



# Exosomal miRNAs in nipple aspirate fluid and breast cancer

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**Background:** Exosomal vesicles transport genetic information between cells and thereby influence the cells they interact with. Exosomes have been detected in multiple body fluids, including blood, milk, urine, pleural fluid, cerebrospinal fluid and the aqueous humor of the eye. We performed a proof of principle study to determine: (I) if exosomes are detectable in breast nipple aspirate fluid (NAF); (II) if exosomes are present, do they contain micro (mi)RNAs; (III) if present and if they contain miRNAs, the ability of the miRNAs to predict: (i) breast cancer; and (ii) response to treatment with the anti-inflammatory/anti-cancer agents vitamin (vit) D and celecoxib.

**Methods:** NAF was collected from 12 healthy women and 20 women with newly diagnosed breast cancer. NAF and matched blood were collected from 5 women before and after daily treatment for one menstrual cycle/one month with low (400 IU) or high dose (2,000 IU) vitD, or high dose vitD plus 400 mg celecoxib. Ten miRs (16, 21, 100, 129, 145, 155, 181, 199, 205, and 212) were measured in NAF and serum samples.

**Results:** We were routinely able to detect exosomes in NAF and serum samples, and in the exosomes to measure multiple miRNAs. Although there was no significant difference in expression based on the presence or absence of breast cancer, miR16 and -155 expressions were higher ( $P=0.030$  for each) in women with node positive compared to those with node negative breast cancer.  $\Delta$ Ct expression was at higher (defined at  $\Delta$ Ct difference  $\geq 1$ ) in serum than matched NAF in healthy women.

**Conclusions:** Exosomes are detectable in NAF. The exosomes contain intracellular information that may be useful for evaluation of the breast.

**Keywords:** Breast cancer; microRNA; exosomes

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## Introduction

Exosomes are small vesicles with a lipid bilayer membrane containing a small cytosolic core. They can transport proteins, DNA, mRNA, miRNAs and lipids from one cell to another, thereby transferring genetic information and influencing the cells that they interact with (1). The content of exosomes may be different from the originating cell based on sorting of the contents within the

exosome (1). Exosomes from malignant cells have been shown to induce neoplastic transformation of normal cells (2). Exosome mediated transfer of miR10b from MDA-MB-231 breast cancer cells increased the ability of immortalized human mammary epithelial cells to develop invasive properties (3). Exosomes isolated from the serum of breast cancer patients (but not those from healthy donors) induced tumor formation in mice when co-injected with nontumorigenic epithelial cells (4). Tumor-derived

exosomes also mediate treatment resistance by transferring drug resistance proteins and miRNAs, by inducing efflux of or encapsulation of cytotoxic drugs, and/or by countering the effect of the drugs (1). Tumor cells appear to release a higher amount of exosomes than nonmalignant cells to influence their growth and spread (1).

### **Body fluid miRNAs, inflammation and cancer**

Exosomes have been detected in multiple body fluids, including blood, milk, urine, saliva, pleural fluid, cerebrospinal fluid and the aqueous humor of the eye. Recently, exosomes from human saliva have been characterized for their mRNA content and 509 mRNAs were found (5). Exosomes containing miRs have been identified in serum from breast cancer patients (6). Exosomes have been identified in the urine of patients with bladder (7), lung (8) and prostate cancer (9). Exosomes isolated from the pleural effusions of lung cancer patients contain proteins related to lung cancer signaling (10). Other reports evaluating body fluids from noncancer patients document exosomes in the aqueous humor of the eye (11) and in cerebrospinal fluid (12).

We analyzed 10 miRNAs (16, 21, 100, 129, 145, 155, 181, 199, 205, 212) in nipple aspirate fluid (NAF) that have been associated with cancer. miR100 is reported to decrease the production of breast cancer stem cells, and its expression in tumors has been inversely correlated with patient survival (13). We previously observed higher expression of miR100 in normal tissue than in hormone sensitive rat mammary tumors (14). miR129 is thought to be a tumor suppressor which is frequently inactivated through methylation (15). Analysis of lymphoma and myeloma cell lines demonstrated methylation in all 13 samples analyzed (15). We observed differential expression (up in tumor but down in normal) in matched mammary tissue after treatment with resveratrol (14).

Downregulation of miR145 was reported to predict postmenopausal breast cancer risk (16). miR155 has been reported to increase cell plasticity and growth (17). When miR155 was overexpressed in B cells, it induced B cell malignancy (18). miR21 appears to play an important role in all phases of breast cancer pathogenesis (19). miR155 and miR21 have been implicated in breast cancer epithelial to mesenchymal transformation, cell migration and invasion control (17,20). Serum levels of miR16 (21), -21 (22), and -155 (23) have been reported to be significantly higher in

patients with breast cancer than controls.

miR181 was evaluated in the serum of 88 patients with CRC and 11 healthy controls. Expression was significantly higher in patients with CRC (24). miR199 (14) and -204 (25) expression were found to be lower in mammary tumors than in matched controls.

miR205 is frequently detected in exosomes from body fluids. It can act both as a tumor suppressor and as an oncogene (26). As a tumor suppressor, miR205 acts as an inhibitor of cell proliferation, migration and invasion. On the other hand, as an oncogene, miR205 promotes tumor initiation and development. miR205 was measured in the serum of 58 breast cancer patients and 93 healthy controls. Levels in the serum of healthy women were higher ( $P < 0.01$ ) than in women with breast cancer (27). A second paper (28) observed the opposite trend, with higher levels of miR205 in the serum of women with breast cancer compared to controls. MCF7 derived exosomes have been reported to have low levels of miR205 (29).

miR212 has been shown to act a tumor suppressor, inhibiting the growth of lung cancer (30) and cervical cancer (31). Many of the same exosomal miRNAs that are present in cancer body fluids are also important in the regulation of inflammation (32), and chronic inflammation is a known risk factor for the development of cancer. We sought to determine if exosomes in NAF contain miRNAs, and the ability of the miRNA concentration to predict (I) breast cancer; and (II) response to treatment with the anti-inflammatory/anti-cancer agents vitamin (vit) D and celecoxib.

## **Methods**

### *Participants and sample collection*

Participants were enrolled in two Institutional Review Board approved studies. This project received ethics approval from the University of Missouri, IRB #1024344, and the University of North Dakota, IRB #200806-372. All participants gave informed consent before taking part. The first study involved 32 women ranging in age from 24–82 years who were undergoing diagnostic biopsy to determine if they had breast cancer. Twenty were recently diagnosed with breast cancer while 12 were found to have benign disease. Seven of the 20 participants with breast cancer had disease spread to their ipsilateral axillary lymph nodes. Four of the women had triple negative breast cancers, while

**Table 1** Patient characteristics

Characteristics	Cancer risk/diagnosis			
	Normal	Family history	DCIS	Invasive
N	10	2	2	18
Age				
Median	42	49.5	54	65.5
Range	24–68	54	40–68	41–82
Stage info				
T0			2	18
T1–3				T1: 12; T2: 5; T3: 1
N0			2	11
N1–2				N1: 5; N2: 2;
M0				18
St 0			2	
St1–3				St1: 9; St2: 7; St3: 2
Receptors				
ER/PR+			2	13
ER/PR–				4
ER/PR unknown				1

No subjects had tumors larger than T3, nodal staging >2, nor stage (St) >3. All women had HER2 negative tumors. DCIS, ductal carcinoma in situ; ER, estrogen receptor; PR, progesterone receptor; HER2, heregulin 2.

most of the remainder were estrogen and/or progesterone receptor (ER/PR) positive (one ER/PR unknown). All participants had HER2 negative tumors (Table 1).

In the second study, matched NAF and serum samples were collected from five healthy women ranging in age from 33–61 years. For both studies, subjects were excluded if they had been diagnosed with cancer and had received definitive treatment (tumor removal, radiation, and or systemic therapy) prior to enrollment. After the participant provided informed consent, nipple fluid was aspirated by a trained physician or nurse clinician using a modified breast pump (33). NAF samples were collected in 50 micron capillary tubes, snap frozen and stored at –80 °C. The samples were batched until analysis. For women in the vitD/celecoxib study, blood was also collected, serum separated and snap frozen until use.

### Isolation and resuspension of exosomes

An exosome isolation kit (System Biosciences Catalog # EXOQ5A-1, Mountain View, CA, USA) was used per manufacturer's instructions. Briefly, NAF samples were diluted in 200 µL phosphate-buffered saline and exosome isolation reagent added, incubated at room temperature, centrifuged, and the excess supernatant removed. The samples were centrifuged a second time to remove additional supernatant. The exosome pellets were resuspended in 200 µL phosphate-buffered saline. The miRNA micro kit (Qiagen) was used to isolate RNA from exosomes per the manufacturer's instruction. RNA was eluted in 14 µL H<sub>2</sub>O.

### miRNA analysis in NAF and serum exosomes

We evaluated the expression in NAF of 10 miRNAs (16, 21, 100, 129, 145, 155, 181, 199, 205, 212). Matched serum was also analyzed in the vitD/celecoxib study. miRNA489, whose expression exhibited minimal variability between participants, was not significantly different in subjects with/without breast cancer nor significantly altered by vitD/celecoxib treatment, was used as a control. Primers were purchased from Qiagen (Valencia, CA, USA). Eight µL total RNA was used to generate cDNA using the miScript Reverse Transcription kit (Qiagen). The miScript SYBR Green PCR kit (Qiagen) was used in real time quantitative PCR for analysis of miRNA expression. RT and qPCR steps were followed according to the manufacturer's instructions.

### Statistical analysis

To evaluate the association of exosomal miRNAs in NAF with breast cancer, we compared expression in women with newly diagnosed breast cancer (invasive or ductal carcinoma *in situ*) with those without. Distributions for each of the miRNAs were first examined and found not to meet assumptions of normality needed for parametric analysis. Therefore, descriptive data are offered as median and interquartile range (IQR). Wilcoxon rank sum tests were conducted to compare the cancer and no cancer groups. Further analysis was done exploring node negative and positive groups within the cancer group. For the smaller sub-sample (less than 30) the exact test alternative was applied. There were no ties in the smaller data set.

**Table 2** NAF mRNA  $\Delta$ Ct data comparing women with and without breast cancer\*

miRNA	No cancer (N=12)		Cancer (N=20)		P value
	Median	25/75%ile	Median	25/75%ile	
16	2.12	-1.91/10.45	2.43	0.23/4.42	0.85
21	1.48	-6.17/3.68	-1.03	-4.02/2.23	0.31
100	9.24	4.40/13.11	8.14	6.37/10.03	0.37
129	7.63	6.24/8.79	7.89	6.38/8.92	0.88
145	7.96	4.68/8.88	8.39	5.14/10.46	0.33
155	6.52	3.88/8.81	4.86	2.31/8.09	0.14
181	10.07	6.65/11.62	7.98	6.40/11.84	0.65
199	11.92	6.79/13.32	11.55	6.62/12.41	0.41
205	3.99	2.26/4.58	3.78	0.74/6.39	0.85
212	9.84	6.63/11.21	9.19	6.75/10.52	0.70

\*, normal includes women without newly diagnosed *in situ* or invasive breast cancer. Breast cancer includes women with newly diagnosed *in situ* or invasive breast cancer. NAF, nipple aspirate fluid.

## Results

### *miRNA expression level in NAF exosomes does not significantly vary in NAF from cancer containing breasts vs. the breasts of women with benign disease, but changes with disease progression*

Among women requiring diagnostic breast biopsy for a suspicious lesion found on breast imaging and/or physical exam, we did not observe a significant difference in the expression of 10 miRNAs analyzed in NAF (Table 2) comparing the cancer and noncancer groups. Among those with breast cancer, there was increased expression of miR16 and miR155 (Table 3) in women with node positive breast cancer compared to those with node negative disease (P=0.030 for both miRNAs).

### *miRNA expression in NAF and serum exosomes in healthy women*

Due to sample size limitations, we did not perform statistical analyses to comparing expression of the 10 miRNAs in NAF or serum before and after vitD +/- celecoxib treatment. There were no notable changes in expression after treatment (data not shown). On the other hand, miRNA expression was at different (defined at  $\Delta$ Ct difference  $\geq 1$ ) in serum than matched NAF for all miRNAs, with higher levels in serum.

## Discussion

One of the limitations of the current methods to diagnose breast cancer is the need for tumor tissue, which requires an invasive biopsy. Once obtained, a limitation of the tissue or cell sample is the intrinsic heterogeneity in breast cancer. Body fluid analysis minimizes the first limitation. NAF collection is totally noninvasive, whereas blood draw, for example, does involve needle access to a subcutaneous vein. Moreover, body fluid biomarkers are in concept a mix of each tumor cell's biomarker expression pattern. As such, if one or more body fluid markers could be validated, they may be more robust than a tissue marker, since tissue sampling and analysis involves only a fraction of the entire heterogeneous tumor burden, increasing the chances that expression will vary from biopsy to biopsy.

We are not aware of a report documenting the detection of exosomes in NAF, nor the presence and measurement of miRNAs. We chose to analyze 10 miRNAs that have been associated with cancer, most with breast cancer (34,35). We were able to detect all 10 miRNAs in every NAF sample. Categorized based on whether the woman had newly diagnosed cancer or not, none were significantly associated with breast cancer.

Prior reports indicate that serum levels of miR16 (21) and -155 (23) are significantly higher in patients with breast cancer than controls. While we did not observe this trend

**Table 3** NAF mRNA  $\Delta$ Ct data comparing women with to women without axillary lymph node spread of their breast cancer\*

miRNA	Node negative (N=13)		Node positive (N=7)		P value
	Median	25/75%ile	Median	25/75%ile	
16	3.00	2.24/5.37	0.21	-4.87/2.19	0.030
21	-0.11	-2.98/2.36	-1.32	-7.99/-0.025	0.31
100	8.18	7.77/10.78	6.54	1.92/9.95	0.24
129	7.96	6.77/8.88	7.33	6.34/9.08	0.44
145	9.57	6.84/10.69	4.99	2.17/9.08	0.056
155	6.79	3.43/8.38	2.35	1.10/5.13	0.030
181	8.33	6.76/12.66	6.40	3.93/8.65	0.16
199	11.88	10.53/12.58	4.66	0.92/12.47	0.067
205	4.60	0.65/8.08	1.14	-0.03/5.78	0.097
212	9.57	7.59/10.79	7.02	4.35/10.055	0.16

\* , normal includes women without newly diagnosed *in situ* or invasive breast cancer. Breast cancer includes women with newly diagnosed *in situ* or invasive breast cancer. NAF, nipple aspirate fluid.

in NAF exosomes, we did observe higher levels of these miRNAs in women with more advanced disease. Specifically, we observed significantly higher levels of miR16 and miR155 in women with node positive compared to those with node negative breast cancer. miRNAs are associated with normal functioning of the lymphatic system, alterations in miRNA155 decrease T and B cell normal functioning, and both aberrant miR16 and miR155 have been associated with lymphomagenesis (36). It is possible that higher levels of these miRNAs in the breast fluid encouraged tumor spread into the lymphatic system, resulting in disease progression.

In summary, we determined if exosomes were detectable in NAF, if the exosomes contained biologic information which could be reliably measured, and if the information obtained would be of potential clinical usefulness. We observed that exosomes are detectable, that they contain miRNAs associated with breast cancer, and that two of the miRNAs were associated with breast cancer progression through the lymphatic system.

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