RESEARCH ARTICLE

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Profiles of microRNAs in Patients with Advanced Breast Cancer Who are Chemoresistant or Chemosensitive to Fluorouracil, Adriamycin, and Cyclophosphamide Treatment

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Abstract

Objective: Locally advanced breast cancer (LABC) is an inoperable breast adenocarcinoma that is commonly treated with neoadjuvant chemotherapy. Neoadjuvant chemotherapy is given to patients as the first step in treatment to reduce tumor size before surgery. However, no biomarkers are currently available to predict early response to chemotherapy. **Methods:** The differential expression of miRNAs between malignant and normal cells from patients with breast cancer reflects tumor dynamics and may reflect an individual's resistance or sensitivity to drugs used in chemotherapy. Ten patients with LABC who responded to chemotherapy, five patients with LABC who did not respond, and three healthy controls were included in this study. **Results:** This study found that miRNAs miR-214-3p, miR-222-3p, and let-7e-5p indicated a patient's sensitivity to neoadjuvant chemotherapy. Whereas miR-20a-5p, miR-27a-3p, miR-424-5p, miR-152-3p, and miR-195-5p suggested a patient's resistance to it. **Conclusion:** Therefore, these findings suggest that miRNAs may serve as predictive biomarkers and potential therapeutic targets in the management of breast cancer.

Keywords: microRNA- breast cancer- FAC chemotherapy- clinical response- neoadjuvant chemotherapy- biomarker.

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Introduction

According to GLOBOCAN data, breast cancer is the most frequent disease (15.3%) and the leading cause of death among women in México [1]. Locally advanced breast cancer (LABC) is an inoperable breast adenocarcinoma that has progressed locally without distant metastases and is commonly treated with neoadjuvant chemotherapy [2]. Despite the new alternatives that are available for the treatment of breast cancer, FAC chemotherapy (treatment with fluorouracil, Adriamycin, and cyclophosphamide) continues to be one of the main treatments used. Neoadjuvant chemotherapy is given as the first step in treatment to shrink the tumor before surgery. However, the efficacy of this treatment is compromised by the development of chemoresistance in some patients, which leads to disease progression. As stated in the Response Evaluation Criteria in Solid Tumors and the World Health Organization criteria, chemoresistance is clinically suspected when no significantly relevant changes occur after 2–3 cycles of neoadjuvant chemotherapy [3]. Clinical and pathological parameters are currently used to select the neoadjuvant chemotherapy regimen for each patient; however, there are no biomarkers that allow for the prediction of early chemotherapy responses [4]. The search for new methods to predict chemoresistance includes studies investigating microRNAs.

MicroRNAs (miRNAs) are small RNAs that play a key role in resistance to chemotherapy by regulating the expression of genes encoding proteins involved in various biological processes of cancer, such as cell survival, migration, apoptosis, drug metabolism, and DNA repair [5–10]. Exposure to chemotherapeutic drugs can alter the expression of miRNAs, and these, in turn, can modulate the sensitivity or resistance of tumor cells to treatment. This involvement demonstrates their clinical utility in the diagnosis, prognosis, and treatment of breast cancer,

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making them promising biomarkers for personalized medicine. Hence, the differential expression of miRNAs between malignant and normal cells in breast cancer could be associated with drug resistance, or with drug sensitivity as a result of the broad influences of miRNAs on multiple cellular processes.

Several miRNAs are involved in the suppression of chemoresistance or the promotion of chemosensitivity (oncosuppressors), whereas other miRNAs are inducers of chemoresistance (oncomiRs). However, the expression of such miRNAs has been evaluated in a single biological matrix [11, 12], at a specific point of treatment [11], or in a single molecular subtype of breast cancer [12, 13]. Nevertheless, miRNAs released from cells into plasma, serum, or tissue act in a different context. Therefore, the use of a miRNA signature could identify resistant or sensitive patients who share a common miRNA expression profile, regardless of the molecular subtype. In this study, we evaluated 84 miRNAs (either known or predicted to change expression during breast cancer initiation or progression) by microarray in plasma and tissue samples from patients. Our goal was to identify a miRNA profile that would potentially allow us to differentiate between patients with chemotherapy resistance and those with sensitivity.

Materials and Methods

Patients and controls

This study was conducted in the Medical Oncology Unit of the Hospital Central "Dr. Ignacio Morones Prieto" (HCIMP) in San Luis Potosí, Mexico; it recruited patients who were newly diagnosed with locally advanced breast cancer and subsequently treated with a first-line neoadjuvant FAC chemotherapy regime, which comprises six cycles of combined treatment with doxorubicin (50 mg/m^2), 5-fluorouracil (500 mg/m^2), and cyclophosphamide (500 mg/m^2) every 4 weeks. After neoadjuvant chemotherapy was completed, patients were classified as resistant (stable or progressive disease) or sensitive (complete or partial response) to chemotherapy according to the Response Evaluation Criteria in Solid Tumors (RECIST). This classification was determined by the oncologists. Additional blood samples from three women with no history of cancer were used as healthy controls. All experiments were conducted following the Declaration of Helsinki, and the study was approved by the Research and Ethics Committee from HCIMP (CONBIOETICA-24-CEI-001-20160427) and the Ethics Committee from the Chemical Sciences Faculty in the Autonomous University of San Luis Potosí, México [14-17]. All participants provided written informed consent. The anthropometric and clinicopathological features of the participants are shown in Table 1.

Collecting samples and processing

Tissue samples were obtained from seven breast cancer patients as part of biopsy procedures (t=0); these included cancer tissues and adjacent normal tissues. The protocol used for this study involved extracting the tissue during the diagnostic visit to the Unit of Gynecologic Oncology (with the diagnosis later confirmed by the Unit of Pathology). All tissues were immersed in RNA later stabilization solution (Thermo Fisher Scientific, CA, USA) and then frozen immediately in liquid nitrogen until use. In addition, before (t=0) and after the patients received chemotherapy treatment (t=4), peripheral blood samples (6 ml in EDTA) were obtained from 15 patients with breast cancer at the Unit of Oncology to assess changes in the miRNA profile. Peripheral blood samples were centrifuged at 2500 RPM for 10 minutes to separate the plasma supernatant and were stored at –80 °C until use. Blood samples from healthy-control participants (n=3) without a history of cancer were selected and included in the study.

Extraction and quantification of miRNA from plasma and tissue

Total RNAs were extracted using QIAzol Lysis Reagent (cat. No. 79306, Qiagen) and then purified using the miRNeasy Serum/Plasma Kit (cat. No. 217184, Qiagen) for plasma samples and using the miRNeasy Mini Kit (cat. No. 217004, Qiagen) for tissue samples, following the manufacturer's instructions. The quantity and purity of each RNA sample were determined using a Synergy™ HTX spectrofluorometer (Biotek Instruments).

Microarray analysis of miRNAs from plasma and tissue

We pooled RNA samples containing miRNAs that were collected under the following conditions:

a) Pooled tissue samples were collected before chemotherapy from resistant patients (T/BCH/R)

b) Pooled tissue samples were collected before chemotherapy from sensitive patients (T/BCH/S)

c) Pooled plasma samples were collected before chemotherapy from sensitive patients (P/BCH/S)

d) Pooled plasma samples were collected after chemotherapy from sensitive patients (P/ACH/S)

e) Pooled plasma samples were collected before chemotherapy from resistant patients (P/BCH/R)

f) Pooled plasma samples were collected after chemotherapy from resistant patients (P/ACH/R)

Next, the miScript II RT Kit (cat. No. 218160, Qiagen) containing 5x miScript HiSpec Buffer was used to perform cDNA synthesis reactions with 100 ng of total RNA from each pool. The miScript miRNA PCR Array Human Breast Cancer Kit (Cat. no. 331221, Qiagen) was used for miRNA expression analysis; it contains reagents that allow 84 miRNAs (known or predicted to change their expression during breast cancer initiation or progression) to be quantified by qRT-PCR in a CFX96 Touch Thermocycler (Bio-Rad, USA).

Analysis of miRNA levels from plasma and tissue samples

Relative miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method for each RNA pool. Samples with expression levels below the limit of quantification (Ct > 35 cycles) were considered to have a non-significant expression of the miRNAs of interest and were excluded from the analyses. Each array contained six different snoRNA/snRNA that were used as normalization controls for the array data (SNORD61, SNORD68, SNORD72,

SNORD95, SNORD96A, RNU6-6P), miRNA reverse transcription control (RTC), and positive PCR control (PPC). The miRNAs of interest were selected according to the following kit specifications: A) The expression level of the miRNA is relatively low (<10) in the control or test group and is reasonably high in the other group (>10). This result suggests that the actual fold-change value is at least as large as the calculated and reported fold-change result. B) The expression level of this miRNA is relatively low (<10) in both the control and test groups. C) The expression level of this miRNA is zero in the control and test groups, which means that its expression was not detected, making the fold-change result erroneous and its profile incomprehensible. Therefore, miRNAs with such low levels were excluded from further analysis.

Identification of interaction networks between microRNAs and genes associated with breast cancer

Using microRNAs associated with sensitivity and resistance, an analysis of interactions between microRNAs and genes expressed in breast cancer tissue was performed using the miRNet platform (https://www.mirnet.ca/). Both experimentally validated target genes and high-confidence computational predictors were considered. To ensure the biological and clinical relevance of the analysis, only interactions involving genes previously reported to be of clinical interest in breast cancer tissue were considered. The obtained interactions were integrated into Cytoscape v3.10 to construct regulatory networks.

Statistical analysis

To evaluate our expression results and identify common and differential miRNAs that were present in each pooled RNA group, we used the online tool VENNY v. 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/); this tool was also used to create Venn diagrams depicting the overlap between chemoresistance and chemosensitive groups. Heatmaps were generated using R software (v4.3.2).

Results

Clinical samples

The study design, the clinical sample collection schedule that was based on the selection criteria, and the classification into each group are shown in Figure 1. Table 1 shows the clinical and pathological features of the patients. The tissue and blood samples that were collected from patients before chemotherapy were classified into chemoresistant and chemosensitive groups. The median age at breast cancer diagnosis was 49±10.5 years for the chemoresistant patients (n=5) and 49±11 years for the chemosensitive patients (n=10). In both the chemosensitive and chemoresistant groups, most patients had ductal carcinoma, a tumor size greater than 3, and a disease stage at diagnosis above stage III. Within the distribution of molecular subtypes, hormonal-type molecular subgroups (luminal A and luminal B) were the most frequent in patients.

Analysis of circulating miRNAs before and after chemotherapy

To analyze the changes in the expression of miRNAs in the plasma of breast cancer patients after chemotherapy, we determined the $\log_2$-fold changes in the miRNA expression of these patients by comparing samples that were collected before chemotherapy. This analysis showed similar changes in some miRNAs in both groups (Supplementary Table 1-3; Supplementary Figure 1); therefore, further analysis was carried out based on the patient's responses to treatment.

Identification of miRNAs deregulated in tissue and circulating blood from breast cancer patients

Subsequently, to identify deregulated miRNAs that were associated exclusively with tumor tissue

Table 1. Clinical Characteristics of Chemoresistance and Chemosensitive Patients

Characteristics	Clinical Response	
	Chemoresistance group (n=5)	Chemosensitive group (n=10)
Age (media±SD)	49 ± 10.5	49.5± 11
Weight, kg (mean±SD)	64.8 ± 11.9	74.1 ± 21.05
Age at menarche (mean±SD)	13.4 ±1.67	13 ± 0.97
Menopausal status		
Premenopausal	3	5
Postmenopausal	2	5
Histological type		
Ductal carcinoma	4	8
Lobular carcinoma	1	0
Mixed	0	2
Tumor Size		
T3	3	4
T4	2	6
Nodal involvement		
N0 -N1	2	1
N2 - N3	3	9
Stage at diagnosis		
III-IV	5	10
Molecular subtype		
Luminal A	2	2
Luminal B	0	3
HER2	2	4
TN	1	1
Histological Receptor		
ER +	3	5
ER –	2	5
PR +	2	3
PR –	3	7
Her2neu +	2	7
Her2Neu –	3	3

Quantitative variables are expressed as mean ±SD and percentage of breast cancer patients. BMI, body mass index; T, tumor stage; N, node stage; TN, triple negative; ER, estrogen receptor; PR, progesterone receptor; *HER2*, human epidermal growth factor receptor 2.

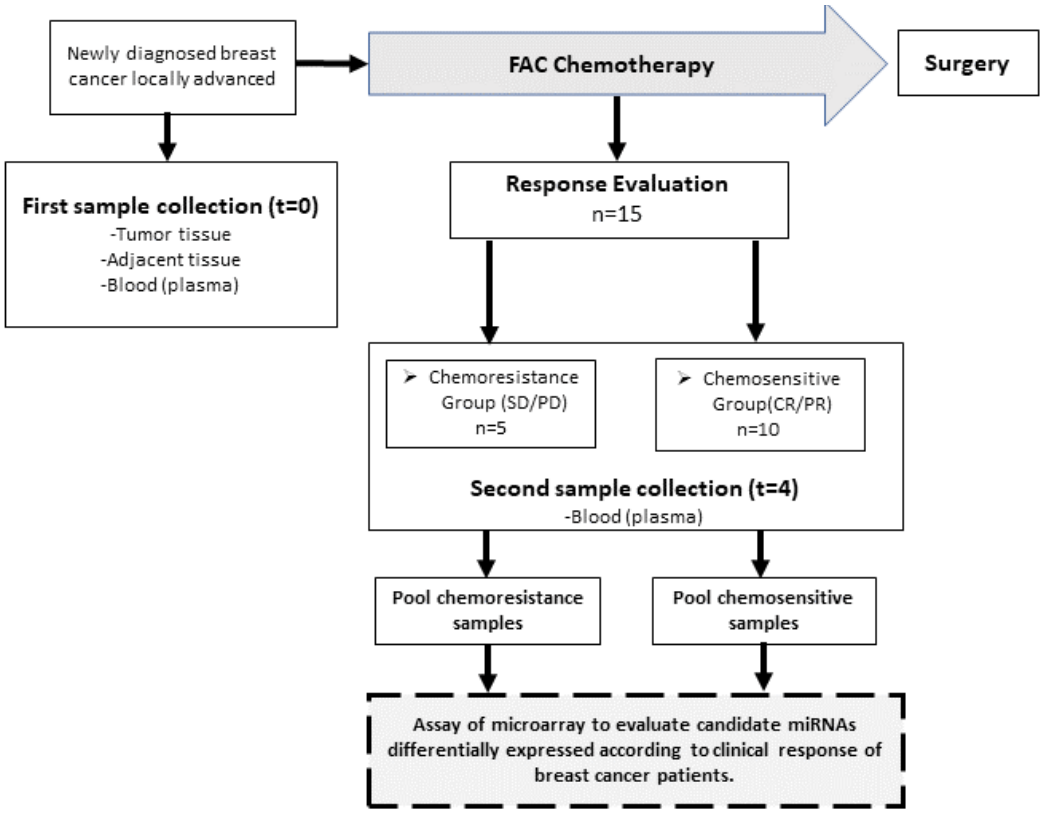

Figure 1. Flow Diagram of the Study and Sample Collection Schedule. Plasma samples from breast cancer patients were collected before and after treatment, classified as chemosensitive or chemoresistant according to each patient's clinical response, and evaluated by microarray.

from resistant and sensitive patients, we compared the pools of RNA from tumor tissues and tumor-adjacent (non-tumorous) tissues. We also performed a miRNA array using pooled RNA samples from tissues that were collected before neoadjuvant chemotherapy from either five patients with chemoresistant breast cancer (T/BCH/R) or ten patients with chemosensitive breast cancer (T/BCH/S), as shown in Figure 2. Using a PCR array method in which the results from each of these groups were compared with those derived from adjacent tissue for the same patients (control), we determined that, before neoadjuvant chemotherapy, 23 miRNAs were deregulated in tumor tissue from sensitive patients, whereas only 20 miRNAs were deregulated in resistant patients. Only three miRNAs were similarly expressed in both groups, and these were excluded from subsequent analysis (Figure 2A). Similarly, the pool of plasma from patients classified as sensitive (P/BCH/S) and resistant to chemotherapy (P/BCH/R) was analyzed in comparison with a healthy subject, discovering that only 11 of the 33 miRNAs (in the sensitive group) and 30 of 52 in the resistant group were found differentially expressed before starting neoadjuvant treatment (Figure 2B).

Chemoresistance miRNA profiles

The next step of the analysis was to identify the miRNAs that were stable components of both plasma and tissue for the group of patients, which could indicate important roles and functions for these miRNAs both inside and outside of the tumor. We determined whether dysregulated miRNAs that were expressed only in the tumor tissue of resistant patients were also released into the patient's plasma. Venn plot analysis was performed on the different data sets obtained in this study, and according to the election results, miR-100-5p, miR-152-3p, miR-195-5p, miR-20a-5p, miR-27a-3p, and miR-424-5p were detected in both biological matrices; consequently, they were identified as resistance miRNAs and selected for inclusion in a more detailed analysis (Figure 3A).

Chemosensitivity miRNA profiles

We followed the strategy described in section 3.4 to detect differential miRNA profiles in patients sensitive to chemotherapy (and theoretically present in the tissue before chemotherapy treatment). We determined which of the miRNAs that were associated with chemosensitivity were present in tumor tissue and also released into the patient's plasma by performing a Venn plot analysis. We found that, of the 24 miRNAs detected in tissue and the 33 miRNAs detected in plasma from patients who were sensitive to chemotherapy, only 3 of them were shared and therefore identified as treatment sensitivity miRNAs: let-7e-5p, miR-214-3p, and miR-222-3p (Figure 3B).

Identification of miRNAs in the circulating blood of breast cancer patients after chemotherapy

Examining the levels of miRNAs that are expressed during neoadjuvant treatment using non-invasive techniques could provide us with important information about the clinical status of the patient; in particular, it

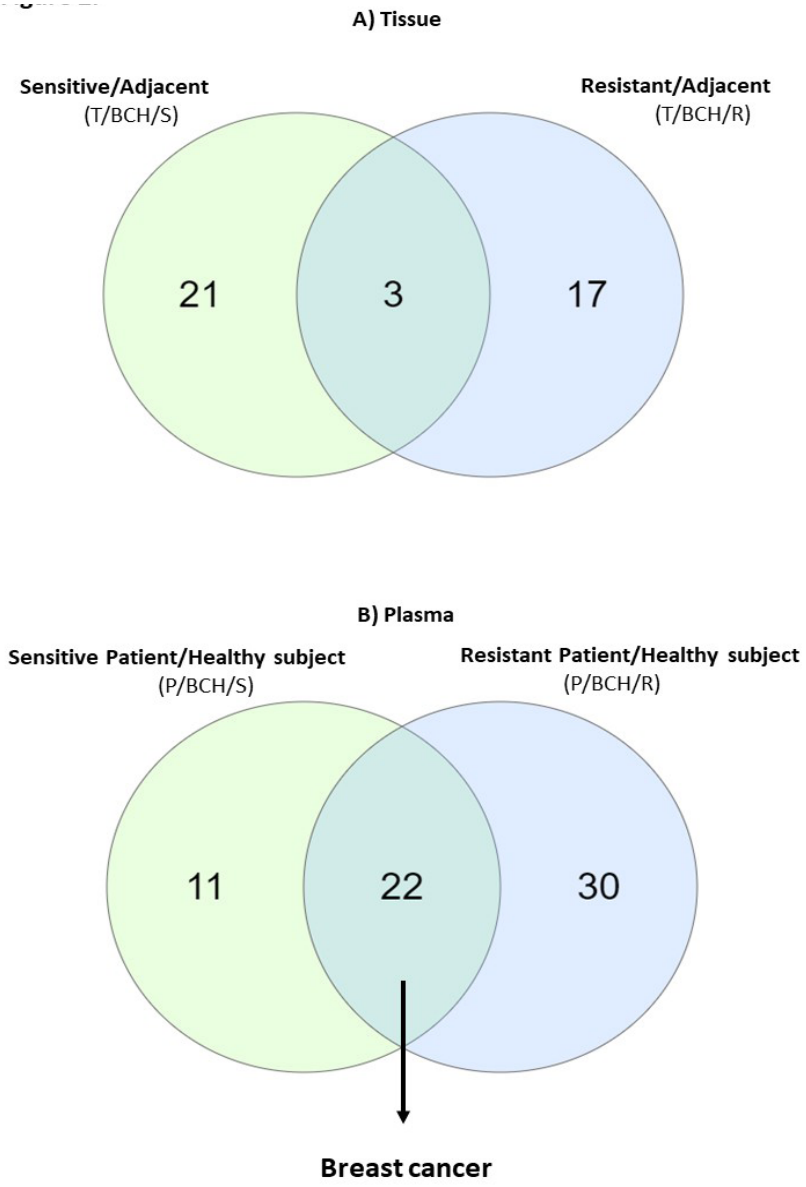

Figure 2. Differential Identification of miRNAs in Tissue and Plasma from Patients Resistant or Sensitive to Neoadjuvant Chemotherapy. A) Venn diagram showing shared microRNAs in sensitive and resistant patients and exclusive expression of microRNAs in the tissue of sensitive and resistant patients before chemotherapy (t=0). B) Venn diagram showing shared microRNAs in sensitive and resistant patients and exclusive expression of microRNAs in the plasma of sensitive and resistant patients before chemotherapy (t=0).

could give an early indication of the patient's response to chemotherapy and how their cancer is evolving. Hence, a heat map was generated to analyze changes in miRNA expression between plasma samples collected before and after chemotherapy from resistant and sensitive patients. This analysis allowed us to detect the expression of the nine candidate miRNAs that we had identified in the analyses described above (Figure 4A-B). In addition, measuring the expression levels of the six miRNAs found in chemoresistant plasma showed that three were upregulated after chemotherapy and three were downregulated after chemotherapy (miR-20a-5p, miR-27a-3p, and miR-424-5p; Figure 4A). In contrast, in the chemosensitive group, only the expression levels of miR-222-3p and miR-let-7e-5p were found to have decreased, and miR-214-3p was downregulated after chemotherapy (Figure 4B).

Interaction network analysis identified a total of 620 target genes regulated by hsa-miR-214-3p, hsa-miR-222-3p, and hsa-let-7e-5p. Of these, 20 genes of biological interest were selected (*PGR, ESR1, ERBB2, GATA3, FOXA1, BRCA1, BRCA2, TP53, MYC, CCND1, EGFR, CDH, MKI67, KRT18, KRT19, STAT3, AKT1, PIK3CA, BCL2)*. The miRNA–gene interaction network constructed in Cytoscape showed a modular organization. On the other hand, the resistance-associated microRNAs, hsa-miR-20a-5p and hsa-miR-27a-3p, regulated a total of 1495 genes in cancerous breast tissue, of which those jointly regulated by both microRNAs were filtered, including *RBSN, PHLPP2, RUNX1, HIF1A, GNB5, ALDH9A1, FOXJ3, CCND1, TP53* and *PPARG* (Supplementary Figure 2).

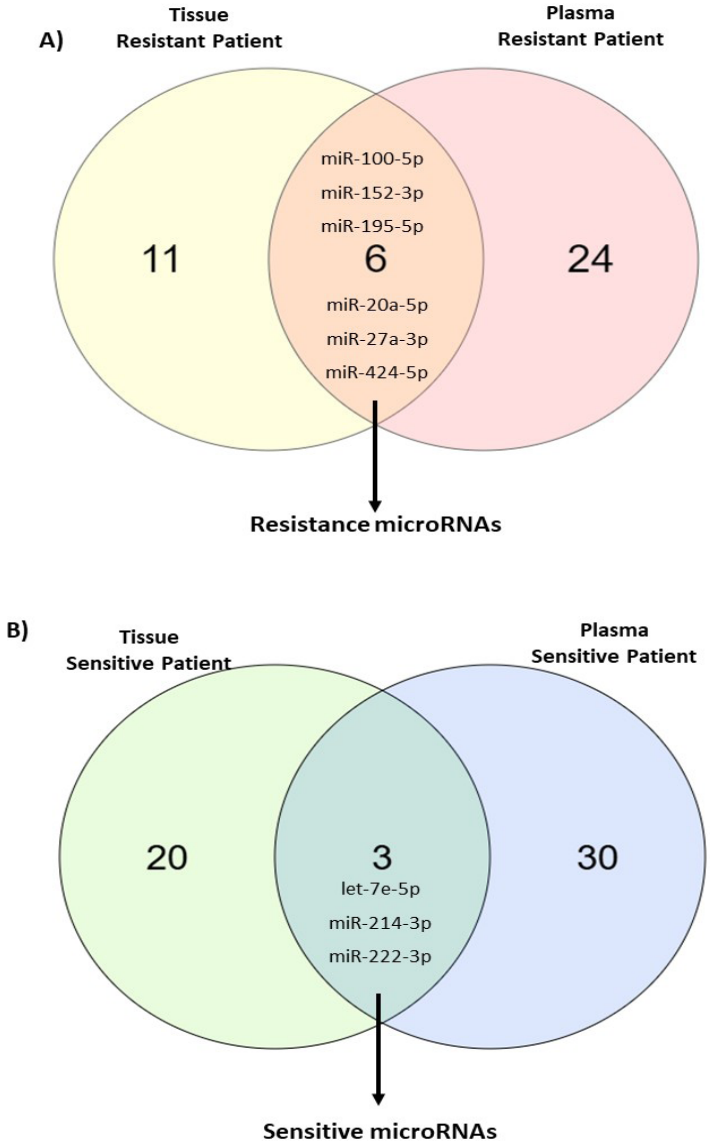

Figure 3. Comparison of miRNAs in Tissue Versus Plasma of Patients Resistant to Neoadjuvant Chemotherapy. A) Venn diagram showing shared expression of miRNAs in tissue and plasma samples from only chemoresistant patients. B) Venn diagram showing shared expression of miRNAs in tissue and plasma samples from only chemosensitive patients.

Discussion

The mechanisms driving chemoresistance in breast cancer include the activation of DNA repair, the epithelial–mesenchymal transition, the overexpression of efflux transporters, genetic polymorphisms, and non-coding RNAs such as miRNAs. Recent studies have shown that dysregulated miRNAs often contribute to the development of chemoresistance in breast cancer and, consequently, metastasis [14]. Likewise, several works reported promising findings on the diagnostic [12–15], prognostic [15, 16], and predictive utility [17]. However, many studies have the limitation that they do not demonstrate through simultaneous evaluation whether the expression of miRNAs is the same in tissues and circulating blood. Furthermore, only a few studies have evaluated the expression profiles of miRNAs before and after neoadjuvant treatment [18].

It is important to note that, generally, an individual miRNA can be characteristic of more than one type of tumor or condition in a patient. Thus, appropriate miRNA combinations must be investigated to establish a breast cancer-specific miRNA profile [19]. In the present study, using miRNA microarray analysis, we analyzed the shared profiles of circulating and tissue miRNAs in breast cancer patients with a predisposition for chemoresistance or chemosensitivity, in order to facilitate earlier and less invasive evaluation than is currently possible. Furthermore, differences in the expression of these miRNAs in plasma samples obtained before and after chemotherapy were described.

Consistent with previously published reports, our study showed that 30% of our patients presented a clinical response of chemoresistance to neoadjuvant chemotherapy, which provided a rationale for identifying predictive biomarkers of patients' chemotherapy responses

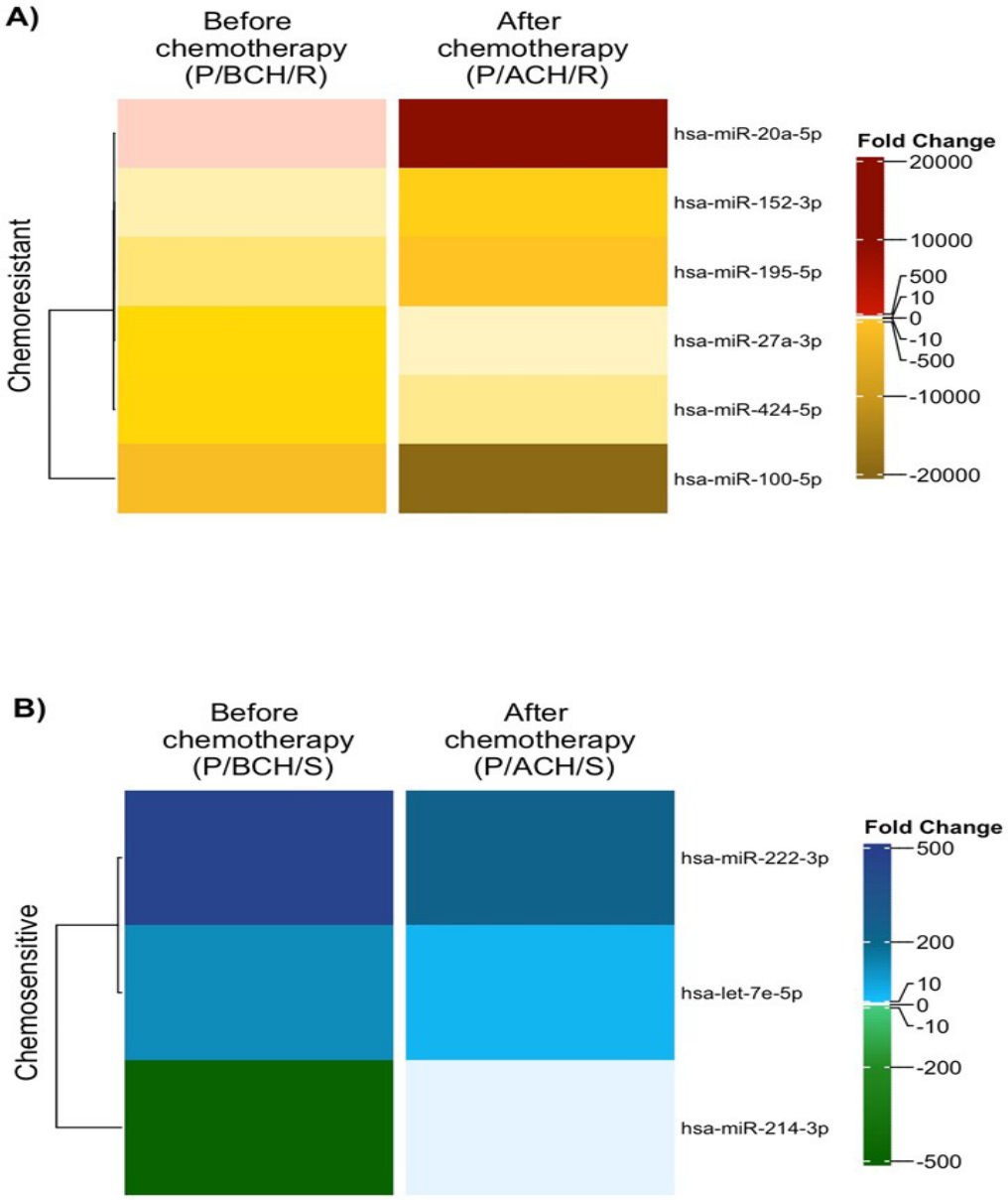

Figure 4. Comparison of miRNAs in Plasma before and after Neoadjuvant Chemotherapy. A) Heatmap of miRNA expression in plasma samples from chemoresistant breast cancer patients. B) Heatmap of miRNA expression in plasma samples from chemosensitive breast cancer patients.

[20, 21]. In the present research, to detect differing miRNA profiles in patients who were either sensitive or resistant to chemotherapy (which, in theory, would be present in the tissue even before developing chemoresistance or chemosensitivity), two pools of tumor tissues from patients with different clinical responses were analyzed first: one pool of resistant patients (T/BCH/R) and another pool of sensitive patients (T/BCH/S).

To discover resistance and sensitivity miRNA profiles in tumors and in circulation, it is important to understand which miRNAs are stable components in both biological matrices. In this context, it is essential to consider that previous studies have shown that only certain miRNAs can be released into circulation freely or though exosomes [10, 22]. Building on this concept, clinical evidence supports the translational value of circulating miRNAs: in a prospective multicenter study, it was found that in patients with locally advanced breast cancer who showed a complete pathologic response to standard neoadjuvant chemotherapy treatment, levels of circulating miR-21 and miR-195 were significantly lower compared with non-responders, thus enabling the identification of patients who will obtain the maximum benefit from chemotherapy. In addition, exosomal levels of miR-21 and miR-105 in the plasma have been reported to be higher in metastatic patients than non-metastatic patients and healthy donors [23], reinforcing the usefulness of circulating miRNAs as dynamic biomarkers associated with tumor progression..

Log_2-fold-change analyses permitted us to address whether there were differences in the expression of miRNAs before and after chemotherapy (Supplementary Figure 1), and we observed that only certain miRNAs showed a significant change in expression. It was then considered important to divide the patients into

chemotherapy-response groups to evaluate which miRNAs were shared in both biological matrices (plasma and tissue). Our results showed that before neoadjuvant treatment, a group of miRNAs (miR-100-5p, miR-152-3p, miR-195-5p, miR-20a-5p, miR-27a-3p, and miR-424-5p) was present in chemoresistant patients both in tissue and in circulation which differed from those observed in chemosensitive patients (let-7e-5p, miR-214-3p, and miR-222-3p). In both chemoresistant and chemosensitive patients, these miRNAs remained in circulation after chemotherapy. These findings could help us understand the tumor's behavior and the potential oncogenic or oncosuppressive roles of the identified miRNA profiles. However, more exhaustive studies are necessary to identify the different mechanisms and signaling pathways, as well as their biological functions, so that they can be used as therapeutic targets or biomarkers to assess the patient's response by monitoring expression levels during neoadjuvant treatment.

The aberrant expression profiles of miRNAs that are present in tumor tissues and in circulation depend in part on the location and type of tumor. In this regard, researchers have previously reported the potential application of miRNAs in breast cancer evaluation development of individualized treatment strategies. A study by Huseyin Akbulut et al. [23] shows that in tumor tissue from a group of patients with unifocal breast cancer, 13 miRNAs were upregulated and 5 miRNAs were downregulated. In contrast, in the multicenter group, only three miRNAs were upregulated and seven miRNAs were downregulated [22].

Barbara N. Borsos et al. performed a study in patients with early-stage breast cancer in which they reported the presence of the combination of miR-15a, miR-16, and miR-221 in the circulation as a possible specific biomarker for treatment selection [19]. As we mentioned, to our knowledge, it is difficult to find a single specific and sensitive miRNA that can be used as a biomarker either at the tumor level or in circulation. Moreover, there is a need to detect markers by noninvasive methods, such as measuring circulating miRNAs. In this regard, identifying and monitoring multiple plasma miRNAs represents a better approach to addressing the possible mechanisms presented in the tumor; in this way, the diagnosis and follow-up of patients could be more precise and real and, in consequence, more valuable for breast cancer liquid biopsy.

To find miRNAs associated with tumor progression and treatment response, we compared the expression of resistance- and sensitivity-associated miRNAs in circulating blood before (t=0) and after (t=4) chemotherapy. After four cycles of neoadjuvant therapy, the expression of some chemoresistance-associated miRNAs (P/ACH/R), including miR-20a, miR-27a, and miR-424, was increased. miR-20a has previously been detected in tumor tissue from breast cancer patients and has been correlated with poor survival. However, unlike in our study, Wengong Si et al. [24] suggest that the ectopic overexpression of miR-20a sensitizes breast cancer cells to a broad spectrum of chemotherapy drugs and suppresses their proliferation through the miR-20a/MAPK1/c-Myc pathway both in vitro and in vivo. In our study, miR-20a-5p was upregulated in both circulation and tissue of breast cancer patients, with increased expression following chemotherapy, which correlates with chemoresistance. Previous experimental data indicated that miR-20a expression in invasive breast carcinomas is associated with a VEGF-related high-risk angiogenic profile that could explain our results [25].

miR-27a is an oncomir that is involved in resistance to chemotherapy (with paclitaxel, cisplatin, 5-fluorouracil, adriamycin, docetaxel, etc.) and is induced by hypoxia in different types of cancer [26]. It is currently known that different pathways converge on miR-27a, negatively regulating both apoptosis and the expression of multidrug resistance transporters (MDR), thus promoting resistance to chemotherapeutic drugs [27]. In contrast, the function of miR-424 is still unclear; studies in breast cancer demonstrate the tumor suppressor role of miR-424-5p and that it is related to higher tumor stages and metastasis through its binding to the DCLK transcript (Doublecortin-like kinase) [28]. Contradictorily, we observed that miR-424-5p plasma levels increased after chemotherapy in chemoresistant patients, which the treatment could have induced. Further studies need to be conducted to explore the roles of these miRNAs in the treatment responses of breast cancers.

In contrast, tumor suppressor miRNAs such as miR-152-3p, miR-195-5p, and miR-100-5p decreased during tumor progression and even after the delivery of neoadjuvant treatment. Both miR-152 and miR-195 have been reported to be tumor-suppressive miRNAs. Specifically, miR-195-5p has been associated with cancer progression, trastuzumab resistance, and poor overall survival [29–31]. In the case of miR-152 with is downregulated in breast cancer [32], also it has been proposed as a highly sensitive and specific marker in liquid biopsies from breast cancer patients that enabling differentiation between stages of the disease (early/intermediate versus advanced) [33].

miR-100 levels have been associated with better overall survival and responses to endocrine therapy in women with operable luminal breast cancers [34]. An increase in miR-100 levels sensitizes breast cancer to paclitaxel treatment [35] and is associated with a more favorable prognosis [36], indicating that this miRNA is involved in altering chemoresistance. In line with these studies, our results also showed low levels of miR-100 in chemoresistant patients after chemotherapy.

The metastatic role of miR-222 has been reported in breast cancer, where upregulation increases the IC_{50} in tumor cells and confers resistance to anthracyclines [37, 38]. In contrast to these findings, we detected miR-222 not in the resistant group of patients, but in the sensitive group, where it exhibited overexpression before the FAC treatment (P/BCH/S). In addition to the context of the sample type used in previous studies (the Adriamycin-resistant breast cancer cell line MCF-7), this difference in results could be due to the small number of patients included in our study as well as the different use of chemotherapy with anthracyclines, fluorouracil, and cyclophosphamide in our patient group.

Let-7 expression has also been associated with cancer initiation and progression [16]. The functions of all members of the let-7 family are generally thought to overlap due to sequence similarity [17]. Interestingly, we found that miR-222-3p and let-7e-5p decreased after neoadjuvant therapy, but only in sensitive patients (P/ACH/S). The findings of the present study provide evidence for let-7e-5p and miR-222-3p as sensitivity markers. In addition, target gene analysis found that solute carrier family member 13 (SLC2A13) is a common target gene for both miRNAs [39]. SLC2A13 expression is downregulated in several types of cancers, including breast and gastric cancer. Therefore, analysis of these miRNAs in combination and their target gene downregulation improves sensitivity and specificity, providing evidence for their use as non-invasive biomarkers in breast cancer progression.

In our study, we observed that the levels of miRNAs associated with sensitivity tended to decrease even more after neoadjuvant treatment (P/ACH/S), except for miR-214-3p. Previous studies have revealed that miR-214 is a suppressed oncogene in breast cancer and other types of cancer [40-42]. The expression of miR-214 is significantly decreased in breast cancer tissues compared to adjacent tissues, and its aberrant expression is negatively associated with Ki-67, a marker of the rate of division of cancer cells in a tumor. Our results also suggest that the increase in plasma levels of miR-214 after treatment might reflect an early apoptotic effect of breast cancer cells and thus sensitivity to FAC, as suggested by Bo Liu et al. [15].

Additionally, to understand the relationship between sensitivity or resistance microRNAs and genes implicated in breast cancer, we constructed and analyzed interaction networks using human breast tumor data. The resulting networks showed that the miRs let-7e-5p, miR222-3p, and miR-214-3p (sensitivity miRs) have common target genes of clinical interest, such as VEGFA, TP53, and CCND1, which could promote treatment response by suppressing growth or angiogenesis mechanisms. On the other hand, only certain resistance-associated miRs, such as miR27-a-3p and miR-20a-5p, were found simultaneously associated with 16 genes, including solute transporters, HIF1A, and TP53, promoting evasion mechanisms such as induced hypoxia or drug efflux. This analysis allows us to confirm the simultaneous participation of miRs as well as the clinical relevance of their evaluation. Therefore, given the aforementioned results and previous evidence, we propose the hypothesis that a multi-miR signature acts cooperatively in the transcriptional regulation of breast cancer.

Finally, it's important to acknowledge that our scope did not include the confirmation of the candidate miRNAs in a larger sample. Therefore, validation studies should be performed in larger cohorts of patients with locally advanced breast cancer, especially to evaluate the prognostic potential of miRNAs associated with sensitivity or resistance to neoadjuvant chemotherapy. Although we did not conduct experiments on miRNA target genes as biomarkers or investigate the molecular mechanisms involved in resistance or sensitivity to treatment, this study lays the groundwork for future experimental research aimed at elucidating these mechanisms and evaluating their involvement, considering the findings observed in our group of patients.

In conclusion, Our results, together with previous reports, suggest that miR-214-3p, miR-222-3p, and let-7e-5p may be protective and indicate a sensitivity to neoadjuvant chemotherapy. In contrast, miR-20a-5p, miR-27a-3p, miR-424-5p, miR-152-3p, mi-195-5p and miR-100-5p could indicate resistance to neoadjuvant chemotherapy in breast cancer, making them biomarkers and potential therapeutic targets. However, given the limited size of our sample, these findings should be interpreted with caution. Although the study provides valuable information, further validation in larger cohorts and diverse clinical settings is required to confirm the potential of these miRNAs as biomarkers and therapeutic targets.

Author Contribution Statement

Eneida Turiján-Espinoza performed data collection, experimental work, and analysis. Data collection and sample preparation were performed by Eneida Turiján-Espinoza and Víctor Manuel Ruíz-Rodríguez. Eneida Turiján-Espinoza wrote the first draft of the manuscript, and all authors gave feedback on the final version. All authors read and approved the final manuscript. Diana Patricia Portales-Pérez conceived and designed the study, contributed to interpreting the results, and drafted and critically revised the manuscript.

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General

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Scientific Approval

This research has been approved in accordance with the guidelines established by the Postgraduate Program in Pharmacy Biological Sciences at the Autonomous University of San Luis Potosí, Mexico.

Conflicts of interest

The authors declare no conflicts of interest related to this manuscript.

Ethics statement

The studies involving human participants were reviewed and approved by the Central Hospital Research Committee (COFEPRIS 14 CI 24 028 083) and the

Committee of Ethics in Research (CONBIOETICA-24-CEI-001-20160427). The patients/participants provided written informed consent to participate in this study.

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