EXPERIMENTAL ONCOLOGY

https://doi.org/10.15407/exp-oncology.2024.03.244

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SIGNIFICANCE OF miRNA-185-5P AND miRNA-424-5P AS PROGNOSTIC MARKERS IN PROGRESSION OF EARLY-STAGE ENDOMETRIAL CANCER

Aim. To compare the expression of miRNA-185-5p and miRNA-424-5p in tumor cells and peripheral blood serum (PBS) of patients with endometrioid carcinoma of the endometrium (ECE) and to evaluate the significance of these biomarkers in cancer progression. **Materials and Methods**. The study was conducted on the samples of peripheral blood serum (PBS) and tumor tissue of 58 patients with stage I ECE using clinical and morphological methods and real-time polymerase chain reaction. **Results.** A significant increase in the levels of circulating and tumor-associated miRNA-424-5p was established in ECE patients with a history of recurrences compared to patients without recurrences. To the contrary, the expression level of miRNA-185-5p increased in the PBS and decreased in the tumor tissue of ECE patients with recurrences compared to the patients without recurrence. In addition, we revealed that the expression levels of the studied miRNAs were associated with the differentiation grade and degree of tumor invasion. We established that miRNA-424-5p levels in PBS could serve as the most significant indicator for predicting the occurrence of recurrence in patients with ECE (AUC = 0.991; Sp 94.0%; Se 99.9%). **Conclusions**. The expression features of miRNA-185-5p and miRNA-424-5p in the PBS and tumor tissue of patients with ECE are associated with the aggressiveness of cancer course and the risk of recurrence.

Keywords: endometrioid carcinoma of endometrium, miRNA-185-5p and miRNA-424-5p expression, peripheral blood serum, relapses.

Endometrial cancer (ECa) is one of the most common tumors of the female reproductive system. ECa is characterized by heterogeneity in terms of the morphological and molecular genetic features that determine the formation of ECa variants with different biological properties. They determine the aggressiveness and variability of the clinical course of the disease, including the initial stage of ECa [1—5].

To date, four molecular subtypes of ECa have been identified, which are characterized by a certain spectrum of mutations and gene expression profiles [1, 6]. However, the authors emphasize that this classification does not fully reflect the hetero-

Citation: Iurchenko N, Glushchenko N, Nespryadko S, Buchynska L. Significance of miRNA-185-5p and miRNA-424-5p as prognostic markers in progression of early-stage endometrial cancer. *Exp Oncol.* 2024; 46(3): 244-252. https://doi.org/10.15407/exp-oncology.2024.03.244

geneity of the molecular changes that occur during the ECa progression, and note the presence of endometrial carcinomas with a different mutator phenotype [7]. In particular, it was established that cancer progression in the endometrium is correlated with the high expression of genes associated with genome instability, serine-threonine kinases ATR and CHK1 [5]. The latter is involved in the cellular response to DNA damage (DDR, DNA damage response) for its restoration [8]. Also, ATR/CHK1 signaling plays an important role in the distribution of genetic material during cell division, differentiation, proliferation, and apoptosis [9—11].

According to the data of recent years, cancer progression is associated with epigenetic disorders. MicroRNAs, which control the expression of genes involved in the processes of proliferation, differentiation, apoptosis, immune response, etc., play a significant role in the generation of such changes [12—14]. Interaction with certain target genes determines the role of miRNA as a tumor suppressor or oncogene. In various nosological forms of cancer, a certain spectrum of miRNAs is expressed, the change of which correlates with the disease aggressiveness [15—18].

Among miRNAs associated with the development of many solid neoplasms, including ECa, miRNA-185 and -424 were identified, whose targets are ATR and CHK1 kinases, respectively.

The expression of miRNA-185 in many cancer types, in particular breast cancer and hepatocellular carcinoma, demonstrates tumor suppressor activity, which is associated with a lower cancer malignancy [19—21]. However, according to some results [22, 23], a high expression of miRNA-185 in breast tumors is correlated with worse patient survival and the progression of colorectal cancer.

To date, it has been shown that miRNA-185 overexpression is detected in tumor cells of the endometrium compared to unchanged tissue [24, 25]. Similarly, a high expression of miRNA-424-5p was detected in colorectal cancer cells, which was correlated with an unfavorable course of the disease [26]. On the other hand, in malignant tumors of the ovary, mammary gland, and hepatocellular carcinoma, a decrease in miRNA-424-5p expression was associated with the progression of these neoplasms [27—29]. The interesting results were obtained by analyzing the expression of miRNA-424-5p in tumor tissue and blood serum of patients with melanoma. The authors noted that high expression of miRNA-424-5p was associated with an unfavorable course of the disease in patients with melanoma compared to the group of patients with low expression of this miRNA [30]. Moreover, the level of miRNA-424-5p expression as a differential diagnostic marker of benign and malignant endometrial neoplasms has been established by [31].

Today, the question regarding the significance of miRNA-185-5p and miRNA-424-5p expressions in predicting ECE aggressiveness remains open.

The aim of our work was to assess the expression of miRNA-424-5p and miRNA-185-5p in tumor cells and peripheral blood serum (PBS) of patients with endometrioid carcinoma of the endometrium (ECE) and evaluate the significance of these biomarkers in cancer progression.

Materials and Methods

The objects of the study were PBS and surgical material samples of 58 patients with stage I ECE by FIGO treated at the Department of Oncology and Gynecology of the National Cancer Institute of the Ministry of Health of Ukraine between 2014 and 2019. 38 patients were without metastases in the regional lymph nodes and 20 patients had recurrences in the regional lymph (para-aortic) nodes during the first 3 years after the initial treatment.

All patients did not receive preoperative therapy and provided informed consent for the use of their biological material for scientific research. According to the conclusion of the Bioethics Commission of the RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, during the research, all necessary ethical standards were observed in accordance with the requirements of the universally recognized international rules within the framework of the Helsinki Declaration of 2008.

Morphological verification of ECE and tumor differentiation grade was performed on preparations stained with hematoxylin and eosin according to the WHO criteria [32].

In the analysis of potential target genes, miRTargetLink2.0 (interactive miRNA target gene) and target pathway networks (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) were used. Data resources: miRNet v.2.0 – the miRNA-centric network visual analytics platform (https://www.mir-

net.ca/miRNet/home.xhtml), mirDIP db – microRNA Integration Portal (https://ophid.utoronto.ca/mirDIP/index.jsp#r), and dbDEMC – Database of Differentially Expressed miRNAs in Human Cancers (https://www.biosino.org/dbDEMC/index) were used to search for miRNAs involved in the regulation of the target genes.

Real-time polymerase chain reaction (PCR) was used to assess miRNA expression. Blood serum samples (5 mL) obtained from the patients with ECE were centrifuged at 1,500 rpm and stored at –80°C until further use as well as with the ECE tissue samples. Total RNA was isolated from serum/tissue samples using a commercial kit "For RNA isolation" from the Ukrainian Genetic Technologies company (Kyiv, Ukraine) according to the manufacturer's recommendations.

The concentration of isolated RNA was determined using a "NanoDrop 2000c" spectrophotometer (ThermoScientific, USA), by measuring the absorption coefficient at wavelengths of 260 and 280 nm. cDNA was synthesized using a TaqMan reverse transcription kit.

qPCR reactions were performed in 96-well plates (Applied Biosystems, USA) using the QuantStudio 5 Dx Real-Time PCR System (ThermoScientific, USA) and the commercial Luna Universal qPCR Master Mix PCR kit (New England Biolabs, USA) according to the manufacturer's protocol.

The sequences of the primers used in the study are shown in Table 1.

The *RNU48* gene was used as an endogenous control. The experiment was performed in three replicates for each sample. The change in miRNA expression compared to the control was calculated by $2^{-\Delta Ct}$ formula [33].

Statistical analysis was performed using Statistica 7.0 software (StatSoft, Inc.) and MedCalc® version 22.016 (MedCalc Software Ltd). The following methods were used: standard descriptive, non-parametric (Mann — Whitney test), and comparison of frequencies of 2 samples (Pearson's $\chi 2$ test). Spearman's rank correlation coefficient (R) was calculated to reveal the relationship between the obtained data. The differences at $p < 0.05$ were considered significant.

To investigate the prognostic significance of the studied biomarkers, we used a receiver operating characteristic (ROC) analysis, including calculations of sensitivity (Se) and specificity (Sp).

Results and Discussion

The general clinical characteristics of patients with ECE and morphological features of tumors are presented in Table 2. The median age of the patients was 60.3 years. Among the examined, the largest number of patients was composed of ones at the ECE stage of IB (44.8%). In 34.5% of patients, relapses of the disease occurred within 1.8—3.0 years (Table 2).

The study of the morphological characteristics of endometrial neoplasms showed that the studied tumors were ECEs mostly of high and moderate differentiation grades (67.2%) and different depths of invasion into the myometrium. It should be noted that in patients without recurrence, G1—G2 tumors predominated (79.0%) without deep invasion into the myometrium (Table 3).

Endometrial carcinomas of patients with relapses were mostly of a low differentiation grade with deep invasion into the myometrium (Table 3).

Earlier, we have shown that in patients with a history of recurrences, the expression of ATR and CHEK1 kinases was significantly higher both at the mRNA and protein levels compared to these indicators in tumors of patients without recurrences [5]. It seemed possible that the detected features of ATR and CHK1 expression in ECE could be related to the expression of their direct epigenetic regulators miRNA-185-5p and miRNA-424-5p. Therefore, we began studying the expression of miRNA-

Table 1. **Primers for miRNAs**

RNU48	forward 5' - AGTGATGATGACCCCAGGTAACTC - 3', reverse 5' - CTGCGGTGATGGCATCAG - 3'
miR-185-5p	forward 5' -GCGCGATTGGAGAGAAAGGCAGT-3' reverse 5' - ATCCAGTGCAGGG TCCGAGG-3'
miR-424-5p	forward5' - GCCAGCAGCAATTCATGT -3' reverse 5' - TATGGTTTTGACGACTGTGTGAT -3'

185-5p and miRNA-424-5p in PBS and ECE tissue samples.

Analysis of the expression level of the studied tumor-associated miRNAs showed an association with clinical and pathological characteristics of ECE patients. Thus, the level of miRNA-185-5p expression in G3 tumors was 4.3 and 2.3 times lower than that in highly and moderately differentiated ECE, respectively. The expression level of miRNA-424-5p was 5.5 times higher in poorly differentiated ECE compared to this index in highly differentiated tumors and 3.0 times higher in moderately differentiated ones (Fig. 1).

A similar trend of the changes in the expression level of these miRNAs was observed depending on the depth of tumor invasion into the myometrium. In endometrial carcinomas with invasion of >1/2 of the myometrium, a significant decrease in the expression level of miRNA-185-5p by 4 times and an increase in the miRNA-424-5p expression by 6.5 times compared to tumors with invasion <1/2 were determined (Fig. 1). Therefore, the decreased miRNA-185-5 expression level and the increased miRNA-424-5p level were characteristic of poorly differentiated and deeply invasive ECE.

Table 2. **Clinical and pathological characteristics of patients with ECE**

Index	Number of patients	
	n	%
Total number of patients	58	100
Average age, years	59.4 ± 0.8	
Age range	38—72	
Clinical stage of the disease		
IA	18	31.1
IB	26	44.8
IC	14	24.1
Recurrence		
Yes	20	34.5
No	38	65.5
Differentiation grade		
G1 (high)	11	19.0
G2 (moderate)	28	48.3
G3 (low)	19	32.7
Depth of tumor invasion in the myometrium		
<1/2	34	58.6
>1/2	24	41.3

Recently, it has been shown that the instability of the genome, which is one of the main characteristics of most sporadic tumors, is associated not only with the accumulation of genetic disorders arising in the genes of the replication and repair systems but also with their aberrant post-transcriptional regulation, which determines cancer aggressiveness [34, 35]. When studying the expression levels of miRNA-185-5p and miRNA-424-5p in ECE of patients depending on the presence of relapses in the anamnesis, we found that the expression level of miRNA-185-5p was 3 times lower ($p < 0.05$), and miRNA-424-5p was 8.2 times ($p < 0.05$) higher in ECE of patients with recurrences compared to these values in ECE samples of patients without relapses (Fig. 2). A similar trend of changes, that is, a decrease in miRNA-185-5p expression, is observed in the progression of such hormone-dependent neoplasms as breast, ovarian, and prostate cancer [19, 20, 36].

Therefore, the studied miRNAs seem to be involved in the regulation of the expression of serine/threonine kinases. The higher ATR expression in ECE of patients with recurrence is associated with the lower expression level of miRNA-185-5. A significantly higher CHK1 expression in ECE of patients with recurrences is associated with a higher expression of miRNA-424-5p. Besides, a higher expression of these miRNAs was found in ECE with decreased differentiation grade and deep invasion.

Table 3. **Clinical and pathological characteristics of patients with ECE depending on the presence of recurrence in the anamnesis**

Index	Number of patients	
	Without recurrences, n (%), n = 38	Without recurrences, n (%), n = 20
Differentiation grade		
G1 (high)	8 (21.0)	3 (15.0)
G2 (moderate)	22 (58.0)	6 (30.0)
G3 (low)	8 (21.0)	11 (55.0)
Depth of tumor invasion in the myometrium		
<1/2	26 (68.4)	8 (40.0)*
>1/2	12 (31.6)	12 (60.0)

Note: * $p < 0.05$ compared to tumors of patients without recurrence (Pearson's $\chi 2$ test)

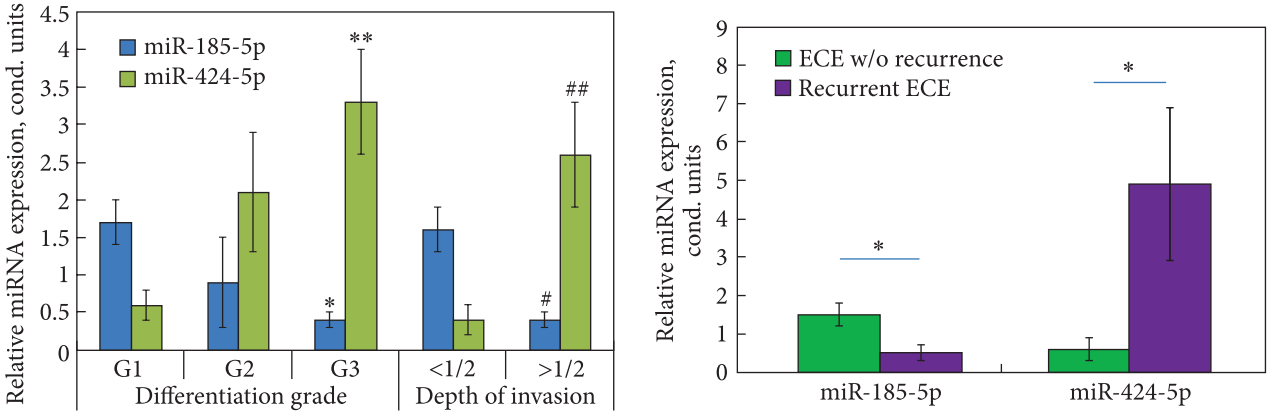

Fig. 1. Comparison of the expression levels of miRNA-185-5p and miRNA-424-5p in ECE cells depending on the clinical and pathological characteristics of the patients. *,** $p < 0.05$ compared to G1 tumors; #,## $p < 0.05$ compared to tumors invading <1/2 myometrium

Fig. 2. Analysis of the expression of tumor-associated miRNAs-185-5p and, -424-5p in ECE patients with and without recurrences. * $p < 0.05$ compared to tumors in patients without relapses

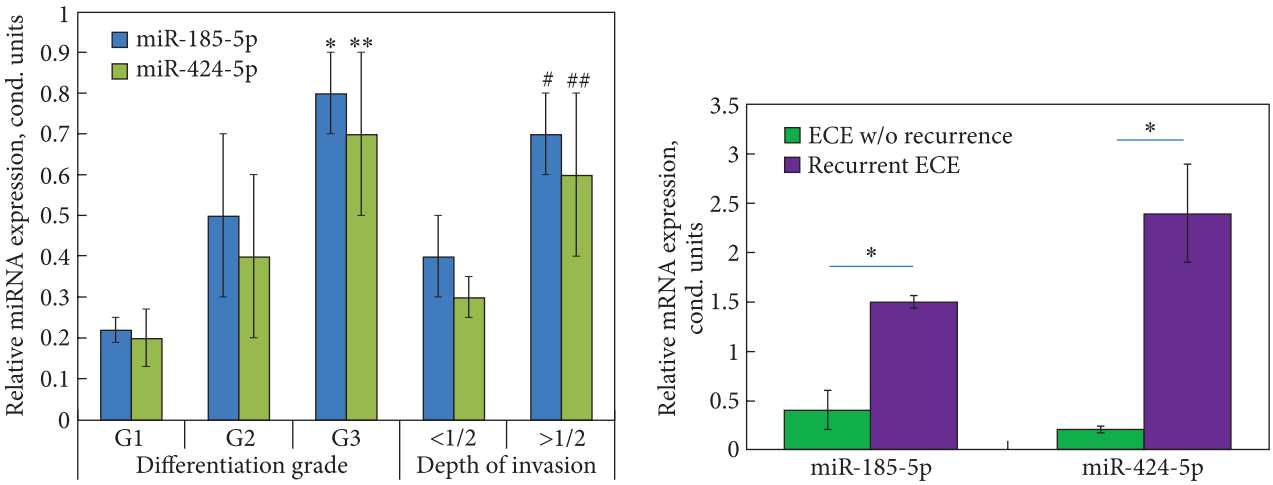

Fig. 3. The expression levels of miRNA-185-5p and miRNA-424-5p in PBS depending on the clinical and pathological characteristics of ECE patients. *,** $p < 0.05$ compared to G1 tumors; #,## $p < 0.05$ compared to tumors invading < 1/2 myometrium

Fig. 4. Comparison of the expression levels of miRNA-185-5p and miRNA-424-5p in PBS of ECE patients depending on the presence of recurrences in anamnesis. $p < 0.05$ compared to the cases without relapses

It is possible that the higher expression level of tumor-associated miRNA-424 in G3-tumors with deep invasion into the myometrium revealed in our study could be associated with the high expression of E2F1 and cancer aggressiveness [37], since miRNA-424 expression is known to be transcriptionally regulated by E2F1, which binds to the miRNA-424 promoter and directly activates its transcription during the G1/S transition. The increased expression of miRNA-424 increases the expression of cyclin-cyclin-dependent kinases and decreases the expression of their inhibitory proteins, which leads to increased proliferation of tumor cells [38].

When analyzing the expression of the studied miRNAs in the blood serum of ECE patients compared to that in the tumor tissue, somewhat different results were obtained. In particular, a significant increase in the expression level of miRNA-185-5p (by 4 times) was found in the PBS of patients with G3 carcinomas, deep invasive neoplasms (almost twice), and relapses (by 3.8 times) compared to corresponding values in patients with highly differentiated tumors, with invasion of <1/2

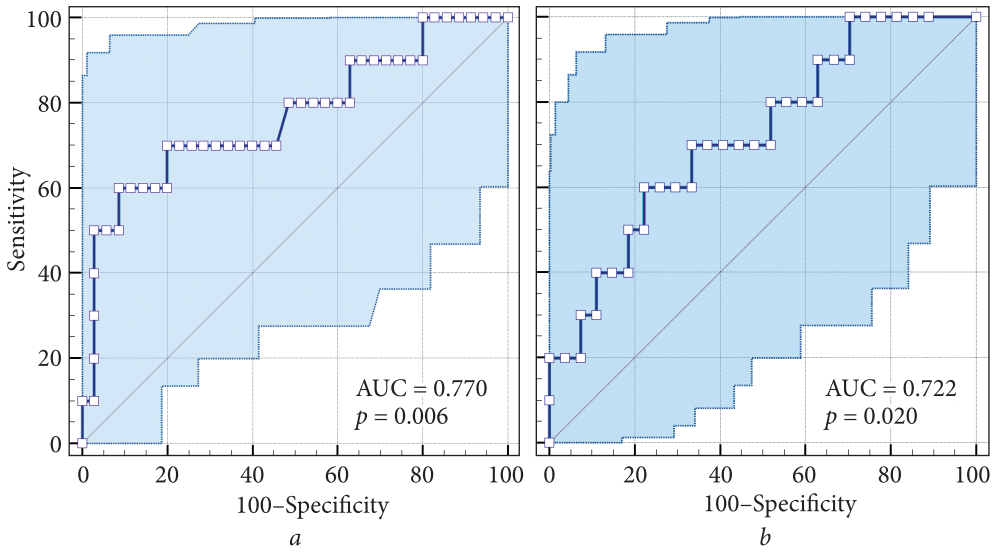

Fig. 5. ROC analysis of expression levels of tumor-associated miRNA-185-5p (*a*) and miRNA-424-5p (*b*)

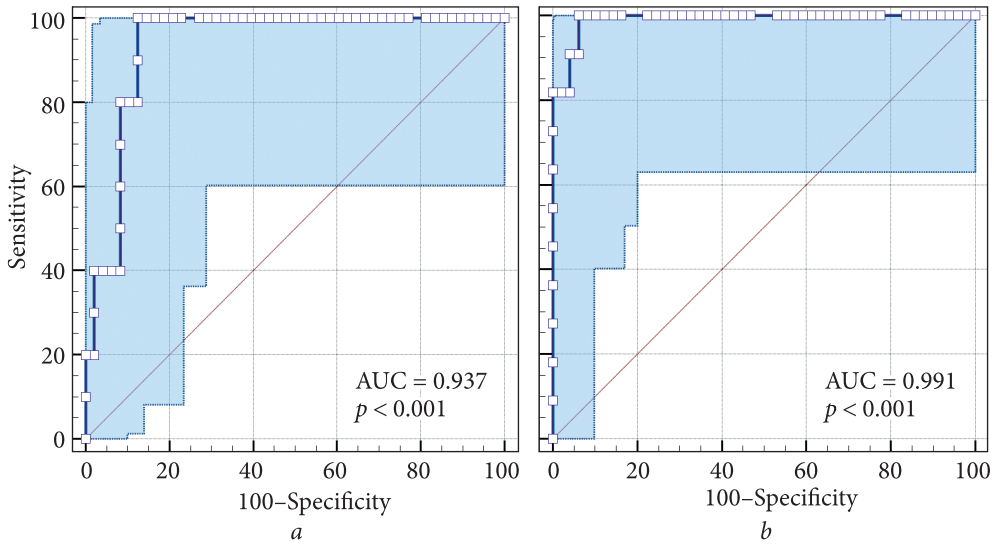

Fig. 6. ROC analysis of miRNA-185-5p (*a*) and miRNA-424-5p (*b*) expression levels in PBS of ECE patients

of the myometrium, and without a history of relapses (Figs. 3, 4). Meanwhile, the expression level of the tumor-associated miRNA-185-5p decreased depending on the specified clinical and pathological features of ECE patients (Figs. 1 and 2).

Recent data have shown that a higher expression of miRNA-185-5p was observed in the blood plasma of patients with colorectal cancer compared to its level in patients with colorectal adenoma and healthy individuals. In addition, an increase in miRNA-185-5p was found in patients with stage III—IV colorectal cancer compared to patients with stage I—II of the disease [23].

At the same time, the expression levels of miRNA-424-5p in the PBS of ECE patients and tumor tissue changed in the same way depending on cancer progression. The expression rate of this miRNA was significantly higher in the PBS of patients with G3 tumors (by 3.5 times) and tumors with deep invasion into the myometrium (twice) compared to the cases of G1 tumors and invasion <1/2 myometrium (Fig. 3).

The expression level of miRNA-424-5p in the PBS of patients with relapses was 12 times higher compared to its values in patients without relapses (Fig. 4).

The obtained data show that the level of expression of tumor-associated and circulating miRNA-185-5p and miRNA-424-5p is a significant factor associated with the morphological and functional

characteristics of neoplasms and the occurrence of recurrence and could therefore be related to the ECE aggressiveness.

This assumption was confirmed by the ROC analysis, according to which the low level of expression of tumor-associated miRNA-185-5p (AUC = 77.0%, Se = 60.0%, Sp = 91.4%; $p = 0.006$) and high expression level of miRNA-424-5p (AUC = 72.2%, Se = 60.0%, Sp = 77.8%; $p = 0.02$) indicated the probability of recurrence in ECE patients (Fig. 5).

According to the results of the ROC analysis, high expression levels of miRNA-185-5p (AUC = 93.7%, Se = 99.9%, Sp = 87.5%; $p < 0.001$) and miRNA-424-5p (AUC = 99.1%, Se = 99.9%, Sp = 94.0%; $p < 0.001$) in the PBS of ECE patients indicate the risk of the recurrence of the disease (Fig. 6).

It should be noted that the ROC analysis revealed that the expression level of miRNA-424-5p in PBS demonstrates the highest prognostic accuracy of the test (Figs. 3 and 6).

Taking into account the association of changes in the level of expression of miRNA-424-5p in tumor cells and PBS of patients with ECE depending on the clinical and pathological features along with the highest prognostic value by the data of the ROC analysis, the level of miRNA-424-5p expression in blood serum is a significant predictor that determines the risk of recurrence.

Today, a lot of publications are devoted to the study of circulating microRNAs as diagnostic and prognostic markers with different levels of significance in ECa [31, 39]. In particular, it was shown that among 6 studied exosomal miRNAs (miR-106b-5p, miR-107, miR-15a-5p, miR-3615, miR-139-3p, and miR-574-3p), the highest AUC = 0.813 was determined for miRNA-15a-5p. The authors noted that miRNA-15a-5p can be used as a diagnostic and prognostic marker for ECa [40]. The results of our study showed that the expression level of miRNA-424-5p in the PBS has a higher prognostic significance for identifying ECE patients with the risk of the disease recurrence.

Thus, the features of miRNA-424-5p expression are associated with cancer progression in the endometrium, which points to its possible use as a prognostic marker of the disease course.

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Submitted: August 03, 2024

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ЗНАЧЕННЯ мікроРНК-185-5p ТА мікроРНК-424-5p ЯК ПРОГНОСТИЧНИХ МАРКЕРІВ У ПРОГРЕСІЇ РАКУ ЕНДОМЕТРІЮ РАННЬОЇ СТАДІЇ РОЗВИТКУ

Мета. Зіставити експресію мікроРНК-185-5p та мікроРНК-424-5p у пухлинних клітинах та сироватці периферичної крові хворих на рак ендометрію та оцінити значення цих біомаркерів у прогресуванні пухлинного процесу. **Об'єкт і методи**. Дослідження проведено на 58 зразках сироватки периферичної крові (СПК) та випадках післяопераційного матеріалу хворих на рак ендометрію (РЕ) I стадії за FIGO (медіана віку: 60,3 роки; діапазон від 38 до 72 років). Методи дослідження: клінічний, морфологічний, полімеразна ланцюгова реакція в режимі реального часу. **Результати**. Встановлено достовірне підвищення показників експресії циркулюючої та пухлино-асоційованої мікроРНК-424-5p у хворих на РЕ з рецидивами в анамнезі порівняно з пацієнтками без рецидивів. Натомість, показано, що рівень експресії мікроРНК-185-5p у СПК та пухлинній тканині ендометрію хворих з рецидивами і без рецидивів має обернену направленість змін. У СПК хворих на РЕ з рецидивами експресія мікроРНК-185-5p зростала, а у пухлинній тканині знижувалася порівняно до її показників у пацієнток без рецидивів. Крім того, визначено, що рівні експресії досліджуваних мікроРНК асоціювались зі ступенем диференціювання, рівнем інвазування РЕ, що свідчить про значення цих мікроРНК у формуванні ступеня злоякісності пухлини та асоціацію з агресивністю перебігу захворювання. Встановлено, що найбільш значимим показником для прогнозування виникнення рецидивів у хворих на РЕ є мікроРНК-424-5p у СПК (AUC = 0,991; Sp 94,0%; Se 99,9%). **Висновки**. Ідентифіковано профіль експресії мікроРНК-185-5p і мікроРНК-424-5p у СПК хворих на РЕ та пухлинній тканині ендометрію, що визначає агресивність злоякісного новоутворення. Отримані дані обґрунтовують можливість використання показників експресії мікроРНК-424-5p для оцінки ризику рецидиву у хворих на рак ендометрію.

Ключові слова: ендометріоїдна карцинома ендометрію, експресія мікроРНК-185-5p і мікроРНК-424-5p, сироватка периферичної крові, рецидиви.