MicroRNA analysis of breast ductal fluid in breast cancer patients

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Abstract. Recent studies suggest that microRNAs show promise as excellent biomarkers for breast cancer; however there is still a high degree of variability between studies making the findings difficult to interpret. In addition to blood, ductal lavage (DL) and nipple aspirate fluids represent an excellent opportunity for biomarker detection because they can be obtained in a less invasive manner than biopsies and circumvent the limitations of evaluating blood biomarkers with regards to tissue of origin specificity. In this study, we have investigated for the first time, through a real-time PCR array, the expression of 742 miRNAs in the ductal lavage fluid collected from 22 women with unilateral breast tumors. We identified 17 differentially expressed miRNAs between tumor and paired normal samples from patients with ductal breast carcinoma. Most of these miRNAs have various roles in breast cancer tumorigenesis, invasion and metastasis, therapeutic response, or are associated with several clinical and pathological characteristics of breast tumors. Moreover, some miRNAs were also detected in other biological fluids of breast cancer patients such as serum (miR-23b, -133b, -181a, 338-3p, -625), plasma (miR-200a), and breast milk (miR-181a). A systems biology analysis of these differentially expressed miRNAs points out possible pathways and cellular processes previously described as having an important role in breast cancer such as Wnt, ErbB, MAPK, TGF-β, mTOR, PI3K-Akt, p53 signaling pathways. We also observed a difference in the miRNA expression with respect to the histological type of the tumors. In conclusion, our findings suggest that miRNA analysis of breast ductal fluid is feasible and potentially very useful for the detection of breast cancer.

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Introduction

Breast cancer is the most commonly diagnosed cancer in women and the second most common cause of cancer mortality. Despite major investments to improve early detection and understanding of its biology, the incidence and mortality of the disease remain surprisingly high (1). Current screening methods rely primarily on imaging techniques, including mammography and ultrasonography. Although these methods have become more sensitive and specific for detecting smaller subtle lesions, early detection remains a challenge in many patients. For this reason, identification of biomarkers that can complement the current imaging methods for early detection of breast cancer continues to be desperately needed.

miRNAs are small (18-24 nucleotides in length) non-coding RNA molecules that regulate the activity of specific mRNA targets, and are involved in a variety of physiological and pathological processes, including carcinogenesis (2). Despite the fact that the research regarding miRNA discovery and characterization in human cancers is still evolving, and despite the discrepancies among different studies, there is a general consensus that miRNAs are excellent candidate biomarkers for human cancers, including breast cancer (3). In addition, it has been shown that miRNAs are stable in archived formalin-fixed and paraffin-embedded (FFPE) tissue samples, and reliably detectable in serum, plasma and other biological fluids (4). These characteristics of miRNAs constitute the basis for using them as new biomarkers with clinical relevance, and such efforts are close to fruition in certain cancers such as pancreatic, lung, and kidney cancers, and cancers with unknown primary origin (4).

The first breast cancer specific miRNA expression signature was reported in 2005, and consisted of 29 differentially expressed miRNAs that were able to differentiate normal

from cancer tissue with 100% accuracy (5). miRNA profiling in breast cancer revealed subsets of miRNAs capable of accurately reproducing the molecular classification of breast carcinomas; other miRNA subsets were associated with clinical and pathological characteristics of breast cancers (6). A recent detailed review of miRNA biomarkers in breast cancer has revealed that, despite the real progress made through numerous studies on the subject, there is still a lack of consensus among studies (4). This is mainly due to the high degree of variability between studies regarding patient characteristics, experimental design, sample preparation, detection methodology and data analysis, thus making cross-studies comparison of the findings difficult to conduct and interpret.

Significant recent efforts focusing on the use of circulating miRNAs as breast cancer biomarkers have yielded some candidate markers. However, none were found to be highly specific or could be validated in independent studies, mainly due to the reasons mentioned above. Another conceptual challenge regarding circulating miRNAs as biomarkers is the uncertainty of the diseased tissue of origin for these miRNAs, suggesting that other biofluids should be investigated such as nipple aspirate fluid and ductal lavage fluid in the case of breast cancer (7). Although these biofluids are obtained through more invasive techniques compared to phlebotomy, they are still less invasive than biopsies and could circumvent the limitations of blood based markers regarding specificity and tissue of origin. Historically, these fluids have been used for cytological evaluation, including immunohistochemistry of breast cancer related markers such as Her2. More recently, reports of several biomarker profiling studies in nipple aspirate or ductal lavage fluids have emerged using proteomic, metabolomic, hormones, and nucleic acid analyses (8-14).

There is a lack of data in the literature regarding miRNA markers in ductal lavage or nipple aspirate fluids. However, there is evidence that these fluids contain sufficient RNA for gene expression screening by microarray studies (13,15), as well as individual gene expression measurement (16). A recent study reported on the analysis of three individual miRNA markers in ductal lavage fluid, as well as gene expression of a candidate gene and array comparative genomic hybridization screening (17).

We investigated for the first time through a real time PCR screening array the expression of 742 miRNAs in the ductal lavage fluid collected from women with unilateral breast cancer. We demonstrated the feasibility of this analysis and its potential for detection of breast cancer.

Materials and methods

Patient population. We enrolled 22 patients with unilateral, biopsy-confirmed, breast tumors [invasive breast cancer (IBC) and/or ductal carcinoma *in situ* (DCIS)], who were scheduled for surgery (mastectomy/lumpectomy) at MedStar Georgetown University Hospital. Patients were identified by the surgeon and offered the opportunity to participate in the study. If they agreed, they were asked to sign an IRB-approved informed consent. Ductal lavage fluid samples were obtained from 22 eligible patients with DCIS or IBC. The DL samples were collected in the operating room from patients with confirmed diagnosis, prior to their surgery. For each patient, two DL samples were obtained: one from the affected breast and the other from the contralateral normal breast (control). Each patient served as her own control.

Ductal lavage. Prior to starting the operative procedure, for each subject, the surgeon obtained breast ductal fluid from the affected breast and the non-affected contralateral breast, using ductal lavage. The ductal lavage procedure was performed as previously described (18), except that the collected fluid was placed in a sterile tube with no preservative solution, and was transferred immediately to the lab, and divided into different aliquots which were frozen at -80˚C for future studies.

One fresh aliquot was used for cytopathology evaluation and to investigate the presence of benign, atypical, or malignant cells, by a certified breast pathologist, using the established criteria for DL cytologic analysis (7).

RNA extraction. Total RNA was extracted from 250 μl of ductal lavage samples using the Qiagen miRNeasy kit and the Ambion RecoverALL Total Nucleic Acid Isolation kit for FFPE, respectively, and the quantity of RNA was assessed using a Thermo Scientific NanoDrop™ Spectrophotometer.

miRNA expression profiling. miRNA profiling was done according to the manufacturer's recommendations using the TaqMan(R) Human microRNA Array Set v3.0 (Thermo Fisher Scientific), a quantitative real time PCR based array containing 742 human miRNAs, 3 endogenous controls to aid in data normalization and one assay not related to humans as a negative control.

Bioinformatics and statistical analysis. A specialized software package DataAssist 3.0 (Thermo Fisher Scientific) was used to process the qRT-PCR data including removal of replicate outliers, normalization using global median method and delta-delta-Ct method for calculating miRNA relative expression level. Further analysis of processed data was performed using MeV 4.8 software package from Dana-Farber Cancer Institute (Harvard University) including filtering, unsupervised hierarchical clustering and statistical group comparison (t-test) as well as principal component analysis (PCA) (19). Groups compared were: DL from affected breasts (lavage tumor, LT) vs. DL from normal contralateral breast (control) lavage control, LC). Statistical group comparison resulted in a list of differentially expressed miRNAs. Differentially expressed miRNAs were analyzed by mapping predicted target genes to the KEGG pathways (20).

Results

Clinical and demographic characteristics of the subjects included in this study are presented in Table I. Most subjects were Caucasians, postmenopausal, with no family history of breast cancer. Most DL samples had insufficient cells for cytopathology evaluation.

Evaluation of miRNA expression was completed on all 44 specimens from the 22 study subjects. All DL samples yielded successful results, showing the ability to analyze miRNA expression in DL fluid. We detected 35 miRNAs expressed in at least 20% of the samples. In order to identify

Table I. Characteristics of the subjects.

Characteristics	N	%[a]
Age (mean ± SD)	53.81 (±12.17)	
Menopause		
Pre	7	31.81
Post	15	68.19
Race/Ethnicity		
A	1	4.54
AA	5	22.73
CA	15	68.19
H	1	4.54
Family history of breast cancer		
Yes	9	40.90
No	13	59.09
Tumor site		
Right	9	40.90
Left	13	59.09
Neoadjuvant chemotherapy		
Yes	2	9.09
No	20	90.91
Histological type		
DCIS	6	27.28
IDC and IDC/DCIS	14	63.64
ILC	1	4.54
Mixed	1	4.54
Stage		
0	5	22.73
I	9	40.90
II	8	36.37
Grade		
Low	1	4.54
Intermediate	10	45.45
High	11	50.00
ER		
Pos	19	86.36
Neg	3	13.64
PR		
Pos	16	72.72
Neg	6	27.28
HER2		
Pos	2	9.09
Neg	15	68.18
Affected breast cytology		
Atypical cells	2	9.09
Benign cells	2	9.09
Insufficient cells	18	81.82

[a]May not add to 100% due to missing values, A-Asian, AA-African American, CA-Caucasian, H-hispanic, DCIS-ductal carcinoma *in situ*, IDC-invasive ductal carcinoma, ILC-invasive lobular carcinoma.

Table II. Differentially expressed miRNAs in DL fluid of subjects with IDC.

miRNA	Log ratio	p-value
miR-126	-2.432	0.024
miR-133b	-2.034	<0.001
miR-181a	2.065	0.029
miR-23b	2.785	<0.001
miR-338-3p	-1.408	<0.001
miR-362-3p	-4.310	<0.001
miR-363	-8.805	<0.001
miR-450b-5p	-5.056	<0.001
miR-500	-3.917	<0.001
miR-548b-5p	-5.509	<0.001
miR-625	-2.816	<0.001
miR-1180	-3.610	<0.001
miR-200a-5p	-3.809	0.039
miR-596	-6.084	0.038
miR-638	-3.043	0.046
miR-645	-2.562	0.020
miR-663b	-3.840	0.045

differentially expressed miRNAs which will discriminate between DL from a breast with tumor vs. a normal breast we performed statistical analysis using t-test. However, expression of microRNA was highly heterogeneous among groups of tumors with different histologies. Preliminary analysis showed that microRNA expression profiles detected in DL fluid were different for tumors of different histological types. When groups were compared based on histology, a statistical comparison identified 20 differentially expressed miRNAs (Fig. 1). Cluster analysis showed that using expression information of only these 20 differentially expressed miRNAs, the histological type of tumors could be accurately identified as samples of the same histological type clustered together (Fig. 1). Principal component analysis (PCA) results also showed that samples of the same histological type were located in clusters that were well separated on a scatter plots (Fig. 2).

To minimize the heterogeneity of observations, the samples were analyzed separately for each histological type. The DCIS, lobular and mixed types (n=8) were excluded from further analysis due to low sample size. Using this approach we were able to discriminate tumor samples from normal paired controls for all invasive ductal carcinoma samples (n=14). This included samples with only IDC, as well as samples with concomitant IDC and DCIS (Fig. 3). This analysis was conducted in two steps. First, we removed all other samples except for the IDC histological types, which were then subjected to a t-test with $p<0.05$. Seventeen miRNAs were differentially expressed between tumor and paired normal samples from the same patients (Table II). Based on the expression profiles of these 17 miRNAs, we found that most of the DL fluid samples from the affected breasts clustered together and most of the normal control DL fluid samples clustered together except for one tumor and 3 normal samples (Fig. 3).

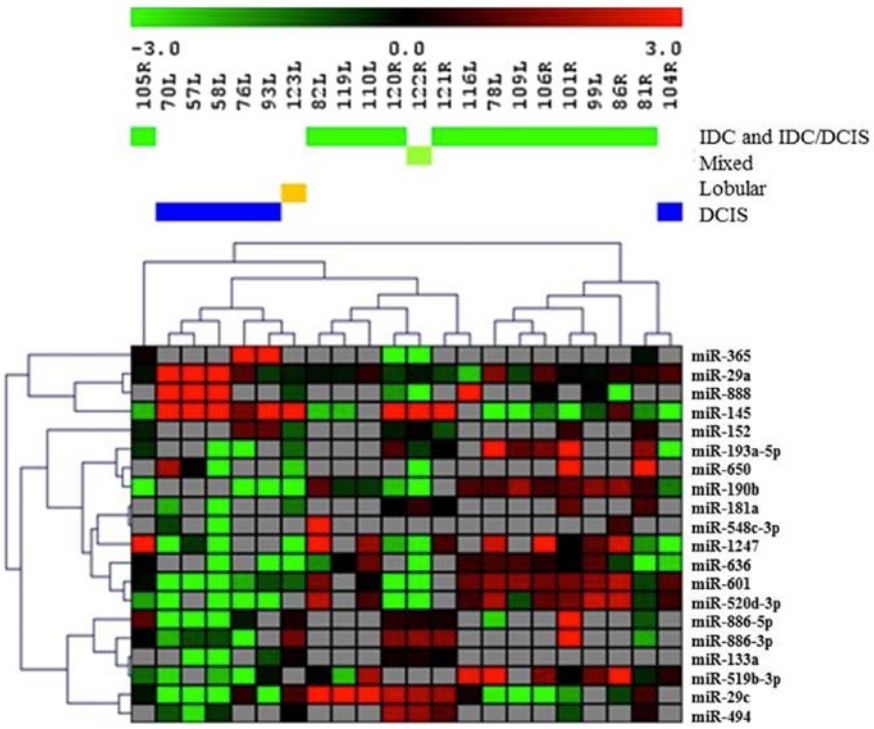

Figure 1. Group comparison based on histology. Ductal lavage fluid from affected breasts only ($p<0.05$).

In order to test if further stratification could improve the clustering of samples, we compared tumor and normal control DL fluid for subjects with IDC and DCIS in their tumors (n=9). Fourteen miRNAs were differentially expressed between tumor and paired normal samples from these patients at $p<0.05$ (Table III). Based on the expression profiles of these 14 miRNAs we found that all samples from tumor DL were clustered together and all of the normal control DLs were clustered together (Fig. 4).

Discussion

Breast ductal lavage fluid is a valuable biological sample obtained using minimally invasive techniques that is mostly used for cytopathological assessment and is still less commonly evaluated for molecular biomarkers of breast cancer. We have shown herein that miRNA screening is feasible in breast ductal lavage fluid obtained from both breasts of 22 women with unilateral breast cancer. miRNA expression was detected in all 44 DL samples. Initial statistical analysis revealed that in order to determine miRNAs that are differentially expressed in the fluid from breasts with tumors vs. normal control breasts, the samples have to be stratified by histological type to minimize heterogeneity of measurements (Figs. 1 and 2). Therefore, because of sample size constraints we focused on invasive ductal carcinoma (IDC) cases, limiting our further analysis to 14 subjects. These included 5 subjects with IDC only and 9 subjects with DCIS and IDC in their tumors.

We identified 17 differentially expressed miRNAs in the DL samples collected from the affected breast compared to the unaffected breast. A list of the differentially expressed miRNAs including several miRNAs that were previously reported as associated with breast cancer is presented in Table II. Most of these miRNAs have been previously identified in breast tumor tissues and cell lines, having various roles in breast cancer tumorigenesis, invasion and metastasis, and therapeutic response, or were associated with several clinical and pathological characteristics of breast tumors. For example, miR-23b and miR-200a were identified as having oncogenic roles in breast cancer, and miR-126, -548b-5p, and -362-3p have been shown to be tumor suppressors (21-25). Other miRNAs that we identified are involved in breast cancer inva-

Table III. Differentially expressed miRNAs in DL fluid of subjects with IDC and DCIS (in the same tumor).

miRNA	Log ratio	p-value
let-7a	4.990	<0.001
miR-181a	2.633	0.028
miR-301a	1.282	<0.001
miR-484	1.916	0.028
miR-520f	-4.176	<0.001
miR-548b-5p	-4.630	<0.001
miR-576-3p	-5.458	<0.001
miR-625	-2.615	<0.001
miR-642	0.697	<0.001
miR-99a	3.224	0.047
miR-1298	-3.284	<0.001
miR-144-5p	-4.765	<0.001
miR-432	2.560	0.027
miR-645	-3.632	0.003

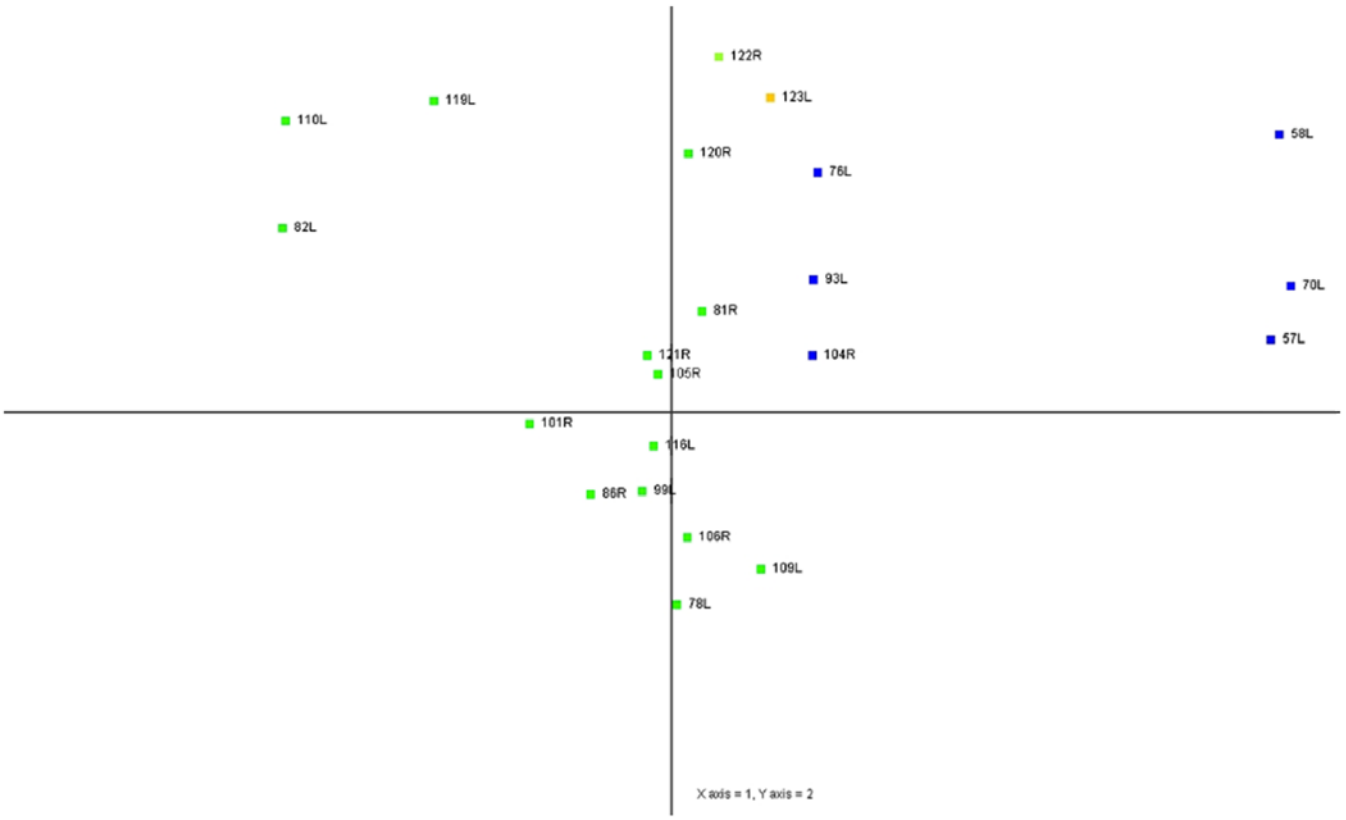

Figure 2. PCA plot. Ductal lavage fluid from affected breasts only. Comparison based on histology. Samples are color-coded based on histological type. Color codes are the same as in Fig. 1.

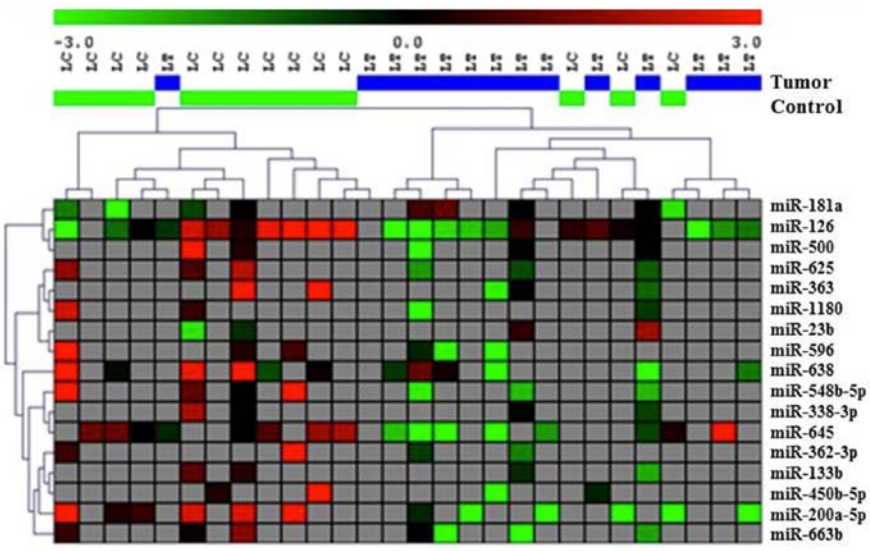

Figure 3. Comparison of miRNA expression in ductal lavage fluid samples from breasts with IDC tumors (IDC alone or IDC and DCIS in the same tumor) compared to ductal lavage fluid samples from normal control breasts ($p<0.05$).

siveness and metastasis (miR-23b, -126, -181a, -200a) (26-29). miR-23b and miR-126 were associated with breast cancer prognosis (21,30), and miR-126, -363, -638, -663 were shown to have a role in the response to therapy in breast cancer patients (31-34). In line with these findings, in our samples, miR-23b and miR-181a were significantly expressed at higher levels in the DL fluid of the affected breast compared to the breast without cancer; all other miRNAs were downregulated in the fluid from the breasts with cancer. Moreover, some of these miRNAs were also found to be differentially expressed between breast cancer patients and normal controls in other biological fluids such as serum (miR-23b, -133b, -181a, 338-3p, -625) (35-40), and plasma (miR-200a) (41). Noteworthy, miR-181a was also identified in breast milk (42).

A systems biology analysis of these differentially expressed miRNAs points to possible pathways and cellular processes

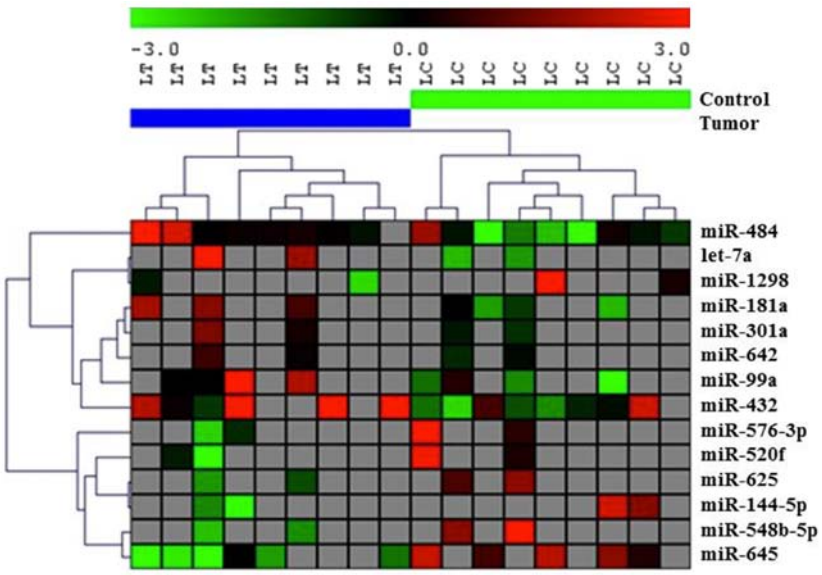

Figure 4. Comparison of miRNA expression in ductal lavage fluid samples from breasts with IDC and DCIS in the same tumor compared to ductal lavage fluid samples from normal control breasts ($p<0.05$).

that have been described as having an important role in breast cancer. Among these, several pathways are hallmarks of cancer molecular signaling including for breast cancer, Wnt, ErbB, MAPK, TGF-β, mTOR, PI3K-Akt, and p53 signaling pathways (data not shown). The most significant top two pathways were Wnt and ErbB ($p<0.0001$).

When restricting the analysis to subjects having both DCIS and IDC in their tumors, we identified 14 differentially expressed miRNAs in the DL samples collected from the affected breast compared to the unaffected breast. A list of the differentially expressed miRNAs including several miRNAs that were previously reported as associated with breast cancer is presented in Table III. Some of these miRNAs are the same as for the entire set of subjects reported in the previous analysis above (miR-181a, -625, -548b-5p, -645), and other miRNAs are specific for this subgroup of subjects (i.e. those with IDC and DCIS), suggesting that there may be a different miRNA molecular signature released from cancer cells in various stages of carcinogenesis as they progress from DCIS to IDC. However, our small sample size of subgroups did not allow for this hypothesis to be specifically tested.

Some of these miRNAs were found to be associated with breast cancer features as well. miR-301a was found to promote breast tumor metastasis (43), miR-520f and miR-99a have tumor suppressor characteristics (44,45), miR-484 and miR-301a were associated with prognosis (46,47), and miR-484, -576-3p, -144 and let-7a were associated with response to therapy in breast cancer patients (48-51). Furthermore, some of these miRs were also found differentially expressed in various biological fluids of breast cancer patients compared to normal controls, such as miR-484 and miR-301a in serum (52,53), miR-144 and miR-301a in blood (54,55), and let-7a in breast milk (56,57).

In the analysis of possible pathways and cellular processes involving these miRNAs, several cancer signaling pathways stand out, some of which have been well-documented in breast cancer based on previous reports, such as ErbB, Wnt, mTOR, MAPK, TGF-β, and PI3K-Akt (data not shown).

This is the first study to investigate miRNA profiling in DL samples, and our study design limits variability compared to classical case-control studies. However, there are certain limitations to consider when interpreting our findings, most important of which is the limited sample size in various strata. This would need to be addressed by future larger studies.

In conclusion, we have shown the feasibility of analyzing miRNAs successfully in the breast ductal fluid obtained by ductal lavage. Our findings suggest that miRNA analysis is potentially useful for the detection of breast cancer using ductal fluid analysis and allows discrimination of tumor histological subtypes as well as detection of cancer vs. normal breast samples. To validate our initial findings a larger study is warranted in order to confirm these preliminary results.

Acknowledgements

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