase in expression of cyclin E1 was reported in breast cancer patients with hereditary mutations in the *BRCA1* gene [35]. At the same time, we established that the proportion of EC patients with amplification of the *c-MYC* gene and high expression of its protein was higher in the group of women with a family history of cancer than in the group with sporadic tumors. There is evidence that certain polymorphic variants in the 8q.24 locus are associated with susceptibility to colon cancer [36]. It was established that colorectal tumors in patients with "familial colorectal cancer type X" are characterized by amplification of the 8q region [37] and increased expression of the *c-MYC* gene [38]. Thus, our findings, along with the results of other researchers, indicated a possible relationship between molecular changes in the *c-MYC* gene, its expression and a family history of cancer. It can be assumed that overexpression of c-Myc protein can lead to genetic instability of endometrial tumor cells in patients with a family history of cancer.

Thus, the present study showed that certain cases of endometrioid endometrial carcinomas are characterized by copy number variations of *HER-2/neu*, *c-MYC* and *CCNE1* genes and high level of the corresponding proteins, which associates with such indicators of tumor progression as a low differentiation grade and deep myometrial invasion. Particularly, amplification of *HER-2/neu* gene and high expression of c-Myc protein was found to be more common in tumors with a low differentiation grade. It was shown that high expression of HER-2/neu and cyclin E1 proteins is more often observed among carcinomas with deep myometrial invasion. Moreover, an increased number of cases with high expression of c-Myc protein was revealed in the group of patients with a family history of cancer. The obtained data indicate the potential possibility of including the studied markers in the panel for determining the EC subtype with an aggressive course of the disease, which can be the basis for the correction of therapeutic measures in the treatment of patients with this form of malignant tumors.

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