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Prostate-specific antigen expression in nipple aspirate fluid is associated with advanced breast cancer

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Abstract

We previously demonstrated that prostate-specific antigen (PSA) is present in breast nipple aspirate fluid (NAF) and its expression is inversely associated with the presence of breast cancer. The purpose of this study was to determine if PSA levels in NAF decrease with disease progression from DCIS to metastatic breast cancer. One hundered and forty-nine women underwent nipple aspiration before or in conjunction with surgery to treat their breast cancer. PSA levels decreased with more advanced disease stage ($P = 0.016$), larger tumor size ($P = 0.031$), and nodal involvement ($P = 0.041$). PSA levels were lower in women with than without distant disease spread ($P = 0.049$). We also evaluated the association of PSA with these clinical parameters based on menopausal status. In general, PSA predicted disease involvement better in pre- than in post-menopausal women. There was no association between PSA and race. Spearman's rank analysis demonstrated that PSA was inversely related to tumor size ($P = 0.009$), nodal status ($P = 0.005$), disease stage ($P = 0.004$), and distant metastases ($P = 0.04$). NAF PSA provides useful prognostic information which may assist with breast cancer treatment.

Keywords: Nipple aspirate fluid; Prostate-specific antigen; Breast cancer

1. Introduction

Present efforts to evaluate the breast directly either through the analysis of tissue or individual cells are hindered because the assessment of these specimens generally requires an invasive procedure. The adult non-pregnant, non-lactating breast secretes fluid into the breast ductal system. This fluid can be obtained through aspiration of the nipple with a modified breast pump. Nipple aspiration has the attractiveness of quickly, painlessly, and non-invasively obtaining both breast epithelial cells, the cells at risk for transformation to breast cancer, as well as secreted proteins, which are concentrated in the fluid. We have obtained nipple aspirate fluid (NAF) from over 900 women during the past 8 years, and demonstrated that secreted proteins in NAF, such as prostate-specific antigen (PSA) [1], can be analyzed and are highly associated with the presence of breast cancer. The purpose of this study is to extend our previous observations by assessing if NAF PSA levels are associated with breast cancer progression.

PSA is made both by normal and malignant human breast tissue [2]. We previously reported that PSA is detectable in NAF and low levels were associated with breast cancer [1]. On the other hand, we did not find that PSA was useful in predicting the presence of residual disease in women after breast biopsy demonstrated cancer with positive or indeterminate margins [3].

NAF provides a source of material which is breast specific, not diluted by the contribution of other organs in the body. We previously demonstrated that secreted proteins such as PSA are present in NAF at levels 100–1000-fold higher than in matched female serum [4]. In this study, we set out to determine if NAF levels of PSA were associated with advanced breast cancer. If markers in NAF predictive of breast

Abbreviations: CC, correlation coefficient; DCIS, ductal carcinoma in situ; IBC, invasive breast cancer; NAF, nipple aspirate fluid; PSA, prostate-specific antigen

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cancer progression are identified, they may prove useful in formulating treatment strategies for women with new and/or recurrent disease.

2. Materials and methods

2.1. Subjects

NAF specimens from 160 subjects aged 30–79 years (mean = 53.6; median = 52) were collected after approval of the Institutional Review Board. These specimens represented all those available at the time of analysis. The procedures followed were in accord with the ethical standards of the Helsinki declaration of 1975. These subjects were recruited either through local advertisement, by word of mouth, or, with the permission of the treating surgeons, by contacting subjects scheduled to undergo breast surgery. In 11 cases, two specimens were collected from the same breast. For these 11 individuals, the mean value of the two PSA results was used for statistical analysis. Subjects included women with histologically proven ductal carcinoma in situ (DCIS) or invasive breast carcinoma (IBC). NAF was collected prior to or in conjunction with the treatment of their disease. NAF was collected from the breast with active disease.

2.2. Aspiration technique

Nipple fluid was aspirated by a trained physician or nurse clinician using a modified breast pump [5]. The breast nipple was cleaned with alcohol, the plunger of the aspiration device was withdrawn to the 7 ml level and held for 15 s. Fluid in the form of droplets was collected in capillary tubes. The quantity of fluid varied from 1 to 200 μl.

2.3. Specimen preparation and analysis

2.3.1. Preparation

Every NAF sample collected was suitable for evaluation. In general, a sample was used to measure total protein and PSA. Samples were collected in 50 μl capillary tubes (generally 1–5 μl per tube). For extraction, the portion of the capillary containing the sample was introduced into a 1.7 ml eppendorf tube and 100 μl of a 0.1 mol/l solution of sodium bicarbonate (pH 7.8) was added. The capillary was then crushed by using a glass rod and the mixture was vortexed to disperse the sample. The crushed capillary was left in the bicarbonate buffer overnight at 4 °C to allow proteins adherent to the glass to go into solution. The mixture was centrifuged at 14,000 × *g* for 5 min and the supernatant used without further dilution.

2.3.2. Analysis

The NAF samples varied both in their total protein concentration and in the volume in the capillary used for marker analysis. For this reason, it has been our practice to determine the concentration of a given protein based on total NAF protein, after controlling for the degree to which the NAF was diluted prior to analysis. Total protein was measured using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). PSA was analyzed using a highly sensitive technique [1]. Briefly, this procedure combines a time-resolved immunofluorometric assay with two monoclonal antibodies and has a detection limit of 1 ng/l.

2.4. Statistical analysis

For purposes of analysis, PSA values were log-transformed (log (PSA+1) for inclusion of samples where PSA = 0 ng/g) to enable us to apply parametric statistics. The unpaired *t*-test was applied when comparing two independent groups. Spearman's rank order correlation was applied to identify which variables were significantly related (PSA versus menopausal status, race, stage, tumor size, nodal status and distant metastasis). All analyses were conducted using the software SigmaStat for Windows version 2.03S (SPSS, Inc., Chicago, IL 60606).

3. Results

3.1. NAF PSA is inversely associated with disease stage, tumor size and nodal status

PSA expression in NAF was highest (Table 1) in women with DCIS (a.k.a. stage 0, mean 1782 ng/g). Expression was significantly lower in women with IBC ($P = 0.016$). Median PSA decreased from stages 1 to 4 tumors, with the lowest levels in stage 4 tumors. Tumor size significantly influenced PSA expression (Table 2) ($P = 0.031$). Women with tumor spread to lymph nodes (Table 3) had significantly lower PSA levels than women without evidence of nodal spread ($P = 0.041$).

3.2. NAF PSA is lower in women with than without distant metastases

Because of the referral patterns to our NAF studies as well as the health of the women involved, the number of subjects with metastatic breast cancer who enrolled in our studies were few. The number of subjects with NAF PSA values who had metastatic breast cancer were only three out of 160. Nonetheless, we observed a significant difference ($P = 0.049$) in expression (no metastases, mean PSA = 874.1 ng/g versus women with metastatic disease, mean PSA = 3.0 ng/g).

3.3. PSA expression predicts nodal status in pre- but not in post-menopausal women

When dividing of subjects was done by menopausal status, in most cases differences previously found to be

Table 1
Mean and median PSA expression (ng/g) based on breast cancer stage[a]

Stage	N	PSA		P-value
		Mean	Median	
Overall				
0	37	1782.5	119.0	0.016
1	39	281.4	48.0	
2	63	838.7	17.6	
3	18	415.1	17.0	
4	3	3.0	0.0	
Total	160			
Pre-menopausal				
0	16	2924.9	392.0	0.069
1	14	484.1	168.5	
2	27	1383.7	17.6	
3	7	409.7	54.0	
4	1	9.1	9.1	
Total	65			
Post-menopausal				
0	21	912.1	83.0	0.128
1	25	167.9	33.0	
2	36	430	17.0	
3	11	418.6	54.0	
4	2	0	0.0	
Total	95			

[a] Stage: statistical significance based on log (PSA + 1) of NAF specimens from women with stage 0 (in situ) vs. stages 1–4 (invasive) tumors.

Table 2
Mean and median PSA expression (ng/g) based on tumor size[a]

Tumor size	N	PSA		P-value
		Mean	Median	
Overall				
0	37	1782.5	119.0	0.031
1	53	468.0	37.0	
2	47	828.8	18.0	
3	17	305.4	12.9	
4	6	390.9	11.0	
Total	160			
Pre-menopausal				
0	16	2924.9	392.0	0.102
1	20	664.2	84.8	
2	21	1469.7	33.5	
3	5	568.6	6.0	
4	3	7.7	3.0	
Total	65			
Post-menopausal				
0	21	912.1	83.0	0.145
1	33	349.1	33.0	
2	26	311.2	17.0	
3	12	195.7	31.5	
4	3	774.2	172.5	
Total	95			

[a] Tumor size: statistical significance based on log (PSA + 1) of NAF specimens from women with T_0 vs. $T_{1–4}$ tumors.

Table 3
Mean and median PSA expression based on nodal status[a]

Nodal Status	N	PSA		P-value
		Mean	Median	
Overall				
0	96	990.7	55.5	0.041
1	61	690.5	14.8	
2	2	8.5	8.5	
Total	159			
Pre-menopausal				
0	37	1753.8	253.0	0.012
1	26	111.7	16.2	
2	2	8.5	8.5	
Total	65			
Post-menopausal				
0	59	512.2	34.0	0.154
1	35	377.7	11.0	
2	0			
Total	94			

[a] Nodal Status: statistical significance based on log (PSA + 1) of NAF specimens from women with N_0 vs. $N_{1–2}$ tumors. Nodal status not available for one post-menopausal subject.

significant were no longer so. One exception was nodal status. Pre-menopausal women without evidence of tumor spread to lymph nodes had significantly higher PSA levels ($P = 0.012$) compared to subjects with nodal spread (Table 3). This difference was not observed in post-menopausal women.

3.4. PSA expression is influenced by menopausal status but not by race

Pre-menopausal women with breast cancer on average had higher PSA values than post-menopausal subjects (mean 1433.3 versus 457.2 ng/g, $P = 0.013$). Race did not significantly influence PSA expression.

3.5. Correlation analysis of tumor and patient variables with PSA

PSA was inversely correlated to tumor size (correlation coefficient (CC) $= -0.205$, $P = 0.009$), nodal status (CC $= -0.220$, $P = 0.005$), stage (CC $= -0.226$, $P = 0.004$), and metastasis (CC $= -0.163$, $P = 0.04$) and menopausal status (CC $= -0.197$, $P = 0.0125$). No significant relationship between PSA and race nor menopausal status was identified.

4. Discussion

We initiated our nipple aspiration studies to identify biomarkers that were associated with breast cancer. The breast ducts of adult non-pregnant women secrete small amounts of fluid [6]. This fluid does not escape because the

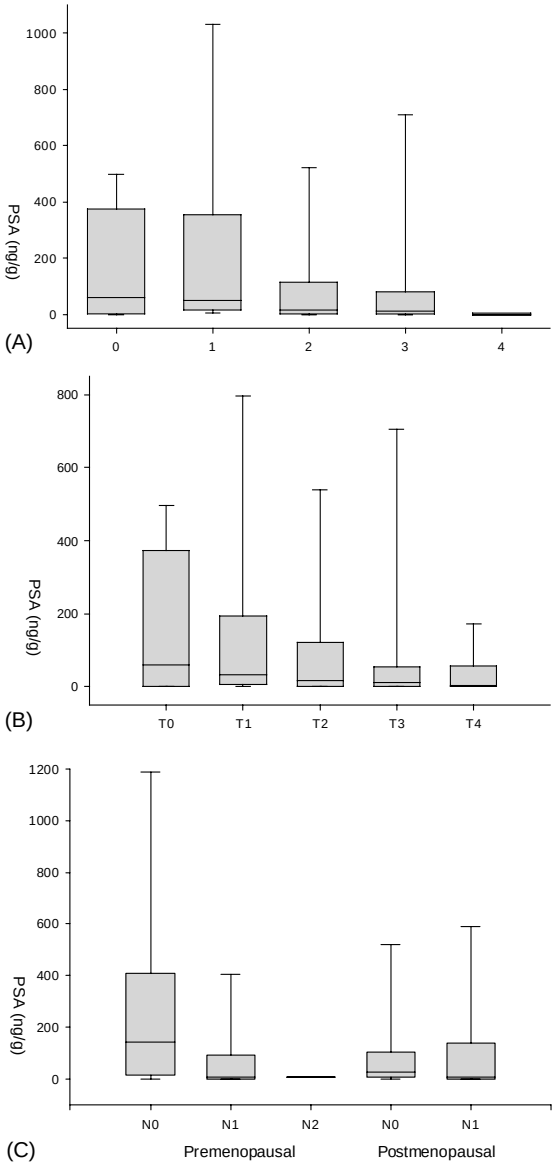

Fig. 1. Box and whisker plots of PSA level based on (A) disease stage, (B) tumor size, and (C) lymph node spread. Seventeen outliers (PSA values > 2000 ng/g) not included.

nipple ducts are occluded by smooth muscle contraction, dried secretions, and keratinized epithelium. Breast fluid can be obtained by nipple aspiration in women without spontaneous nipple discharge with the use of a modified breast pump [7]. This fluid contains several types of cells, including exfoliated breast epithelial cells [8]. Because breast cancer develops from ductal and lobular epithelium, NAF is a potentially useful epidemiologic and clinical research tool.

We found a significant inverse association between PSA levels in NAF and disease stage, tumor size and nodal status (Fig. 1). Our prior reports [1,4,9] compared PSA expression in women with and without breast cancer, information important for disease diagnosis. Our findings in this report provide potentially important information regarding disease prognosis. That there is some overlap in the values in Fig. 1 based on stage, tumor size and nodal status suggests that PSA is likely to prove most useful in assessing prognosis when evaluated in combination with other biomarkers.

We observed that PSA expression was highest among women in the lowest disease category (for stage and tumor size) or when disease was absent (in lymph nodes). PSA expression was lowest for the most advanced disease stage and nodal spread, regardless of menopausal status. The largest tumors in pre-menopausal women had the lowest PSA values. This was not true in post-menopausal women, although our limited sample size requires that we emphasize that this observation is preliminary. Similarly, the dramatic difference in PSA expression among women with and without distant disease spread (mean 874.1 versus 3.0 ng/g) must be viewed with caution until confirmed in a larger sample size.

The mechanism by which PSA decreases with disease progression is unclear. It has previously been proposed that PSA, a serine protease, cleaves insulin like growth factor binding protein type 3 [10]. We have observed that breast cancer risk increases with increasing levels of IGFBP-3, whereas the opposite is true for PSA [11]. Perhaps IGFBP-3 has an increasing influence on PSA as breast cancers grow and spread.

In general, the differences in PSA expression based on advanced disease were greater among pre- than post-menopausal women. This was most notable when evaluating PSA based on nodal spread, where expression was significantly different in pre- but not in post-menopausal women (Table 3). It was observed to a lesser extent when assessing PSA expression and Stage, which approached significance ($P = 0.069$) in pre- but not ($P = 0.128$) in post-menopausal subjects (Table 1). This loss of significance when dividing the data set for stage or tumor size based on menopausal status is likely due to an inadequate sample size. The fact that PSA was a significant indicator of nodal spread both overall and in pre-menopausal women suggests that it is a more important predictor of regional disease spread in these women than it is in predicting regional disease spread in post-menopausal women, or stage or tumor size in either pre- or post-menopausal women.

Interestingly, both when evaluating stage and tumor size, mean values of the earliest invasive lesions had lower PSA expression than slightly more advanced lesions. This was not observed when median PSA values were compared, suggesting that outlier values skewed the mean PSA results.

In prior reports we noted a significant difference in PSA expression based on the subject's menopausal status. We observed a stronger association of PSA with breast cancer in pre- ($P < 0.001$) than in post-menopausal ($P = 0.01$) women [11]. It is our belief that PSA expression is at least partially under the influence of progesterone [12], resulting in increased expression before menopause. While mean PSA values in women with breast cancer were significantly higher ($P = 0.013$) in pre- than in post-menopausal women, the

association was not as great as had previously been reported ($P = 0.002$) for a mixed group of women with and without breast cancer [1].

In the current study, we sought to investigate the association of PSA with menopausal status among a population of all women who have breast cancer. In this data set, pre-menopausal women had higher PSA values (mean 1443.3 ng/g) than post-menopausal women (mean 457.2 ng/g, $P = 0.013$), although the differences are not so great as the differences in PSA of normal women versus women with breast cancer, which are on average one or two logs different. We believe these findings are consistent with earlier results, that differences in PSA expression based on menopausal status are at least partially lost with the development of breast cancer, perhaps through the loss of regulation of PSA by progesterone in the pre-menopausal population.

When we applied correlation analysis to determine which parameters were related to PSA, we found that PSA was inversely correlated to tumor size, disease stage, nodal status and distant metastases. The preliminary findings of decreased PSA expression in patients with advanced breast cancer, if confirmed in a larger sample size, would be especially exciting, given the difficulty clinicians have in predicting which patients have or will develop distant disease. This information, if known with confidence, would significantly impact treatment decisions.

Our results, collected in a large cohort of women with or without breast cancer and in which NAF was obtained in 98% of subjects enrolled, suggest that levels of PSA in NAF are inversely associated with advanced disease. This information extends our earlier findings, which compared PSA expression in women with and without breast cancer. Further studies will help to determine the usefulness of NAF PSA to predict prognosis, as well as response to breast cancer therapy.

In summary, we extended our earlier findings that PSA levels in NAF are inversely associated with the presence of breast cancer to observe that NAF PSA levels are also inversely associated with advanced disease. Further studies are required to confirm these preliminary encouraging findings to determine their potential prognostic usefulness.

References

[1] Sauter ER, Daly M, Linahan K, Ehya H, Engstrom PF, Bonney G, et al. Prostate-specific antigen levels in nipple aspirate fluid correlate with breast cancer risk. Cancer Epidemiol Biomarkers Prev 1996;5:967–70.

[2] Yu H, Diamandis EP, Sutherland DJ. Immunoreactive prostate-specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. Clin Biochem 1994;27:75–9.

[3] Sauter ER, Ehya H, Babb J, Diamandis E, Daly M, Klein-Szanto A, et al. Biological markers of risk in nipple aspirate fluid are associated with residual cancer and tumour size. Br J Cancer 1999;81:1222–7.

[4] Sauter ER, Tichansky DS, Chervoneva I, Diamandis EP. Circulating testosterone and prostate-specific antigen in nipple aspirate fluid and tissue are associated with breast cancer. Environ Health Perspect 2002;110:241–6.

[5] Agurs-Collins T, Adams-Campbell LL, Kim KS, Cullen KJ. Insulin-like growth factor-1 and breast cancer risk in post-menopausal African-American women. Cancer Detect Prev 2000;24:199–206.

[6] Keynes G. Chronic mastitis. Br J Surg 1923;11:89–121.

[7] Petrakis NL, Mason L, Lee R, Sugimoto B, Pawson S, Catchpool F. Association of race, age, menopausal status, and cerumen type with breast fluid secretion in nonlactating women, as determined by nipple aspiration. J Natl Cancer Inst 1975;54:829–34.

[8] King EB, Barrett D, King MC, Petrakis NL. Cellular composition of the nipple aspirate specimen of breast fluid. I. The benign cells. Am J Clin Pathol 1975;64:728–38.

[9] Sauter ER, Welch T, Magklara A, Klein G, Diamandis EP. Ethnic variation in kallikrein expression in nipple aspirate fluid. Int J Cancer 2002;100:678–82.

[10] Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J Clin Endocrinol Metab 1992;75:1046–53.

[11] Sauter ER, Chervoneva I, Diamandis A, Khosravi JM, Litwin S, Diamandis EP. Prostate-specific antigen and insulin-like growth factor binding protein-3 in nipple aspirate fluid are associated with breast cancer. Cancer Detect Prev 2002;26:149–57.

[12] Sauter ER, Babb J, Daly M, Engstrom PF, Ehya H, Malick J, et al. Prostate-specific antigen production in the female breast: association with progesterone. Cancer Epidemiol Biomarkers Prev 1998;7:315–20.