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Original Article

Multi-Drug Resistance against Second-Line Medication and MicroRNA Plasma Level in Metastatic Breast Cancer Patients

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What's Known

- Drug resistance in patients, especially cancer patients, is one of the barriers to achieve effective treatment in the long run. MicroRNAs have a variety of biological roles in the body. Thus, checking their serum levels can help to understand the best therapeutic approaches at different disease stages, especially for cancers. One of their important uses is investigating drug resistance.

What's New

- High plasma miR-663b and low plasma miR-199a were associated with adequate drug response in metastatic breast cancer patients.
- The plasma level of miR-663a was significantly higher in HER2$^-$ breast cancer patients.
- There was no significant correlation between the plasma level of miR-663a and drug resistance.

Abstract

Background: Circulating microRNAs (miRNAs) can help to predict the chemotherapy response in breast cancer with promising results. The aim of the present study was to investigate the relationships between the miR-199a, miR-663a, and miR-663b expression and chemotherapy response in metastatic breast cancer patients.

Methods: This study is a case-control study performed at Yasuj University of Medical Sciences (2018-2021). The expression levels of miR-663a, miR-663b, and miR-199a in the serum of 25 patients with metastatic breast cancer versus 15 healthy individuals were determined by the real-time polymerase chain reaction method. The response to treatment was followed up in a 24-month period. All patients were treated with second-line medications. Two or more combinations of these drugs were used: gemcitabine, Navelbine®, Diphereline®, Xeloda®, letrozole, Aromasin®, and Zolena®. Statistical analyses were performed in SPSS 21.0 and GraphPad Prism 6 software. The expression levels were presented as mean±SD and analyzed by Student's *t* test.

Results: The results and clinicopathological features of patients were analyzed by *t* test. The statistical analysis showed that miR-663a expression was related to human epidermal growth factor receptor 2 (HER2) status and was significantly lower in the HER2$^+$ than HER2$^-$ group (P=0.027). Moreover, the expression of miR-199a and miR-663b was significantly correlated with the response to treatment, in which the expression of miR-199a was higher in the poor-response group (P=0.049), while the higher expression of miR-663b was seen in the good-response group (P=0.009).

Conclusion: These findings state that the high plasma level of miR-199a and the low plasma level of miR-663b may be related to chemoresistance in patients with metastatic breast cancer.

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Keywords ● MicroRNAs ● Breast neoplasms ● Drug resistance ● Neoplasm metastasis

Introduction

Among different types of cancer, breast, lung, and colorectal cancers are the most prevalent cancers in women. Together, these three cancers account for half of all cases. Breast cancer alone

represents 30% of all new cancers diagnosed in women. In the USA, the first cause of cancer death in 2017 was lung cancer among men and women. The second mortality cause in women was breast cancer. Within women with age 20-59 years, breast cancer was the first cause of death. The prevalence of breast cancer in Asian women was 93.3 per 100000 population, and the mortality was 11.4 per 100000 population. It was estimated that during 2022, 1,918,030 new cases of cancer would be detected in the USA, and of these 287,850 are women breast cancer new cases. In this year, the number of death due to cancer is predicted 609,360 cases, and breast cancer is the third cause of death (43,250 deaths).[1] According to the newest assessments, half of the new breast cancer occurrences around the world (1.38 million) and 60 % of deaths from breast cancer (458,000) take place in developing countries.[2] In Iran, the third leading cause of death is cancer following cardiovascular diseases and accidents, and breast cancer is the first prevalent cancer in women.[3]

The global data about different kinds of cancer show that breast cancer poses a serious threat to women's health and life, and its prevalence is increasing every year.[4] Metastatic breast cancer is very dangerous for patients, and their survival rate is very low. Breast cancer metastasis begins with the invasion of cancerous cells to the adjacent tissues, entering the bloodstream or lymphatic vessels, and moving tumor cells to distal tissues.[5] The most rampant tissue of breast cancer metastasis is bone, and up to 50% of patients show this metastasis as the first site of distal metastasis. The second and third metastatic sites of breast cancer are the lungs and liver, respectively.[6] Metastasis to the brain includes about 10-15% of breast cancer patients. This shows the second original cancer location for metastasis to the brain is breast cancer.[7] The therapeutic approaches for metastatic breast cancer have improved progressively based on the development of knowledge about the signaling pathways and understanding of the biological behaviors of cancerous cells.[8] Chemotherapy and radiotherapy are methods for treating metastatic breast cancer in the neoadjuvant setting, before breast conservative surgery or mastectomy and axillary node clearance. The most common drugs are anthracyclines (doxorubicin and epirubicin), taxanes (paclitaxel and docetaxel), fluorouracil (5-FU), and cyclophosphamide.[9] For clinical treatment, therapeutic regimens are planned according to clinical variables and a number of molecular biomarkers, such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and Ki67 antigen.[4] Based on these parameters, breast cancer patients are classified into different subtypes, including luminal A (ER^+ and/or PR^+, Ki67 low, and $HER2^-$), luminal B (ER^+ and/or PR^+, Ki67 high, and/or $HER2^+$), HER2 positive (ER^-, PR^-, and $HER2^+$), and triple-negative (ER^-, PR^-, $HER2^-$). However, in recurrent breast cancer patients, chemoresistance is inescapable.[10] There are various mechanisms involving in drug resistance such as mutations in drug targets, epigenetics diversity, adapted signaling ways and modifications in drug transport to the targets, transfer of anticancer drugs out by transporter proteins, functioning of enzymes involved in drug metabolism and inactivation of anticancer drugs, distinct expression of pro-apoptosis proteins, changes in the expression of genes involved in tumor suppression, or activity augmentation of mechanisms involved in DNA repair. Recent studies have identified that effective therapeutic ways need appropriate biomarkers to guide them. Thus, findings and using effective biomarkers to predict tumor progression and the response to therapy in breast cancer patients are very important.[4, 11-14] Therefore, novel biomarkers could be very helpful for predicting the drug response of breast cancer patients and leading to the best therapeutic plan.[8] If these factors can be detected in plasma samples, they would greatly help in predicting chemotropic responses. One of the potential biomarkers for this purpose is microRNA (miRNA). There are a number of evidence showing that microRNAs (miRNAs) can involve in chemoresistance processes and can be a good biomarker for predicting drug responses, because miRNAs have good stability and practical availability.[15, 16] There are some studies on the use of miRNAs with routine therapies and their ability to involve in therapeutic responses.[17]

MicroRNAs are small noncoding regulatory RNAs with a length of 18–24 nucleotides. They have a central role in the repression of gene expression after transcription by binding to their target mRNAs at three prime untranslated regions (3'-UTR).[4, 18] MicroRNAs have the ability to target more than 100 mRNAs, and they can affect many cellular processes, such as proliferation, cell differentiation, and apoptosis.[19] There are several studies about the role of miRNAs in different kinds of cancers. In a study by Feng and colleagues, miR-630 demonstrated tumor suppressor activity in gastric cancer cells, and this effect was through the blocking of FoxM1 expression thereby causing the inhibition of PI3K/AKT signaling pathway.[20] There is another study about the effect of miRNA-294 on the

PI3K/AKT pathway in bladder cancer showing that miRNA-294 upregulated NRAS and caused activation of this pathway in T24 cells.[21] Some studies demonstrated the effective role of some miRNAs, such as miR-153, miR-148a, miR-206, and some others, in the initiation and development of breast cancer.[22] Other studies showed the role of miRNAs in the response of different kinds of cancers to drugs, for example in breast cancer, gastric cancer, cervical cancer, lung cancer, and so on.[23]

In 2003, it was first identified that miR-199a-5p is the result of two genetic loci. One was chromosome 19 for miR-199a-1, and the other one was chromosome 1 for miR-199a-2.[18] In a study by Zhang and colleagues, it was shown that downregulating miR-199 can promote the invasion of hepatocarcinoma cells.[24] It was proven that MiR-199b-5p has key roles in human cancers and can affect breast cancer progression. A previous study showed that miR-199b-5p could have important roles in the adjustment of some cell functions, such as migration, clonogenicity, and proliferation in breast cancer cell lines. Despite this, the role of microRNA in the prognosis of breast cancer has been less studied.[4]

Moreover, it was shown that miR-663 can target transcripts encoding eukaryotic translation elongation factor 1A2 (eEF1A2) in human MCF7 breast cancer cells, which results in the slower cell proliferation of MCF7 cells.[25] Another study showed miR-663 was increased in multidrug-resistant MDA-MB-231-derived ADM cell line, and this enhancement was accompanied with the decrease of heparin sulfate proteoglycan 2 (HSPG2) and drug resistance.[12]

The present study was planned for serum analysis of miR-663a, miR-663b, and miR-199a-5p in metastatic breast cancer patients and normal people as the control. The relationship between these microRNAs and chemoresistance was investigated.

Patients and Methods

This study is a case-control study. The patients participating in this study were selected from patients referred to Amir Oncology Hospital (Shiraz, Iran, 2018). All patients had metastatic breast cancer and were in the second-line medication phase. These patients did not respond to the first-line medication. These patients were followed for two years by a specialized oncologist. Patients that died during three months after receiving the medication were excluded from the study. Age and sex were matched with the healthy control group. The women were selected by interview, and their family history was checked for chronic and malignant diseases. Subjects with inflammatory disorders, autoimmune diseases, and any endocrine diseases were excluded.

In this study, the sample size was 25 women (mean age=50.96 years), and 15 healthy normal women were selected as the control (mean age=45.06). The sample size in this study was calculated by using the following formula, according to the highest standard deviation reported in a previous study.[26] Type 1 error and type 2 error were considered 0.05 (confidence interval 95%) and 0.2 (power of the test 80%), respectively. Cohen's suggestion for effect size (d) was estimated at 25 persons.

$$n = \frac{2(z_{1-\alpha/2} + z_{1-\beta})^2 \delta^2}{d^2}$$

This study was approved by the Ethics Committee of Yasuj University of Medical Sciences (IR.YUMS.REC.1396.73). All participants gave informed consent before participating in the study.

All healthy women were consulted about healthiness and family history and were tested for serum biochemical factors. A blood collection from women with breast cancer was done before the beginning of the chemotherapy. Chemotherapy included two or more combinations of these drugs: gemcitabine (Sindan, Romania), Navelbine® (Octavius, Italy), Diphereline® (Ipsen, France), letrozole (Iran Hormone, Iran), Xeloda® (Hoffman-La Roche, Swiss), Aromasin® (Pfizer, Italy), and Zolena® (Ronak Pharmaceutical Co., Iran). All patients were followed up after chemotherapy for 24 months, and patients who showed signs of improvement were in the good-response group, but patients who showed no signs of improvement or died were entered in the poor-response group. For every individual, 4–5 mL of venous blood was collected in the blood collection EDTA tubes. Blood plasma (n=25+15) was obtained from the whole blood samples by centrifugation at 1900 ×g (3000 rpm) for 10 min at 4 °C to separate the blood cells. Plasma was aliquoted and stored at -80 °C until total RNA extraction was performed.

Isolation of Total RNA from Plasma and cDNA Synthesis

Total RNA extraction was done using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure. In summary, 1 mL QIAzol Lysis Reagent and 200 µL plasma were mixed. Then, 200 µL chloroform was added, followed by

adding 100% ethanol to the aqueous phase. After they were thoroughly mixed, the sample was transferred into an RNeasy MinElute spin column for centrifugation.

Extracted RNAs were used to synthesize cDNA by using the miRNA first strand kit (Takara, CA, USA) according to the manufacturer's instructions. All primers were designed by the NCBI (National Center for Biotechnology Information), Primer Design software, and their uniqueness was ensured by the NCBI Blast. The primer sequences are shown in table 1. The relative expression of miR-199b-5p, miR-663a, and miR-663b was normalized to the U6 level.

Quantitative Real-time PCR: Quantitative real-time PCR was done using SYBR green master mix, according to the following PCR protocol: initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification at 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec. The amplified fluorescent signal was detected by thermocycler (BioRad CFX96-instrument [BioRad, Hercules, CA]).

Statistical Analysis

All statistical analyses were performed in SPSS version 21.0 (SPSS, Chicago, IL, USA) software and GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, U.S.A.) software. The assessment of normality was done by the Kolmogorov-Smirnov test. Accordingly, the comparisons of the miRNA expression levels between groups were evaluated using Mann-Whitney *U* test. P≤0.05 means the results were significant.

Results

The clinicopathological data of patients are listed in table 2. There was not any triple-negative breast cancer in patients. All the patients were ER^+, and only two samples were PR^-. Moreover, there were no significant differences between case and control groups regarding sex and age (P>0.05).

Association between the Relative Expression of miR-199a, miR-663a, and miR-663b and Clinicopathological Parameters

The expression pattern of miR-199a, miR-663a, and miR-663b in 25 serum samples of patients and 15 normal individuals were calculated according to ΔCt, which was extracted from the output of real-time RT-PCR. U6 primers were used as spiked-in control for normalization. The plasma levels of miR-199 (P=0.05), miR-663a (P=0.011), and miR-663b (P=0.024) in patients were higher than normal persons (figure 1).

Table 1: Primer sequences for real-time PCR

Primer name		Primer sequence
miR-199b-5p	Forward	5'-GCCCGCCCAGTGTTT AGACTAT-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
miR-663a	Forward	5'-TAATAGGCGGGGGCGCC-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
miR-663b	Forward	5'-TAATCCGGCCGTGCCTGA-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
miR-39	Forward	5'-UCACCGGGUGUAAAUCAGCUUG-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
U6	Forward	5'-CTCGCTTCGGCAGC ACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

Table 2: Clinicopathological features in breast cancer patients

Clinical features		Case (n=25)
Age (years)	<50	12 (48%)
	≥50	13 (52%)
HER2 status	Negative	18 (72%)
	Positive	7 (28%)
ER status	Negative	0 (0%)
	Positive	25 (100%)
PR status	Negative	2 (8%)
	Positive	23 (92%)
Metastasis status	Single*	11 (44%)
	Mix**	14 (56%)
Therapeutic response	Good	16 (64%)
	Poor	9 (36%)

*Metastasis to one tissue; **Metastasis to more than one tissue

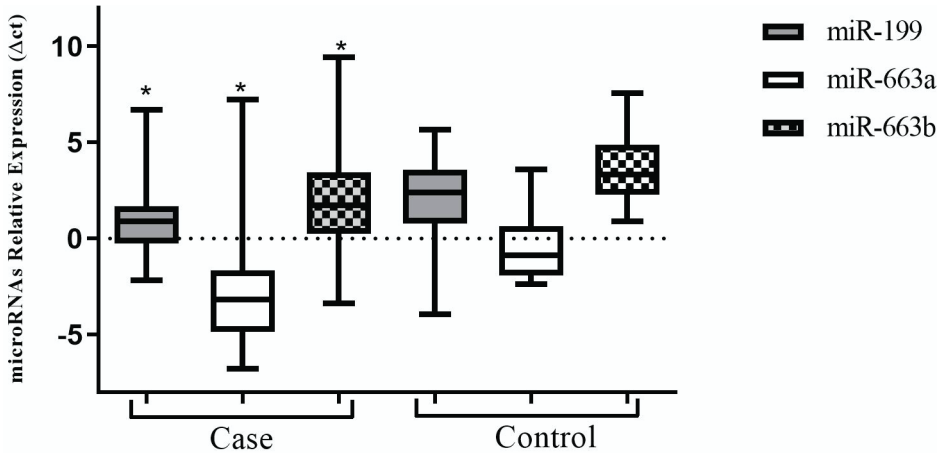

Figure 1: The miRNAs expression levels in patients (case) and normal (control) group show significant differences and a higher expression in patients. Case represents the patient group and control represents healthy normal women. *Indicates significant differences compared with the other group.

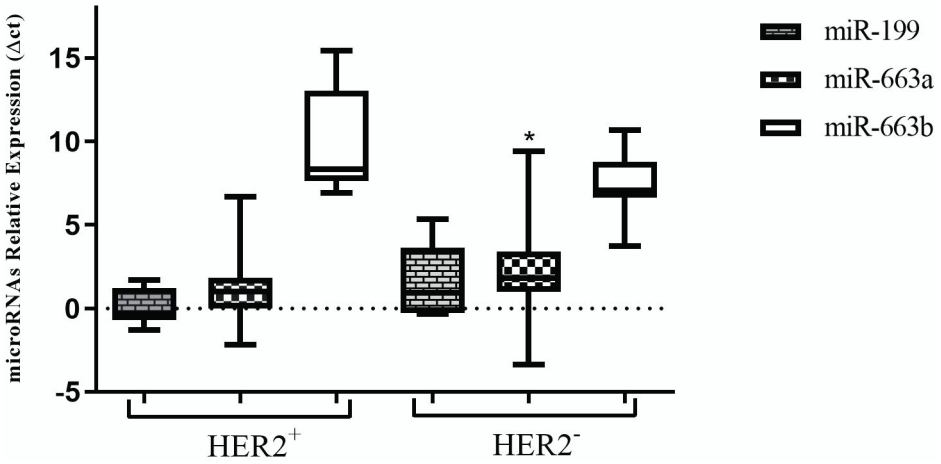

Figure 2: The mean relative miRNAs expression levels in two groups of $HER2^-$ and $HER2^+$ were compared. The $HER2^-$ patients had higher miR-663a than $HER2^+$ patients. *Indicates significant differences compared with the other group.

The data analysis showed that miR-663a expression was related to HER2 status and was significantly lower in $HER2^+$ than in $HER2^-$ group (P=0.027) (figure 1), but the expression of miR-199a and miR-663b showed no significant association with HER2 status (P>0.005) (figure 2).

The plasma miR-199a concentration of patients with a good response was lower than patients with poor response (P=0.049). The plasma level of miR-663b was higher in the good-response group than the poor-response group (P=0.009). Nevertheless, there was no significant association between the expression of miR-663a with the response to chemotherapy (P>0.005) (figure 3).

Correspondence of metastatic status showed that there was no significant correlation between single and multi-locational metastatic samples and the relative expression of miRNAs (figure 4).

Discussion

In this study, it was found that the plasma level of mir-663a was related to the HER2 biomarker. The level of mir-199a was lower in the plasma of patients who responded well to the treatment, and the level of mir-663b in the plasma of these patients was higher than other patients. Drug resistance is a major problem in the treatment of various cancers.

Abnormal miRNA expression has been shown in different kinds of cancer, and these dysregulations of miRNAs contribute to different pathways of cancer pathogenesis such as apoptosis, proliferation, inflammation, cell cycle, and stress response.[27-30] In the present study the plasma levels of the three miRNAs were higher in patients than normal persons, so dysregulation of these miRNAs may be potential biomarkers.

As mentioned above, aberrant miRNA expression can affect a number of molecular pathways; one of these pathways is the chemoresistance pathway. There are many pieces of evidence that show miRNAs might be helpful prognostic markers, and they can affect drug responses in cancers.[30] According to a study

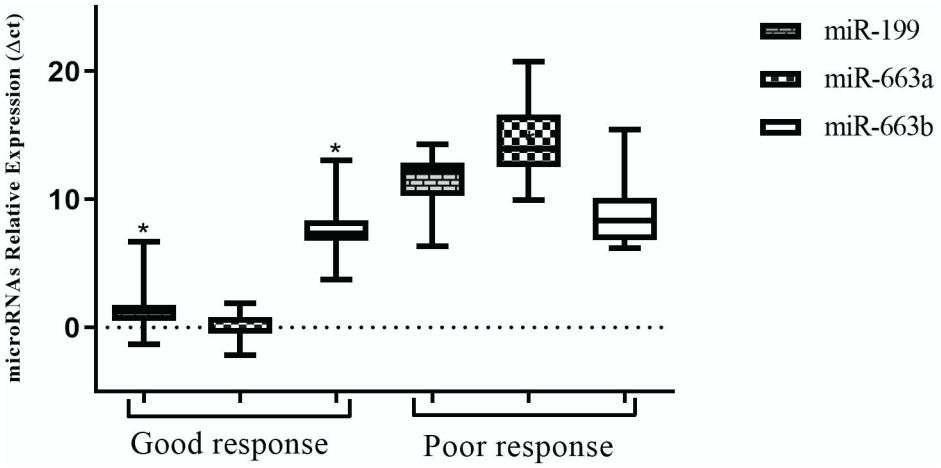

Figure 3: The miRNAs expression levels in good- and poor-response groups were compared. The plasma level of miR-663b was higher in the good-response group, while in the poor-response group, the plasma level of mir-199 was higher. *Indicate significant differences compared with the other group.

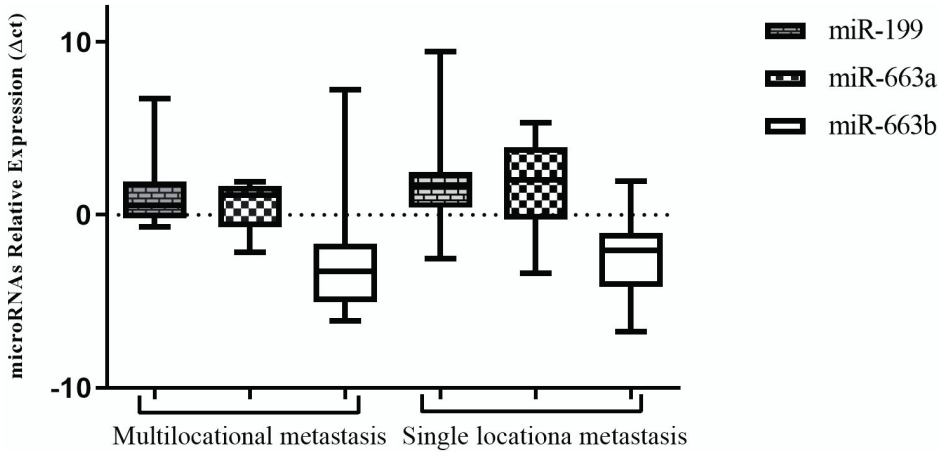

Figure 4: The expression level of miRNAs was compared according to the metastatic locations (single and multi-location). There were no significant differences regarding plasma levels of microRNAs and the location of metastasis. *Indicates significant differences compared with the other group.

by Song and colleagues, the downregulation of lncRNA NEF and upregulation of miR-155 correlate with poor prognosis in triple-negative breast cancer patients. In another investigation, it was shown that miR-214 targets p53/Nanog axis and regulates ovarian cancer stem cell properties, and the decrease in miR-15b or miR-16 is accompanied by an increase in BCL2 levels and the development of drug resistance in gastric and breast cancer cells.[12] In the present study, the plasma levels of the three miRNAs were determined, which were higher in patients than normal group. Thus, dysregulation of these miRNAs might be potential biomarkers. A number of previous studies showed that these miRNAs might involve in drug resistance, and these non-coding RNAs can help in predicting the response to therapies.[12, 31, 32] Therefore, they were selected to investigate the relationship between their plasma level pattern and drug resistance. In this study, all breast cancer patients were in stage 2 or higher and underwent second-line medication, and received a combination of two or more drugs.

Data analysis of the present study showed that the plasma level of miR-199a-5p was higher in poor-response group patients than the good-response group. This data was consistent with a previous study by Mussnich and others. They reported that miR-199a-5p was up-regulated in the cetuximab-resistant human colon cancer cell line by targeting the tumor suppressor gene, PHLPP1.[32] Other studies suggested that multidrug resistance and tumor formation potential can be suppressed by miR-199a, which targets CD44; and quenching of miR-199b-5p is associated with drug resistance in ovarian cancer.[33] These data show that miR-199 has a complex role in tumorigenesis and a possible cancer-specific relationship. TGF-β (transforming growth factor β) pathway has a very important role in drug resistance.[34] This pathway can affect many cellular processes including cell proliferation and differentiation,

extracellular matrix production, and effective immunological responses.[35-37]

The role of the TGF-β pathway in drug resistance in breast cancer was shown previously. Upregulation of genes involving in this pathway can cause drug resistance in breast cancer patients.[38] It was reported that the TGF-β pathway causes downregulation of ATM (Ataxia telangiectasia-mutated) and MSH2 (MutS Homolog 2) genes via miRNA mechanisms. It was suggested that the TGF-β-MSH2 route might induce drug resistance against DNA-degradation drugs.[39] According to the miRPath database, miR-199a can affect the TGF-β pathway. Hence, the downregulation of this pathway can induce this type of drug resistance. Navelbine® and gemcitabine are DNA-degrading drugs, and this mechanism might be responsible for the miR-199a plasma level increase, which might cause a poor response to drugs in breast cancer patients.

Li and colleagues showed that circulating miR-663 can be a good biomarker for drug resistance in breast cancer.[40] In a study, miR-663 was up-regulated in nasopharyngeal carcinoma cells, and its downregulation was identified to act as a tumor suppressor in gastric cancer. However, the role of miR-663 in breast cancer, especially its involvement in chemosensitivity, is still unclear. In a study by Hu and others, it was found that the downregulation of miR-663, as an oncogenic miRNA, enhances drug sensitivity in doxorubicin-resistant cells.[12] In the present study, two types of miR-663 (a and b) were separated, and the results demonstrated that a high plasma level of miR-663b was related to good chemotherapy response. This finding contradicts the findings of Hu and colleagues' study. The difference between the results of these two studies could be due to two reasons: Firstly, in the present study, all patients were treated with second-line medications, and a multi-drug combination was used. Secondly, there were probable differences between the two types of miR-663. It was also shown that out of seven $HER2^+$ patients, five patients were in the poor-response group, two patients were in the good-response group, and the plasma level of miR-663a was significantly higher in the $HER2^-$ group. These data need more investigation.

There were some limitations in this study. One of these limitations was the small sample size due to the unavailability of many samples with the desired specifications. The second one was monitoring of patients for a long time and exclusion of some patients from the study. Due to monitoring all breast cancer cases for more than 18 months, the number of cases that could be in this study was low, and it was very hard to follow all cases until the end of the study. Because of the low number of cases, dividing cases into subgroups according to the type of breast cancer was not done, but we tried to select all cases of the same type. This preliminary study can be a basis for future studies. The other limitation of this study was that the separation of cancer cases into subgroups according to metastasis site was not done. However, they were divided into two groups: single site and more than one site of metastasis. Thus, in future studies, this point can be investigated. Detection of the studied microRNAs was very complicated in healthy individuals, probably due to their low concentrations in serum. Therefore, the number of healthy controls was lower than the number of patients.

Conclusion

According to the results of this study, the plasma concentrations of miR-199a, miR-663a, and miR-663a were higher in breast cancer patients than healthy individuals. High plasma miR-663b and low plasma miR-199a were associated with adequate drug response.

The final data of the present study, as a preliminary study, can show an important relationship between the alteration of microRNA serum levels and drug resistance in breast cancer patients. This study may introduce a new approach for the prediction of breast cancer prognosis after antineoplastic drug therapy based on the determination of miRNAs level in the plasma. In the future, a pattern of the plasma level of some miRNAs could be found to predict how patients respond to multi-drug treatment. According to the present study, the level of miR-199a and miR-663b will be very helpful. However, it needs to be confirmed through further studies.

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Conflict of Interest

Dr. Mehdi Dehghani, as the Editorial Board Member, was not involved in any stage of handling this manuscript. A team of independent experts were formed by the Editorial Board to review the article without his knowledge.

Authors' Contribution

M.D. and M.N: Study concept and drafting; S.M: experimental work, data gathering, and drafting, H.A, B.A, MA.NM, H.S, and R.M: Data analysis, experimental works, and drafting. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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