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Evaluation of human epidermal growth factor receptor-2 and hormonal receptor expression patterns in breast cancer from fine needle aspiration cytology

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Abstract
Introduction: *Breast cancer is the most common cancer among women, accounting for 22% of all female cancers. In breast cancer, the overexpression of human epidermal growth factor receptor-2 (HER-2/neu) is a prognostic marker indicated for stratification of patients for HER-2/neu targeted therapies, while estrogen receptor (ER) and progesterone receptors (PR) are additional prognostic markers in patients with early stage breast cancer and predictive for response to hormonal therapies. Fine needle aspiration cytology (FNAC), can provide enough cellular sample and evaluation of ER, PR and HER2/neu expression patterns has relevant clinical application because the sample can be collected before surgery and help plan management of breast cancer patients. The aim of the study was to evaluate and classify breast cancer by examining HER-2/neu, ER and PR expression patterns using cellblocks.*
Methods: *A prospective cross-sectional study was carried out using FNAC samples from 30 patients with breast cancer. The ER, PR and HER2/neu protein overexpression patterns were determined by immunocytochemistry. Breast cancer cases were considered positive for ER and PR when 10% or more tumor cells were stained. The HER-2/neu overexpression was assessed on a scale of 0 to 3+ and a score of 3+ was considered positive.*
Results: *Estrogen receptor positivity was observed in 50% of breast cancer cases, while PR positivity in 47% of the cases and HER2/neu was overexpressed in 13% of breast cancer cases.*
Conclusion*: Immunocytochemistry performed on cell block is a feasible method for evaluation of ER, PR and HER-2/neu status in breast cancer, especially when the cellblock has adequate tumor cells.*
Keywords: *Breast cancer, human epidermal growth factor receptor-2, estrogen receptor, progesterone receptor, Immunocytochemistry.*

Introduction

Breast cancer comprises of diverse group of neoplasms, which show a broad array of morphological features, genetic changes, and tumor behaviour.[1] These pose a major challenge in patient care as it is difficult to determine the most appropriate therapeutic regimen according to profile of disease characteristics. Given poor prognosis of breast cancer diagnosed at late stages, patient stratification for targeted therapy,[2] remains the most feasible way in improving long survival of patients with breast cancer in Kenya.

Breast cancer is the most frequent cancer among women, and has a prevalence of about 22% of all

female cancers.[3] Globally over 1 million new cases are reported each year, leading to approximately four hundred thousand breast cancer related death annually.[3] In Kenya, breast cancer has an incidence rate of 13.7% and is the most prevalent cancer among women, leading to major cause of morbidity and mortality in women.[4]

The over-expression and/or amplification of HER-2/*neu* in breast cancer is a prognostic molecular marker indicated for patient selection for HER-2/*neu* targeted therapies. [5] The over-expression of HER-2/*neu* is reported in about 20-30% of breast cancer and up regulation of HER-2/*neu* is linked to an aggressive disease process and poor prognosis. [5] A previous study in Kenya reported HER2/*neu* overexpression in 26% of breast cancer among women. [6] The steroid hormonal receptors for ER and PR are markers with prognostic value in early stage cancer of the breast and ER predictive for response to hormonal therapies. Estrogen receptors are positive in about 70% of breast cancer, and are known as estrogen receptor positive breast cancer.[7] Approximately 65% of breast cancer cases positive for ER will be progesterone receptor (PR)-positive.[7] Studies have reported the overexpression of ER/PR among African breast cancer cases with estimates ranging between 18% and 72%.[8,9]

Breast cancer, present a clinical, diagnostic and therapeutic challenge. This is because the treatment decision is based on clinicopathological variables that are prognostic in nature, such as size of the tumor, metastasis to the lymph node and histological grade that are not sufficient for implementation of personalized therapy.[5,10] Although advances in breast cancer chemotherapy have achieved a certain rate of success, studies show that breast cancer ER, PR and HER-2/*neu* positive are difficult to treat and it is now believed that more careful patients stratification using these biomarkers will improve therapeutic outcomes.[5,10] The breast cancer subtypes are classified by assessing the ER, PR and HER2/*neu* status, which includes luminal A (ER/ PR+, HER-2/*neu* -), luminal B (ER/ PR+, HER-2/*neu* +), HER2/*neu* positive (ER-, PR-, HER-2/*neu* +) and triple negative breast cancer (TNBC) is negative for ER, PR, and HER2/*neu*. [11]

Tissue biopsies including core needle biopsies have been the samples of choice for evaluating ER, PR and HER-2/*neu* status.[11] However, the use of fine-needle aspiration cytology samples to evaluate ER, PR and HER-2/*neu* status using a cell block is recommended, especially in metastatic disease where a biopsy may not be advisable, high cost of core needle biopsy especially in poor resource setting [11-13], or when it is not practical to obtain a surgical excision in patients receiving neo-adjuvant therapy. Furthermore, the benefit of using a cell block is that it is cheaper than core needle biopsy, serial sections can be obtained, the use of same antibodies as those of formalin fixed histological sections[11,12] and is also recommended in case of recurrent disease especially in nodal metastasis.

The use of FNAC for determination of ER, PR and HER-2/*neu* expression patterns has not been adequately explored in poor resource setting especially in developing countries including Kenya[9,14], furthermore breast cancer stratification into subtypes based on expression patterns of ER, PR and HER-2/*neu* using FNAC is lacking. Fine needle aspiration cytology is performed by making several passes within the lump using a needle to obtain the cell sample. [11,13] Fine-needle aspiration cytology is readily available, minimally invasive and has been proven to be safe, fast, and cost effective when compared to core needle biopsies especially for aged or frail patients with co-morbidities and those on follow up for neoadjuvant chemotherapy response. To achieve reliable results FNAC of breast cancer should provide adequate cellular material that is representative of the breast mass.[11-13] Fine-needle aspiration cytology samples may be first used as cellular smear for immediate diagnosis, and residual cells (needle washings) can be obtained by rinsing the needle and preserved for subsequent detection of biomarker by immunocytochemistry.

Therefore, the proposed research was aimed at assessing the clinical utility of ER, PR and HER-2/*neu* expression patterns in fine needle aspiration cytology samples from breast cancer patients.

Materials and Methods

Patients Study Population

In this prospective cross-sectional study, a total of 30 fine needle aspiration cytology samples were collected from patients with breast cancer at surgical outpatient clinic of the Kenyatta National Hospital in Nairobi, Kenya between November 2014 and February 2016. All eligible patients recruited in this prospective cross-sectional study were women of 18 years and above who had been diagnosed with breast cancer either through triple assessment (clinical assessment, Imaging techniques (mammography or ultrasonography) and biopsy/fine needle aspiration cytology and had signed a written informed consent before recruitment. Women who had other type of malignancies, or on treatment for breast cancer or those who had undergone mastectomy were excluded. A structured questionnaire was used to interview the participants attending the surgical outpatient (fine needle aspiration) clinic in order to obtain demographic and clinical data as shown in Table 1. The age of the patients ranged from 22 to 100 years, with a mean age of 49.88 ± 18 years and median of 48 years (Table 1). The study protocol was approved by ethical and research committee of the University of Nairobi and Kenyatta National Hospital.

Specimen Collection and Cell block preparation

Fine needle aspiration cytology samples were obtained using a 21 or 23-gauge needle. The material was then placed on a microscopic slide and a second slide was used to spread the material thus obtaining a thin conventional smear, which was immediately fixed in 95% ethyl-alcohol and stained using both papanicolaou staining and haematoxylin and eosin methods for cytomorphological diagnosis of breast cancer and reported using the national cancer institute (NCI) categorization system. [15]

The portion of the remaining aspirate in the needle was used for cell block preparation. Briefly, 10–20 ml of the aspirate was centrifuged and supernatant discarded. Two drops of pooled plasma were added, followed by gentle shaking. Thereafter, two drops of thromboplastin were added and mixed well to activate clotting factor, followed with two drops of calcium ion. The mixture was left to stand for 5 minutes, followed with transfer of the clot to moistened filter paper. The clot was wrapped well, put in a cassette, and then immediately fixed in 10% neutral buffered formalin for at least 6 hours. The sample was then processed using the routine tissue processor and embedded to form a cell block.

HER2/*neu*, ER and PR immunocytochemistry staining

Immunocytochemical staining for ER, PR and HER2/*neu* (DAKO, Glastrup, Denmark) was performed on 4 µm paraffin sections of cellblock following manufacturer's instructions with minor modification. Briefly, the cellblock sections mounted on slides were deparaffinised by use of xylene followed by alcohol washes. This was followed by heat induced epitope retrieval for 20 minutes. The slides were then blocked by Envision Flex Peroxidase blocking reagent for 5 minutes. They were incubated with primary antibody for 30 minutes and only sections for PR staining were incubated with envision Flex Linker for 15 minutes. The sections were incubated with Envision Flex/HRP for 20 minutes, and incubated with labelled Envision Flex 3,3′-diaminobenzidine (DAB) as a chromogen for 10 minutes, and counterstained with hematoxylin for 5 minutes at room temperature. Between incubations sections were washed with Tris-buffered saline. Cover-slipping was performed using the Tissue-Tek SCA coverslipper.

Reporting for immunocytochemistry results

Allred scoring system was used for ER/PR reporting.[16] Allred score takes into account the percentage of tumour positive cells (0–5) and the intensity of immunocytochemical staining (range

0–3). The two scores are added to obtain a final score, which may range from 0 to 8. Tumour cells with a total score of 3–8 are considered positive, whereas those with a score less than 3 are considered negative. Allred scoring stratifies a breast cancer patient's ER/PR status into cancers that are likely to respond to hormone therapy with tamoxifen.[16]

The recommendation by the American Society of Clinical Oncology/College of American Pathologists on the HER2/*neu* -positive status is when (on observing within an area of tumor that amounts to greater than 10% of contiguous and homogeneous tumor cells) there is evidence of protein overexpression. [17] Score 0 and 1+ should be interpreted as negative, Score 2+ must be additionally tested for gene amplification before considering definitive therapy as only about 30% of these cases show gene amplification which is a prerequisite for definitive therapy. Score 3+ may be taken as positive as over 90% of these cases show gene amplification.[17] For cell block adequacy at least 100 tumor cells were counted for assessing the biomarker status.[5,7] Under breast cancer subtypes: Luminal A was defined either ER or PR positive with a negative HER2/*neu* while, luminal B was either ER positive and or PR positive with a positive HER2/*neu*. HER2/*neu* positive was defined as ER & PR negative and with HER2/*neu* positive staining. TNBC was as all ER, PR and HER2/*neu* receptors staining negative.

Data Analysis and Presentation

The results are expressed as a mean ± standard deviation or as median or range. Percentages of the tumor cells stained positive for ER, PR and HER-2/*neu* immunoreactivity were calculated. Spearman's rank correlation, was performed with 95% confidence interval. A *P*-value < 0.05 was considered statistically significant. All data analysis was performed using SPSS, Version 20 (IBM, Armonk, NY, USA).

Results

All patients were of black descent and majority of breast cancer cases occurred between ages 41–60 years, although breast cancer cases below 40 years had a proportion of 37% (Table 1). In terms of laterality, 16 (53%) were located on the left breast, and 14 (47%) on the right breast. Majority of the breast masses were located on the upper outer quadrant (43%) and the size of breast masses ranged from 2 to 14 cm (Table 1). The most frequent breast cancer cytomorphologically was ductal carcinoma 27(90%) (Fig. 1), while axillary nodal positivity was observed in 7(23%) cases (Table 1).

Estrogen receptor (ER) positivity was observed in 15(50%) of breast cancer cases, while progesterone receptor (PR) positivity was observed in 14(47%) of the cases (Fig. 1) and Her-2/*neu* overexpression was reported in 4(13%) cases (Fig. 2).

Receptor expression patterns based on laterality, age, location and size of the breast masses

The ER positivity levels between the left breast and the right breast showed a pearson correlation coefficient, which was significantly higher ($p=0.034$) in the right breast compared to left breast. The PR positivity levels between the left breast and the right breast showed a pearson correlation coefficient, which was significantly higher ($p=0.011$) in the right breast than in the left breast. However, no significant correlation was seen with the HER-2/*neu* expression levels between the left breast and the right breast (($p=0.683$). There was no correlation for ER ($p=0.279$), PR ($p=0.171$) and HER-2/*neu* ($p=0.239$), overexpression levels between age group as well as ER, PR, and HER-2/*neu* overexpression levels between different breast mass locations and mass size (Table 2).

Classification of breast cancer cases on the basis of ER, PR and HER-2/*neu* profile

The Luminal A (ER/PR+, HER-2/*neu*-) profile subtype consisted of 17 (57%) cases, while Luminal B (ER/PR+, HER-2/*neu*+) profile

subtype consisted of 2 (7%) on cell blocks. HER-2/*neu* positive breast cancer subtype comprised of 2 (7%) on the cell blocks (Table 3). The triple negative breast cancer characterized by ER-, PR-, HER-2/*neu*– profile was observed in 9 (30 %) of the breast cancer cases (Table 3).

Table 1: Clinical characteristics of patients with breast cancer

No of Patients	30	
Age of patients	20 - 40	11(37%)
	41 – 60	13 (43%)
	>61	6(20%)
Location of primary breast mass	Left breast	16(53%)
	Right breast	14(47%)
Location of the largest breast	Upper Outer quadrant (UOQ)	13(43%)
	Upper Inner quadrant (UIQ)	6(20%)
	Lower outer quadrant (LOQ)	5(17%)
	Lower inner quadrant (LIQ)	3(10%)
	Periareolar	3(10%)
Size of the largest breast mass	<2cm	2(7%)
	3 - 6cm	16(53%)
	7 - 10cm	6(20%)
	11 - 14cm	6(20%)
Cytodiagnosis of conventional smear	Ductal carcinoma	27(90%)
	Mucinous carcinoma	2(7%)
	Suspicious Lobular carcinoma	1(3%)
Nodal status at diagnosis	Axillary node Metastasis	7(23%)

Table 2: Receptor status based on laterality, location and size of the breast masses (n=30)

	n	ER+		PR+		HER2+	
Age of patients:							
20 - 40	11	3 (27%)		3 (27%)		0 (0%)	
41 – 60	13	6 (46%)	*p=0.279*	8 (62%)	*p=0.171*	2 (15%)	*p=0.239*
>61	6	4 (67%)		3 (50%)		2 (33%)	
Laterality of the breast mass							
Left	16	4 (25%)	*p=0.034*	4(24%)	*p=0.011*	2 (13%)	*p=0.683*
Right	14	9 (64%)		10 (71%)		2 (14%)	
Location of the breast mass							
Upper outer quandrant	13	6 (46%)		5 (38%)		2 (15%)	
Upper inner quandrant	6	3 (50%)		4 (67%)		1 (17%)	
Lower outer quandrant	5	2 (40%)	*p=0.897*	1 (20%)	*p=0.767*	1 (20%)	*p=0.360*
Lower inner quandrant	3	0 (0%)		1 (33%)		0 (0%)	
Periareolar	3	2 (67%)		3 (100%)		0 (0%)	
Size **of** the breast mass							
>2 cm	2	1 (50%)		0 (0%)		1 (50%)	
3-6 cm	16	6 (38%)		8 (50%)		1 (6%)	
7-10 cm	6	4 (67%)	*p=0.820*	3 (50%)	*p=0.908*	1 (17%)	*p=0.736*
11-14cm	6	2 (33%)		3 (50%)		1 (17%)	

ER, estrogen receptor, PR, progesterone receptor, HER-2/neu, human epidermal growth factor receptor 2
Interparametric correlation with ER,PR and HER-2/neu (**pearson correlation coefficient*)

Table 3: Classification of breast cancer cases on the basis of their ER, PR and HER-2/neu profile on cell block (n=30)

ER+, PR+, HER-2/neu–	17 (57%)
ER+, PR+, HER-2/neu+	2 (7%)
ER-, PR-, HER-2/neu+	2 (7%)
ER-, PR-, HER-2/neu–	9 (30%)

ER, estrogen receptor, PR, progesterone receptor, HER-2/neu, human epidermal growth factor receptor 2

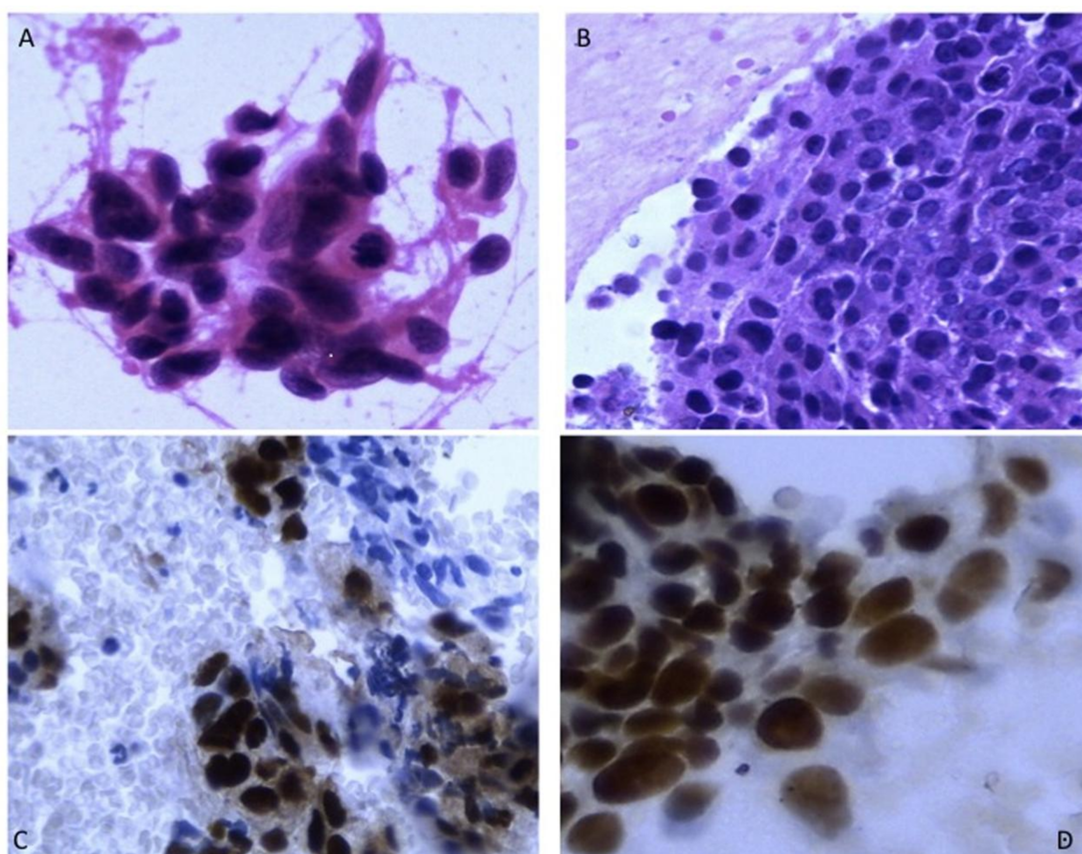

Fig. 1: A. Fine needle aspiration cytology (conventional smear) with discohesive clusters of malignant ductal cells, with marked nuclear pleormophism, hyperchromasia and coarse chromatin (haematoxylin and eosin); **B**. cell block section with clusters of malignant ductal cells, with marked nuclear pleormophism (haematoxylin and eosin). **C.** Progesterone receptor immunostaining on cellblock section. A strong positive reaction for PR is seen on the nuclei of cancer cells (proportion = 3, intensity = 3) (x400). **D** Estrogen receptor immunostaining on a cell block section. A strong positive reaction for ER is seen on the nuclei of cancer cells (proportion = 5, intensity = 3) (x400).

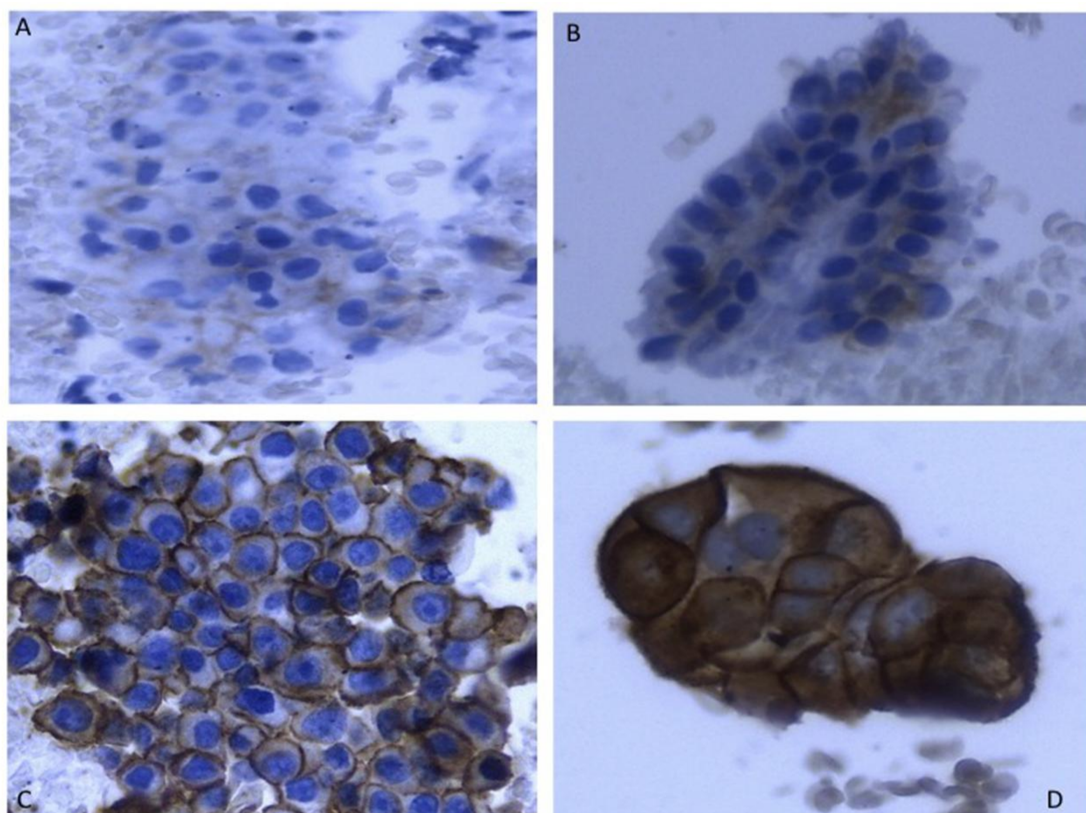

Fig.2: HER2/neu performed on cell blocks. **A,B**. Her2-negative (1+), partial weakly noncircumferential staining, X400. **C,D.** HER2/neu immunostaining on a cell block in a HER2/neu over-expressed breast cancer case. Strong complete membrane and intense staining on the tumor cells of HER2/neu (score 3+) is observed (x400).

Discussion

This study has demonstrated that it is possible to determine the expression patterns of ER, PR and HER2/*neu* in breast cancer using cell block prepared from FNAC for targeted therapy. This study reported ER positivity in 50% of breast cancer cases, while PR positivity was reported in 47% of cases. These expression patterns are within the range of earlier studies, which reported overexpression of ER/PR among African breast cancer cases with estimates ranging between 18% and 72% .[6,8,9, 14] Human epidermal growth factor receptor-2 (HER-2/*neu*) was positive in 13 % of breast cancer cases using cell block. The frequency of HER2/*neu* overexpression varies in Kenyan studies, Bird *et al* reported HER2/*neu* overexpression in 26% of breast cancer cases among women in Kenya,[6] Nyagol *et al*. 20.26%,[8] while a recent study by Sayed *et al* showed 17% of HER2/*neu* overexpression by immunohistochemistry.[9]

The current study reported a 57% proportion of the luminal A (ER+, PR+, HER2/*neu*-) profile of breast cancer using a cell block. A previous study showed a lower proportion (38%) of luminal A in a cell block.[18] While the luminal B (ER+, PR+, HER2/*neu*+) profile of breast cancer subtype comprised of 7% on cell blocks which was slightly higher than earlier study which reported 6% proportion of luminal B using a cell block.[18] The proportion (7%) of HER2/*neu* positive breast cancer subtype in our study was lower than previous work which reported a proportion of 24% using a cell block. [18] The triple negative breast cancer (TNBC) characterized by lack of expression of ER, PR and HER2/*neu* was reported in 30 % of the cases, which is similar to Kurmar *et al*., study which reported 32% proportion of TNBC in cell block. [18] Previous study from Mali reported high TNBC of 46 % of breast carcinomas using biopsies. [19]

The data of this study has demonstrated that it is possible to determine the expression patterns of ER, PR and HER-2/*neu* in breast cancer using cell block prepared from FNAC for targeted therapy. This study is important because previous studies reported simultaneous evaluation of ER and PR status by ICC on cellular material obtained by FNAC, which demonstrated high correlation with results from paraffin sections. [11] Furthermore, the use of immunocytochemistry to assess ER status on paired FNAC and paraffin embedded tissues, showed a reliable correlation, with a range of 61% to 92% rate of concordance. [20]

Therefore, this study has demonstrated that the use of FNAC samples to assess HER-2/*neu*, ER or PR status using a cell block is useful, especially where disease has metastasized and a biopsy may not be advisable. [11-13] Also use of cell blocks in developing countries with poor resources is feasible since it is cheaper than core needle biopsy, serial sections can be obtained and use of same antibodies as those of formalin fixed histological sections. [11-13] We were unable to compare with paired histological biopsies due to unavailability of most samples either from core needle biopsies or excisional biopsies. Although our preliminary data are interesting, due to lack of comparison with biopsy we were unable to control for false negative results which have been reported in previous studies. [18] Also due to small sample size used in this study confirmation of these data in a larger and independent patient population is recommended. In conclusion, immunocytochemistry performed on cellblock is a feasible method for evaluation of ER, PR and HER-2/*neu* status of breast cancer, especially when cellblock has adequate tumour cells.

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