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Biochemistry

A STUDY TO ASSESS THE LEVEL OF FREE PSA IN BREAST CARCINOMA

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ABSTRACT Breast cancer is the most commonly occurring female cancer in the world and second most common cancer India. PSA (Prostate specific antigen) is a 33kDa serine protease expressed at high levels in the epithelium of the human prostate gland. 'free PSA' is that form of PSA that is not bound to an inhibitor..Serum fPSA level is measured in Histologically and clinically proved female breast carcinoma patients by ELISA both presurgically and 15 days post surgically and in control subjects.. Free PSA decreases more dramatically after surgery, strongly indicating that this fraction is produced by breast tumors. PSA can be used as a prognostic indicator of Breast carcinoma and as an indicator of completion of surgery and recurrence.

KEYWORDS : Breast cancer, free PSA, ELISA

Introduction:
Breast carcinoma is the most commonly occurring female cancer in the world.Although incidence rates are higher in the West, breast cancer is the second most common cancer (after cervical cancer) in India.

Urbanization,later age at marriage, age at first birth, reduced breastfeeding, westernization of diet, physical activity patterns, ageing, early menarche and late menopause, family history increase the risk of breast cancer.A history of atypical hyperplasia, lobular carcinoma in situ or ductal carcinoma in situ (as determined by a breast biopsy) increases the risk of developing invasive breast cancer.

Having mutations in BRCA1, a gene on chromosome 17 that controls cell growth or BRCA2, a gene on chromosome 13 that suppresses cell growth, are associated with increased risk of breast cancer.

PSA (Prostate specific antigen) is a 33kDa serine protease [1]expressed at high levels in the epithelium of the human prostate gland. However, studies have demonstrated that PSA can also be secreted in females, especially from prostate equivalent Skene's peri-urethral gland and tissue like breast and ovary (Papotti et al., 1989). PSA is found in normal and hyperplasic breast tissue and majority of breast tumors and breast cysts. Jungchan Nam,Minyoung yoo et al. in 2015 suggested that PSA is a biomarker for breast carcinoma.[2] .'free PSA' is that form of PSA that is not bound to an inhibitor which could comprise both enzymatically active PSA and the inactive 'nicked' form. Using chromatographic techniques it is found that the predominant form of PSA in the sera of women without breast cancer is PSA bound to ACT (serine protease inhibitor alpha 1 antichymotrypsin),whereas free PSA constitutes the predominant molecular form in breast carcinoma. Free PSA decreases more dramatically after surgery, strongly indicating that this fraction is produced by breast tumors. It can be used as a prognostic indicator of breast cancer and as an indicator of completion of surgery and recurrence.

MATERIALS AND METHODS
Study Area: Calcutta National Medical College & Hospital, Kolkata, West Bengal, India in the Departments of Biochemistry and General Surgery.

Study population: Histologically and clinically proved female breast carcinoma patients admitted for surgery.

Study Period: 0ne year

Study design- Case control, non interventional, observational study.

Sample Design: Samples were collected on convenience basis from the surgical ward after obtaining written consent.

Sample size: 50 female breast carcinoma patients and 50 control subjects.

Selection criteria for cases:

Inclusion criteria:
1. Female breast cancer patients who have been newly diagnosed, both clinically and histologically proved by FNAC (Fine Needle Aspiration Cytology).
2. Female breast cancer patients in the age group of 30 to 60 years.

Exclusion criteria:
1. Breast cancer patients who had received any kind of treatment (surgery, radiotherapy, chemotherapy or hormone therapy).Patients treated with any indigenous or traditional Indian medicine e.g. ayurvedic, homeopathic medicines were also excluded.
2. Women with any co morbid conditions like inflammatory disorders, immunological dysfunctions, HIV, tuberculosis, diabetes mellitus, endocrine dysfunctions (thyroid dysfunction, etc.) and gynecological disorders.

Selection criteria for controls :
Control subjects were selected from the healthy female attendants of the patients after proper age matching. First degree relatives of cases were excluded for selection as control population. Subjects with history of gynecological, metabolic, immunological, inflammatory or carcinogenic diseases were not selected as controls.

Parameter to be investigated:Serum fPSA level in breast cancer patients by ELISA both presurgically and 15 days post surgically and in control subjects

Study tools:
1) Centrifuge machine (REMI)
2) ELISA washer and reader (LISASCAN)
3) ELISA kits of fPSA, β estradiol and progesterone
4) Shaker
5) Micropipettes
6) Pipettes of different sizes

STUDY TECHNIQUE:
Patients who were FNAC proved and posted for surgery for excision of the breast lump were selected. After taking proper consent blood was collected preferably in the follicular phase of premenstrual patients. In case of postmenstrual patients blood was collected blood was collected according to their convenience.

Following overnight fasting a fresh morning sample of 5 mL blood was collected from the antecubetal vein and serum thus obtained after centrifugation was taken in aliquots and estimated for fPSA,

From the same patients, blood was again collected 15 days after operation, i.e. modified radical mastectomy (MRM), and the serum was again assessed for the above mentioned tests.

Blood from 50 age matched control subjects were collected and same tests were performed.

Estimation of fPSA by Accubind ELISA
Principle

Immunoenzymometric assay (TYPE 3):
The reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a micro plate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-PSA antibody. Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Reagents
*f-PSA Calibrators – 1ml/vial - Icons a-f

Six (6) vials of references free PSA antigen at levels of 0(a), 0.5(b), 1.0(c), 2.5(d), 5.0(e) and 10.0(f) ng/ml.

Store at 2-8°C. A preservative has been added.

Note: The calibrators, protein based buffered matrix, were calibrated using a reference preparation, which was assayed against the WHO 1st International Standard 96/668.

A. fPSA Enzyme Reagent – 13 ml/vial -
One (1) vial containing enzyme labeled antibody, biotinylated specific free PSA monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

*Streptavidin Coated Plate – 96 wells -
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

*Wash Solution Concentrate – 20 ml -
One (1 vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

*Substrate A – 7ml/vial
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

*Substrate B – 7ml/vial - Icon S^B
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

*Stop Solution – 8ml/vial - Icon
One (1) bottle containing a strong acid (1N Hcl). Store at 2-30°C.

PROCEDURE
*The microplates' wells were formatted for each serum reference, control and patient specimen to be assayed.

*0.050 ml (50µl) of the appropriate serum reference, control or specimen was pipetted into the assigned well.

*0.100 ml (100µl) of the fPSA Enzyme Reagent was added to each well.Microplate was swirled gently for 20-30 seconds to mix and covered.

*The microplate was incubated for 60 minutes at room temperature (20- 27°C).

The contents of the microplate was discarded by decantation or aspiration

*After decanting, the plate was tapped and blotted to dry with absorbent paper.

*350µl of wash buffer (which had been prepared by adding 25 mL of wash buffer solution in 40mL of distilled water) was added and aspirated. It was repeated two (2) additional times. This procedure was carried out in a Elisa Plate washer of (LISASCAN)

*0.100 ml (100µl) of working substrate was added solution to all wells. Again the plates were incubated at room temperature for fifteen (15) minutes.

*0.050ml (50µl) of stop solution was added to each well and mixed gently for 15-20 seconds.

*The absorbance was read in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a micro plate reader of Lisascan.

*The results were read within thirty (30) minutes of adding the stop solution.

Result analysis:

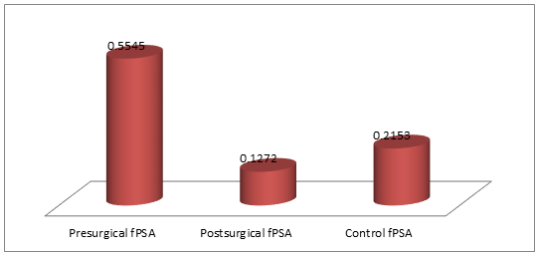
Table 1: Distribution of the mean values serum levels of fPSA in presurgical and postsurgical patients of breast cancer according to their age.

Age Group	fPSA (ng/mL)	
	Pre surgical	Post surgical
30-35	0.70	0.16
36-40	0.61	0.12
41-45	0.37	0.09
46-50	0.30	0.20
51-55	0.24	0.08
56-60	0.22	0.09

From the above table it is apparently visible that fPSA is raised in the lower age groups(30-40) much more as compared with the higher age groups

The decrease in fPSA is more drastic in the lower age group as compared to the higher age groups following surgery.

Table 2: The mean values of fPSA in all cases in ng /mL



fPSA values in ng/mL

Table 3: The line diagram o fPSA values showing a comparison of the values between presurgical, postsurgical and control groups

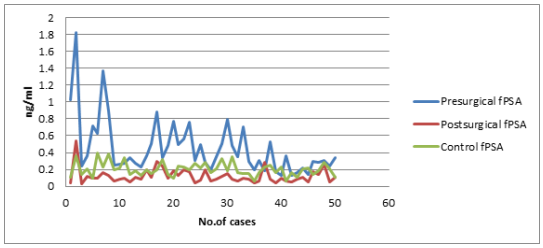


Table 4: ANOVA PostHoc Analysis with Bonferroni correction showing significance of difference between the mean values of study parameters.

Dependent Variable	(I) grouping	(J) grouping	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
fPSA (ng/ml)	1	2	0.33260	0.04017	<0.001*	0.2353	0.4299
		3	0.24980	0.04017	<0.001*	0.1525	0.3471
	2	1	-0.33260	0.04017	<0.001*	0.4299	-0.2353
		3	-0.08280	0.04017	.123	-0.1801	0.0145
	3	1	-0.24980	0.04017	<0.001*	-0.3471	-0.1525
		2	0.08280	0.04017	.123	-0.0145	0.1801

Discussion

The purpose of this study was to measure the serum free PSA (fPSA) level in 50 breast cancer patients presurgically and again 15 days postsurgical. In the present study age of the subjects were between 30-60 years in both study and control group

As shown In the Table no.1 it was observed that fPSA was more in the lower age groups (30-40years), in comparison to the higher age groups. J A Foekens, E P Diamandis et al.[3] suggested that low levels of fPSA were more often found in larger tumours , tumours of older and post-menopausal patients, and in steroid hormone receptor-negative tumours. [3]. In the present study it was found that serum fPSA levels were higher in the lower age groups, and the post surgical decrease was also much more in lower age group. This is in corroboration of the study of Asadi et al (2013), who found that TPSA and fPSA levels were significantly associated with younger age and earlier cancer stage. [4]
From table2,3 and4 the fPSA values in pre and post surgical patients and also in control group were compared. In presurgical patients fPSA value was 0.55± SD 0.33 ng/mL and in post surgical patient it was 0.12 ± SD 0.10 ng/ml. In control group fPSA was 0.21 ± SD 0.10 ng/ml. From the values of fPSA it has been observed that serum fPSA decreases significantly after surgery in breast cancer patients and so it came down significantly compared to the normal control subjects also. This data supported the possibility of fPSA to be used as an indicator of adequate clearance. Moreover, fPSA resurge positively correlates with recurrence. Since fPSA is an indicator of adequate surgery, its reappearance can correctly indicate recurrence of breast cancer. In present study it was found that serum levels of fPSA was significantly raised in the presurgical groups of breast cancer patient in comparison to the control population and post surgical values.

It has been reported that the majority of breast tumour PSA occurs in the free form. It is likely that the tumour produces fPSA that is incapable of binding to serine protease inhibitors such as ACT, given the large molar excess of serum ACT in comparison to PSA [5].Alternatively, breast tumours may produce an endopeptidase, which causes a posttranslational modification (internal cleavage) of PSA produced by the breast, thus preventing complex formation with ACT and increasing the proportion of free PSA [5]. Mashkoor and Asadi(2013)[4] in their study indicated a clinical significance of preoperative measurement of serum TPSA and fPSA in the diagnosis of female breast cancer, and suggested them to be useful marker for monitoring the response to treatment.

Black and Giai (2000)[6] showed that the percentage of breast cancer with free PSA as the predominant molecular form (> 50% of total PSA) in serum was five times higher than that of healthy women or women with benign breast disease and the levels showed significant decrease after surgery.

ANOVA PostHoc analysis in table 4 reflected same findings. The decrease of free PSA was more significant in the younger age group. Data in these tables showed the usefulness of fPSA as a marker for completeness of removal of the tumour as the postsurgical values were even lower than that found in the controls. This may be due to the fact that normal breast also secreted some amount of the hormone and the removal of the diseased breast following modified radical mastectomy resulted in such findings.

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