

Protein Microarray Analysis of Mammary Epithelial Cells from Obese and Nonobese Women at High Risk for Breast Cancer: Feasibility Data

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Abstract

Background: Obesity is a well-established risk factor for cancer, accounting for up to 20% of cancer deaths in women. Studies of women with breast cancer have shown obesity to be associated with an increased risk of dying from breast cancer and increased risk of developing distant metastasis. While previous studies have focused on differences in circulating hormone levels as a cause for increased breast cancer incidence in postmenopausal women, few studies have focused on potential differences in the protein expression patterns of mammary epithelial cells obtained from obese versus nonobese women.

Methods: Protein expression was assessed by reverse-phase protein microarray in mammary epithelial cells from 31 random periareolar fine needle aspirations performed on 26 high-risk women.

Results: In this pilot and exploratory study, vimentin (unadjusted $P = 0.028$) expression was significantly different between obese and nonobese women.

Conclusions: Vimentin is integral both to adipocyte structure and function and to the epithelial-to-mesenchymal transition needed for cancer cell metastasis. Further research is needed to confirm this finding and determine the possible effects of the adipocyte microenvironment on the initiation and progression of breast cancer in high-risk women.

Impact: Differential protein expression patterns obtained from a future expanded study may serve to elaborate the underlying pathology of breast cancer initiation and progression in obese women and identify potential biomarkers of response to preventative interventions such as dietary changes and exercise. *Cancer Epidemiol Biomarkers Prev*; 20(3); 476–82. ©2011 AACR.

Introduction

Obesity is one of the most important known preventable causes of cancer, accounting for up to 20% of cancer deaths in women, with highest body mass index (BMI) category (BMI > 40 kg/m²) conferring higher risk (1). Previous studies have shown an association between postmenopausal breast cancer risk and excessive body

weight, and this association is increased in women with a positive family history of breast cancer (2, 3). In addition, obese women have an increased risk of dying from breast cancer and an increased risk of developing distant metastasis (4, 5).

Studies investigating the molecular basis for the association between breast cancer and increased adiposity have focused on differences in circulating hormones, such as estrogen and insulin, in normal weight versus obese individuals or on how mammary adipocytes and their secreted adipokines may influence tumor cells growth and invasion (6, 7). However, few studies have focused on potential differences in the protein expression patterns of mammary epithelial cells obtained from obese versus nonobese women.

Random periareolar fine needle aspiration (RPFNA) is a research technique used to sample mammary cells from the whole breast of asymptomatic women at high risk for developing breast cancer. It has been shown to successfully predict short-term breast cancer risk in high-risk women (8) and is highly reproducible across institutions

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(9). The presence of atypia in a breast RPFNA confers a 5.6-fold increased short-term breast cancer risk (8).

Reverse-phase protein microarray (RPPM) is a high-throughput proteomic tool, developed to test for dysregulation of protein signaling networks in human biopsy specimens. It uses denatured lysates, so antigen retrieval, which is a limitation for tissue arrays, is not a problem. RPPM sensitivity and analytic robustness allow for very small amounts of mammary epithelial cells to be selected from RPFNA samples via laser capture microdissection and analyzed for differential protein expression patterns (10–12).

The purpose of this brief communication is to relay findings obtained from a pilot and exploratory study aimed at investigating whether mammary epithelial cells obtained from obese (BMI ≥ 30 kg/m²) and nonobese (BMI < 30 kg/m²) women at high risk for developing breast cancer display differential expression of proteins involved in cancer cell growth, survival, and metastasis.

Materials and Methods

Informed consent

This study was conducted among 26 women who underwent RPFNA under Institutional Review Board–approved protocol at the Duke University Medical Center (June 2008–February 2010) and who presented sufficient cells for analysis.

Eligibility

Women were required to have at least one of the following major risk factors for breast cancer: (a) 5-year Gail risk calculation of greater than 1.7%; (b) prior biopsy exhibiting atypical hyperplasia, lobular carcinoma *in situ*, or ductal carcinoma *in situ*; or (c) known *BRCA1/2* mutation carrier. Data for sociodemographic variables, family history of breast cancer, and menopausal status were collected. Women were defined as postmenopausal if they had no menses for more than 12 months in the absence of pregnancy and/or status postbilateral oophorectomy.

BMI calculation

Body weights and heights were clinically measured on a platform scale with a fixed stadiometer. BMIs were

calculated, and the standard cutoff point of 30 kg/m² was used to dichotomize obese from nonobese women (13).

Random periareolar fine needle aspiration

RPFNA was performed as published previously, in accordance with methods established and validated by Fabian and colleagues (8, 14). Cells were classified qualitatively as nonproliferative, hyperplasia, or hyperplasia with atypia. The most atypical cell cluster was examined and scored by the Masood cytology index (Table 1). Morphologic assessment, Masood cytology index scores, and cell count were assigned by a single dedicated pathologist who was blinded to BMI.

Fixation and microdissection of RPFNA cytology

RPFNA samples were immediately placed and fixed in modified CytoLyt (Hologic Inc.) containing 1% formalin for 24 hours at room temperature. Samples were washed with CytoLyt until the majority of red blood cells were removed, followed by storage at 4°C. Cytospin slides were made for each of the samples using a Shandon Cytospin 4 Cytocentrifuge (Thermo Scientific). RPFNA samples were spun onto slides, using Shandon Single Cytofunnels at 420 rpm for 1 minute, which were then immediately placed in 75% ethanol for 30 seconds. The slides were then stained with hematoxylin (Sigma-Aldrich) for 20 seconds, dehydrated in 75%, 95%, and 100% ethanol for 30 seconds each, followed by xylene for 5 minutes, and allowed to air dry. The 75% ethanol and hematoxylin staining solutions were supplemented with Complete protease inhibitor tablets (Roche Applied Science). Approximately 5,000 epithelial cells were microdissected with a PixCell II Laser Capture Microdissection system (MDS Analytical Technologies) and stored onto microdissection caps which were maintained at –80°C until lysed.

Reverse-phase protein microarray

As previously described (15), cellular lysates were printed in triplicate onto nitrocellulose-coated slides, using an Aushon 2470 arrayer equipped with 350- μ m pins. The following cellular lysates served as positive controls for antibody staining: A431 \pm epidermal growth factor, HeLa \pm pervanadate, or Jurkat \pm calyculin. Immunostaining was performed as previously described (15).

Table 1. Masood cytology index

Cell morphology	Cell pleomorphism	Myoepithelial cells	Anisonucleosis	Nucleoli	Chromatin clumping	Score
Monolayer	Absent	Many	Absent	Absent	Absent	1
Nuclear overlap	Mild	Moderate	Mild	Micronucleoli	Rare	2
Clustering	Moderate	Few	Moderate	Micronucleoli	Occasional	3
Loss of cohesion	Conspicuous	Absent	Frequent	Macronucleoli	Frequent	4

NOTE: Sum of Masood scores: <10 , nonproliferative; 11–13, hyperplasia; 14–17, atypia; ≥ 17 , suspicious.

Polyclonal and monoclonal antibodies were purchased from Cell Signaling Technologies, Biosource/Invitrogen, BD Biosciences, and Upstate/Millipore. Each antibody-stained array was scanned and normalized to total protein, as determined by SYPRO Ruby staining. Image analysis was performed as previously described (15).

Immunohistochemical staining and scoring

Cytology specimens were mounted on Superfrost Excell slides (Fisher) and preserved in 95% ethanol. These slides were dehydrated in 95% and 100% ethanol for 30 seconds each and air-dried for 10 minutes. Specimens were fixed in ice-cold acetone for 20 minutes and then air-dried. Rehydration was achieved in TBS for 5 minutes, followed by immersion in 3% hydrogen peroxide for 5 minutes, deionized water, and TBS.

All immunostaining was carried out on Dako Auto-stainer (Dako). Specimens were placed in Background Buster (Innovex Biosciences) for 30 minutes, followed by incubation with mouse monoclonal anti-human vimentin antibody (Dako; M7020) at 1:300 dilution for 45 minutes and single wash in TBS. Detection and visualization were performed with Envision+ and DAB+ (Dako) as per manufacturer's recommendations. Specimens were counterstained in hematoxylin for 30 seconds, dipped in ammonium water 4 times, dehydrated in graded series of ethanol and xylene, and coverslipped. Frozen human tonsil tissue and inflammatory cells served as positive external and internal controls, respectively.

Immunostained specimens were reviewed and scored by a pathologist (J.G.) who was blinded to BMI and patient identity. Samples were evaluated for average staining intensity (0–3) and percentage of positively stained cells (0%–100%). As previously described (16), a HistoScore was calculated for each stained specimen (product of the 2 aforementioned parameters).

Statistics

The Mann–Whitney test was used to compare the relative intensity of each proteomic marker between nonobese and obese samples, given the lack of normal distribution of these variables. To compare difference in the median age between nonobese and obese women, the Mann–Whitney *U* test was performed (Table 2). Fisher's exact test was used to determine the relationships between the two groups and the following variables: race, menopausal status, and Masood cytology. Unsupervised hierarchical clustering analysis of log₂-transformed values for each protein was performed with R/Bioconductor version 2.10.1 software. The values of *P* < 0.05 were considered statistically significant.

Results

Subject characteristics

This study was performed on 31 RPFNA samples obtained from 26 high-risk women. Of these samples, 10 were bilateral and 21 were unilateral. Table 2 provides a list of the characteristics of study subjects according to 2

Table 2. Patient characteristics

	Total (n = 26)	Nonobese (BMI < 30 kg/m ² ; n = 18)	Obese (BMI ≥ 30 kg/m ² ; n = 8)	P
BMI, kg/ m ²				
Mean (SE)	26.9 (6.5)	23.7 (3.1)	34.2 (6.3)	
Median	25.9	22.3	31.3	
Range	19–46.5	19.0–29.7	30.3–46.5	
Age, y				
Mean (SE)	46.7 (8.7)	46.7 (9.2)	46.6 (8.1)	
Median	48.0	47.0	49.5	
Range	26–68	26–68	31–55	0.66
Race, % (n)				
Caucasian	69 (18)	72 (13)	63 (5)	
African American	27 (7)	22 (4)	37 (3)	
Latina	3 (1)	4.5 (1)	0	0.67
Menopausal status, % (n)				
Premenopausal	62 (16)	67 (12)	50 (4)	
Postmenopausal	38 (10)	33 (6)	50 (4)	0.66
Masood cytology, % (n)				
<14	31 (8)	28 (5)	37 (3)	
≥15	68 (18)	72 (13)	63 (5)	0.67

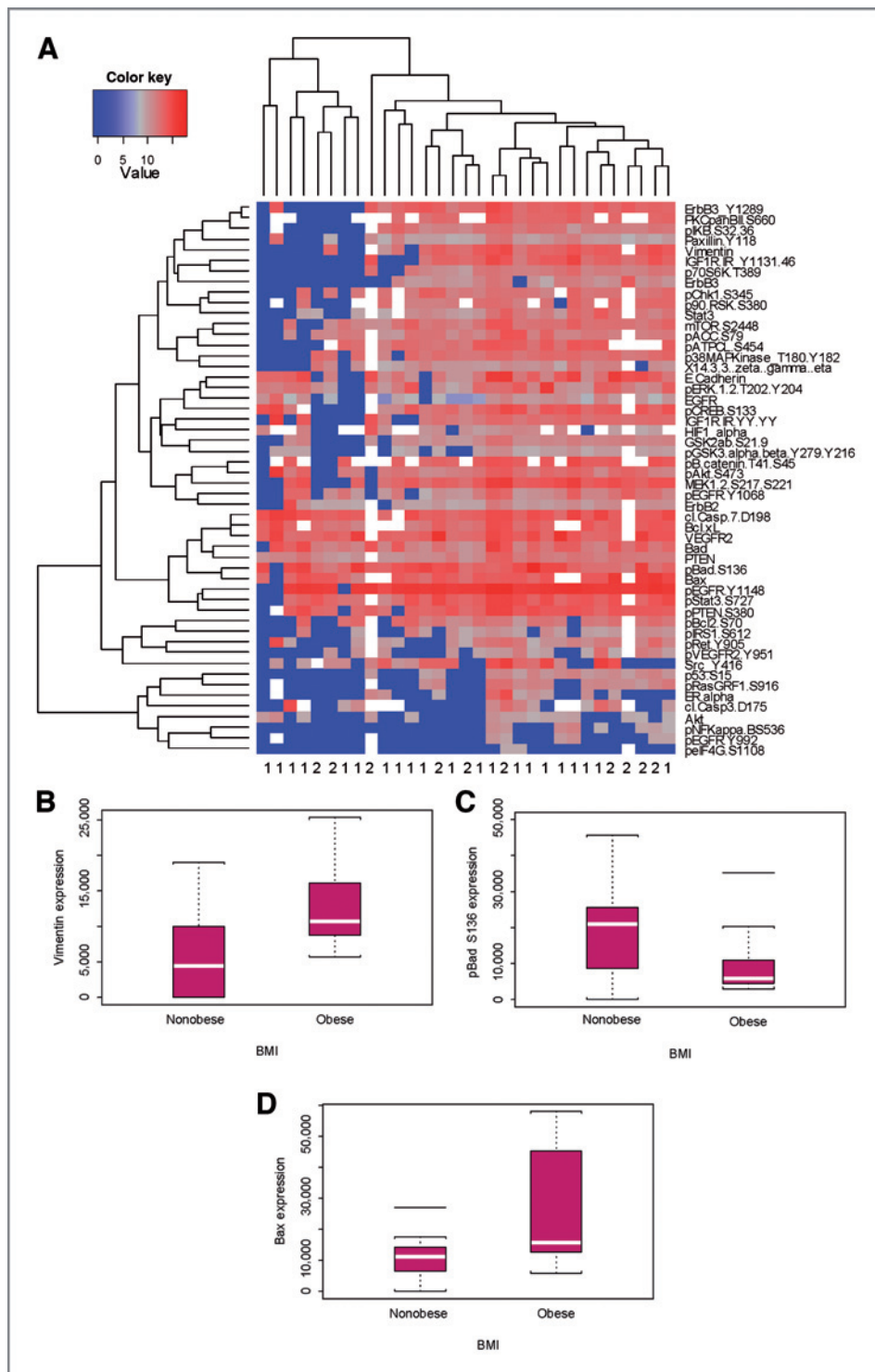
NOTE: Age difference between nonobese and obese women was compared using the Mann–Whitney *U* test. Race, menopausal status, and Masood cytology are compared using Fisher's exact test.

BMI categories, nonobese (BMI < 30 kg/m²) and obese (BMI ≥ 30 kg/m²). We have chosen the BMI cutoff of 30 because of well-established associations between poor prognosis and survival of women in the highest BMI categories (4). In the present study, the median BMI of obese women is 31.3 kg/m², whereas the median BMI of

nonobese women is 22.3 kg/m². These 2 groups of women are not significantly different with respect to age, race, and menopausal status.

RPFNA samples were stratified according to Masood cytology index scores (refs. 8, 14; Table 1) with the following distribution: 3% were nonproliferative, 16%

Figure 1. Protein expression of 52 endpoints in 31 RPFNA samples. A, unsupervised hierarchical clustering analysis of log₂-transformed intensity values of each protein endpoint (rows) are broadly divided by obese and nonobese samples (columns). Nonobese (BMI < 30 kg/m²) and obese (BMI ≥ 30 kg/m²) samples are labeled as 1 and 2, respectively. Shades of blue represent downregulated protein expression, whereas shades of gray and red represent upregulated protein expression. Box plots of (B) vimentin, (C) p-Bad S136, and (D) Bax expression levels in nonobese and obese women.



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were hyperplastic, 74% were atypical, and 7% were suspicious of cancer. These scores were further classified into high and low Masood groups (Table 2), with a

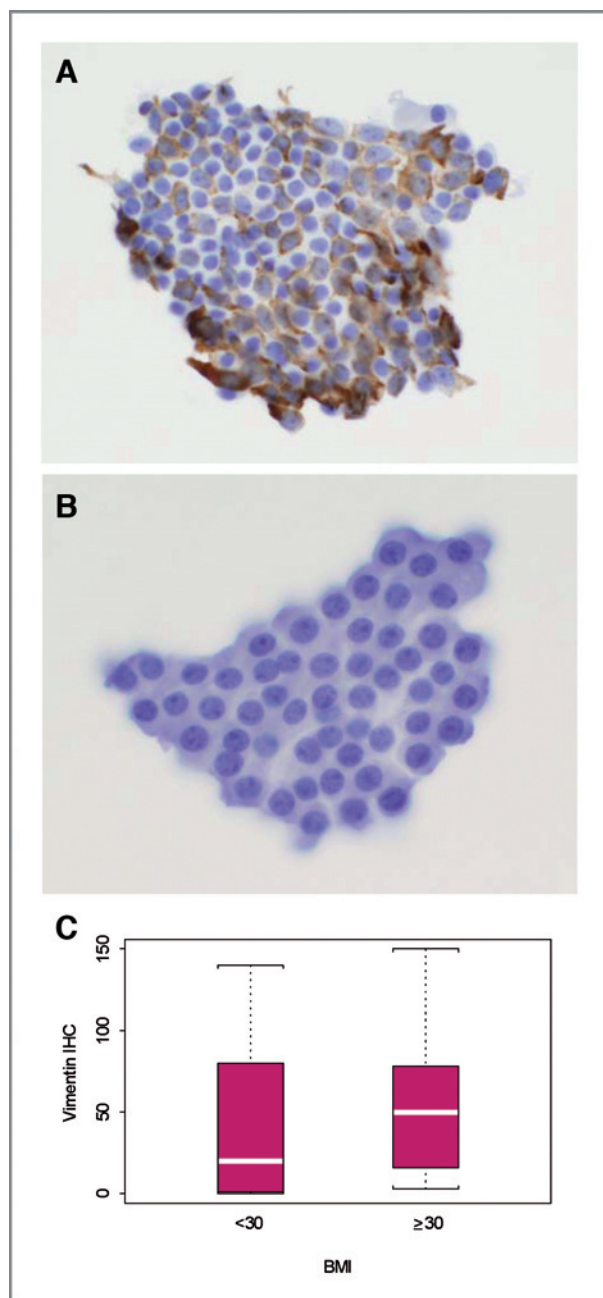


Figure 2. Photomicrographs of representative cytologic samples from obese and nonobese women. Brown cytoplasmic staining indicates vimentin expression. Cytologic sample from obese subject (A) received a HistoScore of 150, with 50% of positive-staining cells and 3+ average intensity score, whereas sample from nonobese subject (B) received a HistoScore of 0, with 0% staining and 0 intensity score. Box plots of HistoScores for vimentin IHC (C) are shown for 20 cytologic samples from 13 nonobese women (BMI < 30 kg/m²) and 7 obese women (BMI ≥ 30 kg/m²). The mean and median HistoScores are as follows: 37 and 20 for nonobese samples; 57 and 50 for obese samples.

Masood score cutoff of 15 for cytologic atypia. The presence of atypia has been used as a surrogate marker of short-term breast cancer risk in high-risk women (8). Here, the short-term breast cancer risk in obese and nonobese women is not significantly different ($P = 0.67$).

Protein expression by RPPM

Figure 1A depicts the expression pattern of log₂-transformed intensity values of 52 of the original 60 total, phosphorylated, and cleaved proteins obtained from all 31 RPFNA samples. Eight proteins did not yield sufficient intensity signal; these were excluded from statistical analysis.

In unadjusted analysis, the expression of vimentin ($P = 0.028$) and phosphorylated Bad (p-Bad) S136 ($P = 0.049$) are statistically different between samples of nonobese and obese women (Fig. 1B and C). However, after adjusting for false discovery rate via the Benjamini-Hockberg method, vimentin and p-Bad S136 expression are no longer statistically significant.

Vimentin immunohistochemistry

We performed retrospective analysis of vimentin expression by immunohistochemistry (IHC) on a limited number of archived RPFNA cytologic samples. A majority of RPFNA samples had been expended in other experiments, and only 20 of 31 samples were evaluable for vimentin expression by IHC. Representative photomicrographs of epithelial cell clusters from obese and nonobese women are shown in Figure 2A and B. Box plot representation of the distribution of HistoScores of nonobese and obese samples is shown in Figure 2C. Because of the limited samples available for retrospective IHC analysis, we were unable to test for correlation between vimentin expression with RPPM and by IHC.

Discussion

In this exploratory study, RPPM analysis allowed us to investigate a wide array of proteins that play a role in cell survival, growth, differentiation, and death. The preliminary results presented herein are a first report suggesting that mammary epithelial cells obtained from high-risk obese women may differ in vimentin expression compared with nonobese women. Vimentin is often used as a general marker of mesenchymal cells and has been shown to play a critical role in epithelial-to-mesenchymal transition (EMT) necessary for cancer cell invasion and metastasis (17). In addition, vimentin is an integral structural component of fat droplet organization in adipocytes, lipid metabolism, and adipogenesis (18–20). Treatment of MCF10A cells with arachidonic acid, a metabolite obtained from a high-fat diet, induced EMT and increased vimentin expression with concomitant decrease in E-cadherin expression (21). Considering the established associations between adiposity, adipokines, and vimentin, further investigation into how adipocyte microenvironment influences mammary epithelial cell

protein expression in high-risk obese women is warranted.

Few members of the apoptotic pathway have been implicated in adipocyte apoptosis induced by leptin, a hormone that regulates food intake and energy metabolism (22). Animal model studies have shown no difference in protein expression of proapoptotic Bax in mammary tumors of obese and nonobese mice that were fed with high-fat diet and low-fat diet, respectively (23). In the present study, Bax expression is not significantly different ($P = 0.055$) in obese and nonobese women (Fig. 1D) whereas pro-survival p-Bad S136 expression is higher in nonobese women (Fig. 1C). In the absence of dietary information at the time of recruitment and RPFNA collection, we cannot attribute the differential expression of these proteins to a recent change in caloric intake. But it is interesting to speculate that diet or exercise may impact apoptotic signaling proteins independently of BMI.

An important limitation of this study is small sample size that does not allow for statistical significance of associations between protein expression and risk factors for breast cancer such as age, menopausal status, and cytologic atypia. Thus, it is not possible to draw definitive conclusion on causal relationship between differential protein expression and breast cancer risk in obese versus nonobese women.

In this study, we did not archive adequate RPFNA cytologic samples for a statistically meaningful comparison of RPPM and IHC data. However, our limited data demonstrate that it is possible to prospectively compare RPPM and IHC analysis of specific proteins. The direct comparison of RPPM data with Western blot analysis and/or IHC is clearly important for the future use of RPPM as a tool for multiplexed cell signaling analysis of human samples. Recent studies by Accordi and colleagues show concordance between RPPM results and Western blot analysis of proteins such as Bcl-2 S70 that are activated in primary leukemia samples with mixed lineage leukemia translocations (24). In the present

study, we lack statistical power to draw definitive conclusion about the correlation between RPPM and IHC data, given the insufficient or small number of samples. Moreover, the IHC that we employed has obvious limitations such as variable cellularity, heterogeneity of staining, and subjective nature of scoring the cytologic samples. In the future, we will address these limitations by using comparable number of cells for IHC and employing image analysis of stained cells to quantify protein expression.

Our future proteomic studies will include a larger cohort with equal number of obese and nonobese women of Caucasian and African American descent and analysis of adipokines to better understand the underlying pathology of breast cancer initiation and progression in obese women. African American women have higher rates of obesity and more aggressive forms of breast cancer, with greater likelihood of dying from breast cancer in pre- and postmenopausal women (25). Future proteomic studies will better delineate whether these 2 factors are causally related and identify potential biomarkers of response to preventative interventions such as dietary changes and exercise.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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