

EXPRESSION OF MICRORNA IN TUMOR CELLS OF ENDMETRIOID CARCINOMA OF ENDOMETRIUM

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Background: It is known that more than half of the genes encoding human proteins are regulated by various microRNAs (miRNAs, miR), the expression of which may be associated with various pathological conditions. At the same time, the question of assessing the relationship between the expression of particular miRNAs and the aggressive molecular subtype of endometrial cancer remains open. **Aim** of the study was to determine the relationship between the expression of miR-34a, miR-125b, miR-142 and miR-101 in endometrioid carcinomas of the endometrium (ECE) and the features of the disease course. **Materials and Methods:** The samples of surgical material of 51 patients with ECE (mean age 59.8 ± 7.1 years), I–III stage were investigated using morphological, immunohistochemical methods, real time polymerase chain reaction (PCR), cytofluorometry. **Results:** In endometrial tumors with high proliferation index ($> Me$), the expression of miR-34a, miR-142 and miR-125b significantly decreased (1.8, 2.7 and 1.5 times, respectively) compared with those in ECE with low proliferation index ($< Me$). The expression of all studied miRNAs was lower in G3 tumors and those that deeply invaded the myometrium compared to G2 carcinomas and tumors with an invasion of $< 1/2$ myometrium and significantly decreased in tumors of patients with low stage III compared with stage I–II. The high ($> Me$) microvessel density in ECE was associated with a significant decrease of miR-125b and miR-101 expression, and the presence of signs of epithelial-mesenchymal transition — with a decreased expression of miR-34a and miR-101. **Conclusions:** The study revealed a significant heterogeneity of expression of miR-34a, miR-125b, miR-142 and miR-101 in ECE, which is associated with changes in morphofunctional characteristics of endometrial carcinoma.

Key Words: endometrioid endometrial carcinoma, microRNAs, miR-34a, miR-125b, miR-142, miR-101.

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According to current epidemiological data, endometrioid carcinoma of the endometrium (ECE) is one of the prevalent oncogynecological diseases in Ukraine and developed countries [1]. In most patients, the disease is detected at the early stages and has a mostly favorable prognosis; however, almost 17% of patients have recurrences or metastases. The significant heterogeneity of ECE in morphological and molecular characteristics affects the variability of the clinical course and requires more sophisticated approaches to ECE diagnosis. Today, according to the recommendations of the European Society of Medical Oncologists, ECE is divided into four molecular subtypes, which are characterized by a certain range of mutations and gene expression profiles, which require different treatment strategies [2–4]. In our previous studies, it was shown that aggressive ECE forms are characterized by higher expression of cyclins E and D1, transcription factor E2F1 and aneuploidy than tumors with low potential for malignancy [5]. The association of variability of the morphological ECE phenotype with changes in the expression of the markers of epithelial-mesenchymal transition

(EMT) was revealed and their relation to possible pathways of tumor cell migration was substantiated [6]. However, despite the progress made in the diagnosis, the search for additional characteristics of the tumor for early detection of highly aggressive ECE forms remains important.

Non-coding RNAs (ncRNAs), which are represented by a large class of evolutionarily conserved RNAs that are not translated into protein products, could be considered as biomolecular epigenetic markers. It is known that a key function of ncRNA is the post-transcriptional regulation of gene expression via activating or inhibiting the mRNA of target genes or disrupting translation processes. Even a single ncRNA can regulate the expression of many mRNAs and thus change the functioning of dozens of genes, including the ones causing malignant transformation of cells [7]. By the transcript length, human ncRNAs are divided into short (miRNA — 18–25 nucleotides), PIWI-interacting RNA (piRNA — 30–35 nucleotides), long (lncRNA — > 200 nucleotides), and interfering RNA (siRNA) — 60–300 nucleotides [8–10].

Recent studies have shown that more than half of the genes encoding human proteins are regulated by miRNAs [11, 12]. They play an important role in tumor progression suppressing or stimulating tumor cell proliferation, apoptosis, EMT, angiogenesis, invasion and metastasis. To date, the list of miRNAs has been determined, the change in their expression is associated with various no-

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Abbreviations used: ECE — endometrioid carcinoma of endometrium; EMT — epithelial-mesenchymal transition; Me — median; miR — miRNA, microRNA; MVD — microvascular density; PI — proliferation index.

sological forms of cancer, and may serve as markers of the disease course and response to cancer therapy [13, 14].

Upon analysis of miRBase [15], we identified four microRNAs (miR-34a, miR-125b, miR-142 and miR-101), the expression of which is associated with the progression of tumors of different genesis. These miRNAs are involved in the regulation of migration and invasion of tumor cells [16–25]. In particular, miR-142 expression has been detected at a lower level in endometrial carcinoma samples than in unaltered endometrial tissue. In addition, miR-142 level in patients without metastases was higher than in patients with metastatic breast cancer, so high expression of this microRNA is considered a predictor of a favorable prognosis [17, 18].

There is conflicting information regarding the expression of miR-125b in tumors of different genesis. A number of authors have shown that in breast cancer miR-125b is a potential tumor suppressor because it inhibits mRNA of *ERBB2* and *ERBB3* genes, i.e. reduces the production of the HER2/neu oncoprotein [19]. Other studies demonstrated that miR-125b as a negative regulator of the expression of suppressor genes, including *TP53* inhibits p53-dependent apoptosis pathway [20, 21].

MiR-101 has been shown to reduce the proliferation of tumor cells by inhibiting the PI3R/Akt/mTOR pathway [22]. In addition, miR-125b and miR-101 have been shown to affect *VEGF* gene expression by exerting an antiangiogenic effect [23]. miR-34a characterized by a wide range of suppressor properties exerts an inhibitory effect on the expression of mRNAs encoding proteins that activate proliferation, angiogenesis, migration and invasion of tumor cells and, in addition, miR-34a activates the expression of the suppressor gene *TP53*, i.e., in contrast to miR-125b, activates p53-dependent signaling [24–26].

It should be noted that so far the role of ncRNA in the progression of endometrial cancer has not been sufficiently studied. There are some studies that determined the expression of several lncRNA and miRNA in the peripheral blood and tumor cells of patients with endometrial cancer [16, 19, 21, 25, 27]. However, the question remains to assess the relationship between the expression of individual miRNAs and the aggressive molecular subtype of endometrial cancer.

In view of the above, the aim of the study was to determine the relationship between the expression of miR-34a, miR-125b, miR-142 and miR-101 in ECE and the course of the disease.

MATERIALS AND METHODS

The samples of the surgical material of 51 patients with ECE, aged from 32 to 78 years (mean age 59.8 ± 7.1 years) were analyzed. The distribution of patients with ECE by the stage of tumor process (International Federation of Gynecology and

Obstetrics) was as follows: stage I — 37 patients (72.6%), stage II — 9 patients (17.6%), and stage III — 5 patients (9.8%). All patients were treated in the Department of Oncogynecology (Head Prof. V.S. Svintsitskiy) of the National Cancer Institute of the Ministry of Health of Ukraine in the period from 2014 to 2018. The patients did not receive preoperative therapy and gave informed consent to the use of their biological material for the research. According to the conclusion of the commission on bioethics R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, during the study all the necessary ethical standards were observed in accordance with the requirements of generally accepted international rules in the framework of the Declaration of Helsinki 2008.

Morphological diagnosis was verified on histological specimens stained with hematoxylin and eosin. The tumor differentiation grade was determined by morphological analysis of histological specimens according to WHO criteria [28].

Real-time polymerase chain reaction (PCR) was used to assess microRNA expression. Total RNA was isolated from ECE samples using a commercial RIBO-prep kit (Amplinsense, Russia). The amount of isolated RNA was determined using NanoDrop 2000c Spectrophotometer (ThermoScientific, USA).

The purity of the isolated RNA was assessed by the ratio of optical absorption values at 260 and 280 nm wavelengths. RNA was dissolved in Tris-EDTA buffer and stored at –80 °C until use. cDNA on RNA template was synthesized using a commercial kit “Reverta-L” (Amplisense, Russia) according to the manufacturer’s protocol. PCR was performed using AppliedBiosystems 7900HT FastRealTime PCR System and the primers listed in Table 1.

The SYBR Green MasterMix M02 kit (UkrGen-Tech, Ukraine) was used to prepare the reaction mixture for PCR. After 12 min of preheating at 95 °C, 25 cycles of amplification were performed as follows: denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. RNU48 microRNA was used as an endogenous control to objectify expression parameters (primer for cDNA synthesis: 5’-CTCTGACC-3’, Forward 5’- AGTGATGATGACCCAGGTAAGTC-3’, Reverse 5’- CTGCGGTGATGGCATCAG — 3’).

The experiment was performed in triplicate for each sample. The change in miRNA expression was calculated by the formula $2^{-\Delta C_t}$, where ΔC_t is the difference in C_t between the studied miRNA and miRNA of endogenous control. The mean for

Table 1. Primers for microRNA

MicroRNA	Sequence
RNU48	5'-CTGCGGTGATGGCATCAG-3'
miR-34a	5'-GTGTGGCAGTGTCTTAGCT-3'
miR-101	5'-GTCACCTGTAGTGTTCCTACTT-3'
miR-125b	5'-GGATTCCCTGAGACCCTAAC-3'
miR-142	5'-GAGGGGTACAGTACTGTGATA-3'
Universal reverse primer	5'-GTGCAGGGTCCGAGGT-3'

triplicates cycle threshold value (C_t) of the studied microRNA was normalized relative to C_t of endogenous control (hereinafter — a.u.). The change in miRNA expression compared to the control was calculated by the formulas of $2^{-\Delta C_t}$.

The proliferative activity of the studied endometrial carcinoma was determined by the proliferation index (PI, %), using the method of flow cytometry [5]. The study was performed on a flow cytometer EPICS-XL (Beckman Coulter, USA).

Microvessels were detected using immunohistochemical method by expression of vascular endothelial marker — antigen CD31 with monoclonal antibody to CD31, clone EP78 (Diagnostic BioSystems, Netherlands). PolyVue HRP/DAB visualization system (Diagnostic BioSystems, USA) was used. 3-diaminobenzidine tetrachloride was used as a chromogen. Cell nuclei were stained with Mayer's hematoxylin.

To determine microvascular density (MVD) in endometrial tumors, the number of vessels in 10 fields of view of the microscope was counted at magnification $\times 100$ (the size of one field of view was limited by a measuring square grid with a side of 1.25 mm). MVD (number of vessels/mm²) was determined by the formula: $MVD = n : 1.56$ vessels/mm², where (n) — the average number of vessels per field of view; 1.56 mm² — area of one field of view. The criteria for evaluating the mentioned indices were as follows: the values of the corresponding marker less than the median (Me) was considered low, and greater or equal to Me — high.

In addition, the obtained data were compared with the results of our previous studies, which determined the expression of EMT markers in the same cases of ECE [6, 29].

Statistical processing was performed using the software package Statistica 8.0 (StatSoft, Inc.) using non-parametric criteria (Mann — Whitney U Test). The differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Morphological analysis of samples of surgical material revealed that all studied tumors were ECE, of which 23 (45.1%) were of moderate (G2) and 28 (54.9%) of low (G3) differentiation grade, 24 (47.1%) tumors invaded $< 1/2$ of the myometrium and in 27 (52.9%) cases there was a deep

($> 1/2$) invasion of the tumor into the myometrium. The studied ECE cases were characterized by variability of PI (8.5–69.2%), on average this index was $31.9 \pm 9.2\%$ (median value (Me) 28.9%).

The mean values of the expression level of the studied miRNAs in ECE were estimated as follows: miR-34a — 10.3 ± 1.7 ; miR-125b — 10.1 ± 1.7 ; miR-142 — 0.30 ± 0.04 and miR-101 — 0.15 ± 0.01 a.u. When comparing the expression of miR-34a, miR-125b and miR-142 with ECE progression indices, it was found that their expression decreased in highly proliferating neoplasms compared with that in tumors with PI $< Me$. In the endometrial tumors with high PI, the expression of miR-34a and miR-142 significantly decreased. miR-101 expression did not depend on whether PI $< Me$ or PI $> Me$ tumors were analyzed (Table 2).

The same direction of changes in the expression of the studied miRNAs in ECE depending on the differentiation grade and the depth of tumor invasion into the myometrium was established. The expression of all investigated miRNAs in G3 tumors was lower than in G2 carcinomas. It should be noted that the expression of miR-34a, miR-125b and miR-142 tended to decrease, and miR-101 significantly decreased by 2.4 times. In ECE that deeply invaded the myometrium, the expression of miR-34a, miR-125b and miR-142 was also reduced compared to tumors with $< 1/2$ myometrial invasion. The most significant changes were observed in the level of miR-101 expression: in tumors with myometrial invasion $> 1/2$, the expression of the marker was reduced by 2.9 times.

It is necessary to note that the expression of miR-34a, miR-125b, miR-142 and miR-101 significantly decreased in tumors of patients with ECE stage III, i.e. in the case of metastasis in regional lymph nodes, and amounted to 3.5 ± 0.1 ; 5.4 ± 0.1 ; 0.1 ± 0.03 and 0.04 ± 0.001 compared with tumors in patients with stage I–II (14.0 ± 0.5 ; 12.3 ± 0.2 ; 0.3 ± 0.006 and 0.3 ± 0.004).

Therefore, we can conclude that the most significant decrease in expression is characteristic of miR-34a, miR-142 and miR-101, which indicates an impairment of their suppressive function in course of ECE progression. To substantiate this conclusion, we compared the expression of miRNAs with such indices of ECE progression as MVD

Table 2. Expression of miR-34a, miR-125b, miR-142 and miR-101 in ECE depending on morphological and functional characteristics

Clinical and pathological parameters	MicroRNA expression level, M $\pm$ m, a.u.			
	miR-34a	miR-125b	miR-142	miR-101
PI $< Me$	13.3 ± 1.9	8.3 ± 1.7	0.43 ± 0.03	0.20 ± 0.02
PI $> Me$	$7.5 \pm 1.3^*$	5.7 ± 1.4	$0.16 \pm 0.02^*$	0.19 ± 0.01
Differentiation grade ECE				
G2	11.4 ± 1.9	11.0 ± 1.8	0.41 ± 0.03	0.19 ± 0.01
G3	7.8 ± 1.6	7.9 ± 1.7	$0.27 \pm 0.02^{**}$	$0.08 \pm 0.003^{**}$
Depth of tumor invasion into the myometrium				
$< 1/2$	12.5 ± 1.8	12.0 ± 1.9	0.31 ± 0.01	0.23 ± 0.02
$> 1/2$	8.2 ± 1.4	8.6 ± 1.4	$0.16 \pm 0.01^{***}$	$0.08 \pm 0.001^{***}$

Note: * $p < 0.05$ compared with the index at PI $< Me$; ** $p < 0.05$ compared with G2 tumors; *** $p < 0.05$ compared with tumors invading $< 1/2$ of the myometrium.

and the presence of EMT signs. It was found that high ($> \text{Me}$) indices of MVD (> 27.6 vessels/ mm^2) were associated with a significant decrease of the expression of miR-125b (2.6 times) and miR-101 (almost 2-fold) (Figure). At the same time, the expression of miR-34a in ECE with high MVD was insignificantly higher (12.4 ± 1.4 a.u.) compared with endometrial carcinomas with low MVD (9.2 ± 1.4 a.u.).

In addition, we compared the expression of miR-34a and miR-101 in ECE cells depending on the expression of EMT markers — E-cadherin and vimentin [6, 29]. In ECE with signs of EMT (E-cadherin $< \text{Me}$; vimentin $> \text{Me}$) expression of miR-34a and miR-101 decrease significantly as compared with ECE without signs of EMT (E-cadherin $> \text{Me}$; vimentin = 0), which may indicate the increased migration capacity of endometrial tumor cells in these cases (Table 3).

One of the key functions of miR-34a is its influence on the expression of L1CAM and CD44 adhesion proteins [24, 30]. miR-101 affects transcription factors ZEB1 and ZEB2, which inhibit the expression of E-cadherin [31], determining the migration and invasion of tumors cells.

Thus, the results of the study indicate the variability of the effects of individual suppressor miRNAs on the progression of ECE, which is probably related to the morphofunctional polymorphism of these tumors. In particular, the decrease in miR-142 expression that we have determined may be associated with high cyclin D1 expression. It was found that the relative expression of miR-142 in endometrial carcinomas with low ($< \text{Me}$) expression of cyclin D1 was higher (0.40 ± 0.02 a.u.) compared to its expression (0.20 ± 0.01 a.u.) in ECE with high ($> \text{Me}$) expression of cyclin D1 [5]. A similar view is also expressed by Su *et al.* [17], who showed that increased expression of miR-142 might be accompanied by suppression of the proliferation by inhibition of the cell cycle activator — cyclin D1 protein. The authors noted that the low level of expression of this miR-142 may be an indicator of an unfavorable prognosis.

It should be noted that, despite the similar patterns of the changes in the expression of the studied miRNAs with the proliferative potential, the

differentiation grade and depth of tumor invasion into the myometrium, we faced some ambiguities comparing the expression of miRNA-34a, miRNA-125b and miRNA-125b with MVD. Thus, miRNA-34a expression was almost unchanged depending on MVD in ECE which contradicts the data of other researchers who showed that this miR-34a is able to inhibit the expression of *VEGF* through the Notch1 signaling pathway [25, 33].

In addition, the data on the expression of miRNA-125b and miRNA-101 in ECE with high and low values of MVD coincided with the literature data, according to which high expression of miRNA-125b leads to regression of blood vessels, and miRNA-101 may reduce angiogenic potential [23, 34–36].

MiR-34a is considered to be one of the important regulators of tumor suppressor gene activity. According to Wang *et al.* [25] and Misso *et al.* [37], high expression of miR-34a in ECE is a marker of favorable prognosis, because miR-34a inhibits the mRNA of the oncogene *c-MYC* and a number of signaling molecules involved in proliferation and migration of tumor cells. Our results indicate that the level of miR-34a expression was significantly reduced in highly proliferating ECE with a decrease in the differentiation grade, deep tumor invasion into the myometrium and in tumors with signs of EMT, i.e. with the progression of endometrial tumors, which reflects the impairment of the p53 signaling pathway, which, in our opinion, is a priority regulator of proliferative activity in the first pathogenetic variant of endometrial cancer [38–40].

In addition, it was found that in endometrial tumors with high expression of *c-MYC* oncogene [41, 42] miR-34a expression is reduced as compared with ECE expressing *c-MYC* at low levels. The obtained results are in agreement with the data of other authors, who believe that high expression of miR-34a is associated with a more favorable course of this form of cancer [25, 37].

Table 3. Comparison of miR-34a and miR-101 expression in ECE cells depending on the expression of EMT markers

The expression level of EMF markers, above or below Me	Expression of miR-34a (a.u.)	Expression miR-101 (a.u.)
ECE without signs of EMT	$9.9 \pm 1.4^*$	$0.16 \pm 0.02^*$
ECE with signs of EMT	4.4 ± 0.3	0.08 ± 0.01

Note: $*p < 0.05$ compared with expression in ECE with signs of EMT.

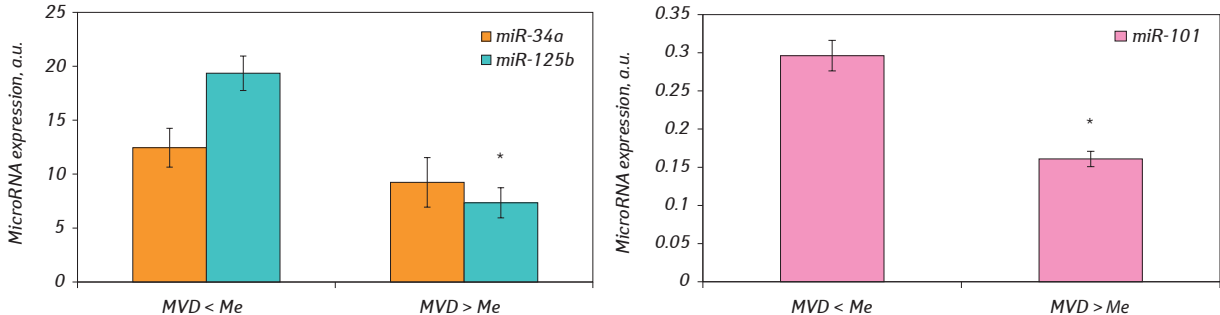


Figure. Comparison of the expression of miR-34a, miR-125b and miR-101 in ECE with low ($< \text{Me}$) and high ($> \text{Me}$) MVD. $*p < 0.05$ compared with the index at MVD $< \text{Me}$

Thus, the study revealed significant heterogeneity in the expression of miR-34a, miR-125b, miR-142 and miR-101 in such morphological variant of endometrial cancer as ECE. Given the results of our previous studies, which showed that the aggressive forms of ECE are characterized by certain morphological and molecular features and changes in the components of the tumor microenvironment, it should be noted that the analyzed suppressor miRs are significant components of the integrated mechanism of ECE progression [40, 43]. Undoubtedly, the question of epigenetic regulation of the ECE progression with involvement of miR with oncogenic functions remains open.

The study expands our understanding of the mechanisms of tumor progression in the endometrium and provides the basis for determining objective prognostic markers of ECE.

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