Detection of Chromosomal Instability in Paired Breast Surgery and Ductal Lavage Specimens by Interphase Fluorescence in Situ Hybridization

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ABSTRACT

Purpose: Ductal lavage is a new modality for collecting exfoliated breast cells with the goal of detecting early neoplasia. The purpose of our study was to evaluate the correlation between cancer-associated abnormalities in breast lesions and exfoliated breast cells collected by ductal lavage.

Experimental Design: We performed histopathologic, cytologic, and molecular cytogenetic analyses on 39 paired cases of surgically excised breast lesions and ductal lavage. Our study demonstrates that cytologic and genetic abnormalities associated with breast cancer progression can be detected in ductal lavage cells collected from women with in situ and invasive breast cancer and suggests that fluorescence in situ hybridization may have superior sensitivity and specificity compared with conventional cytology.

INTRODUCTION

The breast is a renewal tissue in which epithelial cells lining the ductal–alveolar mammary tree are exfoliated into the luminal compartments of the gland. Ductal lavage is a new technique for collecting these cells for the purposes of early breast cancer detection and risk assessment. In this approach, an individual ductal orifice at the nipple is cannulated with a microcatheter to flush the associated ductal system with saline. The harvested fluids contain thousands of epithelial cells that can be evaluated for breast cancer-associated abnormalities. In a recent multicenter clinical trial, the cytologic evaluation of 383 ductal lavage specimens collected from asymptomatic high-risk women revealed abnormalities in 24% of the specimens (1, 2). Of these, 17% were characterized as mildly atypical, 6% as markedly atypical, and <1% as frankly malignant. Although the development of a well-tolerated, minimally invasive procedure to detect abnormal cells in asymptomatic women is promising, the extent to which these cytologic abnormalities represent the different histopathologic stages of breast cancer progression is currently unknown. To examine this relationship, we conducted a paired study to evaluate ductal lavage specimens collected from women immediately before breast surgeries. In a blinded analysis of 39 paired cases, histopathologic diagnoses of the excised surgical specimens were compared with the cytologic classification of the corresponding ductal lavages.

Furthermore, because cytology is subjective, we also analyzed the ductal lavage cells for the presence of genetic alterations associated with breast cancer. Breast cancer progression is characterized by the accumulation of numeric changes on many chromosomes and, in particular, by early and/or frequent increases in copy levels of chromosomes 1, 8, 11, and 17 (3–12). We hypothesized that if these alterations are present with high frequency in the premalignant and malignant surgical breast lesions in our study, we should be able to detect them in ductal lavage specimens harvested from the same women. To test this, we used interphase FISH analysis to measure the frequency of numeric changes on chromosomes 1, 8, 11, and 17 in paired cases of breast tumor touch prep and ductal lavage specimens obtained from women undergoing breast surgeries.

Using this paired case approach, we were able to: (a) demonstrate that breast cancer-associated abnormalities can be
detected in exfoliated breast cells from women with in situ and invasive breast disease; (b) adapt the use of interphase FISH to detect molecular cytogenetic alterations in ductal lavage cells; and (c) validate exfoliated breast cancer cells collected by ductal lavage as targets for biomarker development.

MATERIALS AND METHODS

Ductal Lavage. Ductal lavage was performed on 38 women at the Metro Surgical Associates (Decatur, GA) in the operating room immediately before breast surgeries, including excisional biopsies, duct resections, and mastectomies. One sample was damaged in transport, and bilateral surgeries were performed on two patients, producing a total of 39 paired samples. Informed consent was obtained in all cases. Each nipple was cleaned with Nuprep exfoliant (D.O. Weaver & Co., Aurora, CO), and then subjected to nipple aspiration using an InDuct Breast Aspirator (ProDuctHealth, Inc., Menlo Park, CA) to identify the location of fluid-producing duct orifices. Each productive duct was cannulated with an InDuct Breast Micro-Catheter (ProDuctHealth, Inc.) and lavaged with 10 ml of sterile saline (0.9% sodium chloride, INJ, USP; Abbott Laboratories, North Chicago, IL). Multiple ducts were lavaged in five of the cases, and the most abnormal cytology or FISH result from each of these cases was used for analysis of correlations. Lavage specimens were collected into 15-ml conical tubes containing 7 ml of Cytolyt solution (Cytc Health Corp., Boxborough, MA) and stored at 4°C until shipment in styrofoam containers with ice packs. Specimens were shipped via overnight express service within 1 week of harvest and processed within 24 h of receipt. Half of each specimen was prepared as a ThinPrep sample for cytomorphicologic evaluation, and the other half was processed into drop preparations for FISH analysis.

Cytomorphologic Evaluation. Cytomorphologic evaluation was performed by a Yale School of Medicine Pathologist (D. L. R.) specifically trained in ductal lavage interpretation. Diagnoses were made on each lavage specimen on the basis of the guidelines provided by ProDuct Health, Inc. Specifically, lavage samples were graded on a four-point scale based on the epithelial cell morphology (benign, mildly atypical, markedly atypical, and malignant). Within this scale, benign lavages are characterized by ductal epithelial cells either loose or in clusters showing well-organized architecture and small uniform nuclei with smooth nuclear contours. The chromatin shows a fine pattern, and nucleoli are small if present at all. The atypical specimens show more clusters of cells with less organization, including some with a three-dimensional appearance. The nuclei may be enlarged and irregular but without coarse, granular, and hyperchromatic nucleoplasm. Nucleoli are present and may be somewhat enlarged. The nuclear:cytoplasmic ratio is increased, there is moderate anisonucleosis, and clusters may show a discohesive appearance. The gradation between marked and mild atypia is based on the degree and severity of the criteria above. Finally, malignant cases show a clearcut second population of cells with frank morphological features of malignancy, including enlarged nuclei with markedly irregular shapes and grooves. The chromatin is coarse, granular, and hyperchromatic. Large nucleoli are present. Nuclei are widely variable in shape and size, and the nuclear:cytoplasmic ratio is high. Clusters show marked disorganization in three dimensions. Sometimes cribriform/tubular patterns are seen, and sometimes necrotic material and/or calcifications are present in the background.

Ductal Lavage Drop Preparations. Tubes containing lavage specimens were centrifuged at 600 × g for 5 min to pellet the cells. The cell pellets were resuspended in 20–200-μl volumes of cytospin collection fluid (Shandon, Inc., Pittsburgh, PA). Ten μl aliquots of the resuspended lavage pellets were then transferred to glass microscope slides to create drop specimens measuring ~1 cm in diameter. Drop preparations were air dried and stored at −20°C.

Breast Tumor Touch Preps. Glass microscope slides were pressed against the surface of freshly excised breast lesions, including biopsies, duct resections, lumpectomies, and mastectomies, and immediately placed in cold methanol for 20 min. Slides were then transferred to fresh methanol:acetic acid (3:1) for 20 min, dehydrated in a series of ethanol, and air dried. Slides were stored at −20°C.

FISH. Ductal lavage drop specimens and touch preps were submersed in 95% ethanol for 10 min to remove wax residue from the cytospin collection fluid, air dried, and processed according to the Vysis Urovision pretreatment and hybridization protocols. DNA CEPs complementary for chromosome 1-, 8-, 11-, and 17-specific pericentromeric repetitive sequences (Vysis, Inc., Downers Grove, IL) were directly labeled with SpectrumGold, SpectrumRed, SpectrumGreen, and SpectrumAquamarine fluorophores, respectively, and combined with human placental blocking DNA in CEP Hybridization Buffer (Vysis, Inc.). Three-μl volumes of probe/hybridization mix were applied to each ductal lavage drop preparation and touch prep and then covered with 12-mm circular cover slips (Fisher Scientific, Pittsburgh, PA). Slides were then placed into a HY-Brite thermal unit (Vysis, Inc.) for simultaneous probe/target DNA denaturation at 73°C for 1 min, followed by a 2-h hybridization period at 42°C. After hybridization, slides were washed in 0.4 × SSC/0.3% NP40 at 73°C for 2 min, and 2 × SSC/0.1% NP40 for 1 min at room temperature. After air drying, slides were mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA), coverslipped, and evaluated with epifluorescence microscopy.

Signal Distribution in Normal Breast Cells. Drop preparations were made from cells harvested from subconfluent, mitotically active primary cultures established from reduction mammoplasty surgical tissue specimens (BioWhittaker, San Diego, CA). All four probes were hybridized to these preparations, and signal counts for each probe were determined in 1000 cells.

FISH Scoring. FISH scoring was performed without knowledge of surgical pathology or cytology findings. Because normal cells often represent a small proportion of the thousands of cells within a ductal lavage specimen, scoring a random subset does not always reveal a representative level of abnormalities. As scoring every cell is inefficient, a combination of subjective and objective scoring methods validated previously for detecting exfoliated bladder cancer cells in urine specimens was used (13). Because aneusomy is such a rare event in normal cell populations, the presence of even a very few cells with chromosomal abnormalities within a specimen indicates the
Fig. 1  Breast epithelial cells obtained by ductal lavage (A–D and F–H) and tumor touch prep (E) hybridized with centromeric probes for chromosomes 1 (red), 8 (green), 11 (aqua), and 17 (gold). Surgical pathologic classification of paired tissues: (A), fibroadenoma with florid ductal hyperplasia; B, DCIS; C, IDC; D, IDC; E, DCIS; F, IDC; G, IDC; H, IDC.
FISH Analysis of Paired Breast Tumor/Lavage Specimens

The field was next immediately detected, 60 consecutive large, nonoverlapping epithelial nuclei were scored for signal copy number using the appropriate filters for all four probes. If atypical nuclear cytologies and/or aneusomies were not detected on the initial ×60 scan of the entire field of a specimen, 60 of the largest nuclei were scored for signal copy number. The cutoff points for classifying ductal lavage samples as FISH positive were established by determining the mean per-
Table 2 Summary of ductal lavage findings

<table>
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<th>Cytology</th>
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b. Sensitivity and specificity tables for cytology and FISH

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c. Sensitivity and specificity calculations

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<td>Sensitivity</td>
<td>7/15 (47%)</td>
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<td>Specificity</td>
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RESULTS AND DISCUSSION

The majority of invasive breast cancers are thought to arise from epithelial cells lining the terminal ducts of the mammary tree. These cells are assumed to progress sequentially through a series of histological stages characterized as hyperplasia with and without atypical changes, DCIS, and invasive carcinoma. Although epithelial cell exfoliation occurs regularly in the mammary gland, the extent to which the cells representing the different stages of breast cancer progression are shed into the ductal system and thus accessible to harvest by ductal lavage has not been defined. To study the relationship between the pathologic and genetic features of breast tissue and exfoliated breast cells, we performed histological, cytologic, and molecular cytogenetic analyses on 39 paired cases of breast lesion touch prep and ductal lavage samples (Table 1).

The surgical pathologic diagnoses in this series included 10 IDCs, three ILCs, four DCISs, one lobular carcinoma in situ, 11 papillomas or papillomatosis, and 10 other benign conditions, including hyperplasia without atypia, fibroadenomas, fibrocystic disease, stromal sclerosis, and inflammation. From the 39 cases, evaluable ductal lavage specimens containing enough cells for cytologic and FISH analysis were obtained from 34 and 32 cases, respectively. As shown in Table 2, cytologic analysis of the corresponding ductal lavages detected abnormal (including mild or marked atypia and malignant) cells in 7 of 15 (47%) of the evaluable lavages collected from the malignant cases, versus 4 of 19 (21%) of the evaluable lavages from the benign cases. These findings corresponded to sensitivities and specificities of 47 and 79%, respectively.

Hybridization of the CEP 1, 8, 11, and 17 FISH probes to touch preparations of the freshly excised breast surgery tissues revealed numeric changes in 12 of the samples. Of the 14 malignant touch preps with adequate cells, 11 (79%) were FISH positive at one or more chromosomes. Four of the 12 FISH-positive touch preps were aneusomic for all four chromosomes, and three of these were from IDC cases, whereas the fourth was from a DCIS. The range of aneusomy varied from 1 to 15 copies/cell with three, four, and five copies being the most common.

Numeric changes were also observed in 12 of the ductal lavage samples (Fig. 1). Changes were observed in 10 of 14 (71%) of the malignant cases with evaluable lavages versus 2 of 18 (11%) of the benign cases. The corresponding sensitivities and specificities of FISH for detecting abnormalities in the ductal lavage specimens were 71 and 89%, respectively.

Numeric change at all four chromosomes were seen in three of the ductal lavage specimens, whereas numeric changes at one or two chromosomes, including low levels of monosomy, were observed in nine specimens. Of the three ductal lavages characterized for aneusomy on four chromosomes, one was classified cytologically as malignant and the other two as mildly atypical. Of the nine FISH-positive lavages with changes at one or two chromosomes, five were classified cytologically as benign and four as mildly atypical. In addition, these cases were
generally characterized by lower frequencies of changes, i.e., <20% of the cells showed numeric changes. The cytology and FISH results for the lavages are summarized in Table 2.

Aneusomy is a rare event in normal somatic cell populations, a fact that is underscored by the signal distributions of the four probes (CEPs 1, 8, 11, and 17) in mitotically active cultures of normal mammary epithelial cells (Fig. 2, top panel). The percentages of cells that were monosomic, disomic, trisomic, and tetrasomic for chromosome 17 were 6, 90, 1.5, and 2.5%, respectively. Similar signal distribution patterns were observed in the benign cases (Fig. 2). In contrast, pronounced variation in signal distribution was observed in both the touch prep and
ductal lavage cells from several of the malignant cases (case 14, a DCIS) and (cases 16 and 41, IDCs), as shown in the middle panels of Fig. 2. In the cases in which numeric changes were detected in both the primary lesions and lavages, the levels of aneusomy generally ranged from three to five copies. These patterns demonstrate corresponding rates of chromosomal instability in the breast tumors and ductal lavage cells from the same breast and suggest that the ductal lavage cells are derivative of the breast lesions.

However, the numeric chromosome changes observed in primary lesions were not always present in the corresponding lavages, as is illustrated by case 32 in Fig. 2. The touch prep from this IDC case actually displayed the highest degree of polyosity in the entire series, with a high proportion of cells exhibiting signal counts of >10 copies of chromosome 8. Although one of the lavages from this case was FISH positive, it was characterized by a much lower degree of aneusomy, with a lower magnitude of copy change at only 1 chromosome. Similarly, in a pilot series of paired cases used for protocol development, a high degree of chromosomal aneusomy (including copy changes in the 6–15 range) observed in four large, high-grade invasive ductal carcinomas was not observed in the corresponding ductal lavages. Collectively, these observations suggest that large breast tumors characterized by high nuclear grade and pronounced chromosomal instability may be less likely to shed into the ductal system and thus may not be accessible to harvest by ductal lavage. This interpretation is supported by an earlier nipple aspiration study in which abnormal cytology was not detected in samples obtained from women harboring tumors measuring ≥1 cm (14). The authors of this study suggested that larger breast lesions may extravasate into surrounding tissues instead of shedding cells into the ductal system. If this is the case, some advanced tumors may not be detectable by ductal lavage, although these lesions are likely to be diagnosed through palpation and/or mammography.

The detection of unequivocal, corresponding numeric chromosome changes in a subset of matched specimens in this series validates exfoliated breast cells collected by ductal lavage as targets for breast cancer detection and demonstrates that genetic alterations associated with breast cancer can be measured in these cells. However, the low levels of numeric changes observed in the majority of the cases in this series may not permit sufficiently rapid visual analysis of each specimen for the routine clinical diagnostic and prognostic purposes of early detection and/or risk stratification. The introduction of automated microscopic scanning and analysis systems may enable routine analysis in the future. We chose interphase FISH analysis to measure genetic alterations because ductal lavage specimens are heterogeneous fluids containing up to thousands of nonepithelial cells (1, 15), and an in situ method was thus most suitable. For technical reasons, we focused on numeric increases using centromeric probes, and we chose changes that occur with high frequency in breast cancer (3–12). The suitability of the probes to detect the advanced stages of breast cancer is well illustrated by the robust detection of changes in a high proportion of touch preps in our series. However, in the series of corresponding lavage samples, this probe panel proved effective for detecting a limited window encompassing a subset of tumors that were advanced but still shedding into the glandular lumina. Although numeric changes at chromosomes 1 and 17 have been reported in normal breast tissue adjacent to invasive breast cancer (9), as well as in the early stages of breast cancer progression, these alterations may not be frequent enough for robust diagnostic detection in exfoliated cell populations. The sensitivity of interphase FISH to detect early changes predictive of advancement to invasive disease may be enhanced by focusing on an extended panel of FISH probes that includes the measurement of region-specific allelic losses observed during early breast cancer (16). Although this is technically feasible, these measurements might be enhanced with strategies to enrich the epithelial cell population in ductal lavage specimens and by application of DNA chip technologies to measure a more comprehensive set of changes encompassing aneuploidy, gene amplification, and region-specific losses.

Of interest in this series are the 11 cases classified by surgical pathology as benign papillomas. Although four of the ductal lavages from these cases were classified as cytologically abnormal (three as mildly atypical and one as malignant), none of them displayed numeric chromosome changes, a finding that is consistent with what has been reported for FISH analysis of fine needle breast aspirates (17). In the multicenter ductal lavage trial (1), follow-up imaging and/or surgical exploration of subjects with abnormal cytologic findings revealed a number of papillomas. Papillomas may constitute a diagnostic “gray zone” in ductal lavage specimens, and the application of FISH analysis may help distinguish these benign lesions from invasive lesions, which require distinct surgical interventions.

In summary, our analysis has shown that cytologic and cytogenetic abnormalities can be unequivocally detected in a subset of ductal lavage specimens harvested from women with in situ and invasive breast cancer. Cancer-associated alterations in DNA methylation patterns have been observed in ductal lavage fluids (18), and our observations complement these findings by demonstrating DNA alterations within ductal lavage cells. Our results indicate that interphase FISH may have superior sensitivity and specificity compared with conventional cytology and can be used in conjunction with cytology to confirm and exclude findings, similar to diagnostic approaches in use for the detection of exfoliated bladder cancer cells in urine (13). Our results also validate exfoliated breast cells as targets for biomarker development by demonstrating the unequivocal presence of cancer-associated numeric chromosome changes in these cells. We now hope to extend these preliminary approaches to one of the most important potential applications of ductal lavage: assessment of risk for the development of invasive breast cancer.

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