VIA MEDICA

ORGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2018, vol. 89, no. 8, 414–419

ISSN 0017–0011

DOI: 10.5603/GP.a2018.0071

Serum levels of circulating miRNA-21, miRNA-10b and miRNA-200c in triple-negative breast cancer patients

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ABSTRACT

Introduction: Breast cancer can be classified into five subtypes based on variations in the status of three hormonal receptors that are responsible for the cancer's heterogeneity: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). These classifications influence the choice of therapies (either neoadjuvant or adjuvant), and the range of prognoses, from good (luminal A subtype) to poor (triple-negative cancers).

Objectives: The aim of the study was to compare the serum concentration of selected miRNAs (miRNA-21, miRNA-10b, and miRNA-200c) between in two groups of breast cancer patients with differing ER, PR, and HER2 statuses.

Material and methods: The study was performed on two groups of patients. One group (TNBC) consisted of patients with triple-negative cancer, and the other group (ER(+)/PR(+)) was comprised of patients with positive ER and PR receptors.

Results: The mean level of miRNA-200c was significantly higher in the ER(+)/PR(+) group than in the TNBC group ($p < 0.05$). No statistically significant difference was found between the two groups with regard to the mean levels of miRNA-21 or miRNA-10b.

Conclusions: The level of miRNA-200c was lower in triple-negative patients when compared with the levels in the study's ER/PR positive group.

Key words: miRNA, breast cancer, receptors, biomarker, triple negative

Ginekologia Polska 2018; 89, 8: 414–419

INTRODUCTION

Breast cancer is one of the most often diagnosed malignancies among women in western countries [1]; however, its precise etiopathology remains unclear. Most authors agree that a crucial role is played by hormones, especially estrogen [2], and that the status of the Estrogen receptor (ER), Progesterone receptor (PR) and Human Epidermal growth factor Receptor 2 (HER2) influence the heterogeneity of the disease and form the basis of a five-fold classification of the disease (classification of the disease into five molecular subtypes) [3]. These classifications also influence the choice of therapy (either neo-adjuvant or adjuvant), as well as the prognosis of the disease, ranging from good (luminal A subtype) to poor (triple-negative cancers) [4, 5]. More precise methods of molecular diagnosis will be necessary in future breast cancer therapy to allow for more personalized treatments, because, currently, there are no sensitive and specific non-invasive biomarkers for the diagnosis of the status of the receptors (ER, PR and HER2) or of the molecular pattern of the breast cancer.

A microRNA (miRNA) is a small non-coding RNA molecule. These molecules play a role in regulating gene expression at the post-transcriptional level [6]. Although they are short (20–24 nucleotides), miRNA molecules are involved in the regulation of crucial cell processes such as proliferation, differentiation and apoptosis [7]. Many of the miRNA molecules are known to be important in the pathogenesis and diagnosis of breast cancer [8]. For instance, miRNA-21 and

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miRNA-10b are oncogenic types of miRNA, and the former is over-expressed in breast cancer tissue [9], while the latter influences the migration and invasion of cancer cells [10]. Further, the downregulation of miRNA-200c facilitates cell migration and the creation of remote metastases [11].

Some authors postulate a relationship between miRNA molecules and the expression of ER, PR or HER2 receptors in breast cancer tissue. A study of miRNA-21 and miRNA-10b found a correlation between the expression of the miRNAs and the presence of the HER2 receptor in breast cancer tissue [12]. Yan et al. observed that upregulation of miRNA-21 was significantly correlated with the status of the PR receptor [13]; however, Gao et al. reported a lack of any correlation between miRNA-21 levels and the status of either of the ER or PR receptors [14].

Objectives

In our study we aimed to perform comparisons between the serum concentrations of selected miRNAs (miRNA-21, miRNA-10b and miRNA-200c) in two groups of breast cancer patients with differing ER, PR and HER2 receptor statuses.

MATERIAL AND METHODS

A total of 46 female patients with breast cancer and who had undergone surgery at the Department of Surgical Oncology, of the Medical University of Lodz, Poland, during the period of March to December 2014, enrolled in our study. The ages of the patients ranged from 38 to 72 years, with a mean age of 56.44.

All patients were diagnosed by mammography and ultrasonography. In addition, every case of breast cancer was confirmed by histopathological diagnosis of a specimen obtained by core-needle biopsy.

The histologic diagnosis was based on the World Health Organization criteria, and pathologic staging was performed according to the tumor, node, metastasis classification system revisions of 2002 [15].

No patients displayed clinical symptoms of axillary lymph node metastases. None of the patients had received any treatment before admission to the department. All patients had indications for primary surgical treatment of breast cancer.

The final, postoperative histopathological diagnosis revealed invasive ductal breast cancer in each patient. For the purposes of our study, the patients were divided into two study groups: Triple-Negative Breast Cancer (TNBC) patients, and Positive ER and PR receptor (ER(+)/PR(+))patients. Receptor status was determined by a dedicated pathologist.

Blood samples were collected from the antecubital vein one day before surgery. The samples were centrifuged within 15 minutes with a gravitation force of 1500 g, and the serum was aliquoted and stored at –80°C. The project was approved by the Ethical Committee for Scientific Studies of the Medical University of Lodz, Poland.

Isolation of miRNAs

The total RNA including the circulating miRNA was isolated from the blood serum using an miRNeasy Mini Kit (Qiagen). To ensure complete dissociation of nucleoprotein complexes, 1 ml of QIAzol Lysis Reagent was added to 0.2 ml of serum, mixed by vigorous shaking for 10 s, and incubated for five minutes at RT. All samples were supplemented with 5 pg Caenorhabditis elegans synthetic miRNA-39 (cel-miRNA-39) which was used for normalization of the RNA preparation. Aqueous and organic phase separation was achieved by addition of 200 µl of chloroform. The mixture was vigorously shaken for 15 s, incubated for 3 min at RT and centrifuged at 14,000 g for 15 min at 4°C. Total RNA was precipitated from the upper (aqueous) phase by adding 1.5 volumes of 100% ethanol. Purification of the total RNA extracted was performed with miRNeasy columns (Qiagen) according to the manufacturer's instructions. RNA was eluted from the Qiagen columns in 30 µl of RNase-free water.

MicroRNA (miRNA) quantification

Reverse transcription was performed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems) according to the manufacturer's protocols. Real-time PCR reactions were carried out on 5 µl of total RNA eluate using standard TaqMan® MicroRNA Assays (Applied Biosystems) as a control: hsa-miR-10b (Assay ID 002218), hsa-miR-21 (Assay ID 000397), hsa-miR-200c (Assay ID 002300), and cel-miR-39 (Assay ID: 000200). The reactions were run in duplicate, in volumes of 20 µl using 10 µl TaqMan Universal PCR Master Mix, 1 µl miRNA-specific primer/probe mix, and 1.33 µl RT product. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-Time PCR analysis was applied. Raw Ct was used for analysis.

Statistical analysis

We used Statistica software (version 10.0) for the statistical analysis of our data. The results were presented as mean values. The Mann-Whitney and Kruskal-Wallis tests were used to compare the measured parameters. Pearson's linear correlation analysis was used to identify any independent relationships between serum miRNA concentrations. Normal distribution of the values was confirmed using the Kolmogorow-Smirnow test with the Lillefors test. The values were statistically significant at $p < 0.05$.

RESULTS

In total, 46 female patients were included in our study. The Mean age at diagnosis was 56.44 years (the age range

Table 1. Clinicopathological characteristics of patients with triple-negative (TNBC) and with ER-positive and PR-positive receptors (ER(+)/PR(+)) breast cancer

	TNBC	ER(+)/PR(+)	p Value
Mean age (years)	56.48	52.23	p = 0.321
BMI	26.21	26.78	p = 0.56
Menopausal status			p = 0.393
Pre-menopause	1 (11.11%)	3 (8.10%)	
Post-menopause	8 (88.88%)	34 (91.89%)	
Diabetes	4 (44.44%)	12 (32.43%)	p = 0.311
Ischaemic heart disease	4 (44.44%)	9 (24.32%)	p = 0.062
Hypertension	3 (33.33%)	7 (18.91%)	p = 0.54
Tumor			
T1	2 (22.22%)	15 (40.50%)	p = 0.43
T2	7 (77.77%)	22 (59.45%)	p = 0.387
T3	0	0	
T4	0	0	
Grading			
G1	1 (11.11%)	18 (48.64%)	**p < 0.05**
G2	2 (22.22%)	15 (40.54%)	p = 0.361
G3	6 (66.66%)	4 (10.81%)	**p < 0.05**

TNBC — patients with triple-negative breast cancer; ER(+)/PR(+) — patients with positive ER and PR receptors

was 38–72). To determine the relationship between the serum concentrations of selected miRNAs (miRNA-21, miRNA-10b and miRNA-200c) and the ER, PR and HER2 status of the breast cancer patients, two groups of breast cancer patients were formed: one with triple-negative cancer (TNBC) and the other with positive ER and PR receptors (ER(+)/PR(+)). The characterization and distribution of the two groups is presented in Table 1. No significant differences were observed between the groups with regard to age (p = 0.321), BMI (p = 0.56), or menopausal status (p = 0.393). No significant differences were found in other epidemiological data; nor were there significant difference between the study groups in regard to the incidence of hypertension (p = 0.54), diabetes (p = 0.311), or ischaemic heart disease (p = 0.062). However, the tumors observed in the TNBC group were characterized by significantly lower differentiation than in the other group.

According to the miRNA validation, the mean circulating level (raw Ct) of miRNA-21 was 26.5 in the TNBC group and 26.05 in the ER(+)/PR(+) group (Fig. 1), which was not significantly different (p = 0.447, Pearson's linear correlation analysis). Similarly, no significant difference was found between the two groups' mean levels (raw Ct) of miRNA-10b: 33.5 in TNBC and 33.43 in ER(+)/PR(+) (Fig. 2) (p = 0.397, Pearson's linear correlation analysis). However, the expression level (raw Ct) of miRNA-200c was found to be significantly higher in the ER(+)/PR(+) group (38.78) compared with the TNBC

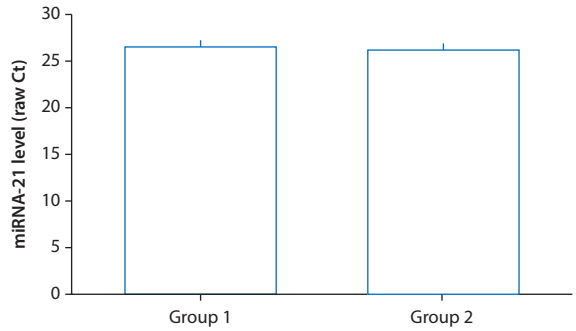

Figure 1. Expression of miRNA-21 (mean ± standard deviation) in serum of breast cancer patients (p = 0.447). Group 1 — patients with triple-negative cancer, Group 2 — patients with positive ER and PR receptors

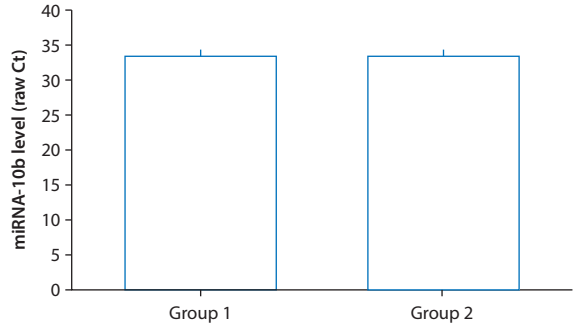

Figure 2. Expression of miRNA-10b (mean ± standard deviation) in serum of breast cancer patients (p = 0.397). Group 1 — patients with triple-negative cancer, Group 2 — patients with positive ER and PR receptors

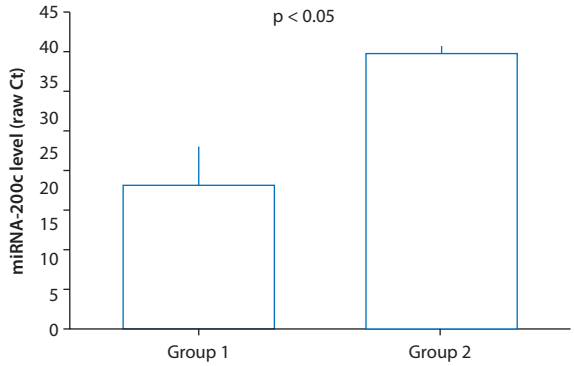

Figure 3. Expression of miRNA-200c (mean ± standard deviation) in serum of breast cancer patients ($p < 0.05$). Group 1 — patients with triple-negative cancer, Group 2 — patients with positive ER and PR receptors

group (20.13), this being a fold change of approximately 1.92 ($p < 0.05$, Pearson's linear correlation analysis) (Fig. 3).

DISCUSSION

In the present study, we researched the serum levels of miRNAs (miRNA-21, miRNA-10b and miRNA200c) to identify possible interactions in triple-negative primary breast cancers. We compared the serum levels of these miRNAs between one group of patients with triple negative cancer and another group of patients with ER/PR-positive breast cancer. The broad contribution of miRNAs to the oncogenesis of breast cancer suggests that they may act as new diagnostic or prognostic factors, as well as therapeutic targets. The chosen miRNAs were known to be dysregulated in breast cancer tissue and had previously been confirmed in *in vitro* studies to play a role in the etiopathogenesis of breast cancer at the molecular level.

The relationship between miRNAs and ER, PR and HER2 receptors expression remains unclear. The few papers that have examined the aforementioned relationship have reported contrary results. Wang et al. postulated miRNAs as a biomarker of the grading and prognosis of breast cancer due to the relative expression of miRNA-21 correlated with hormone receptor expression [16]. However, while Heneghan et al. report up-regulation of circulating miRNA-21 and miRNA-10b in patients with ER-negative breast tumors [17], other authors have found higher miRNA-21 levels in biopsy specimens in ER-positive breast cancer tissue [18]. Another study based on a large population of cancer patients also identified high miRNA-21 expression in ER-negative tissue [19]. Ioro et al. report that high miRNA-21 expression appears to depend not only on ER status but also on tumor diameter, and that miRNA-21 expression was higher in T2/T3 than in T1 tumors [20]. The tumor diameters were more homogeneous in our study group (only T1 and T2). *In vitro* research has found that changes in miRNA-21 expression could be responsible for hormonal therapy resistance [19]. Heneghan et al. postulate the use of miRNA as a marker of breast cancer progression [21].

Hagrass et al. report higher concentrations of circulating miRNA-10b in patients with ER-negative breast cancer [22]. Radojicic et al. report miRNA dysregulation in triple-negative breast cancer: miRNA-21 was up-regulated in triple-negative breast cancer tissue compared with ER/PR-positive tissue and healthy controls; whereas miR-10b and other selected miRNAs were significantly under-expressed. In addition, miRNA-21 dysregulation was associated with worse patient overall survival [23].

Although it is commonly stated that miRNA-21 expression is higher in breast cancer tissue as well as in triple-negative cancers, this was not demonstrated in our study: the group of patients with triple-negative cancer demonstrated no significant difference in the serum level of miRNA-21. However, due to the small number of publications examining this issue, the nature of the level of miRNA-21 in blood serum remains open to question. Other factors may influence the measurement of miRNA-21 in serum. Abrahamsson et al. reported miRNA-21 serum levels to be dependent on hormonal status, with miRNA-21 levels being significantly lower in postmenopausal women [24]. Unfortunately, due to the small number of patients in our study group, it was not possible to confirm this relationship.

MicroRNA-10b is reported in the literature to be down-regulated in triple-negative breast cancer cases compared with normal tissue [23]. However, some authors report a higher level of circulating miRNA-10b in patients with the ER-negative disease [25]. MicroRNA-10b, along with other miRNAs (miR-21, miR-31, miR-125b, miR-130a-3p, miR-155, miR-181a, miR-181b, miR-183, miR-195, and miR-451a) has been proposed as a biomarker for separating triple-negative breast cancer from normal breast tissue [26]. However, our findings indicate that miRNA-10b levels did not differ significantly between the triple-negative breast cancer group and the receptor positive group. Many studies revealed miR-10b expression to be greater in metastatic breast cancer due to its role in the regulation of cell migration and invasion [27]. Both of our examined groups contained metastatic patients, which can influence the serum level of miRNA-10b. Interestingly, Anfossi et al. report increased miRNA-10b serum levels in metastatic HER2-positive patients compared with metastatic HER2-negative patients [28].

Our results confirm that the miR-200c level is downregulated in patients with triple-negative breast cancers [12]. Another study identified lower miRNA-200c in triple-negative breast cancer with metastasis: a more precise analysis showed that this dysregulation resulted from the influence of negative receptor expression on miRNA-200c tissue. The sensitivity and specificity of miRNA-200c as a marker in predicting metastasis among patients with triple-negative breast cancer was found, in that study, to be 87.5% and 81.3% respectively [29].

Unfortunately, the small number of studies on the serum level of miRNA-200c among patients with triple-negative breast cancer prevents any detailed comparison. However, miRNA-200c is part of large miRNA family which also includes miRNA-200a, miRNA-200b, miRNA-141 and miRNA-429. miRNA-200b was researched as a biomarker in breast core needle biopsy. Authors did not state any correlation between miRNA-200b and tissue expression of PR, ER and HER2 [30].

Triple-negative breast cancer is a highly heterogeneous disease. The molecular heterogeneity causes difficulties in creating appropriate targeted therapies for triple-negative breast cancer patients. However, miRNAs could act as biomarkers for indicating therapeutic targets for systemic treatment.

The term for the conversion of hormone receptor status in breast cancer is well known. Namely, it is defined as the difference in the expression of hormone receptors between the primary tumor and metastases and occurs in 18–54% of patients [31]. Determining circulating miRNA -200c could be used as a test prior to the decision on how to treat breast cancer metastases.

CONCLUSIONS

Circulating miRNA-200c was lower in the triple-negative group of patients compared with the ER/PR positive group. More studies on large numbers of patients are needed to explain the relationship between miRNA-200c serum levels and hormone receptor expression in breast cancer tissue.

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