Short Communication

Nipple Fluid Basic Fibroblast Growth Factor in Patients with Breast Cancer

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Abstract

Purpose: **It has been shown that early detection of breast cancer could save lives. Recently, there has been increasing interest in nipple fluid as a potential supplemental avenue for breast cancer diagnosis.**
Experimental Design: **In this study, we determined the levels of an angiogenic factor basic fibroblast growth factor (bFGF) in the nipple fluid of healthy subjects as well as patients with benign breast conditions, those at high risk for breast cancer, and patients with active breast cancer. ELISAs were used to measure bFGF.**
Results: **Nipple fluid bFGF levels were as follows (mean ± SE): 158 ± 17 pg/mL from benign breasts, 561 ± 277 pg/mL from high-risk breasts, and 1,343 ± 441 pg/mL from cancerous breasts. One-way ANOVA showed that the bFGF levels from cancerous breasts were significantly higher than those from benign and high-risk breasts (P = 0.0001 and P = 0.0193, respectively). After logarithmic transformation was applied to the data, high-risk breast bFGF levels were higher than those from benign breasts (P = 0.0028). With a cutoff level of 250 pg/mL, the sensitivity was 79.2%, specificity was 82.5%, and correct diagnosis was 66.4%. The area under the receiver operating characteristic curve was 0.86.**
Conclusions: **We conclude that nipple fluid bFGF levels are progressively elevated in high-risk and cancerous breasts compared with benign breasts. The sensitivity and specificity of this test are promising compared with current breast cancer screening methods, and this test deserves further studies with larger clinical trials. Potential areas of usefulness include the detection of breast cancer risk or breast cancer, as well as the monitoring and/or prediction of the antiangiogenic effect of preventive therapies.** (Cancer Epidemiol Biomarkers Prev 2005;14(12):2995–8)

Introduction

The process of angiogenesis plays a critical role in breast tumor growth and metastasis (1). We were among the first to ask whether the level of any of the angiogenic factors could be detected in bodily fluids, and whether their levels would have clinical relevance (2). Studies from our laboratory and from other institutions have shown that angiogenic factors are significantly elevated in the serum and urine of patients with breast cancer. The levels of certain angiogenic factors have been shown to correlate with the disease stage of the tumor (2). It seems, however, that angiogenic factor levels in the serum may be used to monitor therapy and/or as a predictor of outcome in known breast cancer, but are not sensitive enough to be diagnostic of new breast cancer (3). This is not surprising because the half-life of many angiogenic factors is only a few minutes, and the amount of these factors secreted by the tumor is diluted in the vast volume of blood and urine.

Breast cancer arises from epithelial cells that line the ductal/lobular systems of the milk ducts. Therefore, it makes sense that examination of this ductal system or analysis of its secretions may reveal signs of early cancer (4). We thus expect that the fluid secreted into the breast ducts would contain a much higher concentration of angiogenic factors than serum or urine (2, 3). Our preliminary data indicated that nipple fluid basic fibroblast growth factor (bFGF), a potent angiogenic factor, is significantly elevated in patients with breast cancer in comparison with benign controls (5). Here, we report our more recent results with a larger population of subjects.

Materials and Methods

Subjects. Sample collection was within the guidelines of the University of California at Los Angeles Institutional Review Board, and was conducted from May 2001 to July 2004. The inclusion criteria were as follows: capable of giving informed consent, yield of nipple aspirate fluid from at least one breast, and prior to definitive surgery that would excise the lesion. All subjects underwent a breast physical exam. If indicated, patients may have had a mammogram and/or ultrasound or ductogram.

First, the nipple was wiped with an alcohol prep pad to remove any debris that may occlude the ductal openings. Then, nipple fluid was obtained with manual breast compression in the direction from the chest wall towards the areola. This procedure may be done by the investigator or the patient herself. The nipple-areolar complex may be squeezed manually or with a breast pump and suction syringe apparatus (Cytyc, Boxborough, MA), to encourage nipple fluid yield. Nipple fluids from any and all ductal openings from one nipple were collected with a Pipetman (Rainin Instrument, Emeryville, CA) and placed in Eppendorf tubes (Axygen Scientific, Union City, CA). The nipple fluids obtained were placed in the refrigerator at −4°C, and transported within 4 to 5 hours to be stored in a −70°C secured freezer.

Received 6/3/05; revised 9/27/05; accepted 10/10/05.

Grant support: Susan Komen Breast Cancer Foundation and NIH CA 109603.

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doi:10.1158/1055-9965.EPI-05-0412

ELISA. Measurement of bFGF was done by ELISA according to the manufacturers' instructions (R&D, Minneapolis, MN). We diluted 2 μL of nipple fluid in 98 μL of calibrator diluent (R&D), and this dilution was corrected during the subsequent calculations. The amount of protein in nipple fluid was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Values of bFGF were expressed either as pg/mL directly, or as pg/mg protein.

Data Analysis. Group statistical analysis was done with the Kruskal-Wallis test, and pair-wise comparisons with Wilcoxon test. The nipple fluid bFGF test was also evaluated using measures such as sensitivity, specificity, and correct diagnostic rate.

Results

Subject Characteristics. We collected 128 specimens from 81 subjects classified in the benign group, 48 specimens from 31 subjects classified in the high-risk group, 24 specimens from the cancerous breasts, and 14 specimens from the contralateral noncancerous breasts of 24 subjects with breast cancer. A nipple fluid sample was considered ''benign'' if it was obtained from a breast with a diagnosis of cyst(s) (18), fibroadenoma(s) (5), papilloma(s) (7), or other physiologically normal conditions (98). None of the subjects in the benign group had ever had breast cancer in the past. In the high-risk group, 39 samples came from patients with a high risk for developing future breast cancer, due to family history alone (at least one first-degree relative with breast cancer). Nine samples were collected from subjects with previous precancer lesions such as atypical ductal hyperplasia or lobular carcinoma *in situ*. The 14 samples that came from the noncancerous breasts of patients with current unilateral breast cancer were classified as high risk. We did not include patients with prior breast cancer in this study. The cancer group had only unilateral breast cancer, and thus 24 specimens were collected from the cancerous breasts of 24 patients. Four cases had ductal carcinoma *in situ* only; the 20 invasive breast cancer cases contained three stage 3 lesions, with the rest in stages 1 and 2. The diagnosis was within 2 weeks in the vast majority of the patients in the cancer group, and all diagnoses were within one and a half months. In total, we had 214 nipple aspirate fluid specimens from 136 female subjects.

Group bFGF Levels. We measured bFGF levels and calculated them as pg/mL or pg/mg protein (Fig. 1). In the benign group, bFGF levels were follows: mean 158 ± 17 pg/mL, median 120, range 0 to 1,715, or 0.61 ± 0.07 pg/mg, median 0.46, range 0 to 7.88. In the high-risk group, bFGF levels were as follows: mean 561 ± 277 pg/mL, median 184, range 3 to 17,282, or 2.26 ± 1.29 pg/mg, median 0.64, range 0 to 80.77. In the cancer group, bFGF levels were as follows: mean 1,343 ± 441 pg/mL, median 480, range 42 to 8,726, or 6.28 ± 2.33 pg/mg, median 1.17, range 0.23 to 46.12. One-way ANOVA of the pg/mL data showed a significant group difference ($P = 0.0004$). The bFGF levels from cancerous breasts were significantly higher than those from benign and high-risk breasts ($P = 0.0001$ and $P = 0.0193$, respectively). High-risk breast bFGF levels were higher than those from benign breasts with borderline significance ($P = 0.0601$). Statistical analysis using the pg/mg data set yielded similar results. Because the distribution of bFGF values were highly skewed, logarithmic transformation was applied to the data. One-way ANOVA of this transformed data showed an even greater significant group difference ($P < 0.0001$). The bFGF levels from cancerous breasts were significantly higher than those from benign and high-risk breasts ($P < 0.0001$ and $P = 0.0007$, respectively). High-risk breast bFGF levels were higher than those from benign breasts with statistical significance ($P = 0.0028$). In the benign group, we noted no significant influence on bFGF levels (both pg/mL and pg/mg) exerted by age (Spearman correlation coefficient 0.10; $P = 0.2550$), race (one-way ANOVA $P = 0.06838$), menopausal status (Wilcoxon rank sum test, $P = 0.5930$), or hormone use (Wilcoxon rank sum test; $P = 0.1328$).

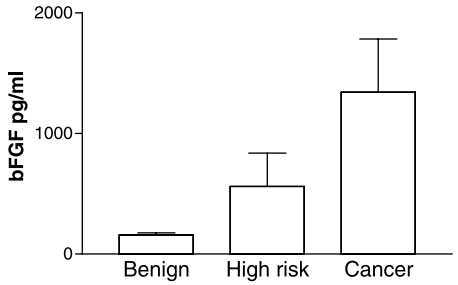

Figure 1. Columns, mean nipple fluid bFGF values (pg/mL) in specimens from benign, high risk, and cancerous breasts; bars, ± SE.

In the cancer group, one had surgical biopsy, nine had large core needle aspiration, five had fine needle aspiration, two had ductogram, and seven had no procedure. When these 17 ''postprocedure'' specimens were compared with 7 ''no procedure'' specimens, there was no significant difference in bFGF levels ($P = 0.42$). When we excluded ductal carcinoma *in situ*, microinvasive disease, and current systemic therapy, there were 13 ''postprocedure'' versus 5 ''no procedure'' specimens. The mean ± SE were 1,469 ± 680 and 2,283 ± 1,083 pg/mL, respectively ($P = 0.54$). Therefore, the bFGF value seemed to be higher if there had been no procedure prior to nipple aspirate fluid collection, but this observation was not statistically significant. It is likely that a prior invasive procedure can cause swelling and bleeding, thereby obstructing the path of the duct containing cancer leading to the nipple. We included the ductogram as a procedure because the contrast used in this radiologic procedure contains sclerosing activity, and may interfere temporarily with the secretion of bFGF into the duct. Indeed, the bFGF values of the two cases with prior ductograms were lower than the cancer group's mean value. Both of these two cases had an eventual diagnosis of ductal carcinoma *in situ*, and were excluded from the second analysis above.

In the benign group, all had nipple aspirate fluid collection prior to any needle or surgical biopsy. We found no significant difference between the bFGF levels from the right versus left breast of the same woman ($P = 0.74$). For this analysis, we used the benign group subjects who had normal physical exams and normal radiological tests (if indicated). We analyzed the benign subgroups of cyst, fibroadenoma, papilloma, and other physiologically normal conditions. The mean ± SE were 187.56 ± 44.77, 238.71 ± 76.32, 424.90 ± 216.85, and 129.87 ± 10.97 pg/mL, respectively. One-way ANOVA showed that there was no significant difference among the three benign diagnosis groups (cyst, fibroadenoma, and papilloma; $P = 0.41$). However, the bFGF level in the papilloma group was significantly higher than the group with physiologically normal conditions ($P = 0.01$, Wilcoxon rank sum test). In the physiologically normal condition group, there were three cases of ''usual type hyperplasia,'' two with apocrine metaplasia, and one with adenosis. For these six cases, the mean ± SE was 130.85 ± 43.33 pg/mL, which was not significantly different from that of the physiologically normal condition group (Table 1A).

In the high-risk group, bFGF levels were follows: 1,524.86 ± 1,214.76 pg/mL for the 14 samples that came from the noncancerous breasts of patients with current unilateral breast cancer, 416.42 ± 166.56 pg/mL for the 9 cases with previous precancer lesions (atypical ductal hyperplasia or lobular

Table 1. bFGF levels in five subgroups of ''benign'' cases and six subgroups of ''high-risk'' cases (mean ± SE, in pg/mL)

(A) Benign group

Benign	Normal	Hyperplasia	Cyst	Fibroadenoma	Papilloma
bFGF	129.87 ± 10.97	130.85 ± 43.33	187.56 ± 44.77	238.71 ± 76.32	424.9 ± 216.85
n	92	6	18	5	7
P	nonsignificant	nonsignificant	nonsignificant	nonsignificant	0.01

(B) High-risk group

Risk	Normal	Hyperplasia	Cyst	Phylloides	Precancer	Contralateral cancer
bFGF	224.44 ± 32.13	244.46 ± 83.03	33.22	1,276.2	416.42 ± 166.56	1,524.86 ± 1,214.76
n	33	4	1	1	9	14
P	nonsignificant	nonsignificant	nonsignificant	nonsignificant	nonsignificant	nonsignificant

carcinoma *in situ*), 244.46 ± 83.03 pg/mL for the 4 cases of ''usual type hyperplasia'' or apocrine metaplasia from patients with a high risk for developing future breast cancer due to family history alone, and 224.44 ± 32.13 pg/mL from 33 samples from the rest of these high-risk patients due to family history and without a pathologic diagnosis. One case with a cyst had 33.22 pg/mL of bFGF, and another with fibroadenoma/phylloides tumor had 1,276.2 pg/mL (Table 1B). Compared with cases from high-risk patients due to family history and without a pathologic diagnosis, there was no significant difference detected in samples that came from the noncancerous breasts of patients with current unilateral breast cancer ($P = 0.48$), cases with previous precancer lesions ($P = 0.52$), nor the four cases of ''usual type hyperplasia'' or apocrine metaplasia ($P = 0.84$).

Analysis of a Nipple Fluid bFGF Test. We calculated sensitivity, specificity, and correct diagnosis variables for different bFGF cutoff levels. With a cutoff level of 250 pg/mL, the sensitivity was 79.2%, specificity was 82.5%, and correct diagnosis was 66.4%. With an equivalent cutoff of 1 pg/mg, the sensitivity was 66.7%, specificity was 81.2%, and correct diagnosis was 78.9%. χ^2 test revealed a statistical significance of $P < 0.0001$. As we adjusted the cutoff level to 132 pg/mL, the sensitivity reached 96% but specificity decreased to 54%, with correct diagnosis of 60.5%. Similarly, for a cutoff value of 0.30 pg/mg, the sensitivity reached 96% but specificity decreased to 36%, with correct diagnosis of 45.4%. Conversely, if the cutoff level was increased to 350 pg/mL, the specificity improved to 93.8% and correct diagnosis 86.4%, at the cost of a lower sensitivity of 54.2%. As the cutoff level was increased to 1.3 pg/mg, the specificity improved to 93.0% and correct diagnosis was 85.5%, at the cost of a lower sensitivity of 45.8%. These analyses are summarized in the receiver operating characteristic curve in Fig. 2, with an area under the receiver operating characteristic curve of 0.86.

Discussion

We show here that the level of nipple fluid bFGF is significantly higher in cancerous breasts in comparison with benign ones, with an area under the receiver operating characteristic curve of 0.86. Furthermore, we observe in this study for the first time that high risk breasts produce an increased amount of bFGF in the nipple aspirate fluids compared with benign ones. This report validates preliminary observations from our pilot work (5) and later work by the Sauter group (6). Here, we use a larger number of subjects and different patient specimens from those previously reported in our pilot study. Also, we now have access to an improved bFGF ELISA with better sensitivity than that used in the previous pilot study. Our test parameters compare favorably with the Sauter study, as seen in sensitivity and specificity: 79.2% and 82.5% in our report versus 89.9% and 69% in Sauter's article, respectively. Such results also compare favorably with the sensitivity and specificity of current screening methods for breast cancer: physical examination (54% and 94%) and mammogram (75% and 92%), respectively (7). Serum breast tumor markers, carcinoembryonic antigen and cancer antigen 27-29, have poor sensitivity and specificity, and are not recommended for screening or diagnostic use in early/local breast cancer (8).

According to the guidelines set forth by the National Cancer Institute Early Detection Research Network, this bFGF nipple fluid test has completed phase 1, and is ready for phase 2 (9). Further studies are warranted, but it does seem that nipple fluid bFGF has the potential to be used to supplement the traditional methods of physical examination and mammogram to screen for breast cancer. A high level of nipple fluid bFGF in a patient with a normal physical exam and mammogram may indicate high risk for breast cancer, and may warrant a more intensive workup or follow-up or even consideration of preventive measures. In the future, we need to proceed to larger prospective studies using this particular method in certain specific populations of women. The validation questions are both scientific and practical. Is this new method statistically valid enough to diagnose or predict breast cancer? And if it is, does this method improve on existing standards of breast cancer diagnosis (physical exam and mammogram), and does it change current breast treatment algorithms? Nipple fluid bFGF aspirate must fulfill both criteria to be useful clinically to women. A possible second use for nipple fluid bFGF may be as a surrogate marker for chemoprevention clinical trials to provide some preliminary insight as to the antiangiogenic effect of the chemopreventive agent (10).

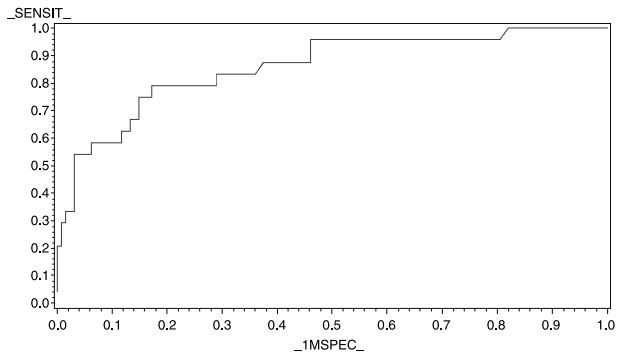

Figure 2. Receiver operating characteristic curve of nipple fluid bFGF values (pg/mL) in specimens from benign and cancerous breasts. Area under the receiver operating characteristic curve, 0.86.

A current major challenge regarding studies of nipple fluid aspirate is the ability to obtain adequate samples. Depending on the investigators' experience and patience, nipple fluid may be elicited in 30% to 98% of eligible subjects. Thus, pharmacologic intervention may be needed in order to increase the sampling adequacy of nipple fluid studies. Normally, the breast fluid is prevented from escaping from the nipple because nipple ducts are occluded by constricting bands of smooth muscle. Various drugs including oxytocin, prolactin, kallikrein, bradykinin, and acetylcholine have been shown to have a relaxation effect on the breast duct smooth muscle (11). We have used nasal oxytocin in a pilot study involving volunteer women, and found that the drug can, in certain cases, increase the yield of nipple fluid aspiration (12). On the other hand, the emerging field of bionanotechnology may soon produce devices that can measure multiple and simultaneous fluid-based as well as cellular markers in the minute amounts derived from nipple fluid aspiration alone (13). Future studies of nipple fluids should use samples obtained from the breast prior to any invasive or semi-invasive biopsy, which is known to release multiple trauma-related factors leading to ambiguity in data interpretation. Furthermore, in premenopausal subjects, care should be taken to account for possible cyclical variability during the menstrual cycle (14).

A second major challenge is the identification of more specific tumor markers. We have observed that bFGF levels are elevated in nipple fluids from pregnant or lactating women (data not shown). There may be other confounding factors, that have yet been identified. Thus, we predict that a panel of multiple nipple fluid markers would be more reliable than one alone. In the near future, more novel breast tumor markers may be identified in the nipple fluid at the earliest stage of breast malignant transformation. Currently, we think that the following nipple fluid factors may prove promising: human glandular kallikreins including prostate-specific antigen (15, 16), insulin-like growth factor and insulin-like growth factor–binding protein (17), Her-2/neu (18), and epidermal growth factor (19). Cellular DNA mutations (20), promoter hypermethylation of important cancer genes (21, 22), and chromosomal changes (23) could also be useful.

In summary, we believe that the breast nipple fluid analysis should be further studied for its potential for adding another important diagnostic tool for breast cancer and for evaluating the antiangiogenic effect of chemoprevention agents (10, 24). This evolving field has promise for future impact on the clinical care of patients with breast cancer.

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Cancer Epidemiol Biomarkers Prev 2005;14:2995-2998.