Prospective Evaluation of a Novel Approach for the Use of a Quantitative Galactose Oxidase–Schiff Reaction in Ductal Fluid Samples from Women with Breast Carcinoma

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BACKGROUND. The galactose oxidase–Schiff reaction (GOS) yields positive findings in a number of malignant solid tumors. The goals of the current study were to develop a novel technique for quantifying GOS reactivity in nipple aspirate fluid (NAF) samples from women with invasive breast carcinoma and to assess the clinical utility of the technique in this setting.

METHODS. Patients with biopsy-proven unilateral invasive breast carcinoma were eligible for study entry. Before definitive surgery, NAF samples were obtained from healthy breast tissue and malignant breast tissue from 23 women with breast carcinoma. Under blind conditions with respect to clinical data, 10 μL NAF samples were applied to a glass fiber membrane and incubated with 100 μL galactose oxidase and 1 mL Schiff reagent. The stain was developed and the color reaction quantitated by measuring hue (shade) and chroma (intensity) using a spectrophotometer.

RESULTS. GOS reactivity was quantitated using two color parameters, hue and chroma. Because chroma varies with concentration, this measurement was adjusted for the concentration of NAF in each sample. After adjustment for NAF concentration, chroma was found to be statistically significantly different in the affected breast tissue sample and the healthy contralateral internal control sample ($P = 0.001$).

CONCLUSIONS. A quantitative measure of GOS reactivity based on spectrophotometric measurement of intensity of color has been developed and was found to be significantly different in the affected breast compared with the unaffected breast in the current population of patients with breast carcinoma. The preliminary results support further exploration of this novel quantitative test in patients with breast carcinoma. ***Cancer* 2004;100:2549–54.** © *2004 American Cancer Society.*

KEYWORDS: nipple aspirate fluid, galactose oxidase–Schiff reaction, breast carcinoma, hue, chroma.

In recent years, there has been increasing interest in the ductal microenvironment, and particularly in the sampling of nipple aspirate fluid (NAF) to assess patients who have or are at risk of developing breast carcinoma. Such approaches may spare patients from having to undergo invasive and costly procedures. Although NAF typically is used for cytologic assessment,[1–3] it also can be used to evaluate potential biomarkers that may be of diagnostic utility.[4–7]

The galactose oxidase–Schiff (GOS) reaction detects D-galactose-β-[1,3]-*N*-acetyl-D-galactosamine (Gal-GalNAc), also known as the Thomsen–Friedenreich (TF) antigen (Fig. 1). This disaccharide moiety

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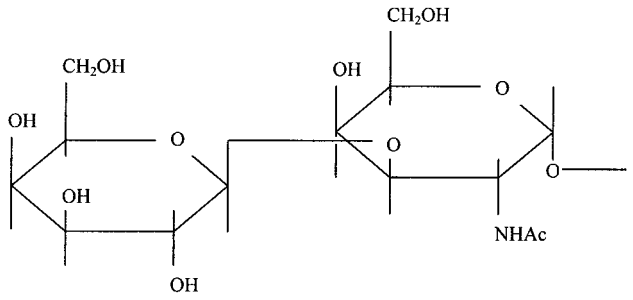

FIGURE 1. Chemical structure of the Thomsen–Friedenreich antigen. Black bars: healthy breast; white bars: affected breast.

has been found in many epithelial malignancies, including breast carcinoma.[8,9] It is noteworthy that it has been found not only in malignant tissue but also in the surrounding healthy tissue, which some speculate is a result of a field effect phenomenon.[10] The utility of the GOS reaction, however, has been limited by the subjective interpretation of color. The goals of the current study were to evaluate a novel quantitative assessment of the GOS reaction[11] in NAF samples from women with unilateral breast carcinoma and to determine the ability of this test to distinguish the affected breast from its healthy contralateral counterpart.

MATERIALS AND METHODS

Patients with biopsy-proven unilateral breast carcinoma who presented to The University of Texas M. D. Anderson Cancer Center (MDACC; Houston, TX) were eligible to participate in the prospective study. The study was approved by the institutional review board at MDACC. Patients who had previously undergone subareolar surgery, which may have disrupted the terminal ductal system in the breast, were excluded. All patients consented to have bilateral nipple aspiration performed.

At the time of definitive surgery, after general anesthesia had been administered, NAF samples were obtained from both breasts. The nipple was cleaned with Omniprep paste (D. O. Weaver, Aurora, CO) to remove any keratin plugs and then wiped with an alcohol pad. Lotion was applied to the breast, and the breast was massaged from the chest wall towards the nipple for 1 minute. The NAF sample was obtained using a handheld suction cup connected to a syringe (Product Health, Menlo Park, CA).[12] Suction was applied to the syringe until the ductal fluid sample was visualized. The NAF droplets were then collected using 10 μL micropipettes (Drummond Scientific, Broomall, PA). The volume of NAF sample obtained from each breast was recorded.

Immediately after collection, the NAF sample was dispensed with 500 μL phosphate-buffered saline containing the protease inhibitors 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (0.2 mM), leupeptin (50 μg/mL), aprotinin (2 μg/mL), and 1,4-dithio-DL-threitol (0.5 mM). The samples were centrifuged at 1500 revolutions per minute for 10 minutes, and the supernatant was collected and stored at -80 °C.

The total protein concentration in the NAF samples was determined by triplicate measurement using the Micro BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). The standards for the protein concentration measurements were diluted in the protease inhibitor solution described above to ensure comparability with the NAF samples.

Aliquots of 10 μL of NAF solution were applied to glass fiber membranes attached to a support of white polystyrene with the approximate dimensions of a microscope slide. The samples were allowed to dry on the membrane and then shipped at ambient temperature to McMaster University (Hamilton, Ontario, Canada) for GOS testing. Investigators were blinded to which breast was affected and which was healthy. They also were blinded to all patient information. Galactose oxidase (100 μL containing 100 units/mL) was delivered by pipette onto the membrane and incubated at ambient temperature for 10 minutes. The slide was then rinsed in deionized distilled water for 1 minute, 1 mL Schiff reagent (Sigma, St. Louis, MO) was delivered by pipette onto the membrane, and the slide was incubated for 1 minute at room temperature. The stain was developed by washing in tap water 4 times, with each wash lasting 10 minutes. The slides were then allowed to dry at room temperature. The color reaction was quantitated by measuring hue and chroma using a portable spectrophotometer (CA22; X-Rite, Grandville, MI) and QA Lite Software (X-Rite). Statistical analyses were performed using SPSS software (Version 10.0; SPSS, Chicago, IL).

RESULTS

Patient Demographics

Fifty patients participated in the trial. The median volume of the NAF sample collected from the healthy breast was 24.5 μL (range, 2–478 μL), and the median volume of NAF sample collected from the affected breast was 18 μL (range, 1–389 μL). The minimum amount of NAF necessary for the GOS test was determined to be 15 μL. Twenty-three patients had $\geq$ 15 μL of NAF sample collected from each breast. These patients constitute the study population of interest. Patients had a median age of 57 years (range, 37–77 years).

TABLE 1
Primary Tumor Features

Feature	No. of patients (%)
Tumor size of invasive component (cm)[a]	
≤ 2	16 (69.6)
2–5	5 (21.7)
≥ 5	1 (4.3)
Palpability	
No	13 (56.5)
Yes	10 (43.5)
Side	
Left	9 (39.1)
Right	14 (60.9)
Tumor location	
Upper inner quadrant	8 (34.8)
Upper outer quadrant	3 (13.0)
Lower inner quadrant	3 (13.0)
Lower outer quadrant	8 (34.8)
Center	1 (4.3)
Grade[b]	
I	1 (4.3)
II	13 (56.5)
III	7 (30.4)
Lymph node status	
Negative	16 (69.6)
Positive	7 (30.4)
Neoadjuvant chemotherapy	
No	17 (73.9)
Yes	6 (26.1)
Stage (AJCC)	
0	1 (4.3)
I	15 (65.2)
II	6 (26.1)
III	1 (4.3)
Estrogen receptor status[c]	
Negative	8 (34.8)
Positive	12 (52.2)
Progesterone receptor status[c]	
Negative	12 (52.2)
Positive	7 (30.4)
HER-2/*neu* status (fluorescence in situ hybridization)[c]	
Negative	15 (65.2)
Positive	4 (17.4)

AJCC: American Joint Committee on Cancer.
[a] One patient (4.3%) had ductal carcinoma in situ only.
[b] Nuclear grade was not specified in 2 patients (8.6%).
[c] Estrogen receptor status was not recorded in 3 patients (13.0%), progesterone receptor status was not recorded in 4 patients (17.4%), and HER-2-*neu* status was not recorded in 4 patients (17.4%).

Primary Tumor Characteristics and Treatment

The primary tumor characteristics of the cohort of 23 patients are shown in Table 1. None of these women had undergone previous subareolar surgery or received radiotherapy to the breast. Two patients had spontaneous nipple discharge, whereas the remaining patients did not. All patients proceeded with definitive surgery after the NAF samples were obtained. Mastectomy was performed for 13 patients, and segmental mastectomy was performed for 10 patients.

TABLE 2
Galactose Oxidase–Schiff Reaction in Nipple Aspirate Fluid

Parameter	Median (range)		*P* value[a]
	Healthy breast	Affected breast	
Volume (μL)	49 (15–478)	66 (15–379)	0.403
Protein concentration (mg/mL)	69.9 (23.0–185.1)	84.8 (24.5–263.0)	0.986
Hue	325.5 (318.3–332.2)	326.5 (317.3–332.2)	0.361
Chroma	27.2 (11.2–43.6)	19.8 (8.6–53.0)	0.080
Adjusted chroma	0.6 (0.1–1.5)	0.3 (0.1–1.4)	0.001

[a] Wilcoxon signed-rank test.

Nipple Aspirate Fluid Analysis

Of the 23 patients with sufficient NAF samples for analysis (≥ 15 μL), the median volume of NAF sample was 49 μL (range, 15–478 μL) for the healthy breast and 66 μL (range, 15–379 μL) for the affected breast. The spectrophotometric assay of the GOS reaction yielded absolute values for hue and chroma. Because chroma (i.e., the intensity of the color reaction) is concentration dependent, the absolute value for chroma was divided by NAF volume (in μL) to obtain a corrected measure. Results for healthy and affected breasts are summarized in Table 2.

We compared healthy breast samples and affected breast samples with respect to volume, protein concentration, and hue and adjusted chroma values to determine whether any of these measures was correlated with the presence of malignant disease. The results of this analysis are shown in Table 2. Adjusted chroma values were significantly different in healthy breast samples compared with samples obtained from affected breasts.

We then sought to determine whether the adjusted chroma values recorded in NAF samples from affected breasts were correlated with any of the other clinicopathologic variables. This analysis is shown in Table 3 and in Figure 2. There was a dramatic trend toward higher disease stage in patients with higher adjusted chroma values ($P = 0.051$). In addition, higher adjusted chroma values were correlated with estrogen receptor (ER)-positive and HER-2/*neu*–negative disease ($P = 0.016$ and $P = 0.027$, respectively).

DISCUSSION

With breast carcinoma being the most common malignancy diagnosed in women today,[12] and with the majority of these tumors arising from the ductal sys-

TABLE 3
Correlation of Adjusted Chroma Values with Clinicopathologic Variables

Feature	Median adjusted chroma value (range)	*P* value
Tumor size of invasive component (cm)[a]		0.115
≤ 2	0.3 (0.1–1.4)	
2–5	0.4 (0.2–0.9)	
≥ 5	0.1	
Palpability		0.148
No	0.3 (0.1–0.9)	
Yes	0.4 (0.1–1.4)	
Grade[b]		0.361
I	0.4	
II	0.4 (0.1–0.9)	
III	0.2 (0.1–1.5)	
Lymph node status		0.720
Negative	0.4 (0.1–0.9)	
Positive	0.3 (0.1–1.4)	
Stage		0.051
0	0.1	
I	0.3 (0.1–0.8)	
II	0.6 (0.2–1.4)	
III	0.1	
Estrogen receptor status[c]		0.016
Negative	0.2 (0.1–1.3)	
Positive	0.5 (0.1–1.4)	
Progesterone receptor status[c]		0.227
Negative	0.3 (0.1–0.9)	
Positive	0.4 (0.1–1.4)	
HER-2-*neu* status (fluorescence in situ hybridization)[c]	0.027	
Negative	0.4 (0.1–1.4)	
Positive	0.1 (0.1–0.3)	

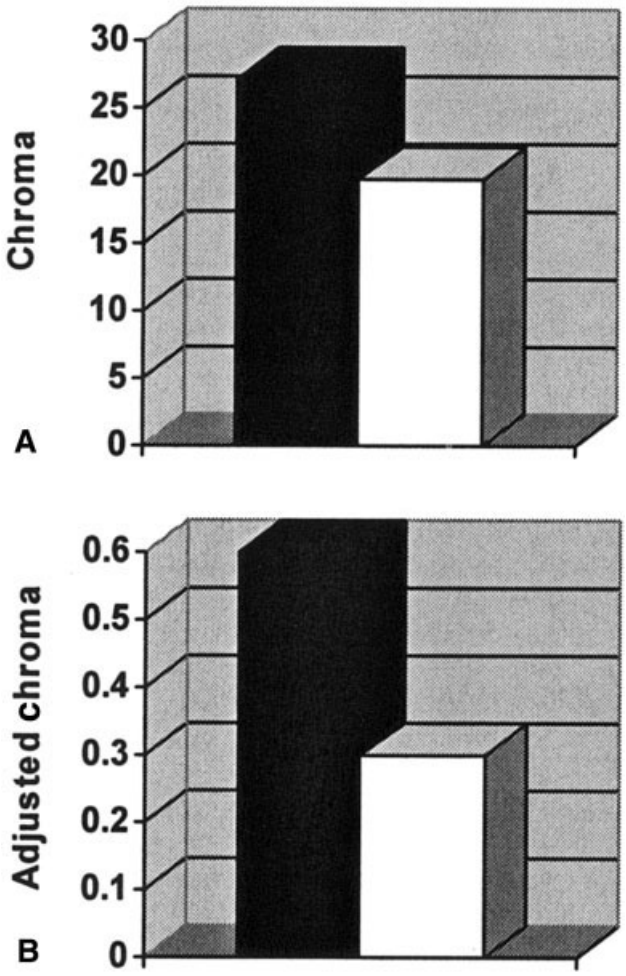

FIGURE 2. Bar graphs depicting (A) chroma values ($P = 0.080$) and (B) adjusted chroma values ($P = 0.001$) in healthy and affected breasts. Black bars: healthy breast; white bars: affected breast.

tem, there has been a surge of research investigating the potential of NAF and ductal lavage fluid to yield novel biomarkers. These techniques sample fluid from the ductal system and may provide insight into the breast microenvironment.

It has been demonstrated previously that NAF may be obtained from up to 95% of healthy women,[13] making this technique widely applicable. Although some centers have used NAF cytology to provide high-risk individuals with information that may influence their decision to receive tamoxifen for chemoprevention,[14] cytologic evaluation relies on the skill of the cytopathologist and thus is not always dependable. NAF cytology is not a sensitive method for detecting invasive carcinoma of the breast.[2] Hence, there is a need for more sensitive and more objective biomarkers that are related to the presence of invasive malignancy of the breast.

The GOS reaction yields positive results in many malignancies, including carcinomas of the lung,[15] pancreas,[16] ovary,[17–19] thyroid,[20] stomach,[21,22] and colon.[23–25] This reaction also produces positive findings in transitional cell carcinoma of the urinary bladder,[26,27] embryonal and yolk sac tumors of the male genital tract,[28] and pheochromocytomas.[29] Immunohistochemical studies have also found that breast carcinoma stains positively for the TF antigen. This phenomenon was first reported in 1975 by Springer et al.[30] and later confirmed by a number of authors who found that breast carcinoma tissue expressed the TF antigen and that no binding of the TF antigen was evident in normal tissue.[31–33]

Detection of the TF antigen in these studies has relied on a variety of reagents, including peanut lectin and monoclonal antibodies (MoAb). Due in part to the variation in the specificity and affinity of these reagents, these assays have not been widely used in clinical practice.[11] The GOS reaction detects the TF antigen via the galactose oxidase enzyme, which re-

acts with the terminal galactose residues of the TF antigen to oxidize the C6 alcohol groups to aldehydes. The Schiff reagent then reacts with the aldehydes to produce a magenta color. This reaction has been studied in breast tissue sections and has been reported to yield positive results in breast carcinoma tissue and negative results in normal breast tissue.[10] Although these results certainly are noteworthy, the findings still rely on the subjective interpretation of the test.

Recently, a novel method for quantification of this reaction was reported.[11] The reported technique essentially uses spectrophotometric methods to quantify the two main components of color: hue (shade) and chroma (intensity). We assessed the ability of this technique to distinguish between healthy and affected breasts in NAF samples obtained from women with unilateral breast carcinoma. We found that when chroma was adjusted for the volume of NAF sample obtained, a statistically significant difference was found between the normal and affected breasts. Although this finding is noteworthy, the current study was relatively limited in size, and further efforts to validate these results are warranted.

In addition, we found that the adjusted chroma value of the NAF sample obtained from the affected breast was correlated with the ER status of the tumor; this finding is supported by previous studies that investigated the detection of the TF antigen using peanut lectin.[34–36] Nonetheless, some studies, such as one in which peanut agglutinin was used for antigen detection, have not observed this correlation.[37] One possible explanation for this inconsistency is that only a combination of estrogen and progesterone may be able to increase binding.[38] In the current study, however, there was a clear correlation between adjusted chroma value and ER status, but not between adjusted chroma value and progesterone receptor status.

The impact of the TF antigen in terms of breast carcinoma prognosis also has been controversial. In a study in which peanut agglutinin was used, Barry et al.[39] found that TF antigen binding had no prognostic relevance. However, other studies involving MoAb have suggested that increased binding is associated with tumor progression.[40] Although we did not find a significant correlation between binding and tumor size, tumor grade, or lymph node status, there was a trend toward increased disease stage among patients with higher adjusted chroma values. These findings, although not statistically significant given the size of the current study, are intriguing and warrant further investigation.

The current study was limited by the relatively small size of the patient population and by the fact that nearly one-half of all patients enrolled did not have sufficiently large NAF samples for GOS analysis. However, this novel quantitative technique is deserving of further research. Studies of this technique are currently being evaluated in bronchial washings from patients with lung carcinoma and in rectal mucus samples from patients with colorectal carcinoma. Sampling of the NAF remains a noninvasive method of assessing the breast microenvironment and may prove to be useful in the discovery of novel biomarkers and in early diagnosis.

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