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ASSOCIATION OF KALLIKREIN EXPRESSION IN NIPPLE ASPIRATE FLUID WITH BREAST CANCER RISK

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Human kallikreins (hK) 2, 3, 6 and 10 are expressed in breast and prostate tissue. hK2 and hK3 (prostate-specific antigen, PSA) are used to screen for prostate cancer. hK6 and hK10 are downregulated in breast cancer compared to normal breast tissue. We demonstrated that levels of PSA in nipple aspirate fluid (NAF) are lower in women with breast cancer than in normal women. We hypothesize that the expression of hK2, 3, 6 and 10 are related and important in detecting breast cancer. The goals of this study are to determine the level of expression of kallikreins in NAF and serum, the association of hK2, 3, 6 and 10 in NAF, and the association of each of the kallikreins with breast cancer. In NAF from 275 women, hK3, 6 and 10 were detectable in ≥ 90% and hK2 in 74% of samples analyzed. NAF levels were highest for hK6 and lowest for hK2, regardless of cancer and menopausal status. hK3 was detectable in 15/29 (52%) and hK2 in 0/29 serum samples collected from 6 women. hK2 and hK3 were concentrated in NAF *vs.* matched serum. The 4 kallikreins were associated with the exception of hK2 with hK6 or hK10. PSA levels were higher in normal pre- than postmenopausal subjects (but not women with breast cancer), whereas levels of hK2, 6 and 10 did not differ by menopausal status. hK2 and PSA were associated with both pre- and postmenopausal breast cancer; hK6 and 10 were not. hK2 and PSA were more associated with pre- than postmenopausal breast cancer. Using logistic regression, PSA and menopausal status provided the best model of breast cancer prediction, with a sensitivity of 91% and specificity of 39%. In conclusion, 4 kallikreins are expressed in NAF. hK2 and PSA, and hK6 and hK10 are highly associated. Higher premenopausal PSA levels suggest the influence of ovarian steroids. PSA shows the most promise in aiding in the early detection of breast cancer.

Key words: *nipple aspirate fluid; kallikreins; breast cancer*

Since its discovery more than 20 years ago, prostate-specific antigen (PSA) has been established as the most valuable tool for the early detection, staging and monitoring of prostate cancer.[1] *KLK2* (encoding for human kallikrein 2, hK2) and *KLK3* (encoding for hK3 or PSA) are 2 members of 15 structurally similar genes of the glandular kallikrein family,[2] which in humans cluster in a 300 kb region on chromosome 19q13.3-13.4.[3] All 15 genes in the locus share significant homologies at both the DNA and protein level, and they all encode for serine proteases with either trypsin- or chymotrypsin-like activity. hK2 shows 79% amino acid sequence identity to PSA (Table I).[4] At the DNA level, hK2 and PSA share 80% similarity.[5] hK2 cleaves pro-PSA to generate enzymatically active PSA,[6] suggesting that hK2 may play a physiologic role in the regulation of PSA activity. PSA and hK2 have been found and measured together in amniotic fluid, breast milk, breast cyst fluid and in malignant and nonmalignant breast tissue.[7]

Both hK2 and hK3 are expressed more in noncancerous than in cancerous prostate tissue, with the degree of downregulation being higher for PSA than for hK2.[8] We previously demonstrated that hK2 was measurable in 53% and PSA in 73% of breast tumor extracts, and they were associated with each other.[7]

The secretion of hK2, similar to PSA, is stimulated by male sex steroid hormones.[9] hK2 was shown to be upregulated in the human breast cancer cell line T47D by both androgens and progestins.[10] We have investigated the steroid hormone regulation of hK2 and PSA in breast cancer cell lines. The expression of each kallikrein varied with the cell line, some producing more PSA and others hK2, but in general it appeared that hK2 was mainly under the control of androgens and progestins, similar to PSA.[11]

The KLK6 gene, encoding human kallikrein 6 (hK6, also known as zyme/protease M/neurosin), is downregulated in cell lines from primary breast cancers, and more so in metastatic lines.[12] hK6 maps close to hK2 and has 37–45% protein homology with other kallikrein genes (Table I). We previously reported the development of an immunofluorometric assay to measure the protein,[13] which is highly specific (no detectable cross-reactivity from PSA and hK2) and able to quantify hK6 protein in various biologic fluids.

hK10 [normal epithelial cell-specific (NES) 1] is expressed in normal mammary epithelial cells and downregulated in most breast cancer cell lines.[14] NES1 is a secreted protein[14] that abolished the ability of tumorigenic cells to grow in an anchorage-independent manner, slowed cell proliferation and reduced the tumorigenic potential of cell lines in a nude mouse model of carcinogenesis.[15] It is decreased in breast cancer and in metastases compared to normal breast tissue and has 33–37% protein homology (Table I) with other kallikreins.[14]

Nipple aspiration is a quick and painless technique to obtain breast fluid noninvasively. Breast fluid contains shed ductal epithelial cells and proteins secreted from the ductal epithelium. We are able to collect NAF from essentially all adult women.[16] Both the cells and extracellular fluid obtained hold promise for use in cancer screening. We previously demonstrated that PSA, which is best known for its use in prostate cancer screening, is expressed at lower levels in the NAF of women with cancer than in normal women.[17]

In summary, there is a family of kallikrein genes, at least 4 of which are present in the breast. In breast tissue, hK2, 3, 6 and 10 have been inversely associated with breast cancer development and progression. It is our hypothesis that these 4 kallikreins are

Abbreviations: BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; hK, human kallikrein; KLK, glandular kallikrein; NAF, nipple aspirate fluid; NES, normal epithelial cell-specific; PSA, prostate-specific antigen.

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TABLE I – KALLIKREIN HOMOLOGY

Kallikrein	Molecular weight (Da)[a]		Protein homology (%)[b]			
	Full length	Mature	hK2	hK3	hK6	hK10
hK2	28,671	26,159		79	45	37
hK3	28,741	26,089			39	33
hK6	26,856	24,500				37
hK10	30,138	26,947				

[a]From SwissProt (www.ebi.ac.uk/swissprot/).–[b]From ExPASy (www.expasy.org).

related and are each important in detecting breast cancer. Although we previously reported the successful measurement of hK6 in NAF,[7,13] this was with a very limited sample size, insufficient to characterize the expression of these markers. To our knowledge, this is the first report of hK10 expression in NAF. The purpose of this study was to determine the level of expression of kallikreins in NAF and serum, the association of hK2, 3, 6 and 10 in NAF, and the association of each of the kallikreins with breast cancer.

MATERIAL AND METHODS

Subject and specimen accrual

Informed consent was obtained and NAF was collected from 275 women using an institutional review board-approved specimen collection study. The approved study was amended to allow 6 women to provide both NAF and serum twice monthly for up to 3 months to assess the ratio of NAF to serum levels of hK2 and PSA over time. The women were between the ages of 24 and 80, with 80% white and 52% premenopausal. NAF measures were recorded as coming from either the right or the left breast. Some subjects had measures from one side only, while others had measures from both sides. For subjects who had measures from both sides, one side was randomly selected for the analyses (along with the corresponding side-specific cancer risk assessment). If multiple measures were available for NAF from the selected side, they were collapsed into a single value (their median). In essence, only one NAF measurement per woman was used in each analysis.

Each breast of a given subject was categorized into 1 of 8 risk groups. The risk groups and number of subjects in each group were as follows: 1, no risk factors (41 women); 2, history of a first-degree relative with breast cancer (38 women); 3, history of hyperplasia without atypia in the incident breast (8 women); 4, history of cancer in the breast contralateral to that being studied (17 women); 5, biopsy-proven atypical hyperplasia (8 women); 6, biopsy-proven lobular carcinoma *in situ* (6 women); 7, biopsy-proven ductal carcinoma *in situ* (29 women); and 8, biopsy-proven invasive carcinoma (128 women). Because there were very few women in risk categories 3 to 6, we collapsed categories 1–6 (noncancer) and 7 and 8 (cancer) for all analyses. NAF was obtained from 97% of subjects who agreed to provide these specimens.

Nipple aspiration technique

Nipple aspiration was performed after obtaining informed consent using a modified breast pump. The technique has previously been described. Briefly, the breasts were warmed with moist towels.[17] The subject then massaged her breasts for approximately 2 min. The nipples were cleansed with a mild soap followed by alcohol. A suction device was then placed first over the right, then the left breast, if present. Suction was created using a 10 cc syringe and held for 10–15 sec, or until the participant experienced discomfort. Fluid in the form of droplets appeared and was collected into glass capillary tubes. NAF total protein content was determined using a Pierce BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Capillary tubes containing NAF were broken in half and placed in 400 μl of a 0.1 M $NaHCO_3$ solution, pH 7.8. The capillary was then crushed with a steel spatula to release the NAF. Samples for analysis were kept at −80°C and batched for analysis.

TABLE II – KALLIKREIN EXPRESSION PROFILE IN NAF AND SERUM

	Subjects	Right breast samples		Left breast samples	
		Total	Number (%) with hK = 0[a]	Total	Number (%) with hK = 0[a]
NAF					
hK2	169	132	35 (27)	139	35 (25)
hK3	273	316	17 (6)	196	17 (9)
hK6	146	95	5 (5)	119	2 (2)
hK10	181	120	12 (10)	137	14 (10)
Serum					
hK2	6	29	20 (100)		
hK3	6	29	14 (48)		

Serum results are listed only once, not by breast as for NAF.
[a]Level is below the limit of detection for the assay.

All analyses were performed under the supervision of a laboratory administrator with over 20 years of experience. Technicians were blinded as to the risk group of each sample. Not all assays were performed on all subjects. Since the PSA assay was developed first, more NAF samples were analyzed for this than for the other kallikreins.

hK2 and PSA

Both hK2 and PSA were measured using time-resolved immunofluorometric assays developed by us.[18,19] hK2 has a detection limit of 6 ng/L, and PSA a detection limit of 1 ng/L. The hK2 assay has less than 0.2% cross-reactivity with hK3. The within-sample coefficients of variation for both the hK2 and hK3 assays were $<$ 10% within the measurement range.

hK6 and hK10

The concentration of both hK6 and hK10 were determined using a sandwich-type time-resolved immunofluorometric assay. The hK6 assay has a detection limit of 250 ng/L,[13] while the hK10 assay has a detection limit of 50 ng/L.[20] Both assays have within-sample coefficients of variation of $<$ 10%. Each assay is specific for the intended protein, lacking cross-reactivity with hK2, hK3, and hK6 (for hK10) and hK2, hK3, and hK10 (for hK6).

Statistical analyses

Because of the skewness of the data, statistical methods based on ranks were employed. For each of the variables, the total number of measurements on each breast and the number and percentage of values that were zero were computed and presented in Table II.

In order to evaluate the association between hK2, PSA, hK6 and hK10 in a given breast, Spearman's correlation coefficient[21] was used. These results are reported in Table III. In order to evaluate the association of the 4 kallikreins based on menopausal status and within the cancer and noncancer groups, the Wilcoxon rank sum test was used (Table IV).

In order to determine the sensitivity and specificity of the kallikreins analyzed in predicting the presence of breast cancer, we developed a logistic regression model using the following variables: hK2, hK3, hK6, hK10, age, menopausal status and race. Of these variables, only hK3 and either age or menopausal status were significant contributors to the model. The best model for predicting the probability of cancer combined menopausal status and hK3.

RESULTS

Ability to measure markers in NAF and serum

PSA, hK6 and hK10 were detectable in $\geq$ 90% of NAF samples from both breasts (Table II). hK6 was the marker measured in the highest percentage of specimens (97%), whereas hK2 was detectable in the lowest proportion (74%). Serum levels of PSA were detectable in 52% of specimens analyzed, whereas hK2 was not detectable in any specimen analyzed.

TABLE III – ASSOCIATION OF KALLIKREINS IN NAF FROM THE RIGHT AND LEFT BREASTS

Kallikrein	hK3	hK6	hK10
hK2			
Spearman correlation	0.41 (0.70)	0.20 (0.21)	0.025 (0.17)
p-value	<0.0001 (<0.0001)	0.10 (0.058)	0.83 (0.13)
Observations	89 (101)	72 (84)	75 (85)
hK3			
Spearman correlation		0.34 (0.20)	0.06 (0.21)
p-value		0.004 (0.068)	0.56 (0.043)
Observations		71 (83)	95 (97)
hK6			
Spearman correlation			0.37 (0.40)
p-value			0.002 (0.0002)
Observations			72 (84)

TABLE IV – ASSOCIATION OF KALLIKREINS WITH BREAST CANCER CONTROLLING FOR MENOPAUSAL STATUS

hK	Menopausal status	Cancer			Noncancer			*p*-value
		n	Median	Range	*n*	Median	Range	
hK2	Pre	38	7.5	0–361	52	43.5	0–1,674	0.005
	Post	50	1.0	0–367	29	21.0	0–418	0.043
hK3	Pre	65	94	0–17,191	77	1523	0–61,040	<0.0001
	Post	91	37.5	0–12,408	40	331.1	0–20,219	0.0002
hK6	Pre	30	20,700	320–85,700	40	10,600	1,900–208,300	0.63
	Post	45	11,900	0–191,700	25	20,800	590–197,600	0.20
hK10	Pre	40	695	0–6,000	47	1,000	0–11,400	0.11
	Post	66	814	0–5,200	28	915	0–4,660	0.23

Kallikreins in ng kallikrein/g total NAF protein.

Kallikreins are concentrated in NAF compared to serum

In a group of 6 women for whom data were available for 3 months, NAF and serum were collected and hK2 and PSA were measured in both sample types. Serum hK2 was uniformly undetectable, whereas median serum PSA was 1.03 ng/L (range, 0–22.7 ng/L). Since we could not detect hK2 in serum, we cannot comment on its relative concentration in NAF, except to say that hK2 was detectable in 74% of NAF samples, with values up to 1,674 ng/g. NAF PSA was concentrated 100–1,000-fold compared to serum. We have previously reported on hK6 (median, 4.4; range, 0–8.1 μg/L)[22] and hK10 (median, 0.6; range, 0.1–1.3 μg/L)[23] expression in serum, suggesting that in NAF hK6 is concentrated 2,500–5,000-fold and hK10 is concentrated 700–1,000-fold.

Association of NAF kallikreins with each other

Based on the protein homology between the 4 kallikreins (Table I), we predicted that the expression of these kallikreins would be significantly associated, and that the degree of association would be related to the degree of protein homology. There was a significant association of kallikreins with each other with the exception of hK2 with hK6 and hK2 with hK10 (Table III). The association was strongest between hK2 and PSA (r = 0.41, right breast; 0.70, left breast) and between hK6 and 10 (r = 0.37, right breast; 0.40, left breast). Each of these associations was significant.

Kallikrein expression based on menopausal status

NAF levels of hK6 were highest and of hK2 were lowest for all groups of women, regardless of cancer or menopausal status (Table IV). Levels of PSA ($p = 0.006$) but not hK2, 6 and 10 were significantly higher in pre- than in postmenopausal women without breast cancer. Menopausal status did not significantly influence the expression of any kallikrein, including PSA, in NAF from women with breast cancer. Interestingly, hK6 levels in subjects without breast cancer tended to be higher in post- than in premenopausal subjects, although the difference was not significant ($p = 0.12$). The range of kallikrein values was also determined in pre- and postmenopausal women. The left breast values were arbitrarily calculated for this analysis. The median range of values (expressed in ng/g) was similar between pre- and postmenopausal women for hK2 (101 *vs.* 104), hK6 (7.6 *vs.* 28.2) and hK10 (1.1 *vs.* 1.33), but was higher in pre- than in postmenopausal women for hK3 (3,975 *vs.* 28.2).

Association of kallikreins with breast cancer

Median NAF hK2 and PSA expression were significantly higher in both pre- and postmenopausal women without than with breast cancer (Table IV). The association was greater for PSA than for hK2, regardless of menopausal status. Neither hK6 nor hK10 levels were significantly different based on whether or not the subject had breast cancer.

Using logistic regression, we determined that the best model to predict breast cancer combined hK3 and menopausal status. With these variables, the sensitivity in predicting breast cancer was 91%, with a specificity of 39%.

DISCUSSION

Because of the success of PSA in screening for prostate cancer and preclinical data suggesting that PSA was expressed in the breast, we determined if the kallikrein was present in NAF and associated with breast cancer.[17] Since our discovery that NAF PSA appears to be a primary candidate marker for breast cancer screening, we sought to determine if kallikreins with known protein homology to PSA might also be associated with the disease.

For any marker to be useful in breast cancer screening, it must be measurable in the vast majority of subjects. Although kallikrein protein sequences suggested that they were secreted, it was not known if our assays would be sufficiently sensitive to detect them, in what fraction of specimens, or what their concentration would be in various subject groups. It was therefore encouraging to find out that each of the kallikreins was detectable in the vast majority of subjects, with all but hK2 being detectable in ≥ 90% of women. On the other hand, our data suggest that assays for serum PSA are only sufficiently sensitive to detect the protein in approximately half of the subjects tested, and hK2 was below the level of detection in all women tested. Both markers await a more sensitive assay before serum can be considered a viable physiologic fluid to evaluate these markers for breast cancer risk.

The 2 kallikreins (hK2 and PSA) whose NAF levels show the highest degree of association are known to be physiologically

linked, since hK2 cleaves pro-PSA to generate enzymatically active PSA.[6] Based on the level of association (Table III), it appears that hK6 and hK10 are more closely associated with each other than they are with the other 2 kallikreins. Whereas PSA appears to be influenced by ovarian steroids based on higher levels in pre- than in postmenopausal women, this was not observed with hK2, 6 or 10. Similarly, the range of PSA values varied with menopausal status, being higher in premenopausal women, whereas the range of values of the other kallikreins did not significantly vary based on menopausal status. This is consistent with our earlier findings[24] that PSA levels vary during the menstrual cycle of premenopausal women and appear to be upregulated in association with the progesterone surge. Indeed, hK6 levels in normal subjects tended to be higher in post- than in premenopausal women, although the difference was not significant. The primary function(s) of hK6 is yet to be determined. Although hK10 has been reported to have a tumor suppressor function in breast tissue,[25] we did not observe an association with breast cancer.

The results of this study support a protective mechanism for PSA in breast cancer. Our logistic regression model suggests that once the level of PSA is known, levels of hK2, hK6 and hK10 do not contribute additional information to the prediction of breast cancer. On the other hand, it is possible that other nonkallikrein biomarkers may improve our ability to predict the presence of breast cancer by analyzing NAF. For example, we previously reported that combining NAF levels of PSA and basic fibroblast growth factor (bFGF) were more predictive than bFGF alone.[26] The finding of lower PSA levels in normal post- than premenopausal women, and the greater inverse association of PSA with cancer in premenopausal women, supports our earlier observation[27] that progesterone may upregulate PSA production. The absence of this regulation by ovarian steroids may make a subject more susceptible to breast cancer. Further studies are needed to confirm this. Our findings suggest that measuring NAF levels of PSA may prove useful to screen for breast cancer.

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