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ASSOCIATION OF miRNA EXPRESSION PATTERN WITH OUTCOME OF LETROZOLE THERAPY IN BREAST CANCER PATIENTS

Breast cancer (BC) remains the most prevalent tumor and the leading cause of death among women worldwide, despite the advancements in diagnosis and new treatments. A significant challenge in BC treatment is the acquired or *de novo* resistance of tumors to systemic therapy. To overcome this obstacle, personalized treatment is needed, with a focus on finding biomarkers capable of predicting the response to therapy. MicroRNAs (miRNAs) have emerged as potential markers due to their diverse clinical applications. **Aim.** To examine the potential prognostic significance of miR-125b-2, -155, -221, and -320a expression in the tumor cells of individuals with hormone-dependent BC before undergoing neoadjuvant hormonal therapy. **Materials and Methods.** The study is based on a retrospective analysis of the treatment outcome of 56 patients with stage II—III locally disseminated hormone-dependent BC. The real-time quantitative reverse transcription polymerase chain reaction was performed on the biopsy material to assess the expression of miR-125b-2, -155, and -221 before neoadjuvant hormonal therapy with aromatase inhibitor letrozole to predict clinical response. **Results.** Most HER2/neu+ BC patients had low levels of miR-155 and miR-221 expression in tumor biopsy **specimens**. Tumors that responded well to letrozole exhibited lower levels of miR-125b-2 and miR-221 compared to non-responsive tumors. **Conclusions.** miR-125b-2, -155, and -221 expression can predict resistance to the letrozole treatment of BC.

Keywords: breast cancer, miRNA, letrozole, biomarker.

Breast cancer (BC) in about 75% of postmenopausal patients is dependent on hormones and relies on estrogen mitogenic effects to promote cancer growth. Endocrine therapies, such as estrogen receptor α (ERα) modulators and aromatase inhibitors (AIs), are the most suitable treatment option for patients with ERα-positive (ER+) BC. Recently, AIs, including nonsteroidal

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inhibitors like letrozole and anastrozole, which prevent estrogen biosynthesis, have demonstrated higher efficacy compared to tamoxifen, a selective ER modulator, in treating postmenopausal patients with ER+ BC. Despite the proven clinical efficacy of AIs, the resistance to treatment can still occur, either *de novo* or acquired, and remains a significant obstacle to successful therapy [1].

Currently, the development of resistance to endocrine therapy is believed to be a gradual process. BC transforms from an estrogen-dependent phenotype, which is receptive to endocrine therapy, to an unresponsive phenotype and eventually to an estrogen-independent phenotype.

The recent research has focused on the molecular mechanisms involved in the switch from steroid signaling to the growth factor signaling pathways in the acquisition of endocrine resistance. The studies have shown the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and/or mitogen-activated protein kinase (MAPK) pathways in BC cell lines and breast tumors. These pathways play a crucial role in survival and may contribute to endocrine resistance by activating kinases in both an estrogen receptor (ER)-dependent and ER-independent manner [2].

There is growing evidence that microRNAs (miRNAs) play a role in estrogen action and the resistance to endocrine therapy, particularly in the resistance to tamoxifen and fulvestrant. The studies have shown that ERα and specific miRNAs have a close relationship, with some miRNAs regulating ERα and ERα negatively regulating the expression of certain miRNAs. Furthermore, miRNAs have been found to repress the expression of ERα transcriptional cofactors adding complexity to their involvement in ERα activity.

Postmenopausal ER+ BC patients are often treated with AIs including letrozole and anastrozole. However, the long-term estrogen deprivation in these patients may result in acquired resistance to AIs, which presents a significant clinical obstacle. Recent studies have demonstrated that miRNAs play a role in AI resistance in ER+ BC, similar to their involvement in tamoxifen and fulvestrant resistance. While the research on the relationship between miRNA regulation and AI resistance in ER+ BC is relatively limited, several specific pathways have been identified as the main targets of miRNA-mediated regulation of AI resistance [3].

Our study aimed to examine the potential prognostic significance of miR-125b-2 -155, -221, and -320a in the tumor cells of individuals with hormone-dependent BC before undergoing neoadjuvant hormonal therapy (NHT).

Materials and Methods

The study is based on a retrospective analysis of the treatment outcomes of 56 patients with stage II—III locally-disseminated hormone-dependent BC who were treated on an outpatient basis at the Transcarpathian Antitumor Center during 2011—2020. All patients were examined using the conventional clinical and laboratory methods according to the standards of diagnosis and treatment of cancer patients approved by the Ministry of Health of Ukraine (Orders No. 140 of July 27, 1998; No. 554 of September 17, 2007; No. 645 of July 30, 2010, and No. 396 of June 30, 2015) and gave the informed consent to the use of their clinical data for scientific purposes. All samples were encoded and depersonalized.

Our study involved patients with hormone-dependent BC who were given NHT consisting of letrozole (2.5 mg/day, 2—4 cycles). The treatment efficacy was monitored using mammography and ultrasound diagnosis according to RECIST 1.1 criteria. All patients received surgical treatment, and some underwent adjuvant polychemotherapy or radiation therapy depending on their risk of progression. The specimens of resected tumors were studied morphologically and immunohistochemically.

The patients in our study were of similar age and mostly menopausal, with infiltrative ductal carcinoma being the most common histological type. Most patients had stage II or III BC; regional lymph node metastases were found in 82% of patients. All tumors were positive for estrogen and progesterone hormone receptors, and 64% were HER2/neu positive (Table 1).

Patients were categorized into two groups based on the clinical response to NHT, using RECIST criteria. The first group included 36 patients who demonstrated a positive response to NHT, where complete tumor regression was not observed, but a partial response was registered in all patients. The second group included 20 women who were resistant to the treatment, of which 14 patients had stabilization of tumor growth, and 6 patients had tumor progression.

Table 1. **Clinical and pathological characteristics of BC patients**

Characteristics	Number of patients	
	n	%
Total number of patients	56	100
Average age, years (range)	56.4 ± 6.1 (43—81)	
Menstrual function is preserved	13	23
Menopause	43	77
Stage		
II	37	66
III	19	34
Presence of metastases in regional lymph nodes		
N0	10	18
N1—N3	46	82
Histological type of neoplasm		
Infiltrative ductal cancer	40	71
Infiltrative lobular cancer	16	29
Differentiation grade		
G1 (high)	8	14
G2 (moderate)	38	68
G3 (low)	10	18
Expression of steroid hormone receptors		
ER+	56	100
PR+	56	100
HER2/neu–	36	64
HER2/neu+	20	36
Molecular subtype		
Luminal A	36	64
Luminal B	20	36

The paraffin-embedded biopsy samples taken before any treatment were used for the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). The procedures for RNA extraction, the sets of primers, and the protocol for qRT-PCR were the same as in our previous study [4]. All values of miRNA expression were presented as $2^{-\Delta CT}$.

The statistical analysis of the obtained data was performed using the STATISTICA 6.0 program. All data were expressed as mean ± SD of at least 3 independent experiments. The differences between the groups were analyzed using Student's *t*-test and ANOVA; $p < 0.05$ was considered significant. The association between the treatment responses and pre-treatment miRNAs levels in biopsy samples was assessed using Pearson's correlation coefficient (r).

Results

We evaluated the expression levels of miR-125b-2 -155, -221, and -320a in BC biopsy samples and examined their association with various clinical and pathological features, as well as the response to AIs NHT.

The average expression levels of miR-125b-2 -155, -221, and -320a were 4.49 ± 2.98, 3.58 ± ± 1.80, 5.66 ± 3.78, and 1.66 ± 1.14, respectively. As in our previous observation [4], we found no significant correlation between their expression levels and the clinical characteristics of the patients, as well as HER2/neu status of the tumors (Table 2). However, when analyzing miRNA expression in terms of high (above median)/low (below median) levels according to clinical-pathological parameters of BC patients, we found that biopsy samples of most HER2/neu+ BC patients

were characterized by low levels of miR-155 and miR-221 expression (Table 3).

We also examined the expression of miRNAs in relation to the response to letrozole treatment (Table 4). Our findings revealed that biopsy specimens of cases with partial response to AIs exhibited significantly lower levels of miR-125b-2 and miR-221 compared to non-responsive tumors ($p < 0.05$). As seen from Table 4 patients with cancer stabilization demonstrated

Table 2. **Expression of miR-125b-2-5p, -155, -221-3p, and -320a-3p in BC biopsy material dependent on clinical- pathological parameters of BC patients before treatment (mean ± SD)**

Parameter	miR-125b-2	miR-155	miR-221	miR-320a
G1	1.10 ± 0.26	4.79 ± 2.31	9.88 ± 5.34	1.97 ± 1.33
G2	5.28 ± 3.59	3.92 ± 1.51	4.71 ± 1.59	1.58 ± 1.26
G3	7.34 ± 2.98	2.83 ± 0.39	6.25 ± 2.75	2.50 ± 0.28
N0	4.17 ± 1.87	3.85 ± 1.43	4.76 ± 2.03	1.69 ± 0.93
N1	4.68 ± 1.39	3.43 ± 1.04	6.17 ± 1.52	1.65 ± 1.29
HER–	5.62 ± 1.79	3.61 ± 1.66	6.55 ± 2.16	1.96 ± 1.15
HER+	1.68 ± 1.42	3.51 ± 1.38	3.44 ± 0.94	0.92 ± 0.78

Table 3. **miRNA expression levels dependent on characteristics of BC patients. The number of patients with higher than median and lower than median levels of corresponding miRNA expression is given**

	miR-125b-2			miR-155			miR-221			miR-320a		
	High	Low	*p*	High	Low	*p*	High	Low	*p*	High	Low	*p*
Age			NS			NS			NS			NS
< 60.0	5	6		6	5		4	7		7	4	
> 60.0	24	21		23	22		24	21		26	19	
Menstrual function			NS			NS			NS			NS
preserved	6	7		8	5		7	6		7	6	
menopause	22	21		24	19		25	18		22	21	
Tumor stage			NS			NS			NS			NS
II	19	18		20	17		18	19		20	17	
III	7	12		8	11		11	8		9	10	
Lymph node metastasis			NS			NS			NS			NS
N0	6	4		5	5		6	4			10	
N1-3	20	26		26	20		25	21			46	
Differentiation grade			NS			NS			NS			NS
G1	3	5		4	4		5	3		5	3	
G2	22	16		18	20		21	17		16	22	
G3	6	4		6	4		5	5		5	5	
HER2/neu expression			NS			<0.05			<0.05			NS
+	19	17		10	26		11	25		22	14	
–	6	14		16	4		12	8		10	10	

1.85 and 1.48 higher levels of miR-125b-2 and miR-221, respectively, compared to patients with partial regression of tumors, but no statistically significant differences were found. Still, the cohort with cancer progression demonstrated a 3.17- and 2.19-fold increase in the mentioned miRNA levels, respectively.

We estimated the correlation between letrozole treatment response and pre-treatment miR-125b-2, -155, -221, and -320a levels in biopsy samples and established that miR-125b-2, -155, and -221 expression levels inversely correlated with the outcome by RECIST 1.1 criteria (r = –0.79, –0.51, and –0.69, respectively).

Discussion

The discovery of AIs as therapeutics for BC was initially serendipitous, similar to the development of tamoxifen. The original objective was to replicate the anti-tumor effect of surgical adrenalectomy by reducing the plasma estrogen levels in postmenopausal women. Since 1974, steroidal AIs have demonstrated the superior efficacy of anastrozole compared to tamoxifen in the adjuvant therapy of hormone receptor-positive BC in postmenopausal women [5].

In a study by Vilquin et al. [2], the miRNA microarray analysis of letrozole- and anastrozole-resistant MCF-7 cells revealed miRNA signatures associated with various signaling pathways, including MAPK, focal adhesion, insulin, ErbB, and mTOR pathways, all converging on the Akt regulation. The resistant cells showed the upregulation of miR-125b that conferred resistance to AIs and promoted aggressive behavior and stem cell self-renewal. The activation of the PI3K/Akt/mTOR pathway played a critical role in mediating the AI resistance via these miRNAs. Conversely, the targeted suppression of miR-125b restored the sensitivity to AIs. The clinical data supported the association of miR-125b overexpression with decreased relapse-free survival in ER+ BC, further highlighting the role of miRNAs, particularly miR-125b, in AI resistance [2].

Several studies claimed that the prognostic value of miR-125b expression levels was more informative than those of the conventional biomarkers such as tumor grade or the receptor status, as revealed by log-rank test [2, 6]. Furthermore, in the multivariate analysis using a backward selection procedure, both miR-125b levels and lymph node status remained independent prognostic markers for recurrence-free survival ($p < 0.05$). Notably, miR-155 and miR-221 expression levels did not show significant associations with recurrence-free survival, although

Table 4. **Association of miRNA expression in biopsy specimens and clinical effect of NHT according to RECIST 1.1. criteria (mean ± SD)**

	Number of patients, n (%)		
	Sensitive	Resistant	
	Partial regression	Stabilization	Progression
Letrozole (n = 56)	36 (64.29)	16 (28.57)	4 (7.14)
	miRNA levels		
miR-125b-2	1.68 ± 0.78	3.12 ± 0.93	5.33 ± 1.16*
miR-155	3.51 ± 1.58	2.99 ± 0.79	4.05 ± 0.74
miR-221	3.44 ± 1.12	5.12 ± 1.22	7.55 ± 2.88*
miR-320a	1.92 ± 0.59	2.82 ± 0.88	1.67 ± 1.05

Note: *$p \leq 0.05$ compared to patients with partial regression

a strong correlation of miR-221 with RECIST percentage was observed (r = 0.61) [2]. These data do not coincide with ours in terms of the association of miR-125b, -155, and -221 expression with the lymph node status.

In 2020, Zedain et al. [7] conducted a study on the association of the serum miR-125b level with lymph node status, the presence of distant metastases, and the response to AIs treatment in Egyptian BC patients. They established that it was significantly higher in metastatic BC patients, but found no association with letrozole and anastrozole response.

Bacci et al. [8] discovered a significant correlation between the miR-155 levels and the response to AI therapy in ER+ BC *in vivo* on xenograft model suggesting that a subset of patients may benefit more from a combination approach rather than AI monotherapy, particularly those with high miR-155 expression. Furthermore, through *in silico* analysis of publicly available datasets, they have established that high levels of miR-155 are associated with poor prognosis in patients treated with tamoxifen. Previous studies have also indicated that miR-155 expression levels in blood samples differed between BC patients and healthy individuals.

There are no specific data in the available literature on the involvement of miR-221 in AIs resistance. On the other hand, there are numerous studies confirming its association with the resistance to tamoxifen, fulvestrant, and some other hormonal anticancer drugs [9].

The miR-221/222 cluster is often upregulated in BC, contributing to the aggressive nature of tumors. The elevated levels of miR-221 have been associated with the increased invasiveness of BC cells and the advanced clinical stages. Furthermore, miR-222 expression has been linked to BC progression and drug resistance, while miR-221 downregulates the specific tumor-suppressor genes. Additionally, the high expression of miR-221 correlated with lymph node metastasis, distant metastasis, and unfavorable prognosis in BC patients. In addition, miR-221/222 expression was found to be implicated in the development of acquired hormonal resistance and was associated with unfavorable clinical outcomes in ER+ BC [10].

Hence, our findings emphasize the potential of utilizing miR-125b, -155, and -221 expression in biopsy specimens as a tool enabling personalized treatment strategies based on patients' stratification.

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АСОЦІАЦІЯ ПРОФІЛЮ мікроРНК З РЕЗУЛЬТАТАМИ ТЕРАПІЇ ЛЕТРОЗОЛОМ У ХВОРИХ НА РАК МОЛОЧНОЇ ЗАЛОЗИ

Рак молочної залози (РМЗ) залишається найпоширенішим типом пухлин та основною причиною смерті серед жінок у всьому світі, незважаючи на прогрес у діагностиці та нових методах лікування. Значною проблемою в лікуванні РМЗ є набута або *de novo* резистентність пухлин до системної терапії. Щоб подолати цю перешкоду, потрібне персоналізоване лікування, зосереджене на пошуку біомаркерів, здатних передбачати відповідь на терапію. МікроРНК (міРНК) стали потенційними маркерами через їх різноманітне клінічне застосування. **Мета.** Дослідити потенційне прогностичне значення експресії мікроРНК-125b, -155, -221 та -320a в пухлинних клітинах хворих на гормонозалежний РМЗ до проведення неоад'ювантної гормонотерапії. **Матеріали та методи.** Дослідження базується на ретроспективному аналізі результатів лікування 56 хворих на локально дисемінований гормонозалежний РМЗ стадій II—III. Кількісну зворотнотранскриптазну полімеразну ланцюгову реакцію в реальному часі виконували на біопсійному матеріалі для визначення експресії мікроРНК-125b, -155 і -221 та зіставляли ці дані з результатами терапії інгібітором ароматази летрозолом. **Результати.** У більшості випадків HER2/neu+ РМЗ у зразках біопсії пухлини виявляли низький рівень експресії мікроРНК-155 і -221. У разі позитивної відповіді на терапію летрозолом рівні експресії мікроРНК-155 і -221 були нижчими, ніж у хворих нечутливих до такого лікування. **Висновки.** Експресія мікроРНК-125b, -155 і -221 асоційована із відповіддю пухлин на терапію летрозолом. Однак функції цих мікроРНК вимагають подальшого дослідження, щоб розширити наші знання про їхню роль і потенційне клінічне застосування при РМЗ.

Ключові слова: рак молочної залози, мікроРНК, летрозол, біомаркер.