Research Article

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Association between 20 serum mirnas and clinicopathological variables in patients with breast cancer

Meme kanserli hastalarda 20 serum mirna'nın klinikopatolojik değişkenler ile ilişkisi

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Abstract

Purpose: Determining microRNAs in breast cancer pathogenesis suggests that it may be beneficial for diagnosis and treatment.

Materials and methods: Patients serum were collected and microRNAs were isolated. Then microRNAs were converted to cDNA. After that, investigated serum levels of 20 microRNAs (*miR-17, miR-21*, *miR-34a*, *miR-105*, *miR-133a, miR-139-5p*, *miR-141*, *miR-143*, *miR-145*, *miR-155*, *miR-200a*, *miR-200b*, *miR-200c*, *miR-203*, *miR-210*, *miR-299-5p*, *miR-365*, *miR-375*, *miR-411*, *miR-452*) in 39 patients with invasive breast cancer were analyzed before and after treatment.

Results: In the analysis results, it is detected that serum levels of *miR-200c* (p=0.030), *miR-375* (p=0.045), *miR-34a* (p=0.042) were markedly higher in the local advanced/metastatic group. *MiR-141* (p=0.062) levels were lower in patients with positive lymph node involvement, whereas *miR-133a* (p=0.037) levels were higher in the same patient group. *miR-105* (p=0.015), *miR-203* (p=0.015), *miR-375* (p=0.033), *miR-145* (p=0.025) serum levels were markedly higher in the progesterone receptor negative group, likewise *miR-105* (p=0.053) levels were high in the estrogen receptor negative group. The high levels of *miR-375* and *miR-133a* were noticeable in human epidermal growth factor receptor-2 positive patients (p=0.037 and p=0.014, respectively). *MiR-143* (p=0.009) and *miR-145* (p=0.017) levels were observed higher in the patient with a ki-67 index >20% (p=0.007 and p=0.015, respectively). It was found that 2 miRNAs (*miR-133a* (p=0.018) and *miR-139-5p* (p=0.004)) were markedly higher in patients in the luminal B group, which were separated by molecular subgroups. Nine of miRNAs that evaluated (*miR-21* (p=0.001), *miR-34a* (p=0.0001), *miR-105* (p=0.0001), *miR-141* (p=0.041), *miR-200a* (p=0.003), *miR-200b* (p=0.0001), *miR-200c* (p=0.0001), *miR-203* (p=0.0001), *miR-452* (p=0.018)) significantly increased and 5 of the miRNAs (*miR-145* (p=0.0001), *miR-365* (p=0.0001), *miR-155* (p=0.0001), *miR-143* (p=0.0001), *miR-299-5p* (p=0.0001)) were significantly reduced post-treatment.

Conclusion: We think that miRNAs may help in evaluating the follow-up and prognosis of invasive breast cancer.

Key words: Invasive breast cancer, circulating miRNA, molecular subtypes, clinical and pathological variables.

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Öz

Amaç: MiRNA'ların meme kanseri patogenezinde rol oynadığının belirlenmesi, meme kanserinin tanı ve tedavisinde yararlı olabileceğini düşündürmektedir.

Gereç ve yöntem: Toplanan 39 invaziv meme kanserli hasta serumundan mikroRNA'lar izole edildi ve cDNA'lara dönüştürüldü. Hastaların tanı anında ve tedavi sonrasında alınan kanlarından, 20 miRNA'nın (miR-105, miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, miR-34a, miR-133a, miR-155, miR-139-5p, miR-143, miR-145, miR-365, miR-299-5p, miR-411, miR-452 ve miR-17) serum düzeyleri analiz edildi.

Bulgular: Analiz sonuçlarında, miR-200c (p=0,030), miR-375 (p=0,045), miR-34a'nın (p=0,042) serum düzeyleri lokal ileri/metastatik grupta anlamlı olarak yüksek saptandı. miR-141'in (p=0,062) serum seviyesi lenf nodu tutulumu pozitif hastalarda daha düşük gözlenirken, miR-133a (p=0,037) seviyelerinin aynı hasta grubunda daha yüksek olduğu tespit edildi. MiR-105 (p=0,015), miR-203 (p=0,015), miR-375 (p=0,033), miR-145 (p=0,025) serum seviyelerinin PR negatif grupta belirgin yüksek olduğu, aynı şekilde miR-105 (p=0,053) seviyelerinin ER negatif grupta yüksek olduğu görüldü. Her 2 pozitif hastalarda miR-375 ve miR-133a seviyelerinin yüksekliği dikkat çekti (p=0,037 ve p=0,014, sırasıyla). MiR-143 (p=0,009) ve miR-145 (p=0,017) seviyelerinin ki-67 indeksi >%20 olan hasta grubunda daha yüksek olduğu ve bu miRNA'ların ki-67 indeksi ile korelasyon gösterdiği gözlendi (p=0,007; p=0,015, sırasıyla). Moleküler alt gruplara göre ayrılan hastalardan luminal B grubunda olanlarda 2 miRNA'nın (miRNA-133a (p=0,018) ve miRNA-139-5p (p=0,004)) anlamlı olarak daha

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yüksek olduğu saptandı. Çalışılan miRNA'lardan 9 tanesinin (miRNA-105 (p=0,0001), miRNA-21 (p=0,001), miRNA-141 (p=0,041), miRNA-200a (p=0,003), miRNA-200b (p=0,0001), miRNA-200c (p=0,0001), miRNA-203 (p=0,0001), miRNA-34a (p=0,0001), miRNA-452 (p=0,018)) tedavi sonrasında anlamlı olarak arttığı, 5 tanesinin (miRNA-155 (p=0,0001), miRNA-143 (p=0,0001), miRNA-145 (p=0,0001), miRNA-365 (p=0,0001), miRNA-299-5p (p=0,0001)) tedavi sonrasında anlamlı olarak azaldığı görüldü.
Sonuç: Sonuçlarımızın, invaziv meme kanserinin takibi ve prognozunu değerlendirmede yol gösterici olabileceğini düşünmekteyiz.

Anahtar kelimeler: İnvaziv meme kanseri, dolaşan miRNA, moleküler subtipler, klinik ve patolojik değişkenler.

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Introduction

The most common cancer among women in the world is breast cancer(BC). According to Globocan data, it constitutes 15% of cancer-related deaths in women in 2018 and 2 million 88 thousand new cases have been reported. Unfortunately, despite the increased multimodal treatment options, cure has not been achieved yet. Although most of the cases are diagnosed at an early stage, the risk of recurrence or metastasis is still high. The clinicopathological data such as age, menopause status, tumor size, lymph node involvement(LNI), Ki-67, hormone receptor status and cerBB2/*Her2* (Human epidermal growth factor receptor 2) status and genetic markers have a great influence on determining the prognosis. In recent years, miRNAs have been included as well as many studies on the biological features of BC, the earlier diagnosing of patients and the treatment choices based on the molecular characteristics of the patients.

MicroRNAs(miRs) are RNA regulators that control gene expression at the 20-21 nucleotide length post-transcriptional level and are not encoded. They pair with messenger RNAs (mRNAs) of protein-coding genes, leading to translational inhibition and degradation of mRNA. In recent years, more than 50% of miRs have been shown to be located in cancer-related genomic areas or regions that are easily broken [1]. Moreover, miRs have been reported to play a significant role in the development, differentiation, proliferation, invasion and metastasis biology of various cancer cells [2]. As the role of miRs in BC pathogenesis is clarified by various studies, it is suggested that BC can be new biomarkers to guide clinicians in evaluating the diagnosis, prognosis, and treatment response. It has been shown that miRNA expressions differ between normal and neoplastic breast tissue, and these are associated with tumor size, proliferation index, hormone receptor status and cerBB2 expression, invasion and metastasis invasion [3]. Another role of miRs in tumor biology is that it is effective in the regulation of tumor suppressor genes and oncogenes. Tumor suppressor miRNAs (Ts miR) inhibit the expression of oncogene miRs, while oncogenes (oncomiRs) are responsible for inhibiting the expression of Ts miRs leading to tumor formation [4]. The most interesting feature of miRs is that a single miR can target hundreds of mRNAs, which leads to disruption of expression of many mRNAs and proteins. These act as oncomiR or Ts miR [5]. However, many miRs that predict the treatment response in BC and affect survival have been identified [6].

The fact that the detection of miRs circulating in cancer patients is technically easily applicable and can be used as a new biomarker creates a field of study in this regard. In this study, we aimed to appreciate the serum levels of 20 most frequently studied miRs that are important in patients with just diagnosed BC, before and after treatment. We compared the measured miRs with the patients' clinicopathological features and then we examined their changes with treatment.

Materials and methods

Patients

Thirty-nine serial patients with BC who have been diagnosed invasive ductal carcinoma histologically and started to treat at the Department of Medical Oncology, Pamukkale University, in Turkey, were included in our study. The clinicopathological variables such as age, menopausal status, hormone receptor (estrogen receptor (ER) and progesterone receptor (PR)) and c-erb B2 status, lymph

node involvement, histologic grade, tumor size, staging and types of treatment were enrolled by analyzing all the medical reports. Patients who have inflammatory carcinoma, age <20 or >80 years, and second tumors were excluded. This study has been approved by the local Ethics Committee of Pamukkale University and all patients were informed about the procedure and written consent was obtained. The sign consents of all participants were taken in accordance with the Helsinki Declaration.

Total miRNA isolation

Serum was obtained by centrifuging the blood taken from patients who applied to Pamukkale University Medical Faculty Medical Oncology Department with the ethical committee dated 20.02.2018 and numbered 04 at 4000 rpm for 5 minutes. Serum samples obtained were obtained using the Qiagen miRNeasy Serum / Plasma Kit (qiagen cat: 217184 Hombrechtion, Switzerland). The miRNAs obtained were stocked at -80°C.

miRNA cDNA synthesis

Poly (A) Polymerase Tailing kit (Cat. No: 903 Richmond, Canada) is used to synthezise cDNA with the abm miRNA cDNA Synthesis. The experiment was continued in according to the protocol of this kit. Approximately 75ng was acquired from the total miR to be obtained. 2 µL of 5X Poly (A) Polymerase Reaction Buffer, 1.5 µL of ATP, 1 µL of MnCl2, 0.5 µL of Poly (A) Polymerase were added to 10 µL of RNase-free water. It was incubated at 37ºC for 30 minutes. After standing on ice for a while, 2 µL miRNA Oligo (dT) adapter was added and incubated for 5 minutes at 65ºC. Briefly, it was incubated for 15 minutes at 42ºC and 10 minutes at 70ºC by adding 1 µL of dNTP, 4 µL of 5X RT Buffer, 1 µL of EasyScript RTase and 2 µL of RNase-free water. The cDNAs obtained were stocked at -80°C.

Real-time pcr (qRT-PCR)

The Rotor-Gene 6000 (Corbett Life Science, Australia) device was used to determine the expression levels of mİRs which all its primers were from abm (Richmond, Canada). qRT-PCR was performed using miRNA qPCR MasterMix (abm, Richmond, Canada). Reaction conditions; 5 µL cDNA, 10 µL miRNA Mastermix, 0.5 µL miRNA primer, 0.5 µL Universal primer were performed as 4 µL dH2O. PCR conditions; 10 cycles of 1 minute at 95ºC, 10 seconds at 95, C/15 seconds at 58ºC/5 seconds at 72ºC], and the melting curve analysis at the accuracy of 0.1ºC between 55ºC and 90ºC. For normalization, normal breast cell line and miR-39 miRNA were used. Real-Time PCR analyses were obtained by calculating the number of copies with the standard curve. Calculated copy numbers were converted into numerical data suitable for analysis by 2 ^^ CT method.

Statistical analysis

We analyzed pre-and-post chemotherapy changes in the plasma levels of twenty BCassociated miRs (*miR105*, *-21, -141, -200 (a,b,c), -203, -210, -375, -34a, 133a, -155, -139-5p, -143, -145, -365, -299-5p, -411, -452, -17*) and clinicopathological parameters by using the chi-square test, Mann-Whitney test and Kruskall-Wallis H test. Spearman's test was used to correlate analysis. We compared the plasma levels of twenty miRs between the preand postchemotherapy samples of each patient by using Wilcoxon signedranks test. As a conclusion, we had two-sided tests and all differences we analyzed were considered non-significant when *P* values were greater than 0.05. The statistical analysis was performed using the SPSS 17.0 software package, version (SPSS Inc. Chicago IL).

Results

Thirty-nine patients with BC were evaluated. Eighteen (46.2%) were early stage (I, IIA, and IIB), 21 (53.8%) were local advanced (IIIA, IIIB, IIIC) and metastatic (IV). The median age at disease onset was 51 years (range: 29-79 years). Twenty patients (51.3%) had premenopausal status. Seventeen (43.6%) patients had a tumor size ≤2 cm, and 22 (56.4%) patients had a tumor size >2 cm. The median tumor size was 22mm (range 0.1-9 cm). Twenty-nine (74.4%) patients had lymph node involvement. Seventeen (43.6%) patients had higher Ki-67 levels than 20%. Twenty-two patients (56.9%) had Luminal A, 8 (20.5%) had Luminal B, 3 (7.7%) had *Her2* positive and 6 (15.4%) had triple negative disease. Patients' clinicopathological variables are shown in Table 1.

Table 1. Clinicopathological variables of patients

Clinicopathological variables	All Patients	
	N	%
Median age (range)	**51 (29-79)**	
Menopausal status		
Pre	20	51.3
Post	19	48.7
Tumor size (cm)		
Median (range)	2.2 (0.1-9)	
≤2	17	43.6
>2	22	56.4
Lymph Node involvement		
Positive	29	74.4
Negative	10	25.6
Ki-67 levels (%)		
≤20	22	56.4
>20	17	43.6
Stage		
Early (I, IIA, IIB)	18	46.2
Local advanced (IIIA, IIIB, IIIC)/metastatic (IV)	21	53.8
Molecular subtype		
Luminal A	22	56.4
Luminal B	8	20.5
Her2 positive	3	7.7
Triple negative	6	15.4

Plasma levels of miRNAs according to clinicopathological variables

We compared miR levels in patients with early (n=18) and local advanced/ metastatic (n=21) BC, and as a result of our research, we found that the plasma levels of *miR-200c* (p=0.030), *miR-375* (p=0.045) and *miR-34a* (p=0.042) were higher in local advanced/ metastatic group than in the early-stage patients. The patients with lymph node involvement had lower *miR-141* (p=0.062) and higher *miR-133a* (p=0.037) than lymph node negative patients. In ER negative patients, *miR-105* plasma levels were found higher (p=0.053) than positive patients. In addition, *miR-105* (p=0.015), *miR-203* (p=0.015), *miR-375* (p=0.033) and *miR-145* (p=0.025) levels were higher in patients with PR negative patients. In cerbB2 positive disease, *miR-375* and *miR-133a* levels were higher than cerbB2 negative disease (p=0.037 and p=0.014, respectively). According to Ki-67 levels, *miR-143* (p=0.009) and *miR-145* (p=0.017) levels were higher in patients with >20%. There were no relationships between miRs and age, menopausal status and tumor size.

We detected a correlation between Ki-67 and the levels of *miR-143* (r=+0.433, p=0.007), and also the levels of *miR-145* (r=+0.397, p=0.015). Besides, there was a strong correlation between the levels of *miR-21*0 and Ca 15-3 (r=+0.435, p=0.008). We have not seen any correlation among miR levels and other clinicopathological variables such as age, albumin, CRP.

Plasma levels of miRNAs according to molecular subtype

In Luminal B patients, *miR-133a* (p=0.018) and *miR-139-5p* (p=0.004) levels were higher than non-Luminal B patients. There was no

association between miRs' plasma levels and other molecular subtypes such as Luminal A, Her 2+ and triple negative subgroups.

Pre- and post-treatment plasma levels of miRNAs

We measured pre and post treatment samples of the plasma levels of miRs. After chemotherapy, the plasma levels of *miR-105* (p=0.0001), *miR-21* (p=0.001), *miR-141* (p=0.041), *miR-200a* (p=0.003), *miR-200b* (p=0.0001), *miR-200c* (p=0.0001), *miR-203* (p=0.0001), *miR-34a* (p=0.0001), *miR-452* (p=0.018) were increased, however the plasma levels of *miR-155* (p=0.0001), *miR-143* (p=0.0001), *miR-145* (p=0.0001), *miR-365* (p=0.0001), *miR-299-5p* (p=0.0001) were decreased. Before and after chemotherapy, miR changes are shown in Figure 1.

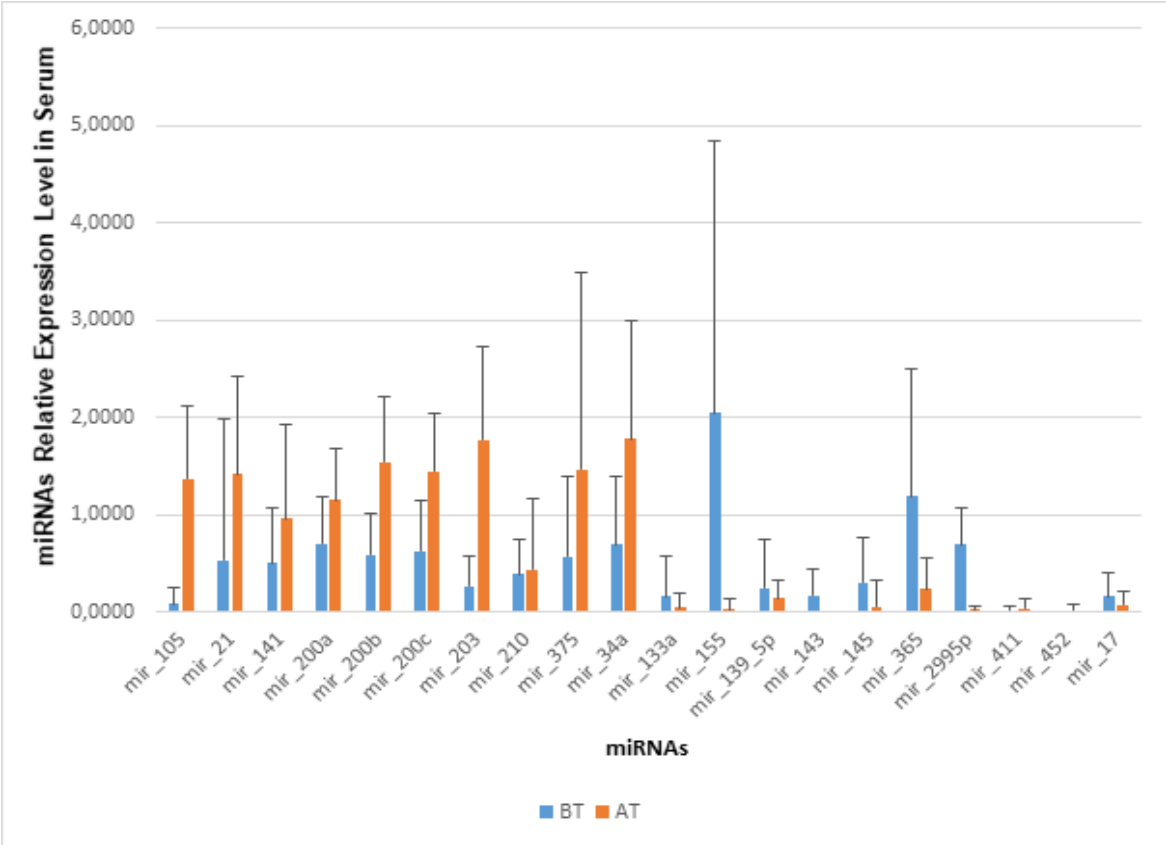

Figure 1. Graphical explanation of miRNA changes before and after treatment. (Before Treatment (BT) After Treatment (AT))

Discussion

In this study, we analyzed 20 miRs (*miR-17, miR-21*, *miR-34a*, *miR-105*, *miR-133a*, *miR-139-5p*, *miR-141*, *miR-143*, *miR-145*, *miR-155*, *miR-200a*, *miR-200b*, *miR-200c*, *miR-203*, *miR-210*, *miR-299-5p*, *miR-365*, *miR-375*, *miR-411*, *miR-452*) expression levels before and after treatment of patients with invasive BC. Serum expression levels of *miR-200c*, *-375, -34a* were markedly higher in the local advanced/metastatic group than in the early stage patients. However, *miR-141* plasma levels were lower in patients with positive lymph node involvement while *miR-133a* levels were higher in this analyze. When evaluated according to hormone receptor states, it was observed that *miR-105* was high in ER and PR negative patients, whereas *miR-203*, *-375, -145* were markedly higher in the PR negative group. Furthermore, *miR-375* and *miR-133a* levels were high in *Her2* positive patients. As the relationship of miRNA with other clinicopathological parameters was examined, we observed that *miR-143* and *miR-145* levels were higher in the group of patients with a Ki-67 index of >20% and these miRs correlated with the Ki-67 index. In patients divided into molecular subtypes, *miR-133a* and *miR-139-5p* levels in luminal B group were markedly higher

than in non-luminal B group. Of the 20 miRs studied before and after treatment, 9 (*miR-105*, *miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-34a, miR-452*) increased markedly after treatment, 5 (*miR-155*, *miR-143, miR-145, miR-365, miR-299-5p*) decreased significantly after treatment.

The most important factors affecting the prognosis of BC are tumor size, lymph node involvement, organ metastasis and molecular subtyping. Circulating miRs have a reliable marker for early detection of lymph node metastasis in invasive BC and staging of the disease has been supported by many studies. In a retrospective study examining miRs associated with distant organ metastasis, it has been shown that *miR-21, miR-184* and *miR-494* are upregulated in patients who develop metastasis and may be useful for future targeted treatments [7]. On the other hand, *miR-199a, miR-29c, miR-424* were found to be higher in patients with invasive BC at an early stage than healthy controls in the study of biomarkers that will facilitate the detection of invasive BC at an early stage [8]. In our study, plasma levels of *miR-375*, *-200c*, *-34a* were markedly higher in the group with local advanced/metastatic disease than in the early stage patients. Madhavan et al. [9] stated that *miR-200c* and *miR-375* levels showed markedly higher expression in BC patients with circulating tumor cells. *MiR-200c* has an important effect on the proliferation, transformation, migration and invasion of the cancer cell. It is also suggested that it regulates epithelial mesenchymal transformation, epidermal growth factor signaling, functions of cancer stem cells, and apoptosis via *p53*. In another study, *miR-200c* and *miR-141* are found higher in metastatic patients than those with localized disease, and it is suggested that *miR-200c* and *miR-141* are regulated by the FOXP3-KAT2B axis [10]. *miR-200c* and *miR-141* were stated that they will be a strong biomarker for determining metastasis for metastatic disease. In a study conducted by Roth et al [11], *miR-10b*, *miR-34* and *miR-155* are higher in advanced BC than early stage. In addition, *miR-200* family (a, b, c) and *miR-210* are higher in patients with metastasis and has been shown to affect survival [12]. However, BC cell cultures examined after *miR-375* inhibition show a decrease in cell proliferation [13]. When *miR-375, -200c, -34a* are evaluated together with other studies that obtained similar results with our study, it may be thought that these miRs may indicate poor prognosis.

Studies have reported that *miR-141*, associated with good prognosis, shows negative correlation with tumor size, lymph node metastasis, cerB-B2 expression levels, and Ki-67 levels. Also, *miR-141* overexpression in vitro has been shown to target ANP32E gene, inhibiting cell growth, proliferation and invasion [14]. In another study, decreased expressions of *miR-141*, *miR-200 family (a, b, c)* levels are detected in BC stem cells. It has also been shown that the *miR-200* family prevents epidermal mesenchymal transformation by suppressing *ZEB1* gene expression, and *miR-200c* also inhibits tumor formation in vivo [15]. However, many studies investigating the *miR-133a* effects on BC pathogenesis have shown the relationship between decreased *miR-133a* levels to advanced clinical stage, lymph node metastasis, and shorter recurrence survival [16, 17]. It has been observed that increased *miR-133a* levels show a Ts effect by decreasing the proliferation, migration and invasion by negative regulation of the *LASP1* gene in vitro [16]. Supporting the studies, in this study, it is predicted that *miR-141*, which is markedly low in patients with positive lymph node involvement, and *miR-133a*, which is found high, may have a positive effect on the course of the disease.

Based on the hormone receptor status, BCs are divided into four groups according to molecular classification: luminal A, luminal B, *Her2* (cerB-B2 positive) and triple negative. Although there is little information about these gene receptors yet, they provide significant benefits in the selection of treatment and in monitoring the response to treatment. For this reason, studies are continuing on miRs showing hormone receptor status and biomarker potential associated with molecular subtypes. In a study, 309 miRs have been identified in 93 breast tumors with different molecular subtypes. In this study, differential miR expression provides an accurate classification of basal and luminal subtypes, and it is shown that the 31 miRNA identified can differentiate different subtypes [18]. Similarly, in a study, the relation between estrogen receptor (ER) with *miR-342, miR-299, miR-217, miR-190, miR-135b, miR-218*; and between progesterone receptor (PR) with *miR-*

520g, miR-377, miR-527-518a, miR-520f-520c and *Her2* with *miR-520d*, *miR-181c, miR-302c, miR-376b, miR-30e* are identified by Lowery et al. [19] *miR-342* and miR-520g overexpression are further analyzed in 95 breast tumors and *miR-342* expression is found high in ER and *HER2* positive tumors and low in triple negative tumors. In a recent study, Piasecka et al. [20] have detected an increase *miR-10b*, *miR-21*, *miR-29, miR-9, miR-221/222, miR-373* and a decrease in *miR-145*, *miR-199a-5p, miR-200* family, *miR-203* and have had a prognostic value in triple negative BC. In this study, we found markedly higher levels of *miR-105* in the ER negative patient group and the levels of *miR-105*, *miR-145*, *miR-203* and *miR-375* were found high in PR negative patients. *miR-375* and *miR-133a* expression levels in *Her2* positive patients were significantly higher. Similar to our study, *miR-105* has been suggested to be upregulated in the plasma of ER/PR and *Her2* negative BC patients [21]. It is stated that *miR-105* activates wnt / p-catenin signaling with SFPRI down regulation and decreases survival by promoting metastasis. Unlike our study, Yu et al. [22] determined that *miR-203* levels increase in ER/PR positive patients compared to the control group and suggested that estradiol can control cell proliferation by regulating miR expression. Another example, the study of Han et al. [23], *miR-145* levels are found to be markedly higher in PR positive patients compared to PR negative patients.

It has been emphasized that, in studies investigating the effect of miRs to development of trastuzumab resistance, which is a monoclonal antibody agent developed against each receptor, the decrease in *miR-375* levels may be responsible for resistance. Studies continue to show that *miR-375* response to the treatment of trastuzumab by targeting insulin-like growth factor receptor 1 (IGF1R) [24]. Other miR study has been done on gastric cancer cells, and *miR-133a* has shown that it inhibits proliferation of stomach cancer cells by reducing ERBB2 expression [25]. Our data contains results that contradict the literature regarding the detected miRs based on the low number of studies performed on hormone receptor status and the number of patients included in our study. It is thought that there may be biomarkers that can be used in determining the subtype of miRNAs determined by the results of larger studies to be conducted in the future, and evaluating the response to anti-Her2 treatment.

However, in comparison with molecular subtypes, we found *miR-133a* and *miR-139-5p* miRs markedly higher in patients with luminal B subtype compared to non-luminal B subtype. It has been reported that *miR-133a* and members of the *miR-139-5p* family inhibit invasion and migration in breast cell culture [21]. In both studies, *miR-139-5p* has been shown that it induces apoptosis in BC cells, causes cell cycle arrest in the S phase, thereby inhibiting invasion and metastasis [26]. As a result, it is stated that *miR-139-5p* and *mir-133a* have significant functions in the development of tumorigenesis and BC and may take place in clinical applications.

Today, the fact that CA-15-3 (cancer antigen-15-3) and CEA (carcinoembryonic antigen), which are the biomarkers used for post-treatment follow-up in BC, are seen as valuable in terms of follow-up in long-lasting metastatic breast cancer patients, there is a need of new biomarkers because they can be false positive for 6-12 weeks due to associated drug-related cell death after treatment and their long half-life. As a result of the correlation analysis we conducted in this analyze, it has been shown that there is a strong correlation between *miR-21*0 and ca-15-3 levels. Although it has (been) shown that *miR-21*0 levels decrease in patients with postoperative BC due to the reduction of tumor burden, when meta-analyses showing the relationship between breast tumors with high *miR-21*0 levels and decreased survival are evaluated together, it can be thought to be used in follow-up and prognosis with a high sensitivity and specificity after treatment [6, 27]. In addition to our study, the correlation of another prognosis marker Ki-67 monitored remarkably high in proliferation index with *miR-143* and *miR-145*, and these two miR clusters in patients with a Ki-67 index of >20%. The correlation of *miR-143* and *miR-145* with the Ki-67 proliferation index are associated with poor prognosis, which have been shown to suppress breast proliferation and invasion of BC cells by inhibition of ERBB3 translation, suggest that existing miRs may not have been elucidated yet [28].

In the literature, there are few miR studies in invasive BC that vary depending on the treatment. We think that miRs expressed

especially in serum or plasma are very valuable in terms of guiding clinicians in the diagnosis and follow-up of the disease. In our study, it is detected that 9 of the 20 miRNAs we looked at in patient serum (*mir-21*, *mir-34a*, *mir-105*, *mir-141*, *mir-200a*, *mir-200b*, *mir-200c*, *mir-203*, *mir-452*) increased after treatment. Alike to this study which examines differences in between miRNAs before and after neoadjuvant chemotherapy in the plasma of 25 BC patients, it has noted that the levels of *mir-34a* increase after treatment and this miRNA is particularly high in 7 patients who partially responded to treatment [29]. This increase has been attributed to the release of *mir-34a* from liver tissues and treatment-related DNA damage due to hepatotoxicity caused by anthracycline-based therapies alongside tumor tissue. Considering that the anthracycline group chemotherapy used in the patient group in our study are influenced by *p53* activation, it can be thought that the increase of *miR-34a* after chemotherapy is realized through the *p53* activation mechanism through treatment-related DNA fractures.

Wang et al. [30] showed that cell motility and migration decreased by approximately 50% of *miR-203* in triple negative BC cells. Data have stated that *mir-203* inhibits proliferation and invasion and acted as a Ts by lowering the levels of BIRC5 and LASP1 proteins. However, it has been suggested that estradiol increases the migration and invasion ability of ER positive BC, which is accompanied by a decrease in *miR-203* levels [31]. Few studies on the relationship between *miR-452* and BC showed evidence that *mir-452* acts as a Ts. Less expression of *miR-452* in BC cells compared to healthy tissues is one of the evidence showing tumor suppressor miRNA properties. However, it has also been shown to suppress cell migration and invasion by targeting RAB11A in BC cells in which *miR-452* is transfected [32]. In our study, the increase in tumor suppressor-bearing miRNAs after treatment may be evidence that patients benefit from treatment, and this may be associated with good prognosis. With studies to support this hypothesis, *miR-141*, *-200a*, *-200b*, *-200c*, *-203* and *miR-452* can be useful as predictive and prognostic miRs in BC and can guide clinicians in follow-up to treatment.

Conversely, Zhou et al. [33] have compared MDA-MB-231 BC cells and MCF-10A healthy breast epithelium, the tight binding protein of *miR-105*, which is expressed and secreted by metastatic BC cells, is ZO-1 (zonula occludens-1) has been shown to be a powerful migration regulator by targeting. While overexpression of *miR-105* causes metastasis and vascular permeability in distant organs, these effects have been found to be reduced by inhibition of *miR-105* in metastatic tumors. For this reason, it has been reported that high *miR-105* levels are associated with the development of metastases and may have predictive and prognostic value for metastatic progression in early-stage BC. Another study has been noted that increased *miRNA-21* expression is markedly associated with poor survival of BC patients, and *miR-21* is involved as an oncomiR targeting Ts miR. It is also suggested to be an effective biomarker that controls uncontrolled cell proliferation, BC cell growth and metastasis caused by programmed cell death 4 (PDLD-4) and tropomyosin 1 (TPM-1). In the same study, an average of 3.2-fold reduction has been observed in *miR-21* levels after treatment [34]. In our study, during the time we followed up, although none of our patients had progression, but one, an unexpectedly significant increase was observed in the *miR-105* and *miR-21* levels, which were functioning as oncomiR, after the treatment. Considering that studies on *miR-105* and *miR-21* strongly correlate these two biomarkers with poor prognosis and tumor aggressiveness, it can be interpreted that patients' follow-up time is insufficient to evaluate progression.

In addition, as a result of our study, we detected 5 miRs (*miR-155*, *-365*, *-143*, *-145* and *miR-299-5p*) that decreased after treatment. Similarly, Sun et al. [35] have detected a decrease in serum *miR-155* levels in 79% of 29 patients diagnosed with breast cancer after 4 cycles of adjuvant chemotherapy and associated the reduction in *miR-155* levels with response to treatment and disease remission. The role of *miR-365* has not been clarified yet in BC and there are studies supporting that it is an oncomiR that increases cell proliferation and migration by targeting ADAMTS-1, an anti-angiogenic gene [36].

The tumor suppressor effect of *miR-143* and *miR-145* in BC is thought to be through the suppression of HER receptors [28]. *MiR-143* has also been shown to reduce proliferation

and migration in other types of cancer [37]. This anti-cancer effect is due to Bcl-2, MYO6, ELK1 and ERK5, which play a role in cell proliferation, apoptosis and migration. Cellular mitogens and stress-activated ERK5 targets proteins that regulate cell proliferation, such as the nuclear factor (NF)-kB, c-myc, and cyclin D1. Moreover, mitogen-activated protein acts on 3 kinase 7 (MAP3K7) or transforming growth factor (TGF-beta) -activated kinase-1. In a study conducted by Zhou et al. [38], *miR-143* is decreased in BC tissue, and p-ERK5, ERK5, p-MAP3K7 and MAP3K7 expressions are increased. It has been suggested that ERK5 and MAP3K7 are the targets of *miR-143*, since the expression of ERK5, p-MAP3K7, MAP3K7 and cyclin D1 are found to be reduced by miR143 upregulation. In addition, there are studies showing that *miR-145* induces apoptosis by activating *p53* and reduces estrogen receptor-α [39]. The role of *Mir-299-5p* in BC patients is not known much. Shevde et al. [40] have been reported that a decrease in *mir-299-5p* levels in BC tumor tissue may cause an increase in osteopontin, a glycoprotein associated with radiotherapy and chemotherapy resistance, invasion and metastasis. The roles of *miR-155*, *-365*, *-299-5p* in BC pathogenesis are not known clearly and *miR-143* and *miR-145*, which have been shown Ts properties in many studies, showed a decrease in our study that is contradictory in the literature. The results of the study show that there is a need to organize studies involving a large number of patients and long follow-up.

Consequently, this study contributes to the findings of previous studies on miRNA levels detected in plasma in BC patients. The major limitation of this study is that it contains a relatively small sample that does not provide enough power to evaluate the relationships between circulating miR levels and clinical features. This study is organized as a preliminary study. We think that it will shed light on future studies with larger sample sizes. In addition, short observation time and insufficient time for survival results are deficient in the prognostic values of miRs that change. Further diagnostic studies are needed for miRNAs with longer follow-up time, greater number of patients, and other predictive and prognostic factors evaluated together.

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Authors' contributions to the article

A.G.Y. and A.Y. constructed the main idea and hypothesis of the study. A.G.Y. and A.Y. developed the theory and arranged/edited the material and method section. A.G.Y., A.Y., A.C.K., A.D. and H.S. have done the evaluation of the data in the Results section. Discussion section of the article written by A.G.Y. and A.Y. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.