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ASSESSMENT OF CIRCULATING miRNA LEVELS IN BREAST CANCER PATIENTS DEPENDING ON CLINICAL CHARACTERISTICS AND CHEMOTHERAPY

Background. Breast cancer (BC) stands out as the most prevalent cancer in women. The levels of miRNA expression before and after chemotherapy are considered a potential indicator for the prognosis of the disease. **Aim.** To study blood plasma miRNA levels in BC patients and to assess their correlation with the menopausal status, disease stage, and molecular BC subtype. **Materials and Methods.** Blood plasma levels of 6 miRNAs (miRNA-25, miRNA-27, miRNA-155, miRNA-200, miRNA-335, and miRNA-497) were studied in 70 BC patients and 18 healthy individuals using RT-PCR. **Results.** miRNA-25, miRNA-335, and miRNA-497 levels were significantly higher in BC patients, while a tendency toward a decrease in the miRNA-27 and miRNA-335 levels in premenopausal patients and high miRNA-27 levels in menopausal patients was established. After neoadjuvant chemotherapy, a decrease in the miRNA-25 and miRNA-335 levels was registered. **Conclusions.** The results indicated that miRNA-25, miRNA-27, miRNA-335, and miRNA-497 deserve attention as markers for assessing the efficacy of treatment of BC patients.

Keywords: breast cancer, miRNA, blood plasma, chemotherapy.

Breast cancer (BC) accounts for 6%—20% of all cancer-related mortality in women [1]. While the number of patients diagnosed with BC has been rising, the mortality rate has stabilized due to advancements in treatment [2].

The spotlight in cancer research has swung towards miRNAs. These short, non-coding RNA molecules are capable of fine-tuning gene expression and play a role in oncogenesis; also they have potential as prognostic markers in cancer

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patients [3, 4]. In varying ways, miRNAs can affect the processes of cancer invasion and metastases. In particular, they can regulate the release of growth factors such as TGF-β, which affects early-stage tumor cell growth and proliferation and promotes the epithelial-mesenchymal transition. Additionally, they can interact with the Wnt/β-catenin pathway, leading to a decrease in E-cadherin expression, the loss of which is associated with oncogenesis [5]. Several miRNAs that facilitate these processes include miRNA-21, miRNA-191, miRNA-10b, and miRNA-155 [5].

Certain miRNAs have been identified as reliable BC biomarkers, particularly, miRNA-30a, miRNA-27a, miRNA-193b, and miRNA-361-5p [4].

Table 1. **Clinicopathological characteristics of BC patients**

Characteristics	Number of patients	
	n	%
Number of patients	70	100
Average age, years	48 ± 4.1	
Reproductive function		
Premenopausal	25	35.7
Menopausal	45	64.3
Stage		
I	23	32.8
II	27	38.6
III	20	28.6
Category N according to TNM		
N_0	24	34.3
N_{1-3}	46	65.7
Histological subtype		
Ductal invasive carcinoma	44	62.9
Lobular invasive carcinoma	18	25.6
Other subtypes	8	11.5
Differentiation grade		
G1	11	15.7
G2	43	61.4
G3	16	22.9
Molecular subtype		
Luminal A	27	38.6
Luminal B	28	40.0
Basal	15	8.6

Copious miRNAs, such as miRNA-17, miRNA-20b, miRNA-21, miRNA-23, miRNA-24, miRNA-27, miRNA-199, miRNA-200, miRNA-342, miRNA-484, and miRNA-638 are associated with resistance to chemotherapy [1, 6]. The potential relationship between miRNA expression levels and the patient's age has been studied, taking into account the molecular subtype of BC, tumor invasiveness, and ability to metastasize [3, 7]. Thus, miRNAs are promising candidates as BC biomarkers, which could increase the diagnostic accuracy and specificity, especially when used in combined panels [5]. However, to discern the relationship between changes in miRNA expression in BC, multipoint and coherent studies should be conducted.

The aim of the study was to investigate the circulating miRNA levels in BC patients and to examine the relationships among miRNAs, menopausal status, disease stage, and the molecular BC subtype.

Materials and Methods

The study was based on the evaluation of levels of 6 miRNAs (miRNA-25, miRNA-27, miRNA-155, miRNA-200, miRNA-335, and miRNA-497) in the blood plasma of 70 patients with stage I—III BC who were treated at the Kyiv City Clinical Oncology Center during 2017—2022 and 18 healthy individuals. All participants gave informed consent for the use of clinical data for scientific purposes. The general clinical characteristics of the patients are presented in Table 1.

The patients underwent from 4 to 6 courses of polychemotherapy: 47 neoadjuvant polychemotherapy (NPCT) in 47 patients and 23 adjuvant polychemotherapy (APCT) in 23 patients. Repeated assessments of miRNA levels after NPCT were conducted in 18 of 47 patients. The molecular BC subtype was established based on the immunohistochemical results.

RNA extraction was carried out using QuickRNA Miniprep Kit (Zymo Research, USA) ac-

cording to the manufacturer's instructions. Endogenous internal control *TBP* and *YWHAZ* gene fragments were used for data normalization. Primer sequences were taken from origene.com resource and synthesized by «UkrGenTech» (Ukraine). For reverse transcription, a one-step mix with revertase and polymerase, namely M08 «One-step RT mix with SYBR Green intercalating dye» «UkrGenTech» (Ukraine), was used according to the manufacturer's instructions. A Bio-Rad CFX-96 amplifier (Singapore) was used for the analysis. The assessment of miRNA expression was performed as copies/ml using Bio-Rad CFX Maestro software.

Statistical analysis was performed using the StatPlus (7.0) software package (AnalystSoft Inc.). The Shapiro — Wilk test was used to test the hypothesis of normal data distribution. To determine the significance of intergroup differences, the non-parametric Mann — Whitney U-test, non-parametric Wilcoxon test, and univariate variance analysis (ANOVA, Fisher's LSD test) were used. The differences were considered significant at $p < 0.05$.

Results and Discussion

Circulating miRNA-25, miRNA-27, miRNA-155, miRNA-200, miRNA-335, and miRNA-497 levels were analyzed in healthy donors and BC patients (Table 2) [1]. No significant differences between the groups were revealed. However, if one accounted for the menopausal status, the picture revealed several differences between the groups.

In healthy women, miRNA-25 levels increased with menopausal status, while in BC patients, miRNA-27 and -335 levels were significantly increased in postmenopause and a trend toward an increase in the miRNA-155 and miRNA-200 levels was observed. Concurrently, miRNA-335 levels in premenopausal BC patients were markedly higher compared to healthy women.

We attempted to identify the relationship between the miRNA levels, treatment tactics, and menopausal status (Table 3). The variations of miRNA-27 and miRNA-497 levels in BC patients in the general patient sample depended on APCT or NPCT treatment and the menopausal status. The menopausal status impacted changes in miRNA-27 and miRNA-497 expression in equal measure for the groups receiving APCT or NPCT. Particularly, the levels of both miRNA-27 and -497 levels were significantly lower in premenopausal women after APCT. At the same time, after NPCT, the miRNA-27 level decreased and miRNA-497 increased within the menopausal sample status.

No reliable relation was found between the levels of miRNA-25, miRNA-27, miRNA-155, miRNA-200, miRNA-335, and miRNA-497 in the blood plasma of BC patients and the stage, grade, and molecular subtype of BC (data not present).

We have also evaluated the changes in the studied miRNA levels after NPCT cycles as a measure of the effectiveness of chemotherapy. Table 4 presents the results of the first and second assessments; the statistical difference was calculated by the non-parametric Wilcoxon test. A statistically significant decrease in miRNA-25 levels was detected after the treatment, and a tendency toward a decrease in the miRNA-335 levels was noted.

The role of miRNA in the development of BC is becoming more obvious and this relationship opens an opportunity for the development of new diagnostic methods and prognostic factors that can improve the treatment results. miRNAs modulate the "stemness" of embryonic stem cells, including cancer stem cells. Namely, miRNA-139-5p has been evaluated as a BC metastasis inhibitor [8]. High levels of miRNA-29b correlate with such features as invasive lobular cancer, stage IV, presence of metastases, and recurrence [9]. The decreased miRNA-139 levels can be rela-

[1] The detailed data with their statistical processing are posted as Supplementary materials at https://harashchenko.com/content/articles/25/tables_en_1.pdf

Table 2. **Average circulating miRNA levels in healthy women and BC patients depending on menopausal status, copies/ml**

miRNAs	Healthy women (n = 18)			BC patients (n = 70)		
	Premenopausal (n = 12)	Menopausal (n = 6)	Total (n = 18)	Premenopausal (n = 25)	Menopausal (n = 45)	Total (n = 70)
miRNA-25	1.2	6.7*	3.0	11.0	35.2	26.2
miRNA-27	2310.0	1.4	1540.0	105.1#	321.5*	237.5
miRNA-155	72.4	3.2	49.3	33.3	62.2	51.3
miRNA-200	88.5	55.4	77.5	172.6	311.9	259.6
miRNA-335	895.3	17.9	602.8	10899.0#	233.0*	6937.3
miRNA-497	3.0	7.8	4.6	112.0#	44.8	70.0

Notes: *$p < 0.05$ compared to premenopausal women by Mann — Whitney test; #$p < 0.05$ compared to healthy women by Mann — Whitney test.

Table 3. **Average circulating miRNA levels in BC patients depending on menopausal status and treatment tactics, copies/ml**

miRNAs	Type of chemotherapy (n = 70)			
	APCT (n = 23)		NPCT (n = 47)	
	Premenopausal (n = 9)	Menopausal (n = 14)	Premenopausal (n = 16)	Menopausal (n = 31)
miRNA-25	1.6	3.0	17.4	49.8
miRNA-27	196.4	959.9*	53.8	33.2*
miRNA-155	1.0	50.6	36.7	67.4
miRNA-200	45.7	128.9	265.4	394.5
miRNA-335	577.1	121.0	145.2	15766.4
miRNA-497	11.3	34.9*	176.7	49.2*

Notes: *$p < 0.05$ compared to premenopausal women by the Mann — Whitney test.

Table 4. **Circulating miRNA levels after NPCT, copies/ml**

miRNAs	BC patients	
	First assessment (n = 18)	Second assessment (n = 18)
miRNA-25	61.1	1.6*
miRNA-27	12.7	3083.6
miRNA-155	94.3	21.5
miRNA-200	239.1	243.1
miRNA-335	27123.3	466.4†
miRNA-497	79.6	35.0

Notes: *$p < 0.05$ as compared to the first assessment by Wilcoxon's test; †$p < 0.05$ as compared to the first assessment by Fisher's LSD test.

ted to Her2/neu expression [10]. A correlation between miRNA-9, miRNA-21, miRNA-126, miRNA-200, miRNA-205, and miRNA-335 and a high risk of metastasis and a poorer prognosis has been established [10—12]. To increase the BC treatment effectiveness, the multiple miRNA changes should be investigated with the development of diagnostic panels of the most significant miRNAs relating to BC molecular subtype and their potential relationship with tumor chemosensitivity [13]. Generally, the detection of the correlations between the miRNA expression and the clinical BC patterns deserves further studies.

Currently, there are scarce data on the changes of miRNA-25 and -27 after chemotherapy of BC. Anwar et al. [14] noticed a significant decrease in the serum miRNA-155 levels in BC patients after surgical treatment and chemotherapy. Fischer et al. [15] established that miR-200 levels in the blood of BC patients were affected by neither APCT nor NPCT. Ali et al. [16] demonstrated that miRNA-335 decreased significantly in BC patients as compared to healthy individuals [17]. The same was established for miRNA-497. These data did not align with our results, and this matter requires more thoughtful examination.

To sum up, based on the analysis of the plasma levels of 6 miRNAs studied, namely miRNA-25, miRNA-27, miRNA-155, miRNA-200, miRNA-335, and miRNA-497, in healthy women and BC patients, we suppose that these miRNAs could be considered potential diagnostic BC markers useful for the assessment of the effectiveness of BC treatment.

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ОЦІНКА ЗВ'ЯЗКУ РІВНІВ ЦИРКУЛЮЮЧИХ мікроРНК
ІЗ КЛІНІКО-ПАТОЛОГІЧНИМИ ХАРАКТЕРИСТИКАМИ ТА ЧУТЛИВІСТЮ
ДО ХІМІОТЕРАПІЇ ХВОРИХ НА РАК МОЛОЧНОЇ ЗАЛОЗИ

Стан питання. Рак молочної залози (РМЗ) є найпоширенішою онкопатологією в жінок у світі. Сьогодні рівні експресії мікроРНК до і після хіміотерапії розглядаються як потенційний індикатор, що може вказувати на перебіг різних онкопатологій, включаючи РМЗ. **Мета.** Дослідити рівні мікроРНК у плазмі крові хворих на РМЗ та оцінити їх зв'язок із менопаузальним статусом хворих, стадією захворювання та молекулярним підтипом новоутворень. **Матеріали та методи.** Рівні 6 мікроРНК у плазмі крові (мікроРНК-25, -27, -155, -200, -335 та -497) досліджено у зразках 70 хворих на РМЗ та 18 здорових осіб з використанням методу полімеразної ланцюгової реакції в реальному часі. **Результати.** Встановлено, що рівні мікроРНК-25, -335 і -497 були достовірно вищими у хворих на РМЗ порівняно із аналогічними показниками в жінок без онкопатології. Продемонстровано тенденцію до зниження рівнів мікроРНК-27 і мікроРНК-335 у пацієнток із пременопаузою та підвищення рівнів мікроРНК-27 у пацієнток із менопаузою. Виявлено зниження рівня мікроРНК-25 та мікроРНК-335 у плазмі крові хворих на РМЗ після неоад'ювантної хіміотерапії. **Висновки.** Отримані результати свідчать, що мікроРНК-25, мікроРНК-27, мікроРНК-335 і мікроРНК-497 можуть використовуватися як маркери для оцінки ефективності лікування у хворих на РМЗ.

Ключові слова: рак молочної залози, мікроРНК, плазма крові, хіміотерапія.