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Tumor MicroRNA Expression Profiling Identifies Circulating MicroRNAs for Early Breast Cancer Detection

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BACKGROUND: The identification of novel biomarkers for early breast cancer detection would be a great advance. Because of their role in tumorigenesis and stability in body fluids, microRNAs (miRNAs) are emerging as a promising diagnostic tool. Our aim was to identify miRNAs deregulated in breast tumors and evaluate the potential of circulating miRNAs in breast cancer detection.

METHODS: We conducted miRNA expression profiling of 1919 human miRNAs in paraffin-embedded tissue from 122 breast tumors and 11 healthy breast tissue samples. Differential expression analysis was performed, and a microarray classifier was generated. The most relevant miRNAs were analyzed in plasma from 26 healthy individuals and 83 patients with breast cancer (36 before and 47 after treatment) and validated in 116 healthy individuals and 114 patients before treatment.

RESULTS: We identified a large number of miRNAs deregulated in breast cancer and generated a 25-miRNA microarray classifier that discriminated breast tumors with high diagnostic sensitivity and specificity. Ten miRNAs were selected for further investigation, of which 4 (miR-505-5p, miR-125b-5p, miR-21-5p, and miR-96-5p) were significantly overexpressed in pretreated patients with breast cancer compared with healthy individuals in 2 different series of plasma. MiR-505-5p and miR-96-5p were the most valuable biomarkers (area under the curve 0.72). Moreover, the expression levels of miR-3656, miR-505-5p, and miR-21-5p were decreased in a group of treated patients.

CONCLUSIONS: Circulating miRNAs reflect the presence of breast tumors. The identification of deregulated miRNAs in plasma of patients with breast cancer supports the use of circulating miRNAs as a method for early breast cancer detection.

Breast cancer is the most prevalent cancer in women worldwide, and although mortality rates are decreasing, it ranks second among the most common causes of cancer death in women *(1)*. Because stage of the disease at diagnosis is associated with prognosis, efficient diagnostic tools for early detection are keys to survival. Although mammography is the most reliable way to detect breast cancer, it has some limitations *(2–4)*. Biopsy is the method used to establish a definitive diagnosis, but it is an invasive procedure. Serum tumor markers such as carcinoembryonic antigen or cancer antigen 15–3, although promising at the time of their identification, are not recommended by the American Society of Clinical Oncology and other expert panels for screening or diagnosis of breast cancer because of their low diagnostic sensitivity in early stages of the disease *(5)*. Consequently, there is an urgent need for diagnostically sensitive, specific, and noninvasive markers for early breast cancer detection.

Recent evidence demonstrates that microRNAs (miRNAs)[10] could emerge as biomarkers for the diagnosis and prognosis of different diseases, including breast cancer. MiRNAs are small, evolutionarily conserved, noncoding RNAs 18–25 nucleotides in length that reg-

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[10] Nonstandard abbreviations: miRNA, microRNA; FFPE, formalin-fixed, paraffin-embedded; LNA, locked nucleic acid; FDR, false discovery rate; SVM, support vector machine; AUC, area under the curve; qRT-PCR, quantitative real-time PCR.

ulate gene expression by sequence-specific base pairing on the 3′-untranslated region of target mRNAs, resulting in mRNA degradation or inhibition of translation *(6)*. MiRNAs have an important function in many cellular processes such as proliferation, differentiation, and apoptosis, and thus, their alteration can lead to malignant transformation. Different miRNA expression profiles have been reported in numerous types of tumors; indeed, it seems that miRNA expression profiling can be a more accurate way of classifying tumors than gene expression profiling *(7)*. Lately, circulating miRNAs have been attracting the attention of researchers because they are highly stable, resistant to degradation, and easily obtained by noninvasive procedures *(8)*. Their stability can be partially explained by 2 mechanisms: protection of secreted miRNAs by the membrane of vesicles of endocytic origin called exosomes *(9)* and stabilization of secreted miRNAs by their association with RNA-binding proteins *(10)*. Moreover, there is evidence that some miRNAs are selectively released from malignant mammary epithelial cells while being retained by nonmalignant cells *(11)*, and certain circulating miRNAs are differentially expressed in the serum and plasma of patients with breast cancer compared with healthy individuals *(12–14)*, suggesting that these molecules could provide a noninvasive tool in the diagnosis of breast cancer. Nevertheless, the analysis of circulating miRNAs is just beginning. Few studies have compared plasma and tumor miRNA expression. Moreover, little is known about miRNA expression levels after tumor resection or treatment, which would show if there is a correlation of circulating miRNAs with tumor dynamics.

In this study, we aimed to identify miRNAs deregulated in breast tumors and analyzed their potential as noninvasive biomarkers for early breast cancer detection.

Materials and Methods

ETHICS STATEMENT

The study was performed in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients before sample collection, and the study was approved by the ethics committee of Instituto de Salud Carlos III (Madrid), Hospital Virgen del Rocío and Virgen de la Macarena (Sevilla), and Hospital Monte Naranco (Oviedo).

PATIENT AND HEALTHY CONTROL SAMPLES

Tumor tissue and blood samples were collected from patients with breast cancer and healthy women of the same ethnicity (white Spaniards) in 5 Spanish institutions: Hospital Virgen de la Macarena and Hospital Virgen del Rocío (Sevilla), Hospital Monte Naranco and Biobanco del Principado de Asturias (Oviedo), and Sistemas Genómicos (Valencia). Clinicopathologic characteristics were retrieved on each patient and are shown in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue8.

Table 1. Summary of the samples used in this study.

Sample and series	n	Platform
FFPE tissue	133	
Breast tumor	122	
Training	61	Microarray
Test	61	Microarray and qRT-PCR
Healthy breast tissue	11	
Training	7	Microarray
Test	4	Microarray and qRT-PCR
Plasma	339	
Breast cancer patients	197	
Discovery before treatment	36	qRT-PCR
Discovery after treatment	47	qRT-PCR
Validation before treatment	114	qRT-PCR
Healthy women	142	
Discovery	26	qRT-PCR
Validation	116	qRT-PCR

For microarray profiling, we obtained 122 formalin-fixed, paraffin-embedded (FFPE) breast tumors from patients undergoing surgery for breast cancer. All samples were histologically confirmed by 2 pathologists (R. González-Cámpora and P. Menéndez) and divided into training (n = 61) and test (n = 61) sets. In addition, for control samples, we obtained 11 healthy breast tissue samples after breast reduction surgery from healthy women with no family history of cancer.

To study selected candidate miRNAs in blood, we obtained plasma samples from 83 patients with breast cancer and 26 healthy women. Plasma samples from patients with breast cancer were divided into 2 groups: those obtained at the time of diagnosis, before any curative practice such as surgery, radiation, or systemic therapy (n = 36), and those obtained after treatment (n = 47). In addition, we obtained a validation set of plasma samples from 114 patients with breast cancer before treatment and 116 healthy women (Table 1).

BLOOD PROCESSING AND miRNA EXTRACTION FROM PLASMA

EDTA blood samples were processed for plasma within 1 h of collection. Blood was centrifuged at 3000*g* for 20 min at 10 °C, followed by further centrifugation of the supernatant at 15 500*g* for 10 min at 10 °C to remove cell

debris. Plasma was stored at −80 °C until use. Total RNA was extracted from 250 mL plasma with an miRNeasy Mini Kit (Qiagen) and the modified Exiqon protocol, which includes the addition of MS2 RNA (Roche), a carrier RNA that ensures the highest and most consistent yield of RNA in the samples. The final elution volume was 50 μL.

miRNA MICROARRAY

FFPE blocks were analyzed by 2 pathologists (R. González-Cámpora and P. Menéndez), and the tumoral area was identified and macrodissected. Total RNA was extracted from 3 sections of 30-μm thickness with an miRNeasy FFPE Kit (Qiagen). We assessed RNA quality and quantity with a NanoDrop Spectrophotometer (NanoDrop Technologies). Total RNA was hybridized on locked nucleic acid (LNA)-based miRNA microarrays (seventh generation) containing probes for 1919 human miRNAs in quadruplicate, including 1894 miRNAs from miRBase release 18.0 and 25 hsa-miRPlus not included in miRBase (Exiqon). Briefly, 300 ng RNA was treated with calf intestine phosphatase and fluorescence-labeled (Hy3) with a miRCURY LNA™ microRNA Hi-Power Labeling Kit (Exiqon) and hybridized to miRCURY LNA microRNA arrays over 16 h at 56 °C with Agilent SureHyb-enabled hybridization chambers and a rotating oven. Arrays were then washed, dried, and scanned with an Agilent G2565AA Microarray Scanner System. We measured fluorescence intensities on scanned images with Agilent Feature Extraction software, version 10.7.3, according to the modified Exiqon protocol. The microarray dataset is publically available at the National Center for Biotechnology Information's Gene Expression Omnibus database http://www.ncbi.nlm.nih.gov/geo/ under GEO accession number GSE58606.

MICROARRAY DATA ANALYSIS

We used the "normexp" method to subtract the microarray background. To normalize the dataset, we performed quantiles between array normalizations. Replicate probes were merged by their mean profile, and miRNAs with low expression variation across samples (<0.03) were excluded, reducing the number of miRNAs to 698. To obtain a cluster of the data, we performed unsupervised hierarchical clustering with Gene Cluster software (http://rana.stanford.edu/software) using average linkage clustering, Pearson correlation, and uncentered metrics. We used Java Tree View (http://jtreeview.sourceforge.net) for image visualization. Differentially expressed miRNAs were obtained by applying linear models with R limma package (Gordon Smyth; Bioconductor project, http://www.bioconductor.org), implemented in the POMELOII tool (http://asterias.bioinfo.cnio.es/). To account for multiple hypotheses testing, the estimated significance level (*P* value) was adjusted with Benjamini and Hochberg false discovery rate (FDR) correction *(15)*. FDR <0.05 was set as the threshold to select significantly differentially expressed miRNAs.

BUILDING A miRNA MICROARRAY CLASSIFIER

To identify the smallest set of miRNAs that better discriminated breast tumors from healthy breast tissues, we generated a miRNA microarray classifier using samples from the training set (61 breast tumors and 7 healthy breast tissues). The predictor was built with the 698 miRNAs used in the differential expression analysis, and the most relevant miRNAs were chosen by correlation feature selection. We evaluated the performance of different methods previously shown to function well with microarray data *(16, 17)*: support vector machines (SVM), *k*-nearest neighbor, and random forest, which are included in the Prophet tool (http://babelomics.bioinfo.cipf.es/). The classification performance was evaluated by 5-fold cross-validation repeated 10 times. We selected classifiers producing the minimal root median square error and maximal accuracy, Matthews correlation coefficient, and area under the curve (AUC). To validate the performance of the selected classifiers, we used the samples from the test set (61 breast tumors and 4 healthy breast tissue samples). Diagnostic sensitivity and specificity values were estimated on the basis of the confusion matrix.

miRNA VALIDATION BY PCR

We quantified the expression of the most relevant miRNAs by quantitative real-time PCR (qRT-PCR) with a miRCURY LNA Universal RT microRNA PCR system (Exiqon) according to the manufacturer's protocol. qRT-PCR was applied first to FFPE samples from the validation set to validate the most relevant miRNAs, and second to plasma samples with the objective of analyzing the status of the selected miRNAs. In the case of FFPE tissues, 12 ng total RNA was reverse-transcribed with universal poly-T primers in 30-μL reactions. In the case of plasma samples and because of the low RNA concentrations, RNA amounts were used on the basis of starting volume rather than RNA quantity, and 6 μL total RNA was reverse-transcribed with universal poly-T primers in 20-μL reactions. The remaining steps were common for FFPE tissues and plasma. cDNA was diluted 10× and amplified by qPCR with miRNA-specific primers optimized with LNA. The amplification conditions consisted of an initial step at 95 °C for 10 min, followed by 50 cycles of 10 s at 95 °C and 1 min at 60 °C. We detected miRNA expression levels using the ABI Prism Sequence Detection System 7900HT (Applied Biosystems). Because it appears in the literature as a widely used endogenous control for miRNA qRT-PCR and was stably expressed among our samples, we used miR-103a-3p to normalize miRNA expression from

both FFPE tissues and plasma. Relative expression was calculated by use of the comparative cycle threshold (ΔΔCt) method implemented in qBasePLUS software (Biogazelle). For detailed methodology, see online Supplemental Methods.

STATISTICAL ANALYSIS

We performed statistical analysis with GraphPad PRISM 5 software and SPSS software package, version 17.0 (IBM). Briefly, the Kolmogorov–Smirnov test was used to analyze the normal distribution of the miRNA expression levels, and the unpaired *t* test or Mann–Whitney test was applied when appropriate to evaluate differences in miRNA expression between 2 groups. MiRNA discrimination potential was analyzed by computing ROC curves and calculating AUCs with corresponding 95% CIs, as well as the optimal specificity and sensitivity values. In all the analyses, a 2-tailed *P* value <0.05 was considered statistically significant.

Results

miRNA EXPRESSION PROFILING OF BREAST TUMORS AND HEALTHY BREAST TISSUES

To identify miRNAs associated with breast tumors, we explored the expression of 1919 human miRNAs in 122 primary breast tumors and 11 healthy breast tissue samples with LNA-based microarrays. After filtering the data to remove miRNAs with low expression variation across samples (<0.03), we obtained 698 miRNAs for further analysis. Unsupervised hierarchical clustering (see online Supplemental Fig. 1) showed that miRNA expression profiling separated breast tumors from healthy breast tissues, although a perfect stratification was not observed according to the molecular subtype of the samples.

To detect significantly deregulated miRNAs in breast tumors, supervised analysis was performed. A total of 194 miRNAs showed significant differential expression (FDR <0.05) between breast tumors and healthy breast tissues: 117 were upregulated in breast tumors, whereas 77 were downregulated. Eleven of these differentially expressed miRNAs had a >2-fold change (Table 2).

Table 2. Eleven miRNAs with the greatest difference in expression between breast tumors and healthy breast tissues (FDR <0.05, fold change ≥2).

			Median	
miRNA	FDR, adjusted *P*	Fold change	Healthy breast tissue	Breast tumor
miR-125b-5p	1×10^{-7}	4.1 ↓	10.9	8.6
miR-21-5p	9.07×10^{-5}	3.2 ↑	6.5	8.3
miR-3613-3p	7.16×10^{-5}	3.1 ↑	7.3	8.7
miR-4668-5p	0.000194	2.8 ↑	6.6	8.2
miR-4516	1.77×10^{-5}	2.5 ↓	11.0	9.7
miR-548as-3p	0.000167	2.4 ↑	6.4	7.7
miR-3656	2.00×10^{-7}	2.2 ↓	9.4	8.4
miR-4488	1.80×10^{-6}	2.2 ↓	8.8	7.7
miR-5704	5.04×10^{-5}	2.2 ↑	7.3	8.3
miR-141-3p	0.003198	2.0 ↑	7.3	8.0
miR-638	0.000536	2.0 ↓	8.7	7.9

miRNA SIGNATURE FOR BREAST TUMOR PREDICTION

To identify the smallest set of miRNAs discriminating breast tumors from healthy breast tissues, a miRNA microarray classifier was generated with samples from the training set. To identify the most representative miRNAs, we used correlation feature selection and 5-fold cross-validation repeated 10 times to estimate how accurately the predictive model would perform in an independent data set. We used different algorithms that have been shown to function well with microarray data: SVM, *k*-nearest neighbor, and random forest. We selected SVM because it showed the best performance, producing the minimal root median square error and maximal accuracy, Matthews correlation coefficient, and AUC (see online Supplemental Table 2). With this algorithm, we generated a 25-miRNA signature for breast tumor prediction with 100% diagnostic sensitivity and 83% specificity. To validate the discrimination potential of the miRNA signature, we classified an independent series of samples (test set) in a blind approach. Patients in the test set were correctly identified with 100% diagnostic sensitivity and 100% specificity. The list of miRNAs that compose the signature is shown in Table 3.

Table 3. Diagnostic performance of the 25-miRNA signature[a] for the prediction of breast tumors and healthy breast tissue.

Dataset	n	Accuracy, %	Sensitivity, %	Specificity, %
Training		98	100	83
Breast tumor	61			
Healthy tissue	7			
Test		100	100	100
Breast tumor	61			
Healthy tissue	4			

[a] miR-125b-5p, miR-3613-3p, miR-4668-5p, miR-3656, miR-5704, miR-3676-3p, miR-3196, miR-3941, miR-585, miR-1264, miR-200a-3p, miR-1273g-3p, miR-5581-3p, miR-877-5p, miR-96-5p, miR-744-3p, miR-2276, miR-342-5p, miR-760, miR-203, miRPlus-A1086, miR-185-5p, miR-20b-5p, miR-4521, and miR-4692.

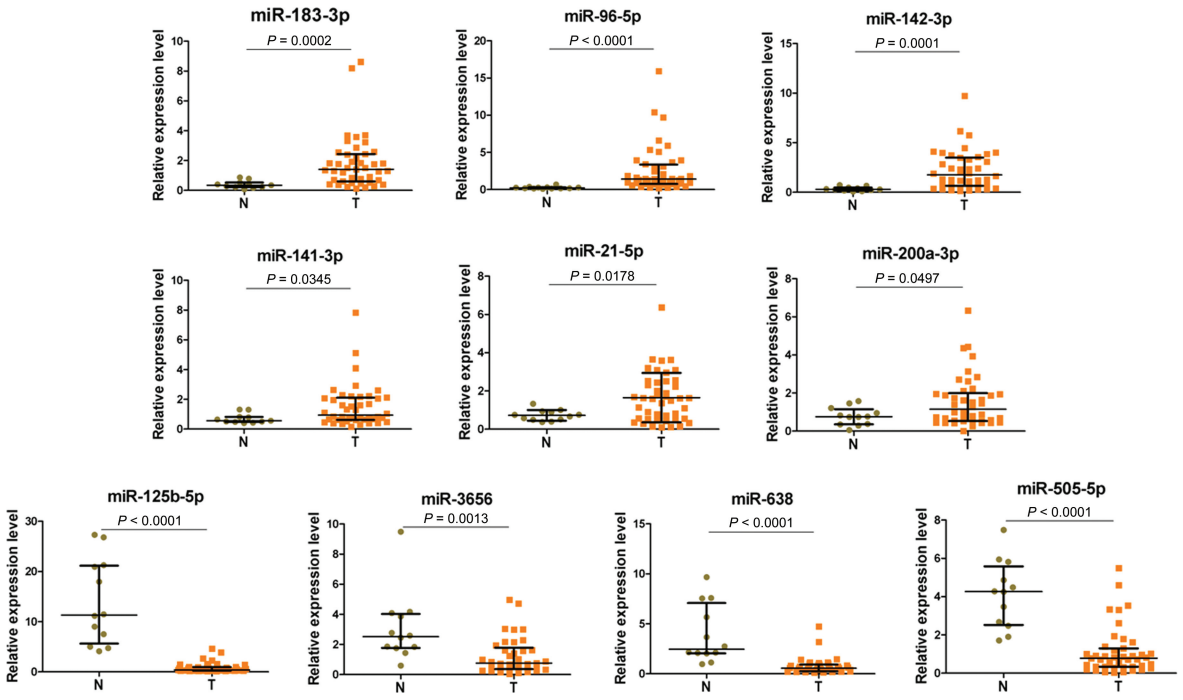

Fig. 1. Validation of most relevant miRNAs by qRT-PCR in breast tumors and healthy breast tissues.

Scatterplots show relative expression levels of miR-183-3p, miR-96-5p, miR-142-3p, miR-141-3p, miR-21-5p, miR-200a-3p, miR-125b-5p, miR-3656, miR-638, and miR-505-5p in 44 breast tumors (T) and 12 healthy breast tissues (N).

VALIDATION OF THE MOST RELEVANT miRNAS BY qRT-PCR

We selected miRNAs with the smallest FDR and highest fold change when comparing breast tumors and healthy breast tissues and tried to validate them by qRT-PCR in 44 tumors from the test set and 12 healthy breast tissues. We selected 19 miRNAs: 10 from the breast tumor classifier and 9 from the differential expression analysis. Unfortunately, 8 were discarded owing to unspecific amplification or no amplification (miR-3613-3p, miR-4668-5p, miR-5704, miR-1264, miR-5581-3p, miR-548as-3p, miR-3686, miR-4419b), and therefore 11 could be analyzed: 5 from the breast tumor classifier and 6 from the differential expression analysis. Statistical analysis led to the validation of all ($P < 0.05$) except miR-1273g-3p. Hence miR-183-3p, miR-96-5p, miR-142-3p, miR-141-3p, miR-21-5p, and miR-200a-3p were confirmed to be significantly upregulated in breast tumors, whereas miR-125b-5p, miR-3656, miR-505-5p, and miR-638 were confirmed to be downregulated (Fig. 1).

FROM TUMOR TO PLASMA: ANALYSIS OF CIRCULATING miRNAS

To evaluate the expression of the selected miRNAs in plasma, amplification by qRT-PCR was performed in 26 healthy individuals, 36 breast cancer patients before treatment, and 47 breast cancer patients after treatment. Among 10 miRNAs analyzed, 9 had detectable expression levels in plasma; only miR-183-3p could not be detected. Comparison of the expression levels in pretreatment breast cancer patients and healthy individuals led to the identification of 5 differentially expressed miRNAs in plasma: miR-3656, miR-505-5p, miR-125b-5p, miR-21-5p, and miR-142-3p. In addition, although not statistically significant, miR-96-5p showed a trend (P value <0.1). Interestingly, some of the miRNAs analyzed were deregulated in opposite directions compared with tumors. That is, miR-21-5p, miR-142-3p, and miR-96-5p were overexpressed in both breast tumors and plasma from breast cancer patients, whereas miR-3656, miR-505-5p, and miR-125b-5p were downregulated in breast tumors but upregulated in plasma from patients with breast cancer (Fig. 2). A selective release of certain miRNAs from tumors to plasma might be the explanation for this discordance.

Next we compared the expression levels of the 5 significant miRNAs and the 1 with P value <0.1 in patients with breast cancer before and after treatment. Although miR-125b-5p, miR-142-3p, and miR-96-5p did not show a significant change in expression, the expres-

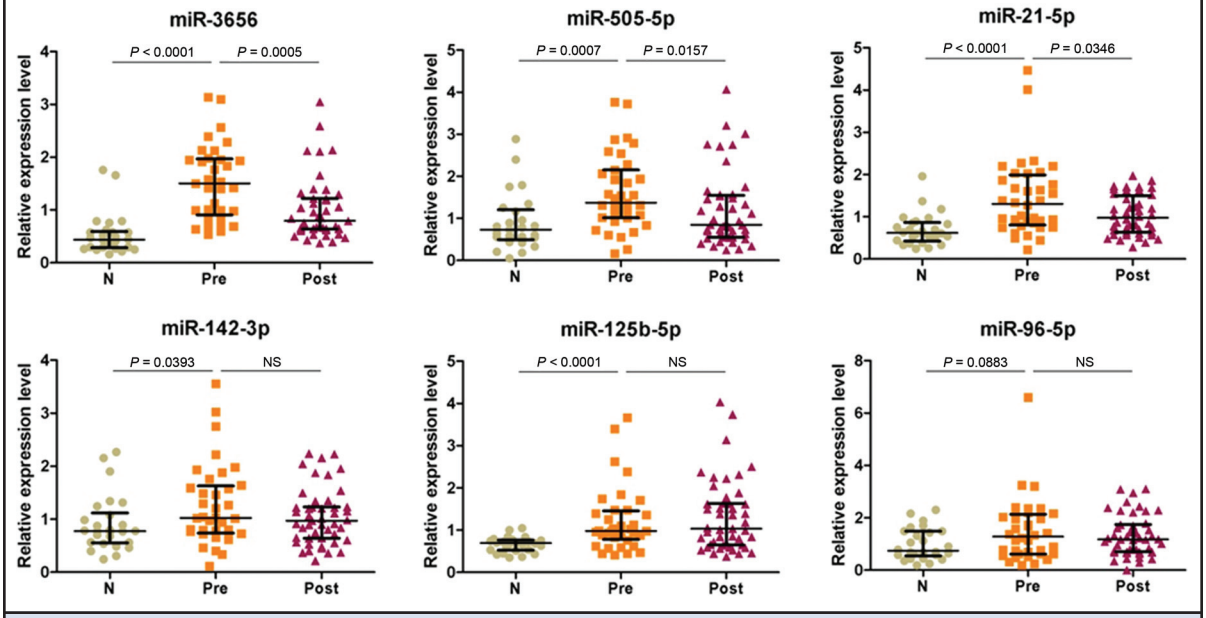

Fig. 2. Relative expression levels of circulating miRNAs deregulated in the plasma of 36 breast cancer patients before treatment (Pre) in comparison with 26 healthy individuals (N).

In addition, miRNA plasma concentration was quantified in 47 breast cancer patients after treatment (Post).

sion of miR-3656, miR-21-5p, and miR-505-5p was significantly reduced in plasma after treatment, suggesting that these miRNAs may be sensitive to changes in tumor mass (Fig. 2).

MARKER VALIDATION

The 5 significant miRNAs identified were then subjected to validation in a second set of plasma from 114 pretreated breast cancer patients and 116 healthy women. Because miR-96-5p showed a trend in the previous analysis, we decided to include this miRNA in the validation. The expression levels of miR-505-5p, miR-125b-5p, miR-21-5p, and miR-96-5p were confirmed to be significantly overexpressed in the plasma of breast cancer patients (Fig. 3A). ROC curve analysis was performed to evaluate the diagnostic utility of these miRNAs. The resulting curves showed that miR-505-5p and miR-96-5p were the most valuable biomarkers for discriminating patients from healthy individuals, with AUCs of 0.7213 (95% CI 0.6558–0.7867, $P < 0.0001$) and 0.7167 (95% CI 0.6507–0.7827, $P < 0.0001$), respectively, and diagnostic sensitivity and specificity at the optimal cutoff of 75% and 60% for miR-505-5p and 73% and 66% for miR-96-5p. miR-125b-5p and miR-21-5p showed AUCs of 0.6368 (95% CI 0.5642–0.7093, $P < 0.03699$) and 0.6070 (95% CI 0.5336–0.6803, $P < 0.03742$), respectively (Fig. 3B).

Discussion

Altered miRNA expression has been associated with several types of human cancer, including breast cancer. Here, we analyzed the expression levels of 1919 human miRNAs in a large series of breast tumors and healthy breast tissues and identified miRNAs deregulated in breast cancer with potential utility as diagnostic biomarkers. We first performed unsupervised hierarchical clustering of our samples and observed a clear separation between breast tumors and healthy breast tissues, confirming previous studies suggesting that miRNA expression profiling could be used to classify breast tumors *(7, 18)*. We detected common expression profiles in tumors having the same molecular subtype, but also clusters of samples belonging to different subtypes. One possible explanation could be that miRNAs are regulating multiple processes in the cell, and our samples could be grouping not just according to their molecular subtype but also according to other tumor characteristics.

To identify miRNAs associated with breast tumors, we performed a supervised analysis. The large amount of deregulated miRNAs identified in our set of breast tumors highlights the important role that miRNAs play in breast tumorigenesis. Among the most deregulated miRNAs, miR-125b-5p and miR-21-5p have been repeatedly associated with breast cancer *(18, 19)*. MiR-21-5p is known to function as an oncogene by targeting

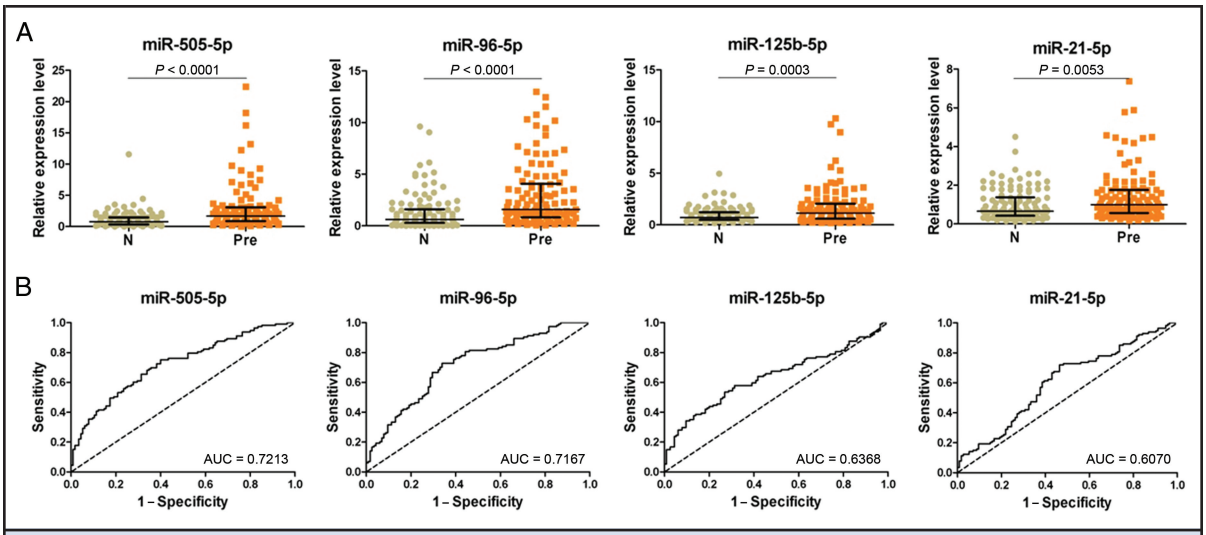

Fig. 3. Circulating miRNAs validated overexpressed in the plasma of 114 breast cancer patients before treatment (Pre) compared with 116 healthy women (N).

(A), Relative expression levels of miR-505-5p, miR-96-5p, miR-125b-5p, and miR-21-5p with their corresponding *P* values. (B), ROC curves for each miRNA and the resultant AUC.

tumor suppressor genes including tropomyosin 1, programmed cell death 4, and phosphatase and tensin homolog, leading to cell proliferation and inhibition of apoptosis and regulation of cancer invasion and metastasis in breast cancer *(20–22)*. MiR-125b-5p is upregulated in many cancers but downregulated in others, such as breast cancer, and controls many different cellular processes by targeting numerous transcription factors such as ETS1, E2F3, and BCL3 *(23–25)*. In addition, we have identified new miRNAs that have not been associated with breast cancer before, such as miR-3613-3p, miR-4668-5p, miR-4516, miR-548as-3p, miR-4488, miR-3656, and miR-5704, expanding the knowledge on miRNA deregulation in breast cancer.

We have also generated a 25-miRNA microarray classifier that discriminates breast tumors, independently of tumor subtype and stage, from healthy breast tissues with high diagnostic sensitivity and specificity. The diagnostic accuracy of 0.98 suggests excellent classification ability. The validation of our classifier in an independent set of samples with similar diagnostic sensitivity and specificity confirms the ability of the miRNA signature to differentiate breast tumors. In addition, considering that almost three-quarters of the patients in the training and the test sets have stage I or II breast cancer, this signature could be especially indicated for early-stage breast cancer discrimination. Because these miRNAs might be relevant for breast cancer detection, we decided to investigate their expression in plasma.

A strong correspondence between microarray expression and qRT-PCR was observed, as 91% of the explored miRNAs were validated ($P < 0.05$). These results are in line with those reported by Git et al., where a high correlation (0.82–0.92) between Exiqon platform and qPCR was described *(26)*. However, the validation of significant tumoral miRNAs in plasma was more controversial. Among 10 miRNAs analyzed in the discovery set, 5 were found to be differentially expressed in the plasma of patients with breast cancer, and 1 showed a trend. However, only half of these 6 miRNAs were deregulated in the same direction as in tumors (miR-21-5p, miR-142-3p, and miR-96-5p). Dissimilar patterns of miRNA expression between tumor and plasma have been reported recently. Chan et al. *(13)* performed miRNA profiling of tumors and sera from breast cancer patients and healthy individuals and observed 73 miRNAs deregulated in breast tumors and 85 in plasma. However, only 21 were in common in both tissues, and 13 of them were deregulated in opposite directions *(13)*. Similarly, Pigati et al. studied the liberation of miRNAs from malignant and nonmalignant mammary cells into body fluids and suggested that miRNAs are released from breast cancer cells in a selective manner, and therefore, extracellular and cellular miRNAs profiles are different *(11)*. Because circulating miRNAs might have different origins, isolation of tumor-derived exosomes and analysis of their miRNA content could be a novel strategy to explore secreted miRNAs. Nevertheless, characterization of marker proteins that allow enrichment of tumor-derived exosomes over healthy exosomes is required.

After validation in a second series of plasma from untreated patients and healthy women, miR-505-5p,

miR-125b-5p, miR-21-5p, and miR-96-5p were confirmed to be significantly deregulated in both tumor and plasma from pretreated patients. Therefore, we hypothesized that these miRNAs might be candidates for noninvasive breast cancer detection. Overexpression of circulating miR-21-5p has been described in breast cancer patients *(27–30)* but also in other cancers such as esophageal, gastric, colorectal, and lung *(31)*. This result shows the potential utility of circulating miR-21-5p as a broad-spectrum biomarker for the detection of various cancers and not specifically for breast cancer diagnosis. MiR-125b-5p has also been reported to be upregulated in the serum of breast cancer patients *(28, 32)* and has been associated with chemotherapeutic resistance, nonresponsive patients having higher expression levels. Finally, upregulation of miR-96-5p and downregulation of miR-505-5p have been reported in breast tumors and related to increased cell proliferation *(33, 34)*, but this is the first time that their circulating levels are associated with breast cancer, both being overexpressed.

ROC curve analysis showed the discrimination potential of these 4 miRNAs, with AUCs ranging from 0.6070 to 0.7213, and with miR-505-5p and miR-96-5p being the most valuable biomarkers for discriminating patients with breast cancer from healthy individuals. In addition, the expression levels of miR-505-5p and miR-21-5p were significantly reduced after surgery/treatment in our first series (training set), supporting the potential utility of these miRNAs to monitor treatment response and highlighting their clinical value for breast cancer detection and surveillance.

In conclusion, we have identified a large number of deregulated miRNAs in breast tumors, many of them not previously reported, and have generated a miRNA signature that discriminates breast tumors from healthy breast tissues with high diagnostic sensitivity and specificity. Furthermore, we have reported and validated the overexpression of miR-505-5p, miR-125b-5p, miR-21-5p, and miR-96-5p in the plasma of patients with breast cancer and demonstrated the potential utility of these miRNAs as noninvasive breast cancer biomarkers. An advantage of our study is that most of the patients had early-stage breast cancer at the time of sample collection, which highlights the relevance of the identified miRNAs in early breast cancer detection. Although these results are promising, prospective studies on larger cohorts of patients are required to confirm the diagnostic role of these miRNAs.

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