Thomsen-Friedenreich and Tn Antigens in Nipple Fluid: Carbohydrate Biomarkers for Breast Cancer Detection

Senthil R. Kumar,[1] Edward R. Sauter,[2] Thomas P. Quinn,[1] and Susan L. Deutscher[1,3]

Abstract

Purpose: Novel biomarkers would facilitate early and accurate diagnosis of breast cancer. The Thomsen-Freidenreich (TF) and Tn antigens are aberrantly glycosylated carbohydrate cancer-associated antigens found in ~80% of adenocarcinomas. Both TF and Tn are expressed on cell-surface glycoproteins and glycolipids. Nipple aspirate fluid (NAF) is concentrated in secreted proteins and lipids from cells that give rise to cancer. The objective of this study was to determine if NAF from breasts with cancer contains elevated levels of TF and Tn compared with NAF from normal breasts. A sensitive and specific antigen capture immunoassay for TF and Tn detection in NAF was developed for this purpose.

Experimental Design: Fifty NAF samples, 25 from breasts with cancer and 25 from normal breasts, were examined. Antigen capture immunoassays were done on the samples using monoclonal antibodies that specifically recognized either TF or Tn antigen in NAF. These antibodies captured serially diluted NAF samples, and the concentration of TF or Tn was determined by comparing absorbance values against a standard curve generated from standard sources of TF or Tn.

Results: TF and Tn were detected in 19 of 25 and 20 of 25 NAF samples from breasts with cancer, respectively, compared with 0 of 25 and 1 of 25 NAF samples from breasts without cancer ($P < 0.001$ for both TF and Tn). In 92% of the cancerous breast NAF samples tested, either TF or Tn was found.

Conclusions: Simultaneous measurement of TF and Tn in NAF may facilitate the noninvasive detection of breast cancer and warrants further study.

Breast cancer is the most commonly diagnosed noncutaneous cancer and the second leading cause of cancer death in women. Early breast cancer detection is critical to successful treatment. Standard breast cancer screening includes physical examination and mammography. Breast cancer diagnosis requires morphologic changes in breast cells obtained by diagnostic fine needle, core needle, or surgical biopsy. Mammography and physical examination overlook up to 40% of early breast cancers. Given the inadequacies in mammography and the pain and expense of diagnostic surgical biopsies, new approaches for improved breast cancer detection are being explored. Less invasive techniques, including nipple aspiration, are under assessment to improve breast cancer detection (1). Nipple aspirate fluid (NAF) provides concentrated secreted proteins and lipids from cells in the breast ductal epithelium—cells that give rise to cancer (2). Malignant NAF cytology and ploidy predict the presence of breast cancer (1), and increased levels of growth factors in NAF of women with breast cancer have been documented (3–5). Thus, NAF may provide an enriched source of predictive biomarkers for early breast cancer detection or prognosis.

Early detection and timely therapy would be facilitated by the identification and utilization of breast cancer-associated antigens in clinical testing of NAF. However, only a small set of breast tumor-associated antigens have been identified and their use in noninvasive breast cancer screening has been surprisingly limited (3–5). Glycoantigens are potential biomarkers for breast cancer risk assessment and disease detection. Epithelial cancer cells exhibit increased cell surface expression of mucin-type antigens with aberrant O-glycosylation. Among such antigens, the Thomsen-Friedenreich (TF; Gal β1→3GalNAc-) antigen and its biosynthetic precursor, Tn (GalNAc-), are displayed on cell-surface proteins and lipids in 70% to 90% of adenocarcinomas including those of the breast, prostate, and ovary (6–10). Whereas the function of Tn remains unclear, TF has been shown to be involved in tumor cell adhesion and migration (11). Both TF and Tn have been used clinically as prognostic indicators of cancer and have been detected immunologically in primary breast cancer tissues, lymph nodes, and distant metastases (7–9, 12). The finding that TF and Tn carbohydrates are present on both epithelial cell surface proteins and lipids (6) prompted us to question whether NAF from breast cancer patients may contain high levels of TF and Tn compared with normal NAF.

Authors' Affiliations: Departments of [1]Biochemistry and [2]Surgery, University of Missouri, and [3]Harry S. Truman Memorial Veterans Hospital, Columbia, Missouri
Received 1/20/05; revised 6/6/05; accepted 7/8/05.

Grant support: Department of Defense grant DOD DAMD 17-03-1-0509 (T. Quinn).

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Requests for reprints: Susan Deutscher, Department of Biochemistry, University of Missouri, M743 Medical Sciences Building, Columbia, MO 65212. Phone: 573-882-2454; Fax: 573-884-4597; E-mail: deutschers@missouri.edu.

doi:10.1158/1078-0432.CCR-05-0146

Materials and Methods

Demographic and clinical information were available for all subjects. Twenty-four percent (6 of 25) of women with NAF collected from a cancerous breast were premenopausal, versus 52% (13 of 25) of women with NAF collected from a breast without cancer (Table 1). All subjects evaluated except one were white. Two NAF samples from the cancer group and four NAF samples from the no cancer group were derived from patients with a prior history of breast cancer in the contralateral breast. Six women from each group had a family history of breast cancer (Table 1). Forty percent of NAF samples collected from a breast with cancer were obtained before needle or surgical biopsy, whereas all but one of the samples (24 of 25) from a breast without cancer were collected before biopsy. Of these 24 latter women, 16 did not undergo breast biopsy. Of the benign biopsies obtained in the other nine women, five were classified as normal and four with benign proliferative changes. In the cancerous breast group, there was one subject with precancer, one with *in situ* cancer, and 23 with invasive breast cancer.

We employed competitive inhibition ELISAs for quantitation of both TF and Tn in NAF. After obtaining written consent to participate in a University of Missouri Institutional Review Board–approved clinical trial, NAF samples were collected from patients requiring diagnostic breast biopsy to evaluate an abnormal mammogram and/or a palpable breast mass. Asymptomatic women were also recruited as controls to our ongoing nipple aspiration trials. Nipple fluid was aspirated as previously described (1). Briefly, the breasts were warmed with moist towels. The nipples were cleansed with a mild soap followed by alcohol. A suction device was then placed first over the breasts. Fluid was collected into 50 μL glass capillary tubes. The samples were stored at −80°C and batched for analysis.

Fifty NAF samples, 25 from breasts with cancer and 25 from normal breasts, were examined in a blind study. The immunoassays were done on the samples using monoclonal antibodies to expressly capture the TF and Tn antigens in NAF. Antigen capture, in contrast to less specific chemical methods for mucin NAF analysis (13, 14), allowed for the detection of TF and Tn. The monoclonal antibodies (anti-TF A78-G/A7, NeoMarkers, Freemont, CA; anti-Tn V 1053, Biomeda, Foster City, CA) were applied to Nunc-immunomaxi 96-well plates (Nunc A/S, Roskilde, Denmark) for 4 hours at 37°C. After washing in TBS containing 0.1% Tween 20, and blocking with this solution containing 2% bovine serum albumin, 100 μL of diluted (1:50-1:500) control and cancer NAF samples were added and incubated for 4 hours at room temperature. After washing in TBS containing 0.1% Tween 20, biotinylated asialofetuin (a standard source of TF) or asialo-ovine submaxillary mucin (a standard source of Tn) was added. Washed wells were incubated with avidin/peroxidase complex and peroxidase activity was measured by incubation in 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate system (Sigma, St. Louis, MO). The absorbance was read at 405 nm with an ELISA plate reader (Bio-Tek, Winooski, VE). The concentration of TF or Tn in NAF was determined by interpolation of the absorbance values against a standard curve done with different dilutions of biotinylated asialofetuin or asialo-ovine submaxillary mucin. Values of <15 ng/μL (corresponding to an absorbance of <0.04) were considered as zero (not detectable).

Table 1. Demographic and clinical characteristics

	Cancer	No cancer
Menopausal status		
Premenopausal	6	13
Postmenopausal	19	12
Race		
White	24	25
Black	1	0
Prior history of breast cancer*	2	4
Family history†	6	6
NAF collection relative to diagnostic biopsy‡		
Before	10	24
After	15	1
Biopsy results§		
No biopsy	0	16
Benign	0	9
Fibrocystic changes	0	3
Hyperplasia	0	1
Normal breast tissue	0	5
Atypical ductal hyperplasia (precancer)	1	0
Ductal carcinoma *in situ*	1	0
Invasive breast cancer	23	0
Stage I	5	0
Stage II	12	0
Stage III	5	0
Stage IV	1	0

*Prior history of breast cancer in the contralateral breast. Additionally, in the cancer group, two women had synchronous bilateral breast cancer, which are not included in the table above.

†Family history is defined as one or more first-degree relatives with a history of breast cancer.

‡Additional subjects in both cancer and no cancer categories had biopsies for prior breast complaints. Biopsies more than 3 months before NAF collection were not considered. Some subjects in the no cancer category had never undergone breast biopsy.

§Of the 23 breasts with invasive cancer, 19 were ductal and 4 were lobular subtype.

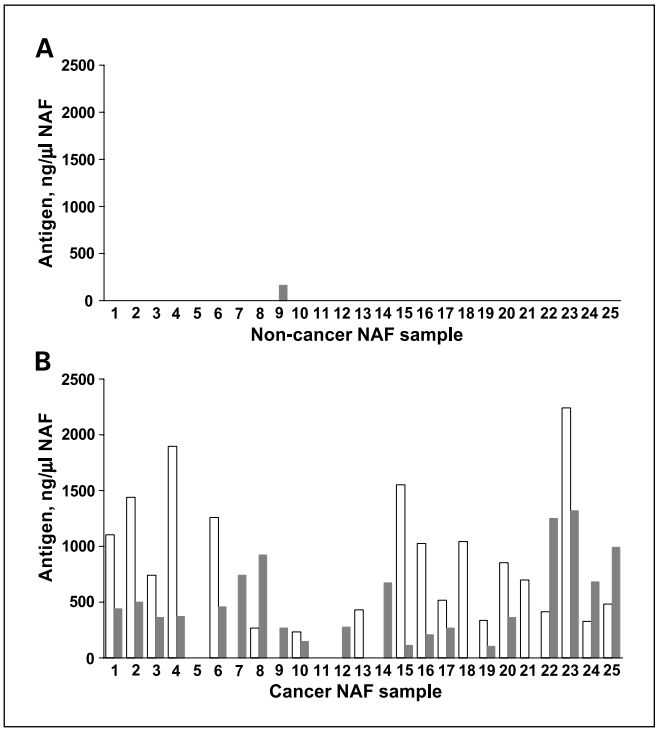

Fig. 1. Concentration of TF (□) and Tn (■) antigens in noncancer (*A*) and cancer (*B*) NAF as determined by competitive inhibition ELISA. Values of <15 ng/μL were considered as zero (not detectable). Statistical significance between the values obtained for noncancer and cancer NAF values was determined by Student's *t* test (TF, $P = 0.0007$; Tn, $P = 0.0002$).

Results

There was a significant association of the presence of TF and Tn in NAF with the presence of breast cancer. NAF samples from the 25 normal breasts exhibited no detectable TF and only one sample had detectable but very low levels of Tn (Fig. 1A). This sample (Fig. 1A, no. 9) was from a patient with a breast classified as noncancerous by NAF cytology and mammography and follow-up mammography 18 months later. Cancer was detected in the contralateral breast. In contrast, TF or Tn was detectable in 23 of 25 (92%) NAF samples from 25 breasts with precancer or cancer (Fig. 1B). It was of interest to examine whether Tn co-occurred with TF in NAF because the Tn monosaccharide is a biosynthetic precursor of the TF disaccharide. The antigens did not always co-occur in that 16 of 25 (64%) NAF samples from breasts with cancer had detectable levels of both TF and Tn. In the breast cancer NAF samples, the median concentrations of TF and Tn were 480 ng/μL (range, 0-2,240 ng/μL) and 360 ng/μL (range, 0-1,320 ng/μL), respectively (Table 2). In noncancer NAF samples, the median concentration of TF was 0 ng/μL (range, 0), and that for Tn was also 0 ng/μL (range of 0-168 ng/μL; Table 2). Statistical analyses using the Student's t test showed differences in the NAF levels of TF (P = 0.0007) and Tn (P = 0.0002) between breasts with and without cancer. Although the protein concentration in NAF ranged from 0.01 to 0.85 mg/μL, the TF and Tn antigen levels normalized for either NAF protein concentration or for standard volume (per microliter) of NAF yielded similar results in terms of increased amounts in cancer versus noncancer samples, suggesting that the increased levels observed in the breast cancer patients were independent of NAF protein concentration (Table 2). Neither TF nor Tn was detected in corresponding serum samples from cancer or noncancer (data not shown).

Discussion

New approaches for improved breast cancer detection are continually being explored. One approach involves the analyses of NAF biomarkers for the noninvasive detection of breast cancer (1, 2). Levels of carcinoembryonic antigen have been found to be higher in NAF from breasts with invasive cancer than from benign breasts, but its usefulness in cancer detection is limited due to poor test sensitivity (15, 16). Significant increases in high basic fibroblast growth factor levels have been reported in the NAF of breast cancer versus noncancer patients (3, 5). Nonetheless, subjects without breast cancer often had detectable levels of basic fibroblast growth factor, indicating that the antigen is not entirely cancer specific. Therefore, it was of interest to explore antigens that have been more closely associated with breast cancer, in terms of tumor growth, progression, adhesion,

Table 2. TF and Tn in NAF of cancer patients

Patient	TF		Tn	
	TF level (ng/μg NAF protein)	Total antigen concentration (ng/μL* NAF)	Tn level (ng/μg NAF protein)	Total antigen concentration (ng/μL NAF)
1	9.0	1,100	3.7	440
2	12.0	1,440	4.2	500
3	4.1	740	2.0	360
4	16.4	1,900	5.1	370
5	0.0	0	0.0	0
6	30.0	1,260	11.6	460
7	0.0	0	11.3	740
8	1.8	270	5.9	920
9	0.0	0	10.0	270
10	2.1	230	1.2	150
11	0.0	0	0.0	0
12	0.0	0	11.8	280
13	17.9	430	0.0	0
14	0.0	0	1.1	670
15	6.5	1,550	0.5	113
16	1.3	1,030	0.3	210
17	1.8	520	0.8	270
18	10.0	1,040	0.0	0
19	2.4	340	0.7	100
20	2.4	850	1.0	360
21	7.3	700	0.0	0
22	0.9	412	2.6	1,248
23	5.6	2,240	3.3	1,320
24	0.9	325	1.9	684
25	1.6	480	3.5	990

*Values indicate the amount of antigen present in 1 μL of undiluted NAF.

and/or metastasis, as improved biomarkers for breast cancer detection in NAF. Our previous work, as well as that of others, has shown that TF and Tn play important roles in cell adhesion, tumor cell growth, and migration (6–9, 11, 17). Studies on Tn antigen in breast ascitic and pleural effusions indicated that Tn was elevated in breast cancer patients and was associated with MUC1, a major breast mucin (12). These findings suggest that TF and Tn may occur in NAF from women with early stages of disease. Indeed, the one subject in our study with atypical ductal hyperplasia had elevated Tn in NAF. One limitation of the current study was the lack of samples from subjects with precancerous lesions. We are planning a new study to compare NAF samples from patients with benign lesions without atypia to NAF samples from subjects with atypical ductal hyperplasia.

The results discussed here clearly showed that there were significantly higher levels of both of these antigens in breast cancer versus noncancer NAF samples. The finding that ~90% of the breast cancer NAF samples examined contained measurable levels of TF and/or Tn is consistent with immunohistochemical analyses of these antigens in biopsy materials (18). Importantly, TF and Tn were not detectable in the vast majority of noncancerous NAF, suggesting that these antigens are cancer specific. Such specificity was not achieved in NAF analysis using galactose oxidase-Schiff reactions (13). These findings are in keeping with earlier studies that revealed that noncancer cells display sialylated forms of TF and Tn that are not involved in tumor growth and progression (6, 19). Future studies will analyze TF and Tn in NAF from patients with precancer and different stages of breast cancer. The analysis of TF and Tn in NAF may prove useful as an adjunct to mammography and physical examination to noninvasively screen for primary or recurrent breast cancer.

Acknowledgments

We thank Marie Dickerson for technical support.

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Clin Cancer Res 2005;11:6868-6871.