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ASSOCIATION OF CIRCULATING MIR-21, -205, AND -182 WITH RESPONSE OF LUMINAL BREAST CANCERS TO NEOADJUVANT FAC AND AC TREATMENT

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Aim: **To identify the association of serum miRNAs with neoadjuvant polychemotherapy response in patients with breast cancer of luminal A and B subtypes.** ***Materials and Methods:*** **We analyzed the expression levels of circulating miR-21, -155, -182, -373, -199a, -205, -375 in serum of 182 breast cancer patients using real-time polymerase chain reaction. Each case was characterized by TMN criteria using morphological and immunohistochemical analyses.** ***Results:*** **Serum levels of miR-205 and -375 are associated with the response of luminal A tumors and miR-205 and -21 with the response of luminal B tumors to neoadjuvant polychemotherapy in fluorouracil + doxorubicin + cyclophosphamide and doxorubicin + cyclophosphamide regimens. In addition, we found correlation of miR-155, -182, -199a, -375 with 3-year relapse-free survival of patients. Based on the obtained data, we developed innovative prognostic and predictive panels to assess the drug sensitivity of tumors and lower the risk of breast cancer recurrence, which would significantly improve the treatment outcomes and the quality of life of patients.** ***Conclusions:*** **Serum levels of miR-155, -182, -199a, -205, -375 can be used as predictive and prognostic panel for monitoring BC course in Ukrainian population.**

Key Words**: miRNA, breast cancer, resistance.**

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The current trend in cancer treatment is the development of non-invasive prognostic procedures based on molecular analysis techniques examining the samples of biological fluids, primarily circulating blood [1, 2]. This approach does not require material from the tumor tissue and does not depend on the localization of the tumor [3]. Particular attention is given to miRNAs as they are the major regulators of genes involved in carcinogenesis [4]. Much of the research in recent years has been devoted to the study of changes in miRNA expression in various pathological conditions including cancer [5].

The relatively inexpensive and highly sensitive real-time polymerase chain reaction (RT-PCR) method for assaying miRNA is now used widely in routine laboratory practice. miRNA is an ideal biomarker that can be promptly implemented in clinical practice, as it does not require additional equipment and staff training.

The impairment of miRNA expression may be caused by mutations or methylation of genes encoding miRNAs. miRNAs, depending on their role in carcinogenesis, are conditionally divided into oncogenic and oncosuppressive. A miRNA is oncogenic if its target is an oncosuppressor gene, and, conversely, a miRNA is oncosuppressive if its target is an oncogene. The same miRNA can be both oncogenic and tumor-suppressive depending on the target gene as well as on the histological origin of the cells in which it is expressed [6]. Increasing level of oncogenic miRNAs leads to increased proliferation, invasion, angiogenesis and/or reduced apoptosis activity and a differentiation level that promotes the formation of malignant tumors. Increased expression of oncosuppressive miRNAs, on the contrary, inhibits the growth and migration of tumor cells and promotes the induction of apoptosis [7–9]. According to the literature as well as the results of our own studies, the changes in the expression of a specified set of miRNAs in different types of cancer correlate with tumor progression [10–12].

Numerous studies in recent years have shown that the development of malignant tumors is accompanied by a change in the ratio of specified miRNAs not only in tumor cells but also in biological fluids (circulating miRNAs) [13]. Providing communication between tumor and host, these miRNAs are involved in the systemic response of the organism to the development of a neoplasm. The circulating miRNAs are stable — they are included in lipoprotein complexes, apoptotic bodies, microvesicles, or exosomes that protect them from nucleases [14]. Due to such properties, miRNAs can be easily detected by routine molecular methods. The stability of these molecules and changes in the level of their expression in oncogenesis make it possible to use extensively the circulating miRNAs as markers for the diagnosis and prognostic purposes in cancer treatment [14, 15].

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Abbreviations used: AC – doxorubicin + cyclophosphamide; BC – breast cancer; FAC – fluorouracil + doxorubicin + cyclophosphamide; IHC – immunohistochemical; NPCT – neoadjuvant polychemotherapy.

Numerous basic studies in recent years as well as the clinical observations demonstrated that circulating miRNAs as prognostic markers are not less informative than the corresponding target proteins in primary tumor cells [16]. That is why numerous miRNAs, involved in regulation of P-glycoprotein, glutathione S-transferases and other resistance-related proteins are the objects of high interest [17].

Breast cancer (BC) therapy involves several neoadjuvant and adjuvant regimens. In addition, in the presence of hormonal receptors in tumor tissue, hormonal therapy are used. Combinations of anthracyclines such as doxorubicin, daunomycin and epirubicin, and taxanes such as paclitaxel and docetaxel represent the most common treatment strategies for common BC. Among the neoadjuvant regimens, combinations of paclitaxel, docetaxel, doxorubicin, epirubicin, cyclophosphamide, and fluorouracil are most commonly used [18].

The prevalence of BC (the third place in female deaths worldwide) has contributed to the active search for miRNAs associated with the development of drug resistance in this form of cancer. To date, a number of microRNA panels have been developed for neoplasms of this localization that are effectively used in clinical practice. In particular, there are more than 6,949 patents registered in the world for the use of circulating miRNAs as markers of BC course [19].

Zhao *et al.* [20] demonstrated that high levels of miR-221 in the blood plasma of BC patients are associated with a worse response to neoadjuvant polychemotherapy (NPCT) with anthracyclines and taxanes. Wang *et al*. [21] demonstrated that elevated levels of miR-4530 in blood serum of BC patients indicate high-efficiency NPCT based on taxanes and anthracyclines. Other researchers have found that using of circulating miR -125b, miR-19a, and miR-205 as indicators allows predicting the response of BC to chemotherapeutic treatment with anthracyclines and taxanes with high accuracy [22, 23]. Shao *et al*. [24] have demonstrated that the panel of three miRNAs (miR-200a, miR-210, miR-451) predicts with high accuracy (80%) the sensitivity to taxanes and anthracyclines in patients with advanced BC.

It should be noted that changes in serum miRNA levels are a secondary manifestation of the tumor process as these changes occur either as a result of the release of miRNAs from the tumors or as a result of the host response to the development of the tumor.

Thus, the above facts confirm the need to use circulating miRNAs to predict the course and monitor the effectiveness of BC therapy. Analysis of the current literature data indicates that the profile of circulating miRNAs is associated with the development and progression of BC, the course of the disease and the survival rates of patients [25].

Fundamental studies of the role of miRNAs in biological processes and their importance in the development of pathologies have allowed the formation of databases that collect information about the verified and experimentally confirmed target genes of most miRNAs, as well as the signaling pathways in which they are involved. There are many resources in which potential target mRNAs (www.mirdb.org, www.microrna.org, www.microrna.gr/tarbase, www.targetscan) are predicted *in silico* based on the already known data of the regulatory role of miRNAs (DianaTools, TargetScanHuman 7.2, etc.). There are also resources to collect experimentally validated data on the role of miRNAs in tumor growth (http://www.mirbase.org/, http://www.oncomir.org/). Based on the above resources, we have selected a panel of miRNAs that have been linked to breast carcinogenesis and proven to have oncogenic (miR-21, -155, -182, -373) and oncosuppressive (miR-199a, -375, -205) properties in BC. The aim of our study was to identify the association of serum miRNAs with NPCT response in patients with BC of luminal A and B subtypes.

MATERIALS AND METHODS

The study was based on a retrospective analysis of the results of the examination, treatment, and survival of 182 BC patients, stage II–III, who were treated at the Kyiv City Clinical Oncology Center during 2013–2017 and gave informed consent to the use of clinical data for scientific purposes. All samples were encoded and depersonalized.

The stage of cancer was determined according to the international tumor classifications (TNM, 7th edition, 2009). According to the case records, all patients were completely examined (the general clinical, biochemical, laboratory tests, ultrasound of the abdomen, mammography, X-rays of the chest cavity, puncture biopsy of tumors) according to the standards of diagnosis and treatment of cancer patients approved by the Ministry of Health of Ukraine (№ 140 of 27.07.1998; № 554 of 17.09.2007; № 645 of 30.07.2010; and № 396 of 30.06.2015).

Depending on the clinical indications, all BC patients, stage II–III underwent organ-saving surgery or radical mastectomy according to Madden and NPCT according to the standards of treatment approved in Ukraine. The treatment courses included 2–6 cycles of fluorouracil + doxorubicin + cyclophosphamide (FAC) or doxorubicin + cyclophosphamide (AC) regimen with an interval of 21 days. NPCT efficacy was evaluated every 2 cycles according to mammography according to RECIST 1.1 criteria.

General clinical characteristics of patients and the results of the morphological and immunohistochemical (IHC) study of tumors are presented in Table 1.

As can be seen from Table 1, the age of patients ranged from 24 to 81 years (mean age — 56.1 ± 5.4). The menstrual function was maintained in 80 (43.96%) of patients. BC stage II was in 110 patients and stage III — in 72 patients. A comprehensive examination (radiological, ultrasound, laboratory) revealed 112 (61.54%) cases with metastases in regional lymph nodes. Morphological examination of biopsy material revealed infiltrative ductal carcinoma in 125 (68.68%) patients and infiltrative lobular cancer in 57 (31.32%)

Table 1. General clinical characteristics of BC patients

Characteristics	Number of cases	
	N	%
The total number of patients	182	100
Age (years)		
Mean	56.1 ± 5.4	
Range	24–81	
Menstrual function		
Active menstrual cycle	80	43.96
Menopause	102	56.04
Stage (TMN)		
II	110	60.44
III	72	39.56
Lymph node metastasis (TMN)		
N0	70	38.46
N1–N3	112	61.54
Morphological type		
Infiltrative duct cancer	125	68.68
Infiltrative lobular cancer	57	31.32
Grade		
G1	42	23.08
G2	82	45.05
G3	58	31.87
Molecular subtype		
Luminal A	115	63.18
Luminal B	67	36.82

cases. The tumors differed by their grade, most of them were of moderate differentiation (45.05% of patients).

Based on IHC studies, we identified two molecular subtypes: luminal A (ER+, PR+, Her2/neu–) and luminal B (ER+, PR+, Her2/neu+). Most cases (115; 63.18%) were of luminal A molecular subtype.

Morphological examination, IHC analysis, RNA isolation, real-time polymerase chain reaction, and statistical analysis were performed as described earlier [26].

RESULTS AND DISCUSSION

Expression of circulating miRNAs in blood serum of BC patients with different clinical and pathological characteristics. We analyzed the expression of circulating miR-21, -155, -182, -373, -199a, -205, and -375 depending on the main clinical and morphological characteristics of BC patients (Table 2). The difference of miR-21, -155, and -182 expression in patients with stage II and stage III was found to be significant ($p < 0.05$). Furthermore, the level of miR-182 was significantly higher in patients with the involvement of the regional lymph nodes.

We examined the expression of circulating miR-21, -155, -182, -373, -199a, -205, and -375, depending on the molecular subtype of tumors, and found no significant difference in patients with luminal A and luminal B subtypes (Table 3).

Circulating miRNAs expression in patients with different sensitivity of luminal BC to NPCT. FAC and AC regimens are the most used regimens of neoadjuvant treatment. The allocation of patients with luminal A and luminal B subtypes to FAC or AC regimens of chemotherapy is shown in Table 4.

When the levels of miR-21, -155, -182, -373, -199a, -205, and -375 in serum of patients with varying outcome of NPCT (complete remission, partial response, no response or disease progression) were analyzed, miR-205 and -375 were shown to be most reliable markers for assessing the sensitivity of tumors of the luminal A subtype. Luminal A tumors in patients with high levels of miR-205 (> 4.0) and low miR-375 (< 0.3) were found to be more sensitive to FAC and AC regimens (Table 5). In most patients with serum levels of miR-205 in the range from 0.1 to 3.0 and miR-375 levels in the range of 0–0.2 worse response to treatment was observed. For miR-21, -155, -182, -373, and -199a no significant correlation with sensitivity of luminal A BC was established.

The study of blood serum of patients with luminal B subtype BC showed that samples with sensitive tumors were characterized by the expression of miR-21 and -205 below 2.0 and above 3.0, respectively. The levels of miR-21 above 3.0 and miR-205 below 2.5 were determined in the serum of patients with NPCT-resistant tumors (Table 6). For miR-155, -182, -373, -199a, and -375, the association between sen-

Table 3. Expression of serum miRNAs in patients with the different molecular subtypes of BC, fold change

miRNAs	Luminal A	Luminal B
	Oncogenic	
miR-21	4.2 ± 2.4	4.8 ± 1.8
miR-155	3.8 ± 1.3	2.9 ± 1.9
miR-182	4.6 ± 2.2	4.6 ± 2.1
miR-373	6.6 ± 1.3	5.7 ± 1.6
	Oncosuppressive	
miR-199a	0.3 ± 0.2	0.5 ± 0.2
miR-375	0.2 ± 0.1	0.1 ± 0.1

Table 4. Number of BC patients treated with different NPCT regimens

Molecular subtype	Number of patients
Luminal A	115
FAC	61
AC	54
Luminal B	67
FAC	34
AC	33

Table 2. Relationship of serum microRNAs expression with clinical and morphological features of breast cancer patients in the general group, fold change

miRNA	Oncogenic				Oncosuppressive		
	21	155	182	373	199a	375	205
				TNM stage			
II	3.1 ± 1.1	1.9 ± 0.8	3.5 ± 0.7	5.5 ± 1.1	0.5 ± 0.2	0.7 ± 0.6	2.7 ± 1.4
III	6.1 ± 0.5*	4.2 ± 0.8*	5.4 ± 1.0*	6.9 ± 1.1	0.2 ± 0.1	0.5 ± 0.3	2.2 ± 1.3
				Metastases to regional lymph nodes			
N0	3.5 ± 1.1	4.4 ± 0.4	2.9 ± 1.0	5.6 ± 0.8	0.5 ± 0.2	0.7 ± 0.6	2.1 ± 1.0
N1–3	5.4 ± 1.7	2.5 ± 0.4*	6.5 ± 1.9*	6.7 ± 1.0	0.3 ± 0.1	0.4 ± 0.2	3.5 ± 0.7
				Grade			
G1	3.7 ± 0.8	3.8 ± 0.5	3.3 ± 0.9	5.7 ± 1.0	0.5 ± 0.2	0.6 ± 0.6	2.0 ± 0.9
G2	4.5 ± 0.8	3.7 ± 0.9	3.9 ± 1.6	5.9 ± 1.0	0.4 ± 0.2	0.8 ± 0.6	2.2 ± 1.7
G3	5.1 ± 1.9	4.8 ± 0.5	4.9 ± 2.0	6.5± 2.2	0.3 ± 0.2	0.6 ± 0.6	2.9 ± 1.0
				Histological type			
Ductal cancer	4.5 ± 1.7	3.8 ± 1.2	4.5 ± 1.7	5.9 ± 1.6	0.4 ± 0.2	0.6 ± 0.4	2.1 ± 1.1
Lobular cancer	4.4 ± 2.5	3.5 ± 1.9	4.7 ± 1.7	5.7 ± 1.3	0.4 ± 0.3	0.6 ± 0.5	2.3 ± 1.57

*$p < 0.05$

Table 5. The distribution of patients with Luminal A subtype by the level of expression of circulating miRNAs depending on the clinical effect of NPCT

	Clinical evaluation of NPCT with RESIST criteria 1.1.			
Level of miRNA (fold change):	Complete remission n = 28/100%	Partial response n = 35/100%	Stable disease n = 30/100%	Disease progression n = 22/100%
205 > 4.0 375 < 0.3	28/100%	25/71.43%	6/20.0%	0
205 < 3.0 375 < 0.2	0	10/28,57%	24/80.0%	22/100%

Table 6. Distribution of patients with luminal B subtype by the expression level of circulating miRNAs depending on the clinical effect of NPCT

	Clinical evaluation of NPCT with RESIST criteria 1.1.			
Level of miRNA (fold change):	Complete remission n = 19/100%	Partial response n = 21/100%	Stable disease n = 14/100%	Disease progression n = 13/100%
205 > 3.0 21 < 3.5	28/100%	16/76.19%	2/14.28%	0
205 < 2.5 21 > 4.5	0	5/23.81%	12/85.72%	13/100%

sitivity of the luminal B subtype BC to NPCT was not detected.

Expression of circulating miRNAs and the survival of BC patients with various molecular subtypes. We analyzed the 3-year disease-free survival of BC patients taking into account the expression of miR-21, -155, -182, -373, -199a, -205, -375 in serum. In luminal A BC patients with the expression of miR-182 > 5.5, miR-199a < 0.2, 3-year relapse-free survival turned out to be lower than in patients with miR-182 < 5.1, miR-199a > 0.34 (Fig. 1). Disease relapses in the group of patients with luminal B tumors were observed 32.4% more often in cases with a profile of miR-155 > 3.0, miR-375 < 0.15 than in patients with expression of miR-155 < 1.5, miR-375 > 0.41 (Fig. 2). For other studied miRNAs, we found no correlation with survival rates.

To sum up, we profiled seven miRNAs in blood serum samples of 182 BC patients treated by NPCT in FAC and AC regimens. We have demonstrated the association of serum miR-205 and -375 levels with the sensitivity of luminal A BC to FAC and AC therapy. The prognostic value of miR-182 and -199a for patients with luminal A of BC has been proved. The expression levels of miR-205 and -21 in serum of patients with BC are associated with the sensitivity of tumors of the luminal B subtype to FAC and AC regimens. The correlation between the indicators of overall and disease-free survival of patients with luminal B BC with the levels of miR-155, -375 in the serum was established.

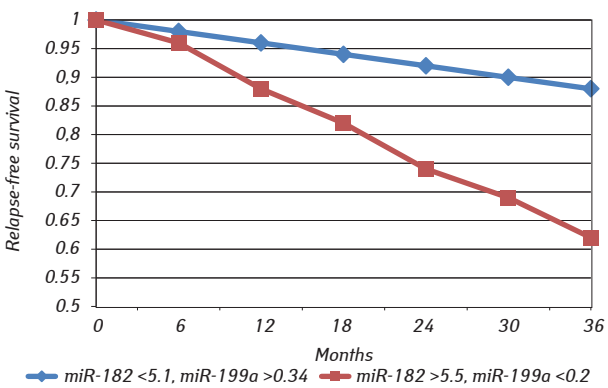

Fig. 1. 3-year relapse-free survival of patients with luminal A BC depending on miRNA expression in serum. Kaplan — Meier survival curve based on miRNAs expression (p = 0.005; Log-rank test)

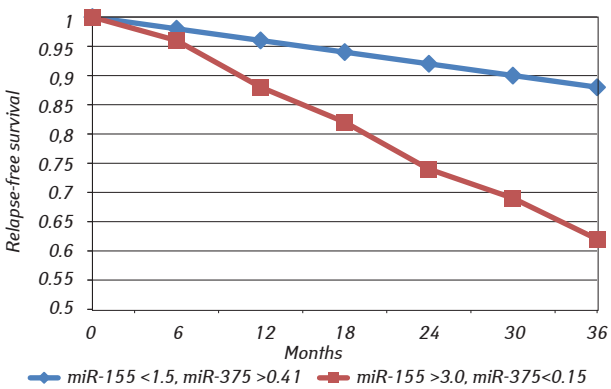

Fig. 2. 3-year relapse-free survival of patients with luminal B BC depending on miRNA expression in serum. Kaplan — Meier survival curve based on miRNAs expression (p = 0.005; Log-rank test)

In several studies the association of miR-21 [27], -155 [28], -182 [29], -373 [30], -199a [31], -205 [32], -375 [33] with clinical-pathological features of BC have been shown. In present study, only several characteristics such as stage and lymph node metastases were accompanied with statistically significant differences in specified miRNAs levels [34]. This inconsistency may be caused by population differences since most studies cited above were conducted in East-Asian populations, in several cases — in American populations characterized by the mixed gene pool in contrast to the local Caucasian population in our study. Nevertheless, an examination of the association of serum miRNA levels with response to NPCT proved that levels of miR-21, -205, -375 can be used as predictive markers for FAC and AC therapy regimens in different patients worldwide [35, 36].

Considering obtained results we can speculate that miR-155, -182, -199a, -205, -375 can be introduced into clinical practice after clinical trials on more diverse sample collection, as well as markers of relapse-free survival.

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