An Improved Breast Epithelial Sampling Method for Molecular Profiling and Biomarker Analysis in Women at Risk for Breast Cancer

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BACKGROUND: There is a strong need to define the molecular changes in normal at-risk breast epithelium to identify biomarkers and new targets for breast cancer prevention and to develop a molecular signature for risk assessment. Improved methods of breast epithelial sampling are needed to promote whole-genome molecular profiling, increase ductal epithelial cell yield, and reduce sample cell heterogeneity.

METHODS: We developed an improved method of breast ductal sampling with ductal lavage through a 22-gauge catheter and collection of ductal samples with a microaspirator. Women at normal risk or increased risk for breast cancer were studied. Ductal epithelial samples were analyzed for cytopathologic changes, cellular yield, epithelial cell purity, quality and quantity of DNA and RNA, and use in multiple downstream molecular applications.

RESULTS: We studied 50 subjects, including 40 subjects at normal risk for breast cancer and 37 subjects with non-nipple aspirate fluid-yielding ducts. This method provided multiple 1.0 mL samples of high ductal epithelial cell content (median ≥8 samples per subject of ≥5,000 cells per sample) with 80%–100% epithelial cell purity. Extraction of a single intact ductal sample (fluid and cells) or the separate frozen cellular component provided DNA and RNA for multiple downstream studies, including quantitative reverse transcription- polymerase chain reaction (PCR) for microRNA, quantitative PCR for the human telomerase reverse transcriptase gene, whole-genome DNA amplification, and array comparative genomic hybridization analysis.

CONCLUSION: An improved breast epithelial sampling method has been developed, which should significantly expand the acquisition and biomarker analysis of breast ductal epithelium in women at risk for breast cancer.

KEYWORDS: breast cancer, breast ductal epithelium, breast duct sampling, normal breast epithelium, breast epithelial profiling

CITATION: Danforth et al. An Improved Breast Epithelial Sampling Method for Molecular Profiling and Biomarker Analysis in Women at Risk for Breast Cancer. *Breast Cancer: Basic and Clinical Research* 2015:9 31–40 doi:10.4137/BCBCR.S23577.

RECEIVED: January 7, 2015. **RESUBMITTED:** February 15, 2015. **ACCEPTED FOR PUBLICATION:** February 17, 2015.

ACADEMIC EDITOR: Goberdhan P. Dimri, Editor in Chief

TYPE: Original Research

FUNDING: This study was funded by the Intramural Research Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. This project was funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract number HHSN261200800001E. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: DD received a research grant from the American Cancer Society: Research Grant #189903 Mechanism of Selective MicroRNA Release from Cancer Cells. Other authors disclose no potential conflicts of interest.

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Introduction

Breast cancer develops from the progressive accumulation of mutations in "driver" genes, which confer a proliferative advantage to the cells. Clonal expansion of these cells results in an enlarging field of "cancerized" cells with increased susceptibility to the acquisition of additional mutations[1–3] and increased risk for breast cancer. Studies of normal epithelial cells from breast tissue of women at risk for breast cancer indicate that the cancerized field may occupy large portions or even the entire breast.[4–6] Importantly, analysis of normal at-risk breast tissue has identified cytologic and molecular abnormalities indicative of breast carcinogenesis, which correlate with increased risk for breast cancer, including atypical epithelial cells,[7,8] DNA methylation,[9] loss of heterozygosity/allelic imbalance,[5,10] accumulation of p53 protein,[11] aneuploidy, and overexpression of epidermal growth factor receptor.[4,12] These findings may represent early changes in breast carcinogenesis and encourage further characterization of these at-risk breast tissues.

Identification of cytologic and molecular profiles of normal at-risk breast tissue could play in important role in developing molecular signatures for risk assessment, in the identification of new targets for breast cancer prevention, in the selection of women for prevention therapy, and in defining the molecular changes of early breast carcinogenesis. The study and characterization of at-risk normal breast tissue requires sampling methods that provide adequate ductal material for a comprehensive genomic analysis from women at risk for breast cancer. Two important sampling methods—nipple aspirate fluid (NAF) analysis, and random periareolar fine-needle aspiration (RPFNA) of breast

tissue—have established the role of cytologic markers, especially epithelial atypia, and molecular markers (as described above) as confirmation of a subject's high-risk status. NAF, however, while identifying a specific duct with atypical epithelium, contains a median of only 120 epithelial cells per sample.[13] RPFNA predominantly samples women at high risk[14] and makes available four slides of 5,000 cells, each fixed in CytoLyt®/formalin.[15] Additional techniques that expand cellular yield while minimizing cell heterogeneity and that are applicable to a broad subject population are needed to complement the important risk assessment and biomarker findings of these methods.

Breast ductal lavage (BDL), a technique in which the breast duct is cannulated at the nipple surface and lavaged with a balanced salt solution, has been utilized for breast ductal studies and has several important features for collection of ductal contents: 1) it provides direct access to the duct. 2) Ductal material from a duct with atypia is available for direct analysis of the microenvironment of atypical cells. 3) It allows collection of the complete ductal sample, including ductal fluid and ductal epithelial cells. 4) The anatomic distribution of breast ducts has been defined, aiding in the selection of ducts for cannulation and for interpretation of the extent of the breast that has been sampled.[16,17] 5) The complete duct, including the terminal ductal lobular units, can be accessed.[18] Ductal lavage has characteristically been performed with the Cytyc microcatheter (Cytyc Corp, Boxborough, MA, USA), which, however, has also resulted in heterogeneous cell samples with limited epithelial cell content. These difficulties appear to be explained by the dimensions of the microcatheter and anatomy of catheter placement. The microcatheter measures 1.5 cm, and when completely seated in the nipple, the end of the 1.5-cm catheter is at the proximal or midportion of the lactiferous sinus (which lies immediately beneath the nipple) depending on the length of the nipple (usually 1.0–1.2 cm) and the length of the lactiferous sinus (usually 0.4–0.9 cm).[19] The sinus, which is lined by stratified squamous epithelium and may also contain foam cells, was therefore frequently lavaged by this catheter. The lactiferous sinus is also considered to be distensible (as a reservoir for milk) and may promote accumulation of the introduced lavage fluid in the sinus rather than in the duct, therefore limiting lavage of the duct and further promoting cellular heterogeneity of the sample. Large volumes (10–20 mL) of saline were typically infused and a single sample collected, which thus resulted in considerable dilution of the ductal contents. We reasoned that ductal sampling could be improved considerably by using a catheter that traversed the lactiferous sinus and directly accessed the ductal columnar epithelium, thereby enhancing cellular yield and epithelial purity of the ductal sample, while simultaneously facilitating collection of multiple, smaller samples. We therefore investigated lavage through an open 22-gauge angiocatheter with a length of 2.5 cm and collected the contents with a microaspirator (Solos Endoscopy, Inc, Boston, MA, USA) as they refluxed into the hub of the catheter. This open system allowed collection of multiple ductal samples of high epithelial cellularity and epithelial content with minimal heterogeneity, suitable for multiple RNA and DNA molecular profiling studies in normal-risk and high-risk subjects.

We now review our experience with 50 consecutive subjects undergoing ductal epithelial sampling with this improved technique. The research conducted in this study has been approved by the IRB, Intramural Research Program, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. The research was conducted in accordance with the Declaration of Helsinki.

Materials and Methods

Patient population. Fifty consecutive subjects were studied between 2009 and 2012. All women were participating in a clinical trial (#NCT00028340) approved by the National Cancer Institute's institutional review board and all gave informed consent. Eligibility criteria included women at either normal risk for breast cancer or increased risk as defined by the presence of unilateral breast cancer, lobular carcinoma in situ, atypical ductal hyperplasia, or a Gail Index ≥1.67%. Women in the study had a normal breast examination, received a negative mammogram result within the past 12 months in the case of women ≥30 years of age, were without any exogenous estrogen use, and had not undergone previous periareolar surgery or breast implants. Breast cancer subjects must not have received systemic therapy for at least 3 months before study inclusion.

Breast ductal lavage. BDL for the first 36 subjects was followed immediately by breast ductal endoscopy performed under intravenous sedation in the operating room according to the clinical protocol. Ductal endoscopy and sedation were then discontinued, and for the remaining 14 subjects, ductal lavage was performed in the operating room as a single procedure under topical LMX 4% xylocaine ointment applied to the nipple surface for 30–40 minutes and held in place with a loosely applied Op-site film. For all 50 cases, ductal lavage was performed as follows: the nipple surface was examined for NAF using a First Cyte aspirator (Cytyc Corp). If present, the associated duct was selected for lavage. If no NAF was present, a ductal orifice was identified using the FirstCyte UltraSlim Dilator (Cytyc Corp), followed by a FirstCyte Tapered Dilator. A 2-0 prolene suture was then passed into the duct, and a 24-gauge plastic intravenous catheter (Introcan Safety, B Braun Medical, Bethlehem, PA, USA) lubricated with 1% xylocaine ointment was passed over the prolene suture into the ductal orifice and then replaced with a 2.5-cm 22-gauge intravenous catheter, which was completely seated in the duct. The prolene suture was then removed, and using a tuberculin syringe, a 0.2- to 0.3-mL aliquot of 1% plain xylocaine solution was gently instilled into the duct and left in place for 3–4 minutes. The tuberculin syringe was removed, allowing the fluid and ductal contents to reflux into the hub of the catheter; the fluid was then collected with a microaspirator (Solos Endoscopy, Inc) and transferred to a sterile 1.5-mL microcentrifuge tube or

a 12-mL centrifuge tube. The instillation and collection was repeated, and when 1.0 mL of lavage fluid was collected, the microcentrifuge tube was sealed and placed immediately on ice. The decision to collect 1-mL aliquots was made arbitrarily, and larger aliquots could easily be collected. In this manner, multiple samples of ductal components were collected from the duct. Xylocaine solution was used initially for a total of 1.5 mL, and it was then replaced with normal saline for the remainder of the instillation and collections. The samples were then processed as described below.

Cytologic analysis and cell counting. A 100-µL aliquot of each 1.0-mL sample was taken, placed in PreservCyte, and a ThinPrep slide made for cytopathologic analysis. The cytologic diagnostic categories included insufficient cellular material for diagnosis (<10 epithelial cells), negative, mild atypia, severe atypia, suspicious for malignancy, or malignancy. The epithelial cell content of each sample was determined as previously described.[20,21] A representative slide was restained by immunochemistry using the pan cytokeratin antibody AE1/AE3 (Dako, Carpinteria, CA, USA), and detection was performed using a Ventana autostainer to confirm the epithelial origin of the cells.

Analysis of DNA and RNA from ductal lavage sample. Ductal lavage samples collected in 1.5-mL microcentrifuge tubes were immediately centrifuged at 364 × *g* at 4°C for 5 minutes, the supernatant removed and placed into a separate tube, and both the supernatant and cell pellet were frozen on dry ice and stored at −80°C. Samples were processed in the following manner for analysis of RNA and DNA content: a) The frozen pellet was placed in lysis buffer and RNA and DNA were extracted as described below. b) The frozen pellet was combined with 10 volumes of RNAlater (Life Technologies, Grand Island, NY, USA), mixed gently, incubated at room temperature for 1 hour and then overnight at 4°C, centrifuged at 5,000 × *g* for 10 minutes, the supernatant discarded, and the cell pellet frozen for subsequent lysis and DNA and RNA extraction. c) The cell pellet, before freezing, was either placed immediately in 350 µL of lysis buffer, vortexed, and frozen on dry ice, or was combined initially with RNAlater and processed as in b) above. Cell pellets were then lysed in 350 µL RLTPlus lysis buffer (Qiagen Corp, Valencia, CA, USA) containing 1% β-mercaptoethanol (BME) and the DNA and RNA were extracted using the AllPrep Micro Kit (Qiagen Corp) according to the manufacturer's instructions. d) For those lavage samples in which the initial lavage sample was placed in a 12-mL centrifuge tube, an aliquot (100 µL) was removed for cytologic review, and the remaining 900 µL of whole ductal lavage suspension was combined directly with 3.5 mL RLTPlus lysis buffer containing 1% BME, vortexed, and frozen on dry ice. Efforts were also made to treat the intact (unseparated) BDL suspension with RNAlater; however, the large volume of solution that was required resulted in inadequate cell recovery, and therefore this approach was not feasible. This limitation has been noted by the manufacturer.

For DNA and RNA extraction, lysates were passed over a DNA Mini column (Qiagen Corp) and RNA was extracted from the flow-through with phenol/chloroform and resuspended in RNase-free water. The DNA was purified according to the manufacturer's instructions and eluted with elution buffer (EB). DNA and RNA preparations were then stored at −80°C.

Purified DNA samples were analyzed spectrophotometrically, and purified RNA samples were analyzed both spectrophotometrically and by an Agilent Bioanalyzer using a RNA 6000 Pico Chip (Agilent Corp, Santa Clara, CA, USA).

Quantitative reverse transcription-polymerase chain reaction for microRNA. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to identify three microRNA (miRNA) species commonly expressed in breast tissues—miR-16, miR-451, and miR-720—was performed using the TaqMan MicroRNA Reverse Transcription Kit (ABI, 4366597) according to the manufacturer's instructions and as previously described.[22,23] The following thermal cycling program was used: 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C, and a final hold step at 4°C. The complementary DNA mixes were aliquoted into qPCR plates and realtime PCR was performed on an ABI 7500 PCR system (Applied Biosystems) with a program of 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantitation was performed using the ABI 7500 detection software v1.4 with correction for amplification efficiency based on an exponential model of PCR.[24,25]

Quantifiler analysis of DNA. DNA samples (10 ng) were analyzed by the Quantifiler method (AB Biosystems, Foster City, CA, USA) to assess for a) the presence of inhibitory substances and b) the suitability of DNA for qPCR amplification. The Quantifiler method utilizes a FAM™ dye-labeled probe (6-carboxyfluorescein) for amplification of a human telomerase reverse transcriptase (*hTERT*) gene and a VIC™ dye-labeled probe for amplification of a synthetic DNA template (a synthetic sequence not found in nature) to test for the presence of DNA amplification or PCR inhibitors. Real-time PCR was conducted according to the manufacturer's instructions. Positive amplification for both analyses was indicated by cycle threshold (C_t) values <40.

DNA amplification and array comparative genomic hybridization. Whole-genome amplification (WGA) of the DNA samples (approximately 10 ng) was done using the REPLI-g Mini Kit (Qiagen Corp), and the amplified DNA was verified using a Nanodrop instrument (Thermo Scientific, Waltham, MA, USA) or a 3% agarose gel to determine the quality and quantity of the product. Amplified DNA (2.5 µg) and sex-matched control DNA (Promega, Madison, WI, USA) were digested using AluI (Promega) and RsaI (Promega) for 2 hours and then purified using a QIAprep Spin Miniprep Kit (Qiagen Corp) before being labeled for 2 hours with Cyanine (Cy) 3 dye-labeled deoxyuridine triphosphate (Cy3-dUTP) and Cy5-dUTP (Promega), respectively, in a random priming reaction using Bioprime Array CGH Genomic Labeling Module

(Life Technologies, Carlsbad, CA, USA). Unincorporated nucleotides were removed using Microcon YM-30 columns (Millipore, Bedford, MA, USA) before both Cy3- and Cy5-labeled DNA were combined. Human oligonucleotide-based microarrays (4 × 44 K, Agilent Technologies, Santa Clara, CA, USA) were hybridized for 40 hours at 65°C, washed with the manufacturer's recommended solutions, and scanned (G2565BA, Agilent Technologies). The resulting file was then extracted using the Agilent Feature Extraction™ software (Agilent Technologies) before analysis using the Nexus Copy Number (BioDiscovery, Hawthorne, CA, USA).

Results

Subject characteristics. The characteristics of the 50 study subjects are summarized in Table 1. The women were predominantly premenopausal, with an equivalent distribution of Caucasian, Hispanic, and African American subjects. Forty subjects were at normal risk for breast cancer. Among the high-risk subjects, there were four cases of breast cancer, two of whom had not received any systemic therapy and two had previously received chemotherapy, one of which included Femara. Among the high-risk subjects, the risk for breast cancer was the highest at the time of ductal lavage.

Ductal sampling and cytopathologic review of ductal samples. BDL was performed in 48 subjects (96%). In two subjects, a ductal orifice could not be identified. The majority of subjects (74%) did not have NAF-producing ducts, and a ductal orifice was identified with the UltraSlim Dilator. LMX 4% xylocaine ointment applied topically for 30–40 minutes provided the best local anesthesia. Ductal sampling was routinely performed with the 22-gauge angiocatheter seated completely in the duct. Gentle instillation of 0.2–0.3 mL of saline, rather than larger amounts, was found to be most effective, and collection of aliquots with the microaspirator from the hub of the angiocatheter was easily performed. Total time for collection of 10–12 aliquots was usually 45 minutes. The ductal lavage was well tolerated. In some cases, there was mild discomfort with the instillation of saline, probably representing distention of the duct. If this occurred, it was usually after multiple aliquots had been collected. There were no complications.

Each ductal lavage sample was examined for epithelial cell cytopathology and content. Representative slides illustrating epithelial cell content and confirmation by cytokeratin immunostaining are shown in Figure 1. Twelve subjects had ductal epithelial atypia on cytopathologic review, the majority of which were described as mildly atypical epithelial cells. Among these 12 cases of epithelial atypia, 3 occurred in high-risk subjects and the remainder occurred in women at normal risk for breast cancer. The findings for ductal cell yield are described in terms of the number of cells per sample and are summarized in Table 2. It can be seen that the ductal sampling method in an individual frequently provided multiple samples of high cellular content. For example, among the 48 subjects undergoing ductal lavage, 42 subjects produced one or more samples of ≥5,000 cells, and a median number of eight such samples (≥5,000 cells) per subject. Thirty-seven subjects (77%) had a median five samples of ≥10,000 epithelial cells per sample. When the samples were examined for the percentage of cells represented by epithelial cells (homogeneity of the sample), and using samples of ≥5,000 cells for calculations, it was found that a median eight samples per subject contained ≥80% epithelial cells, and a median six samples per subject contained ≥90% epithelial cells. Many samples comprised 99%–100% ductal epithelial cells (Fig. 1). We did not find any difference in cell yield between subjects with atypia compared to those without atypia.

Table 1. Demographic characteristics of subjects.

CATEGORY	INCIDENCE
Subjects	50 subjects
Menopausal status	
Premenopausal	44 subjects
Postmenopausal	6
Ethnicity	
Caucasian	16 subjects
African American	15
Hispanic	16
Asian	3
Age	
Median	43 years
Mean	41.8 ± 1.2
Range	25–63
Risk for breast cancer	
Normal risk	40 subjects
High risk	10 subjects
Breast cancer	4
ADH/LCIS	4
Gail > 1.67%	2
Nipple aspirate fluid	
Absent	37 subjects
Present	13
Ducts lavaged	
1 duct	35 subjects
2 ducts	13
No ductal lavage	2*
Cytologic findings	
ICMD	1
Negative	35
Mildly atypical cells	9
Atypical cells	3
Severe atypia	0
Suspicious for malignancy	0
Malignancy	0

Note: *Ductal orifice could not be identified.

Figure 1. Cytopathologic illustration of epithelial cell content in ductal lavage samples. A ThinPrep Papanicolaou-stained slide of the cellular content for samples collected either using the original Cytyc microcatheter (**A**) or using the angiocatheter described in the present report (**B**). Panel A illustrates heterogeneity of the cell sample, showing both epithelial cells and foam cells with the original sampling method. The slide in (**B**) illustrates a more homogeneous population of ductal epithelial cells obtained with the current method. The slide in (**C**), same duct as in B, was immunorestained with cytokeratin antibodies to confirm the epithelial cell content.

We found the characteristics of the samples collected to be comparable between ductal lavage performed under iv sedation vs ductal lavage performed under topical xylocaine. Among the latter 14 cases, the median number of total samples collected was 10 (range: 2–11; 86% of subjects had ≥6 samples collected). Thirteen subjects (93%) had ≥1 sample of 5,000 cells, with a median of six such samples, and 13 subjects had samples (of 5,000 cells) that comprised ≥90% epithelial cells.

Analysis of DNA and RNA from ductal lavage samples. We next studied the DNA and RNA extracted from individual fresh or frozen cellular pellets and, to evaluate the combined analysis of the ductal components, we studied DNA and RNA extracted from the intact BDL suspension, which consisted of ductal fluid, cells, and extracellular components including exosomes. The findings of these studies are summarized in Table 3.

DNA analysis. Lysis and extraction of either the frozen pellet or intact BDL suspension produced DNA of high purity (confirmed by ratio of absorbance at 260 nm and 280 nm) and good yield (Table 3). Immediate lysis of the fresh cell pellet did not appear to provide any advantage over processing of the other two sample preparations. DNA samples were studied on agarose gels, which demonstrated high-quality, high-molecular-weight genomic DNA (Fig. 2A). Five DNA samples from different subjects, including two frozen pellets and three fresh BDL suspensions, were analyzed by the Quantifiler method (AP Biosystems) to test for the presence of inhibitory substances and suitability for qPCR. This demonstrated that the C_t for amplification of the synthetic template was <40 in all cases (mean: 27.32; range: 26.5–28.9), confirming the absence of PCR inhibitors in the lavage preparations. The C_t values for *hTERT* were also <40 in all cases (mean: 26.5; range: 23.3–28.0), indicating good amplification of the target sequence. There did not appear to be any difference in the quantity or quality of DNA extracted from frozen pellets vs DNA extracted from fresh BDL suspensions.

The quality of the DNA for subsequent analysis was further tested by whole-WGA and array CGH. Ten nanograms of genomic DNA was amplified, yielding microgram quantities of high-molecular-weight fragments for DNA extracted from both frozen pellets and the intact BDL suspension, and confirmed by agarose gel electrophoresis (data not shown). Hybridization of amplified DNA to Agilent human 4 × 44 K CGH arrays showed high-quality arrays for both types of sample preparation (frozen pellet or BDL suspension), with no differences noted (Fig. 3). Interestingly, occasional nonrandom gains were seen on chromosomes 6, 12, and 19 in all samples. The following genes are more highly gained in the respective segments: chromosome 6 – *APOM*; chromosome 12 – *ARHGAP9, NDUFA4L2, CDK2, RHEBL1, TUBA1A, PRPH, LOC100335030, RACGAP1, LASS5, METTL7B*;

Table 2. Frequency of cell yield in ductal lavage samples.

CELL YIELD/SAMPLE	≥1000 CELLS	≥2000 CELLS	≥5000 CELLS	≥10,000 CELLS	≥20,000 CELLS
Number subjects	45 subjects*	43 subjects	42 subjects	37 subjects	22 subjects
Median number of samples/subject	12 samples	10 samples	8 samples	5 samples	2 samples
Range, number of samples/subject	1–16 samples	1–15 samples	1–13 samples	1–11 samples	1–6 samples

Notes: *Number of subjects with ≥1 sample of this cellular content; for example, 45 subjects had 1 or more samples consisting of ≥1000 cells, and among those subjects the median number of samples of ≥1000 cells was 12 samples/subject.

Table 3. Preparation of breast ductal lavage samples for DNA/RNA analysis.

SAMPLE	N	CELL COUNT RANGE (CELLS/ SAMPLE)	NUCLEIC ACID 260/280		NUCLEIC ACID CONTENT SPECTROPHOTOMETER		CONTENT BIOANALYZER
			DNA	RNA	DNA	RNA	RNA
Frozen cell pellet	14	7,850–20,020	2.06 (0.07)*	1.49 (0.03)	598 ng (69.4)	381.1 ng (59.8)	40.9 ng (10.3)
Frozen cell pellet, RNAlater post-freezing	4	7,090–12,180	1.79 (0.04)	1.59 (0.10)	874 (75.1)	479.1 (20.9)	81.6 (51.3)
Fresh cell pellet	5	1,500–4,810	1.60 (0.13)	1.33 (0.07)	205.1 (38.0)	98.3 (59.0)	2.32 (0.71)
Fresh cell pellet, RNAlater pre-freezing	12	1,130–12,890	1.65 (0.12)	1.55 (0.09)	147.9 (29.8)	149.2 (32.1)	34.3 (11.3)
BDL intact suspension							
≤5000 cells	10	1,530–4,430	2.10 (0.25)	1.63 (0.08)	190.5 (58.7)	311.1 (76.4)	266.0 (79.4)
>5000 cells	12	6,400–29,100	1.96 (0.08)	2.00 (0.13)	391.5 (51.3)	333.1 (123.8)	441.1 (160.4)

Notes: *Number in parenthesis indicate SEM.

chromosome 19 – *C19orf55*, *HIPK4*, *BCAM*, *RTN2*, *DHX34*. Whether these gains represent artifacts of the amplification procedure or real genomic abnormalities of these normal breast epithelial samples needs to be determined.

RNA analysis. The RNA preparations from fresh or frozen cell pellets and from the BDL suspension were analyzed spectrophotometrically and by Bioanalyzer. The findings are summarized in Table 3. Gel electrophoresis of total RNA indicated good-quality RNA (Fig. 2B). The quantity of RNA extracted from the frozen pellet or the BDL suspension was often greater than that extracted from the fresh pellets undergoing immediate lysis. Similar to the findings for DNA, immediate lysis of the fresh pellet did not appear to provide any advantage over processing of the other sample preparations. It was noted that the quantitative values determined for RNA from the cell pellets were often higher when measured spectrophotometrically (260 nM) than when measured by the Bioanalyzer (Table 3). This may be due to

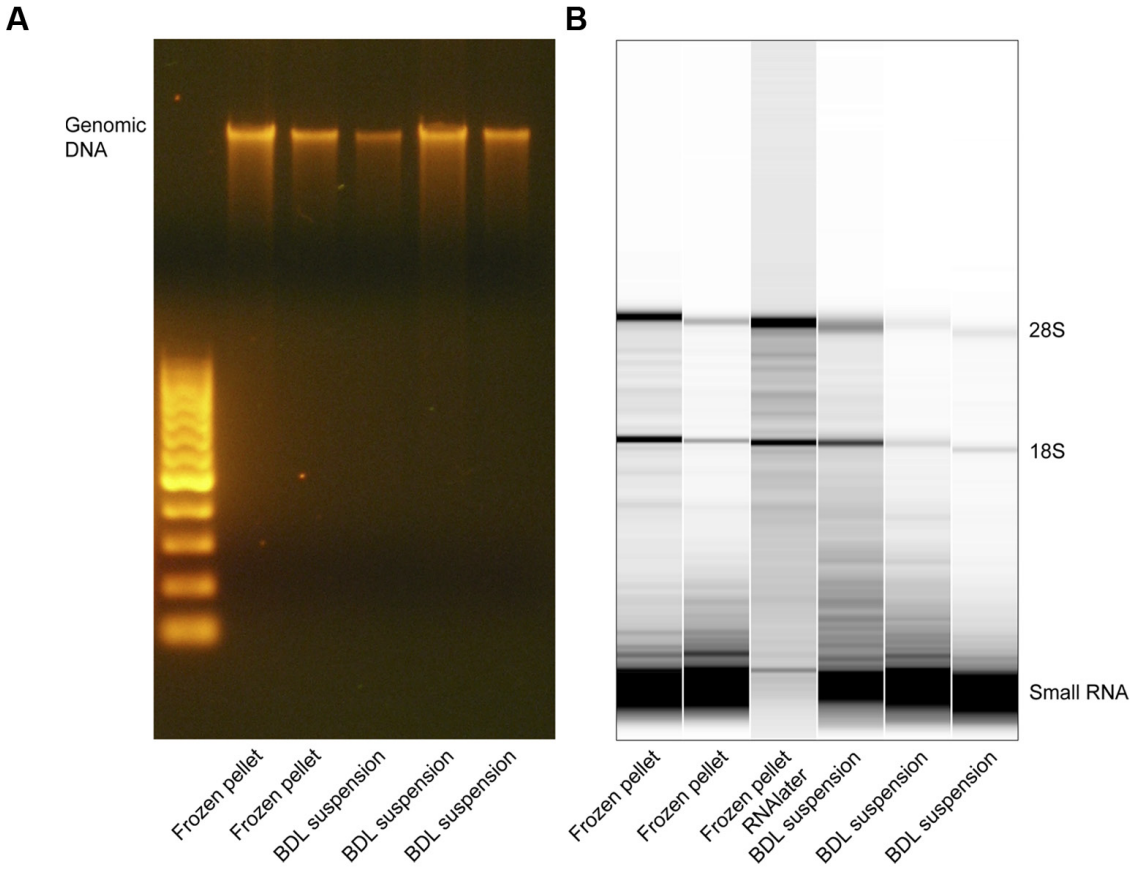

Figure 2. Gel electrophoresis of DNA and RNA samples. Agarose gel electrophoresis of intact genomic DNA from the indicated ductal lavage samples (**A**). Lane 1 indicates separation of 100-bp markers. Bioanalyzer gel electrophoresis of total RNA from the indicated ductal lavage samples (**B**).

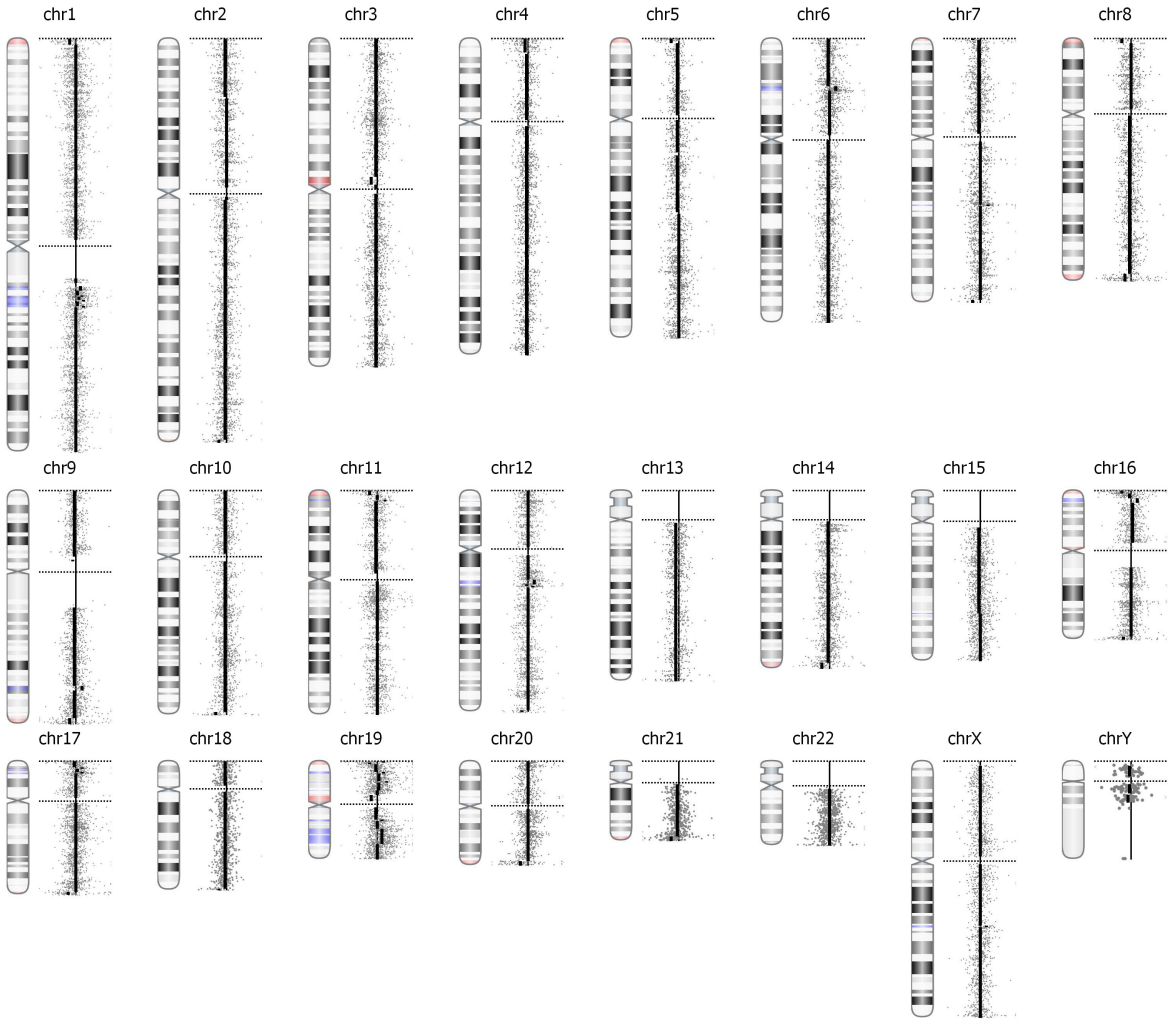

Figure 3. Array CGH of ductal lavage whole-genome-amplified DNA. This array is representative of DNA extracted from either the frozen pellet or the intact lavage suspension. Nonrandom gains are noted in chromosomes 6, 12, and 19.

reduced sensitivity of the spectrophotometer at low concentrations (<2 ng/μL) or may represent residual guanidinium isothiocyanate (GI; a component of the extraction buffer), which also absorbs at 260 nM and which would result in a higher spectrophotometric value for RNA. This difference was not present during processing of the BDL suspension, wherein extracted samples were precipitated with isopropanol and washed with ethanol. No differences were noted, however, in the downstream analysis of miRNA between the frozen pellet and the BDL suspension (see below), indicating no interference by any residual GI.

Analysis of bioanalyzer electropherograms of samples lysed and extracted without RNAlater, whether as frozen pellets or as the intact BDL suspension, revealed a prominent low-molecular-weight (LMW) peak in the range of 50–150 nt, corresponding to small RNA species (Fig. 4A). Additional ethanol was used in the extraction of RNA from samples to facilitate collection of these species. The presence of the peak was not considered to represent degraded RNA. There was no evidence of degradation in the electropherograms in the regions before or between the ribosomal peaks and no shift in the position of the 18S and 28S ribosomal peaks as is seen with degradation. Ribosomal 28S/18S ratios were >1.0 in all cases, with low baseline between peaks. An interesting finding in the comparison of these methods was the absence of this LMW peak in the pellets treated with RNAlater (Fig. 4B). This was seen consistently. The explanation for the loss of the LMW peak when RNAlater was used is not clear. The small RNA species may possibly be removed with the RNAlater, which is discarded after treatment.

miRNA analysis. The quality of the RNA samples was then studied with qRT-PCR for the presence and amount of three miRNAs that are known to be present in breast ductal epithelium, namely, *miR-16*, *miR-451*, and *miR-720*. We tested six sample preparations of ductal lavage from five subjects: frozen epithelial cell pellets without RNAlater stabilization (two samples), a frozen cell pellet with RNAlater stabilization (one sample), and intact BDL suspension (three samples; Fig. 5). All three miRNAs amplified well, indicating high-quality RNA. The abundance of the three miRNAs

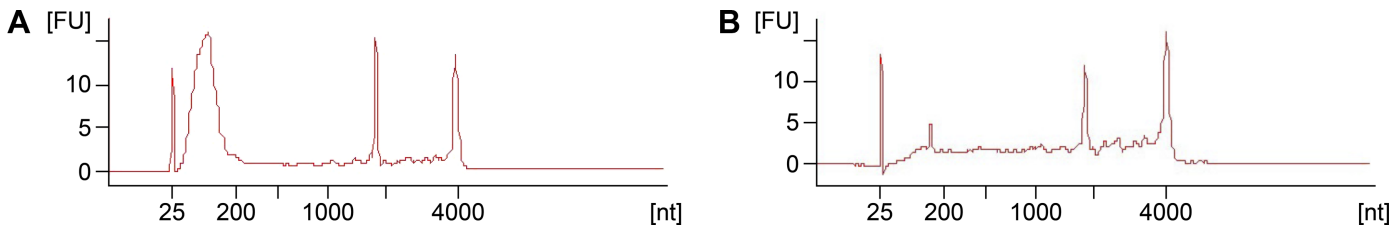

Figure 4. Electropherogram of RNA from ductal epithelial samples. Total RNA was extracted from ductal lavage samples and examined by Agilent 2100 Bioanalyzer nanoassay. Electropherograms for RNA extracted from a frozen pellet without RNAlater treatment (**A**) or from a frozen pellet treated with RNAlater (**B**) are depicted. FU, fluorescence units. The peak at 25 nt is an internal standard.

relative to each other within each sample was consistent across the six samples, *miR-16* and *miR-720* being the most abundant and *miR-451,* the least (Fig. 5). Interestingly, two samples were collected by ductal lavage from the same duct in a subject, but at two time points 6 months apart: the first sample a frozen cell pellet processed with RNAlater stabilization, and the second an intact BDL suspension. The relative abundance for the three miRNAs was comparable between the two samples, supporting reproducibility of the samples and comparable quality of the RNA. These findings would also suggest that small RNAs identified on the electropherogram did not interfere with amplification and identification of miRNAs by qRT-PCR. Conversely, miRNA is typically <40 nt in size, and loss of the LMW peak did not appear to influence identification of miRNA species in our extractions.

Discussion

In this report, we describe a breast epithelial sampling method that increases epithelial cell yield and cell homogeneity for molecular profiling, which should significantly expand the analysis of at-risk breast tissue for biomarkers and for molecular changes of early breast carcinogenesis. The technique allows for collection of multiple aliquots of the entire contents of the ductal microenvironment, including ductal fluid with its multiple components,[14,26–28] and provides for a median of eight samples of ≥5,000 epithelial cells per sample, and a median six samples consisting of ≥90% pure epithelial cells. Samples are proven to be free of inhibitory substances. Importantly, each aliquot may be analyzed fresh or stored frozen, and each is suitable for multiple downstream applications, including qRT-PCR for miRNA analysis, qPCR for the *hTERT* gene, WGA, and array CGH. Cytopathologic analysis and cell counts are available for each aliquot to aid in identification of proliferative changes and atypia, as well as in the subsequent selection of samples for molecular studies. The collection of multiple samples also provides additional material for future correlative or confirmatory studies. The technique is easily performed under topical anesthesia, and prestudy preparation and poststudy management of the subject are minimal.

We studied ductal samples prepared as either intact BDL suspensions, consisting of ductal fluid, cells, and intraductal components, or as individual cell and fluid preparations. Extraction of the intact BDL suspension was designed to provide a composite sample of DNA or RNA from all ductal components, while at the same time potentially minimizing losses due to separation. Molecular profiles for DNA (qRT-PCR, array CGH) and RNA (miRNA) were seen clearly in both preparations. Analysis of individual frozen ductal fluid or frozen cell pellets, on the one hand, allows for selection of samples based on cell counts, epithelial cell purity, and cytologic findings. Studies of the intact BDL suspension, on the other hand, may be well suited for the analysis of molecular abnormalities present in both ductal fluid and ductal cells, such as DNA methylation[29–31] and DNA structural changes including loss of heterozygosity.[32] Interestingly, we have previously shown that miRNAs characteristic of human mammary epithelial cells (*miR-451, miR-720,* and *miR-1246*) are present in the exosomes of ductal fluid.[22] Analysis of the intact BDL suspension might also provide an opportunity to study both exosomal and intracellular miRNAs. Lastly, because these samples are also collected as sterile suspensions of breast epithelial cells, one would anticipate these may also be suitable for the development of breast epithelial cell lines and from women at different risks for breast cancer, a much-needed resource.

The sampling technique developed in this report should complement other sampling methods such as NAF and RPFNA, which have identified atypical epithelium and other biomarkers in high-risk subjects. The improved technique allows one to target NAF-producing ducts or the ductal systems in the upper inner or upper outer quadrants of the breast, which are sampled by RPFNA,[5,15] to provide additional material for analysis. In addition, in the present study, 80% of the subjects were at normal risk for breast cancer, and 74% of ducts studied were of the non-NAF–yielding type, indicating a technique that is useful for ductal analysis in low-risk and high-risk subjects and in NAF-yielding as well as non-NAF-yielding ducts. Epithelial samples from low-risk subjects and non-NAF-yielding ducts may also serve as a useful control for NAF and RPFNA studies. It has also been estimated that 50%–70% of women who develop breast cancer have no identifiable risk factors,[33,34] emphasizing the importance of the present technique to be able to collect and study breast ductal epithelium from women not (by traditional criteria) at

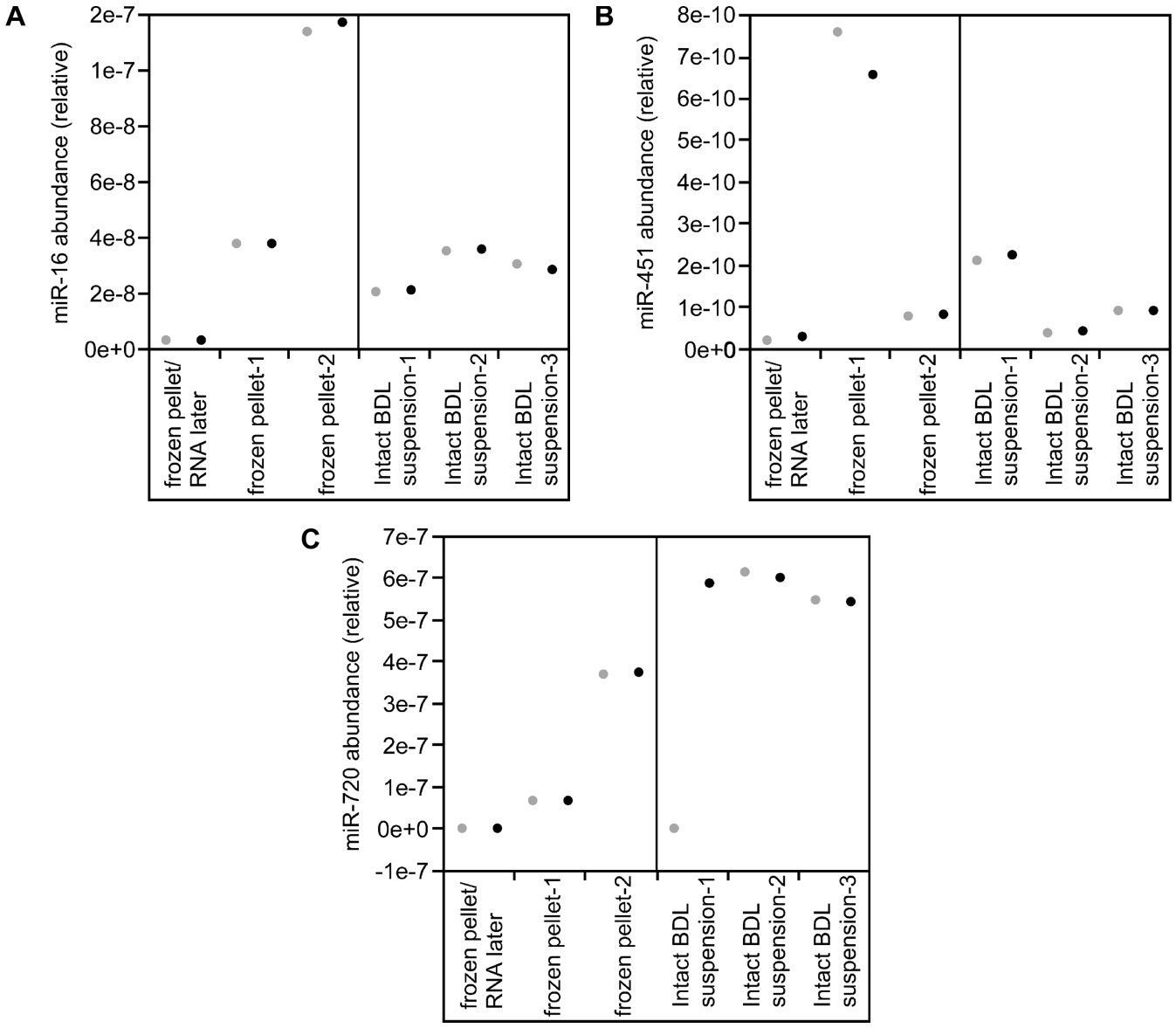

Figure 5. Relative abundance of miRNA in ductal lavage samples. Total RNA was studied by qRT-PCR for expression of the indicated miRNAs, miR-16 (**A**), miR-451 (**B**), and miR-720 (**C**), in the respective ductal lavage sample preparations. Duplicate measurements were performed on each preparation (gray and black markers).

increased risk for breast cancer. Identification of molecular abnormalities and potential biomarkers in these later categories of women could have a significant impact on the conduct of prevention studies for breast cancer.

An important question is the extent to which the breast is being sampled with this technique. It has been shown that one ductal system drained fully 23% of the total breast volume and the largest of three duct systems drained 50.3%.[16] Others have found that the proportion of the breast drained by a single fluid-yielding duct ranged from 13% to 68%.[17] Going and Moffat[16] pointed out that if these ducts that open accessibly on the surface of the nipple do communicate with larger duct systems, than even a small number of ducts could give diagnostic or therapeutic access to much of the breast parenchyma. At the same time, their studies[16] found eight other collecting ducts accounted for only 1.6% of the total breast volume, and thus lavage of these ducts might provide limited information. Together, these studies suggest the possibility that lavage of a breast duct may provide ductal epithelium from a significant portion of the breast. The present technique lends itself to the study of more than one duct. Lavage of two or more ducts may provide an even more comprehensive analysis of the cancerized field.

In summary, we have developed a breast epithelial sampling method that significantly expands our ability to study breast epithelium in women at normal risk and at increased risk for breast cancer. This should enhance our ability to analyze these tissues to develop biomarkers for risk assessment and breast cancer prevention, as well as to define the molecular changes in early and preneoplastic breast carcinogenesis.

This method complements other sampling techniques including NAF and RPFNA and, together, facilitates our ability to define the molecular changes and biomarkers associated with cytologic epithelial changes and the cancerized field in breast carcinogenesis. Lastly, it is important to note that the sampling method described is viewed primarily as a research application for characterizing at-risk breast epithelium. Its role in the management of at-risk women cannot be determined from these studies and must await future trials.

Acknowledgments

The authors thank Keith Brosky (Cytotechnologist [CT]), Dana Erickson (CT), and May Owens (CT) for processing the samples, Patricia Fetsch (Medical Technologist) for performing the immunostains, and Mark Roth, MD, for analyzing some of the samples. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

Author Contributions

Conceived and designed the experiments: DND, AW, DW, TR, DD. Analyzed the data: DND, AW, DW, TR, DD, AF, SAP. Wrote the first draft of the manuscript: DND, AW, TR, DD, AF. Contributed to the writing of the manuscript DND, AW, DW, TR, DD, AF, SAP. Agree with manuscript results and conclusions: DND, AW, DW, TR, DD, AF, SAP. Jointly developed the structure and arguments for the paper: DND, AW, DW, TR. Made critical revisions and approved final version: DND, AW, DW, TR, DD. All authors reviewed and approved of the final manuscript.

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