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Abstract.

Breast cancer is a heterogeneous disease with a variable clinical course, morphological and clinical features. In clinical observation, we attempted to study the correlation between clinical characteristics of breast cancer patients and plasma miRNA-497 concentration as a possible pathogenic molecular disease factor and a diagnostic indicator. It was established that miRNA-497 levels were significantly higher in the plasma of premenopausal compared to menopausal women, while the opposite is true for healthy women. We did not find a link between miRNA-497 levels and tumor size and clinical stage, though a weak positive correlation between miRNA-497 levels and the N0-N3 stage was noted, with a pronounced increase at the N3 stage, which was reliable in the group of patients after APCT. miRNA-497 levels after the first and second courses of NPCT did not differ statistically significant. There was no correlation between miRNA-497 concentration and the molecular subtype of breast cancer, and the difference between patients with HER2+ type and the triple-negative type was not convincing due to the small patient sample size. Also, no connection was found between the analyzed miRNA-497 levels and follow-up results, and positive initial results require additional research and analysis. In conclusion, analysis of miRNA-497 levels can be useful in the study of the molecular type and stage of breast cancer. Prospects for further research are in analyzing this indicator in a larger sample of breast cancer patients, obtaining remote follow-up results, and comparison with other types of miRNA.

Key words. miRNA-497, breast cancer, blood plasma, chemotherapy, metastasis.

Introduction.

miRNA is a set of heterogeneous non-coding small RNAs, which play a role in the regulation of gene expression of mRNA processing [1]. Disruptions in miRNA regulation can be a factor in the development and progression of oncologic diseases [2]. The number of studies that indicate the role of miRNA-497 in the development of breast cancer (BC) presented in the literature is insignificant [3]. In particular, the role of miRNA-497 in suppressing BC cell proliferation and invasion is already established [3,4], including triple-negative BC [5]. The level of miRNA-497 expression was higher in normal breast tissue compared to BC tissue [4]. Low miRNA-497 expression is closely correlated with a lower degree of tumor differentiation, positive HER2 expression, and a higher frequency of lymph node metastasis, progressing clinical stage, and lower overall 5-year patient survival rates [6,7]. The literature also presents conflicting data on the antitumor role of miRNA-497 in various breast cancer cell lines [8,9]. Though, there is data supporting the role of miRNA-497 as an oncogenesis suppressor, impaired synthesis of which can be a cause of the development of oncological disease. For the possibility of explaining the role of miRNA-497 in the development of breast cancer, and the prognostic value of its concentration in blood plasma we conducted a study of levels of this indicator in the blood of patients with different stages of BC.

Aim of the study.

To study miRNA-497 levels in the blood plasma of BC patients in comparison to relatively healthy donors, and also the correlation of these levels with the clinical course of oncological disease. For the possibility of explaining the role of miRNA-497 as an oncogenesis suppressor, impaired synthesis of which can be a cause of the development of oncological disease. For the possibility of explaining the role of miRNA-497 in the development of breast cancer, and the prognostic value of its concentration in blood plasma we conducted a study of levels of this indicator in the blood of patients with different stages of BC.

Materials and Methods.

88 patients were included in the study, aged 30 to 74 years old: the main group consisted of 70 BC patients; comparison group included 18 people (relatively healthy women). Patients with BC in combined treatment received 4 courses of neoadjuvant polychemotherapy (NPCT) and 6 courses of adjuvant polychemotherapy (APCT). Median age at the time of diagnosis was 45 years old. The distribution of women by age up to 45 years and older was carried out. Tumor volume was calculated using the formula, \( V=(a\times b)^2 \times 2 \) [10], where \( a \) — tumor height; \( b \) — tumor width; \( c \) — tumor width. RNA extraction was carried out according to the manufacturer's instruction (Manufacturer: «Ukrgentech» (Ukraine); endogenous internal control – TBP and YWHAZ gene fragments covering the range from the smallest to the longest fragments.

Primer for Real-time PCR synthesized by Metabion (Germany), sequence:

One-step mix with reverse transcriptase and polymerase – M 08 «One-step RT mix with SYBR Green intercalating dye» «Ukrgentech» (Ukraine). Amplification according to the manufacturer's instructions. A Bio-Rad CFX-96 amplifier (Singapore) was used for the analysis. Bio-Rad CFX Maestro assay and quantification program.

Calibrators for quantification of internal controls:

1 – TCCCTTTGCCTTGCACTCCCCACAGACTATTTCTCTCA- TCCATATTACTGACCAAATCTCTCC – 60 bp
2 – TGACACAGGAGCCAGAGTGACACAACGAGTCAGAC- GACTGCGACAAATATGCTAGAAGTTTACAGAAGGCTGAC- GAGATTGATTCTCAGCTTGTGAGCCTTGATGTG - 132 bp
Quantitative assessment method:
1. An exogenous (added to the sample before extraction at a known concentration) synthetic internal control was used to estimate the level of extraction A035, by comparing the applied concentration and the concentration after the extraction in quantitative indicators calculated using the Bio-Rad CFX Maestro software. As a result, the extracted coefficient ranged from 0.7 to 0.9, samples in which the extraction coefficient was below 0.7 were subject to repeated extraction.
2. For quantification, synthetic calibrators were used as positive controls for each group of reactions separately in duplicates and two dilutions with a two-order difference of 100 and 10,000 copies, respectively, and using Bio-Rad CFX Maestro software, copy per reaction was obtained (duplicate, samples performed in doubles).
3. To determine the number of copies per milliliter: Copies/ml = Amount of miRNA in the reaction (instrument data in copies/reaction)/Extraction coefficient×Number of reactions from one eluate×Number of extractions from one ml – gene fragment.

Statistical analysis was performed using the SPSS 17.0 software package (SPSS, Chicago, Illinois, USA). Data were presented as mean ± SEM. Univariate analysis of variance (ANOVA, Bonferroni post hoc test) and Mann–Whitney U-test were used to determine the significance of intergroup differences. Spearman’s test was used to assess the correlation of miRNA-497 with clinical and pathological features of breast cancer. Differences were considered statistically significant if p<0.05.

Results.
miRNA-497 levels differed in pre- and postmenopausal women (<45 and ≥45 years old) (Table 1). If the difference in miRNA-497 levels was not significant in healthy women over 45 years old, it was significant for BC patients. In the general sample of patients under 45 years of age, a significantly higher rate was established (p=0,01), this pattern was confirmed in subgroups of patients who received APCT (p=0.05) or NPCT (p=0.02). At the same time, miRNA-497 levels were significantly higher in premenopausal than in menopausal women. These results can indicate a link between miRNA-497 expression level and the development of BC and the role of age factor in the probability of disease occurrence. The age dependence remains insufficiently understood, because of the small sample size, which is especially true of healthy women, that was investigated, but acquired results indicate a connection between plasma miRNA-497 levels and BC in premenopausal women.

The correlation of the BC clinical stage with miRNA-497 levels was analyzed and no difference was found between the 1st and subsequent stages of the disease (Table 2). At the same time, the probability of the difference in miRNA-497 levels between the groups of patients with the 2nd and 3rd stages in the APCT subgroup was p=0.06, and between the groups with the 1st and 3rd stages in the NPCT subgroup was equal to p=0.07. These results can be viewed as a correlation tendency of the breast cancer stage with an increase in plasma miRNA-497 levels. After clustering patients according to age, this trend was noted in the category of women under 45 years old, namely, the trend of plasma miRNA-497 level increase in the 3rd stage of cancer.

Correlational analysis was conducted (Spearman’s criterion) and the intergroup difference was assessed while taking into account the disease stage according to TNM classification. A weak positive correlation between miRNA-497 level and N stage was detected r=0,26; p=0,03. In the general patient sample, the miRNA-497 level increased statistically significantly at the N3 stage (the tendency was also present while comparing N0-N2), and the result of linear regression R2=0.04. There was no discrepancy between the compared treatment groups. Obtained results can be interpreted this way: there is a probability of a positive relationship between plasma miRNA-497 levels and breast cancer stage, though this statement requires further research on larger patient samples.

There was no association between miRNA-497 level and tumor size (r=0,11; p=0,35). In our observations, there is no difference in the general sample of the studied values as well as in the subgroups of the two treatment regimens. Although a trend towards an increase in miRNA-497 level was noted with a tumor volume ranging from 1.0 to 5.0 cm3 (at the same time, the value of variance increases significantly; hypothetically, the difference could be probable if there were more cases in the general sample).

Univariate analysis of variance and group differences according to the non-parametric Mann–Whitney test of miRNA-497 levels in cases of four molecular cancer subtypes were performed: luminal A (n=13), luminal B (n=34), HER2+ (n=4), triple-negative (n=19). No statistically significant difference was found between the comparison groups (high variance in each group). An insufficient sample of analyzed HER2+ cases does not allow for confidence in the obtained statistical assessment results in this molecular subtype patient group. That is, in this study, a reliable link between miRNA-497 and molecular subtype was not established.

We attempted to analyze the dependence of treatment results on the initial miRNA-497 level at the time of diagnosis (Table

| Table 1. miRNA-497 levels in breast cancer (BC) patients (Mean(SEM)×10⁴). |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Indicator/patient group     | Healthy women               | Breast cancer patients       |
| miRNA-497 level             | p <45 vs ≥45                | APCT                        | p <45 vs ≥45                | p healthy vs ill            | NPCT                        | p <45 vs ≥45                | p healthy vs ill            | All patients               |
| Age                         | n=18                        |                             |                             |                             |                             |                             |                             |                             |
| ≥45                         | 4,6(1,4)                    | 3,0(1,2) n=12               | 11,3(6,4)                   | 0,05                        | 176,8(85,0)                 | 0,02                        | 117,2(56,2)                 | 0,01                        |
| ≤45                         | n=6                         | 7,8(3,2)                    | 34,9(31,0)                  | **0,04**                    | 49,3(3,2)                   | **0,04**                    | 44,8(24,3)                  | 0,01                        | 0,18
increased miRNA-497 levels on stage N3 compared to N1-2, and in the group after APCT the difference was statistically significant (N0 vs N3 $p=0.02$; N2 vs N3 $p=0.05$).

Analysis results demonstrated a statistically significant link between miRNA-497 level and initial outcome as well as with 2-3-year treatment follow-up analysis, though the clinical significance of this data requires further research.

Repeated evaluation of plasma miRNA-497 levels after a course of NPCT ($n=18$) did not show a statistically significant difference between repeated observations ($p=0.50$).

Hence, there is a link between plasma miRNA-497 levels

3). For that purpose, patients were separated into two categories: stabilization, improvement, no recurrence or metastasis ($o0+$); deterioration, metastasis, relapse, and death ($o1+$). With the research factor set that way, a moderate correlation was found regarding the initial result ($r=0.30$; $p=0.01$) and the result of a 2-3-year follow-up ($n=70$) ($r=0.38$; $p=0.01$).

Patients with an unfavorable treatment outcome ($n=6$) had I ($n=1$), II ($n=1$), III ($n=2$), and IV ($n=1$) stages. It should be taken into count, that in observed patients a weak positive link ($r=0.25$; $p=0.04$) between miRNA-497 levels and regional lymph node involvement was present. In the BC group after NPCT a tendency toward

### Table 2. The difference in plasma miRNA-497 levels of breast cancer (BC) patients depending on clinical disease course and chemotherapy regimen (Mean(SEM)×10³).

<table>
<thead>
<tr>
<th>Indicator/miRNA-497 level</th>
<th>Breast cancer patient group and probability level</th>
<th>APCT</th>
<th>p</th>
<th>NPCT</th>
<th>p</th>
<th>All patients</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>9.3(5.9)</td>
<td>1.0(0.9)</td>
<td>0.33</td>
<td>94.9(53.4)</td>
<td>NA</td>
<td>138.0(84.3)</td>
<td>NA</td>
</tr>
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<td>2</td>
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<td>0.12</td>
<td>0.06</td>
<td>19.1(8.5)</td>
<td>NA</td>
<td>23.7(10.1)</td>
<td>0.07</td>
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<td>157.7(139.8)</td>
<td></td>
<td></td>
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<td>4</td>
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<td></td>
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<tr>
<td>Tumor size, cm³</td>
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<td>&lt;1.0</td>
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<td></td>
<td>33.0(30.1)</td>
<td>1.0</td>
<td>37.4(22.2)</td>
<td>1.0</td>
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<td>1.0-2.0</td>
<td>7.0(5.8)</td>
<td>224.1(211.3)</td>
<td>0.02</td>
<td>184.3(112.7)</td>
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<td>41.3(14.1)</td>
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<tr>
<td>2.0-5.0</td>
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<td>0.92</td>
<td>1.0</td>
<td>184.3(112.7)</td>
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<td>41.3(14.1)</td>
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<tr>
<td>≥5.0</td>
<td>54.2(47.9)</td>
<td>1.0</td>
<td>0.93</td>
<td>114.0(67.0)</td>
<td>1.0</td>
<td>17.7(15.8)</td>
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<tr>
<td>0</td>
<td>8.7(4.2)</td>
<td>196.9(165.6)</td>
<td>0.60</td>
<td>318.3(145.4)</td>
<td>218.3(145.4)</td>
<td>NA</td>
<td>0.03</td>
</tr>
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<td>39.8(25.6)</td>
<td>1.0</td>
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<td>218.3(145.4)</td>
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<tr>
<td>3</td>
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<td></td>
<td>18.1(12.9); Me=1,3</td>
<td>18.1(12.9); Me=1,3</td>
<td>NA</td>
<td>0.04</td>
</tr>
<tr>
<td>Luminal A</td>
<td>18.1(12.9); Me=1,3</td>
<td></td>
<td></td>
<td>18.1(12.9); Me=1,3</td>
<td>18.1(12.9); Me=1,3</td>
<td>NA</td>
<td>0.04</td>
</tr>
<tr>
<td>Luminal B</td>
<td>55.6(27.7); Me=3,5</td>
<td></td>
<td></td>
<td>55.6(27.7); Me=3,5</td>
<td>55.6(27.7); Me=3,5</td>
<td>NA</td>
<td>0.04</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>148.2(78.1); Me=2,7</td>
<td></td>
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<td>148.2(78.1); Me=2,7</td>
<td>148.2(78.1); Me=2,7</td>
<td>NA</td>
<td>0.04</td>
</tr>
</tbody>
</table>

NA – not analyzed, not evaluated statistically.
and more advanced disease stage with the involvement of regional lymph nodes in BC patients. miRNA-497 levels were significantly higher in premenopausal women with BC, did not differ between chemotherapy regimens, and did not differ between the first and second NPCT courses.

Discussion.

The assessment of blood plasma parameters is gaining more and more importance in clinical oncology, and new biomarkers are being researched, such as miRNA, for monitoring healthy and ill people [11]. Our study attempted to discover a new way in the area of breast cancer study and diagnosis. Our results show that high plasma miRNA-497 levels are connected to breast cancer and correlate with the progression of the disease. Though there is no correlation with tumor size, the link with tumor cell invasion into the regional lymph nodes is present. Our data also indicates that miRNA-497 level increases significantly in patients younger than 45 years old, while it is lower in older patients, but does not correspond with that of healthy women. These results can be useful in the study of the disease, though they differ from earlier data, which needs to be investigated further. Particularly, published data indicates a decrease in miRNA-497 expression in breast cancer, which then explains the loss or decrease of the role of miRNA-497 as a cell proliferation suppressor [10]. On the contrary, in the analyzed patient sample we found a link between high miRNA-497 levels and a tendency for disease progression.

A low level of miRNA-497 expression was observed in patients with triple-negative breast cancer [12]. Our work did not reveal a dependency of plasma miRNA-497 levels on the molecular type of breast cancer, though a correlation tendency with the triple-negative type was present. Evidently, the sample size of our study was not sufficient for discerning such a correlation, but that does not mean that it cannot be established by further research.

Expression levels of some miRNAs can become a biomarker in predicting the outcome of oncologic disease. Certain miRNA panels can potentially become prognostic biomarkers for breast cancer. In that fashion, some data indicates a potentially better treatment prognosis for cervical and colorectal cancer patients in the case of high miRNA-497 expression levels [8,13]. However, in the case of breast cancer, similar observations were not supported by data sighted in open sources. We attempted to analyze the correlation between miRNA-497 levels in the blood of patients, who received different chemotherapy treatment regimens and we did not find a significant difference before or at the beginning of treatment and after a course of chemotherapy. Analysis of the results, which were obtained based on correlational analysis, indicated an association between higher miRNA-497 levels and a worse condition or prognosis of the disease in breast cancer patients.

Comparison of acquired results with data represented in the literature [6] demonstrated certain differences, which can be explained by multiple variables present in each study, such as differing population patient samples, different chemotherapy regimens, and other factors of patient treatment. In the end, it should be noted, that the potential research possibility of plasma miRNA-497 levels requires further observations and statistical studies.

Conclusion.

1. The correlation between plasma miRNA-497 levels and age in women with breast cancer states that miRNA-497 level increases significantly in premenopausal women, while the opposite tendency is true for healthy women.

2. A weak positive correlation between plasma miRNA-497 levels of breast cancer patients and regional lymph node metastasis was noted. The level of miRNA-497 increased at the N3 stage in patients after APCT.

3. Tumor molecular subtype and received immediate treatment does not affect blood plasma miRNA-497 levels of breast cancer patients.

REFERENCES


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| Table 3. Correlation between initial miRNA-497 levels and treatment results. |
|-----------------|------|-----|
| Indicator (VAR vs. VAR) | Rho | p   |
| 497-RNA vs. Initial results (improvement=1; no change=2; increase=3) | 0.30 | 0.01 |
| 497-RNA vs. Treatment outcome (improvement/stabilization/no metastasis =0; relapse/prolongation/metastasis=1) | 0.38 | 0.00 |
| 497-miRNA vs. N0-N3 | 0.25 | 0.04 |
| 497-miRNA vs. Stage (I-IV) | 0.23 | 0.05 |
| Follow-up results (stabilization=1; relapse, death=2 vs. N0-N3) | 0.04 | 0.73 |
| Follow-up results (stabilization=1; relapse, death=2 vs. Stage) | 0.09 | 0.45 |
| Initial results (improvement/decrease=1; no change=2; increase=3) vs. Treatment outcome (improvement/stabilization/no metastasis =0; relapse/prolongation/metastasis=1) | 0.47 | 0.00 |
| Treatment outcome (improvement/stabilization/no metastasis =0; relapse/prolongation/metastasis=1) vs. N0-N3 | 0.15 | 0.21 |
| Treatment outcome (improvement/stabilization/no metastasis =0; relapse/prolongation/metastasis=1) vs. Stage | 0.17 | 0.17 |

Резюме
Рак молочної залози є гетерогеним захворюванням з варіаційним клінічним перебігом, морфологічними та молекулярними особливостями. У клінічному спостереженні зроблено спробу дослідити зв’язок між клінічною характеристикою хворих на РМЗ та рівнем міРНК-497 у плазмі як можливою патогенетичною молекулярним фактором захворювання та діагностичного показника. Встановлено достовірно вищі рівні міРНК-497 у плазмі крові хворих жінок у пременопаузальному віці порівняно з менопаузою, тоді як у здорових жінок відмічено протилежну тенденцію. Ми не виявили залежності між рівнем міРНК-497 і розміром пухлин та стадією раку (Grade), але відмічено слабку позитивну кореляцію між рівнем міРНК-497 та стадією N0-N3, виражену тенденцію зростання показника на стадії N3, яка у групі хворих після АПХТ була достовірною. Рівень міРНК-497 після першого та повторного курсу НПХТ не мав статистично значущої різниці. Не виявлено різниці показника між молекулярними типами РМЗ, а деяка різниця між хворими з HER2+ та тричінегативний не була переконливою з причини невеликої вибірки таких пацієнтів. Також не виявлено зв’язку між проаналізованим рівнем міРНК-497 та віддаленими результатами, а позитивні безпосередні результати потребують додаткових досліджень та аналізу. Як заключення, аналіз рівня міРНК-497 може бути корисним у дослідженнях молекулярного типу та стадії РМЗ. Перспективи наступних досліджень полягають у аналізі цього показника у більшій кількості спостережень хворих з РМЗ, віддалених результатів та порівнянні з іншими типами міРНК.

Ключові слова: міРНК-497, рак молочної залози, плазма крові, хіміотерапія, метастазування.