

ORIGINAL ARTICLE

Evaluation of Biomarkers (Estrogen Receptor, Progesterone Receptor and HER-2) Immunostaining on Fine Needle Aspirates in Carcinoma Breast: A Prospective Observational Study

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HOW TO CITE THIS ARTICLE:

Sayan Kundu, Rama Saha, Jayati Chakraborty. Evaluation of Biomarkers (Estrogen Receptor, Progesterone Receptor and HER-2) Immunostaining on Fine Needle Aspirates in Carcinoma Breast: A Prospective Observational Study. Ind J of Path: Res and Practice 2025; 14(3) 97-106.

ABSTRACT

Background: Breast cancer remains one of the most prevalent cancers affecting women worldwide, with early detection and accurate biomarker evaluation playing pivotal roles in prognosis and treatment planning. Immunohistochemical analysis of biomarkers such as Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER-2/neu) on tissue samples is standard practice. However, the use of fine needle aspiration (FNA) cytology as a minimally invasive method for evaluating these biomarkers is gaining attention for its potential diagnostic utility and advantages. Aims and objectives of this study were to study the expression of biomarkers ER, PR, HER2 in FNAC smears of breast carcinoma and core needle biopsy, to evaluate whether there is a concordance or discordance in expression of these biomarkers in FNAC smears as compared to that of core needle biopsy and to assess the utility of FNAC in preoperative assessment of biomarker status in breast carcinoma.

Methods: It was a hospital based prospective study. Forty cases of breast carcinoma of which both FNAC and core needle biopsy were performed were included in our study.

Results: We studied 40 patients of histopathologically proven Breast Carcinoma. When compared to Immunohistochemistry, there was 82.5% diagnostic accuracy of Immunocytochemistry (ICC) done on FNAC for ER. For PR and HER2 diagnostic accuracy of ICC was 87.5% and 77.5% respectively.

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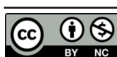
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➤ Received: 24-06-2025 ➤ Accepted: 21-07-2025



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Conclusion(s): ICC done on alcohol fixed smears shows high concordance with IHC done on CNB and may be routinely performed in all suspected cases of breast cancer.

KEYWORDS

• Breast Carcinoma • Fine Needle Aspiration Cytology • Estrogen Receptor • Progesterone Receptor • HER2

INTRODUCTION

Breast cancer is the most common malignancy in Indian women and its incidence is continuously increasing in recent years.¹ There is a potential epidemic of breast cancer in the next decade. Breast cancer is also the most common cause of cancer death in women. Breast cancer is the most frequently diagnosed cancer in women worldwide.

In many regions, breast cancer incidence has overtaken the carcinoma cervix, mostly due to changing life style trends. According to IARC, collectively, USA, India and China account for almost one third of global breast cancer burden. Among these countries, India have the maximum number of deaths due to breast cancer. In USA there has been constant

decrease in deaths due to breast cancer. The reason behind this is larger proportion of women present at the early stage of the disease. It is important to diagnose breast cancer at its early stage to prevent cancer death and increase survival rate.

Prognosis of breast cancer depends on stage of the disease, histologic type, histological grade and Estrogen receptor (ER), Progesterone receptor (PR) and HER2/neu expression. Breast cancer is a heterogeneous disease having many morphological and molecular genetic subtypes. Molecular genetics by gene expression profiling (GEP) has classified breast cancer into four distinct subtypes Luminal A, Luminal B, HER2/neu and basal like. Expression of biomarkers classify these tumors as follows:²

Immuno-profile	Luminal A	Luminal B	HER2/neu	Basal Like
ER, PR	ER and/or PR +ve	ER and/or PR +ve	ER -ve PR -ve	ER -ve PR -ve
HER2 and others	HER2 -ve. Low Ki67<14%	HER2 +ve. or HER2 -ve Ki67 >14%	HER2 +ve	HER2 -ve CK5/6 and/or EGFR +ve

Aims and objectives of this study were to study the expression of biomarkers ER, PR, HER2 in FNAC smears of breast carcinoma and core needle biopsy, to evaluate whether there is a concordance or discordance in expression of these biomarkers in FNAC smears as compared to that of core needle biopsy and to assess the utility of FNAC in preoperative assessment of biomarker status in breast carcinoma.

MATERIALS AND METHODS

It was a Hospital based prospective study that commenced after obtaining permission from institutional ethical committee. Forty cases of breast carcinoma of which both FNAC and core needle biopsy were performed between February 2016 to October 2017 were included in our study. The sections with fixation artefact were excluded from the

study.

Cytologically diagnosed breast carcinoma patients who were undergoing core needle biopsy were selected irrespective of age. The site and size of the lump and axillary lymph node enlargement, if present were noted by clinical examination.

The data were collected from the cytology request form and directly from the patient and relatives of the patient. Data was collected in a case record form which was filled in for every specimen. The form included clinical findings such as tumor size, tumor site, and lymph node status.

Immunocytochemistry was done for ER, PR and HER2 on FNAC smears and IHC for ER, PR and HER2 done on core needle biopsy sections.

FNAC was performed using 21G needle and 2-3 passes were done from each case. In cases of breast carcinoma presenting with palpable axillary lymph node, FNAC of the lymph node was performed. FNAC slides were stained by Leishman Giemsa and Papanicolaou stain. Slides were examined and cases positive for breast carcinoma were selected for core needle biopsy. ICC smears were fixed in 95% alcohol for a period of 30 minutes and further ICC was performed.

Core needle biopsy of the breast lump was performed by 14G disposable core biopsy instrument having penetration depth 22 mm. Specimen of core needle biopsy was fixed in 10% neutral buffered formalin for 24 hours and then stained with haematoxylin and eosin.

Four to five micrometer thick sections were cut from the paraffin block for histopathology and IHC.

The following primary antibodies were used for IHC

Immunostain	Clone
ER	Rabbit monoclonal antibody SP1
PR	Rabbit monoclonal antibody Y85
HER2	Rabbit monoclonal antibody SP3

The percentage of immunoreactive cells was determined by visual estimation and quantification. Quantification was done by Allred scoring system.

Allred scoring for estrogen and progesterone receptor evaluation.

Positive Cell %	Proportion Score	Intensity	Intensity Score
0	0	None	0
<1	1	Weak	1
1 - 10	2	Intermediate	2
11 - 33	3	Strong	3
34 - 66	4		
≥ 67	5		

Scores of 0-2 are considered to be Negative. Scores 3-8 are considered to be Positive.

Known positive and negative sections for ER and PR included in the study as external control for IHC, Known HER2 positive validated by FISH served as positive control for HER2 for IHC. FNAC samples of fibroadenoma served as positive control of ER and PR for

immunocytochemistry (ICC).

Reporting results of HER2 testing by IHC according to CAP protocol of Template for Reporting Results of Biomarker Testing of Specimens from Patients with Carcinoma of the Breast.³

Result	Criteria
Negative (Score 0)	No Staining Observed OR Incomplete faint / barely perceptible membrane staining ≤ 10% of Invasive Tumor Cells
Negative (Score 1+)	Incomplete, faint / barely perceptible membrane staining >10% of Invasive Tumor Cells
Equivocal (Score 2+)	Incomplete and / or weak to moderate circumferential membrane staining in > 10% of Invasive Tumor Cells OR Complete, intense circumferential membrane staining in ≤10% of Invasive Tumor Cells
Positive (Score 3+)	Complete, intense circumferential membrane staining in >10% of Invasive Tumor Cells

In this study, for reporting of ICC, the same criteria as of IHC was used for ER, PR and HER2.

All procedures performed in the current study were approved by IRB and/or national research ethics committee (ESIPGI/MKT/IEC/5/2016) in accordance with the 1964 Helsinki declaration and its later amendments.

RESULT

A prospective study was performed with forty cases of FNAC diagnosed breast malignancy presenting to the Department of Pathology, ESI PGIMS, Manicktala for core needle biopsy of the lesion.

IHC on formalin fixed paraffin embedded tissue sections from core needle biopsy (CNB)

and ICC on alcohol fixed FNAC smears were done on every case. IHC of core needle biopsy was considered gold standard and the result of ICC was evaluated and compared with that of IHC on CNB. In the present study the mean age of the study population was 51.98 years with an age range of 31 to 85 years. Maximum number of the cases was seen in the age range of 41-50 years (40% cases) (Table 1).

Table 1: Age Distribution

Age	No. of cases (N=40)
31-40	6 (15%)
41-50	16 (40%)
51-60	10 (25%)
61-70	5 (12.5%)
>70	3 (7.5%)
Total	40 (100%)

In the present study incidence of breast carcinoma was found to be slightly higher in left breast than right breast. (Table 2)

Table 2: Frequency distribution table of tumor laterality

Laterality	No of cases (n=40)
Right	19 (47.5%)
Left	21 (52.5%)

Upper outer quadrant was most commonly involved by breast carcinoma. (Table 3)

Table 3: Frequency distribution table of tumor site:

Site	No of cases (N=40)
Central	10 (25%)
Upper Outer	17 (42.5%)
Upper Inner	4 (10%)
Lower Outer	6 (15%)
Lower Inner	3 (7.5%)

Majority of the cases presented at T2 tumor stage (47.5%), however a significant proportion of cases presented with skin involvement and staged as T4 (30%). (Table 4)

Table 4: Frequency distribution table showing distribution of cases according to T stage (Tumor size):

Tumor Stage	No. of Cases (N=40)
T1	1 (2.5%)
T2	19 (47.5%)
T3	8 (20%)
T4	12 (30%)

In all cases of breast carcinoma presenting with palpable axillary lymph node, FNAC

of the lymph node was performed and FNA smears were examined to detect any lymph node metastasis. Majority of the cases presented with lymph node involvement (55%). (Table 5)

Table 5: Distribution of cases according to lymph node Involvement:

Lymph node metastasis	No of cases (n=40)
Present	22 (55%)
Absent	18 (45%)

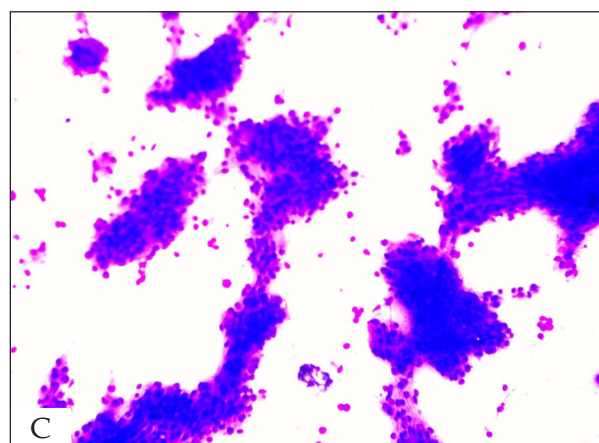
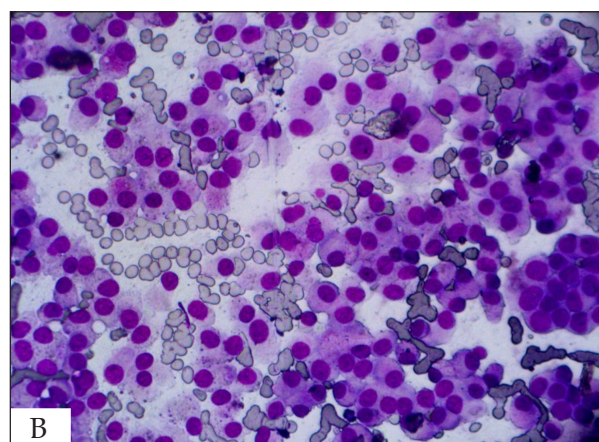
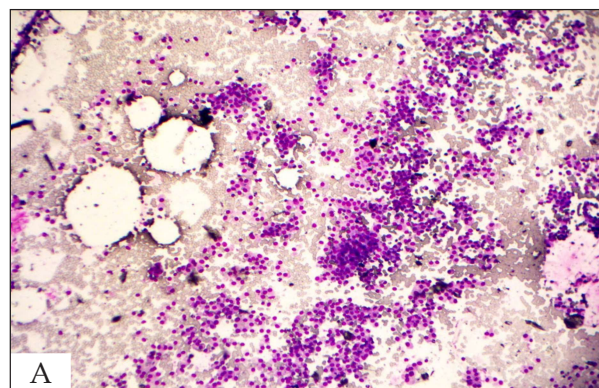


Figure 1: FNAC smears of Breast Carcinoma:

A. Invasive Ductal Carcinoma NOS Leishman Giemsa stain 10x.
B. Invasive Ductal Carcinoma NOS Leishman Giemsa stain 40X.
C. Papillary Breast Carcinoma Leishman Giemsa stain 10X.

Both in FNAC and CNB the most common tumor type was Infiltrating ductal carcinoma of no special type (IDC NST). (Figure 1) One case showed features of papillary carcinoma and two cases showed mucinous differentiation in both FNAC and CNB. The cases were later diagnosed as Invasive papillary carcinoma and Mucinous carcinoma respectively on MRM specimen.

On Immunohistochemistry of core needle biopsy of the 40 cases, 47.5% cases were found to be ER-Positive, 67.5% cases were PR-Positive and 45% cases were HER2 Positive. HER2 was equivocal in 5% of the cases. (Table 6) (Figure 2)

Table 6: ER, PR, HER2 status on CNB (IHC):

Biomarker Status	No. of Cases (N=40)
Estrogen Receptor Status:	
ER Positive	19 (47.5%)
ER Negative	21 (52.5%)
Progesterone Receptor Status:	
PR Positive	15 (37.5%)
PR Negative	25 (62.5%)
HER2 Receptor Status:	
Negative	20 (50%)
Equivocal (2+)	2 (5%)
Positive (3+)	18 (45%)

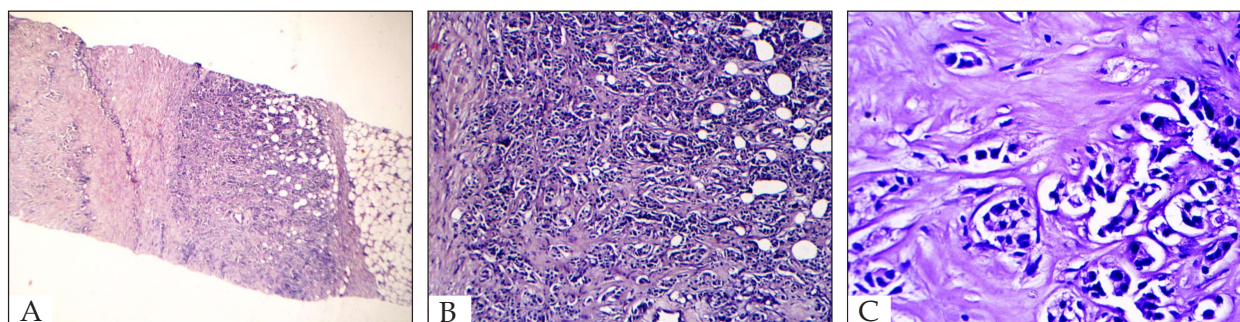


Figure 2: Core Needle Biopsy of Breast Carcinoma A. H & E stain 4x, B. H & E stain 10X, C. H & E stain 40x

The most predominant molecular subtype was Luminal A (40%). (Table 7)

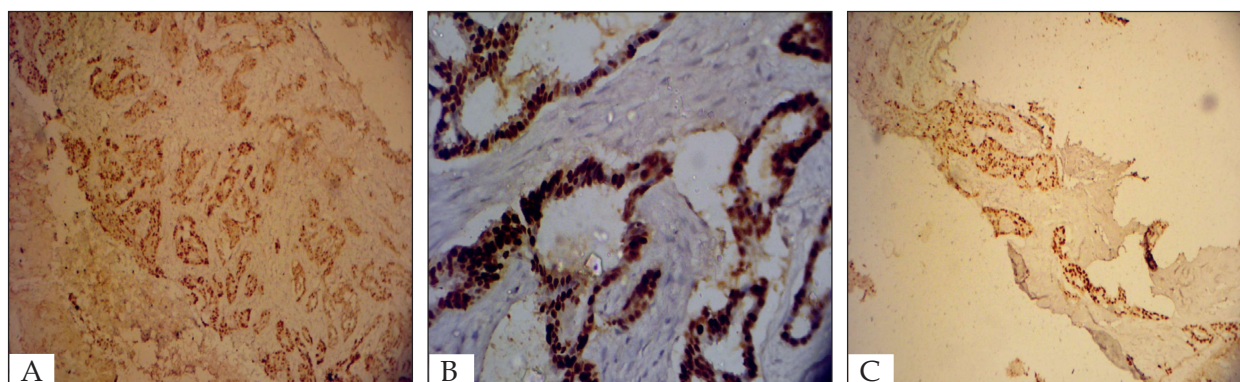
Table 7: Molecular subtype of breast carcinoma by IHC

Subtype	Number of Cases (N=40)
Luminal A	16 (40%)
Luminal B	5 (12.5%)
HER2/neu	13 (32.5%)
Triple Negative	6 (15%)

Findings of Immunocytochemistry of ER, PR and HER2 on FNAC smears (Figure 3 & 4) are presented in Table 8.

Table 8: ER, PR, HER2 status on FNAC smear (ICC)

Biomarker Status	No of Cases (N=40)
Estrogen Receptor Status:	
ER Positive	14 (35%)
ER Negative	26 (65%)
Progesterone Receptor Status:	
PR Positive	12 (30%)
PR Negative	28 (70%)
HER2 Receptor Status:	
Negative	28 (70%)
Equivocal (2+)	1 (2.5%)
Positive (3+)	11 (27.5%)



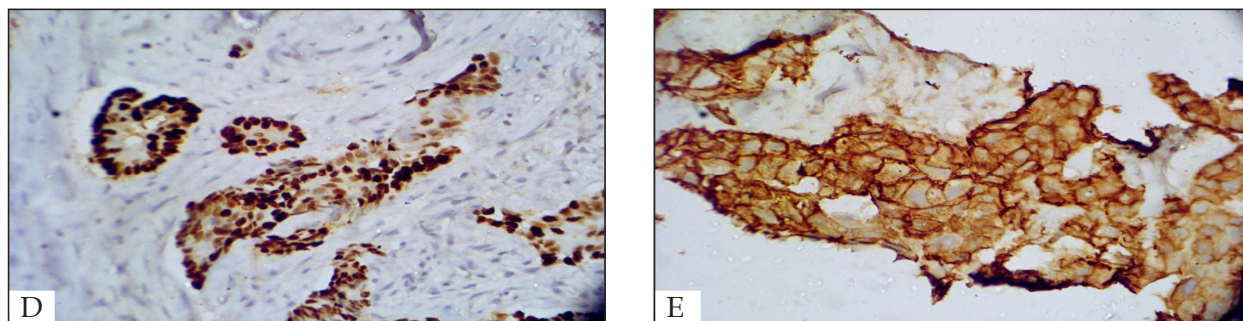


Figure 3: Immunohistochemistry on Core Needle biopsy:
A. ER Positive 10X, B. ER Positive 40x, C. PR-Positive 10x, D. PR-Positive 40X, E. HER 2 (3+) 40X

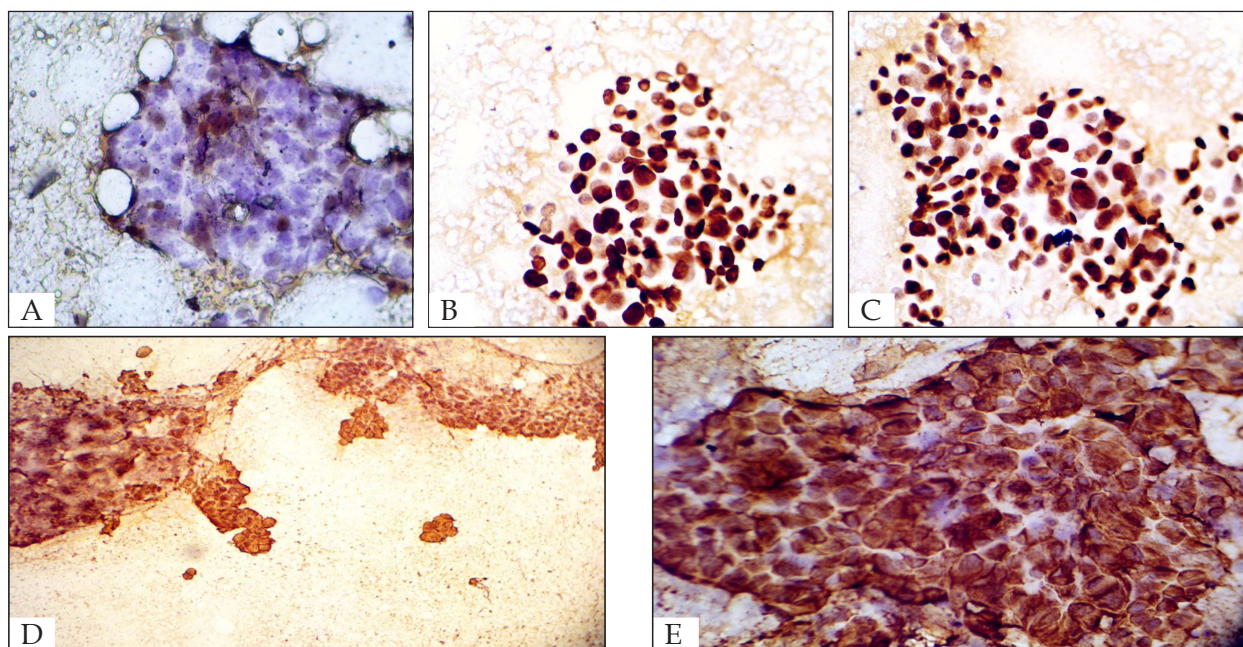


Figure 4: Immunocytochemistry on FNAC material of Breast Carcinoma.
A. ER-Negative 40X, B. ER-Positive 40X, C. PR - positive 40X, D. HER 2 (3+) 10X, E. HER 2 (3+) 40X.

In this study, IHC performed on core needle biopsy of breast lump was considered as Gold Standard for evaluation of ER, PR and HER2 status. The sensitivity, specificity, positive predictive value and negative predictive value of ICC on FNAC smear were determined accordingly.

Table 9: IHC for ER on CNB v/s ICC for ER on FNAC

ER Status	CNB Positive	CNB Negative
FNAC Positive	13	1
FNAC Negative	6	20

1) IHC for ER on CNB v/s ICC for ER on FNAC (Table 9):

$$\text{Sensitivity} = 13 \times 100 / (13 + 6) \% = 68.42\%$$

$$\text{Specificity} = 20 \times 100 / (20 + 1) \% = 95.23\%$$

$$\text{Positive predictive value} = 13 \times 100 / (13 + 1) \% = 92.85\%$$

$$\text{Negative predictive value} = 20 \times 100 / (20 + 6) \% = 76.92\%$$

$$\text{Diagnostic accuracy} = (13 + 20) \times 100 / 40 \% = 82.5\%$$

Cross tabulation of FNAC- ICC and CNB IHC for ER shows p value 0.00025. The result is significant at $p < 0.05$.

2) IHC for PR on CNB v/s ICC for PR on FNAC (Table 10):

Table 10: IHC for PR on CNB v/s ICC for PR on FNAC

PR Status	CNB Positive	CNB Negative
FNAC Positive	11	1
FNAC Negative	4	24

Sensitivity = $11 \times 100 / (11 + 4) \% = 73.33\%$
 Specificity = $24 \times 100 / (24 + 1) \% = 96\%$
 Positive predictive value = $11 \times 100 / (11 + 1) \% = 91.66\%$
 Negative predictive value = $24 \times 100 / (24 + 4) \% = 85.71\%$
 Diagnostic accuracy = $(11 + 24) \times 100 / 40 \% = 87.5\%$

Cross tabulation of FNAC- ICC and CNB IHC for PR shows the p-value is $< .00001$.

The result is significant at $p < 0.05$.

3) IHC for HER2 on CNB v/s ICC for HER2 on FNAC (Table 11):

Table 11: IHC for HER2 on CNB v/s ICC for HER2 on FNAC

HER2 Status	CNB Positive	CNB Negative	CNB Equivocal
FNAC Positive	11	0	0
FNAC Negative	6	20	2
FNAC Equivocal	1	0	0

Sensitivity = $11 \times 100 / (11 + 6) \% = 64.70\%$
 Specificity = $20 \times 100 / (20 + 0) \% = 100\%$
 Positive predictive value = $11 \times 100 / (11 + 0) \% = 100\%$
 Negative predictive value = $20 \times 100 / (20 + 6) \% = 76.92\%$
 Diagnostic accuracy = $(11 + 20) \times 100 / 40 \% = 77.5\%$

DISCUSSION

Breast cancer is one of the most frequently diagnosed cancers in India and its incidence is rising, especially in urban areas. Early diagnosis and prompt treatment is very important to reduce the cancer burden and provide longer disease free survival. FNAC and CNB are two most widely used diagnostic modalities for breast carcinoma diagnosis. Preoperative assessment of biomarker status is very important in locally advanced breast carcinomas who are the candidates for neo-adjuvant chemotherapy (NACT). Currently CNB is the preferred method of determining the ER, PR and HER2 status in breast carcinoma preoperatively. FNAC is a cheap, cost effective, reliable diagnostic modality for establishing diagnosis of breast carcinoma but can also be used to assess biomarker status.

Age:

Age is one of the important risk factors for development of breast cancer and an important factor for management of the cases. According to National Cancer Registry Programme, peak age of breast cancer in India is 50-69 years.^[1] In our study, the age range was found to be 31-85 years with mean age 51.98 years and most common age group 41-50 years.

Sex:

Male population comprises only 1% of the all diagnosed breast carcinomas. However, in our study, no male breast carcinoma was found.

Site:

The current study shows upper outer quadrant is the most common site which is in concordance with the literature.^[4]

Tumor Size:

The measured greatest dimension of the tumor is one of the most significant prognostic markers. It determines the tumor (T) component of the currently used TNM staging of breast carcinoma. Survival decreases with increasing tumor size.^[2] In the present study, the most common tumor size was between 2 cm to 5 cm.

T Stage:

T stage is an important prognostic factor in breast carcinoma. It is largely dependent on maximum tumor dimension (Tumor size). However involvement of skin or chest wall by the tumor of any size upgrades the t stage to T4 stage. In our study the commonest t stage was T2 (47.5%), however a large proportion of cases (30%) presented with skin involvement and thus at T4 stage.

Nodal Status:

Nodal status is the most important prognostic factor in breast carcinoma. The present study documented that majority of the patients (55%) presented with lymph node metastasis at the diagnosis. Data from developed countries showed that most of the breast cancer patients do not have any metastasis at diagnosis. Indian studies, however documented higher percentage of lymph nodal involvement in breast cancer patients.¹

Histologic type:

Histology is another important prognostic factor. The most prevalent histological type as documented in the literature is IDC-NST (Invasive ductal carcinoma no special type) accounting for 83.09%.⁵ In the present study, two cases (5%) showed mucinous differentiation in FNAC and CNB and one case (2.5%) showed papillary pattern. Further mastectomy supported the diagnosis of FNAC and CNB in all three cases. All the other cases (92.5%) were of invasive carcinoma of no special type (IDC NST) on both FNAC and CNB findings.

Hormone receptor status and Immunohistochemistry on CNB:

Hormone receptors namely ER and PR expression is an important prognostic factor in breast carcinoma. HER2 expression is also an important prognostic factor in breast carcinoma and overexpression of HER2 is associated with worse prognosis as compared to ER/PR positive HER2 negative tumors. Triple negative breast carcinoma has the worst overall survival.² The present study shows that 47.5% cases were positive for ER and 37.5% cases were positive for PR which is in keeping with the studies performed in India.^{5,6}

Immunocytochemistry on FNAC smears:

It also shows that ICC done in FNAC smears have satisfactory correlation with IHC done on CNB. In the present study ER and PR shows 82.5% and 87.5% concordance with the IHC done on CNB respectively. The sensitivity of ICC for ER and PR is 68.42% and 73.33% respectively. Specificity, positive predictive value and negative predictive value for ER are 95.23%, 92.85% and 76.92% respectively. For PR on ICC, specificity, PPV and NPV lies at 96%, 91.66% and 85.71% respectively. For both ER and PR, there is statistically significant correlation for ICC when compared to IHC result.

Six out of nineteen cases were false negative for ER on ICC and 4 out of 15 cases were false negative for PR on ICC. This can be attributed to many reasons. Low cellularity of the smear is an important factor. Fixation plays a major role and improper fixation and type of fixative used may affect the result of ICC. A variety of fixatives have been used in previous studies like periodate lysine paraformaldehyde

at room temperature, a formalin acetone sequence at -10°C, 95% alcohol, 10% buffered formalin at room temperature, Mixture of ethanol, isopropanol and polyoxyethylene.⁷ An ideal fixative is that which can be used for both morphological examination and immunocytochemistry. Another important cause of low sensitivity can be inadequate antigen retrieval.^[8] Loss of smear and loss of cells during antigen retrieval is a major drawback in ICC. In this study, on three instances, smear was lost during antigen retrieval and the study was performed on reserved slides. Other attributable causes can be tissue heterogeneity, inadequate tissue sampling and the methodological pitfalls. In the present study HER2 expression by tumor cells on ICC shows 64.70% sensitivity, 100% specificity and positive predictive value. The diagnostic accuracy for HER2 is 77.5%.

Zeng *et al* conducted a similar study in Breast carcinoma metastatic to bone and they found that concordance for ER, PR and HER2 was 89%, 67% and 93%, respectively between FNA-CB and CNB pairs from 27 patients.⁹

One similar study was done by Toi *et al* which showed that the Sensitivity of ICC on FNAs for ER, PR, and Her-2neu was 49%, 28.8%, and 46%, respectively, while specificity was 84.5%, 90.6%, and 86.6%, respectively, with a fair agreement on kappa statistics. Her-2neu positivity on CNB versus FNA had a moderate agreement.¹⁰

Another study was done by Bansal *et al* on Comparative Evaluation of Immunohistochemical Expression of Estrogen Receptor, Progesterone Receptor and HER2 in Fine Needle Aspiration Cell Blocks and Surgical Biopsies in Primary Breast Carcinoma. Immunostaining assessment on cell block and their corresponding tumor tissues showed a good concordance: ER (92%), PR (92%) and HER2 (93.75%). Taking histology as the final outcome, the sensitivity of ER, PR and HER2 on cell block was 92.30%, 86.36% and 91.67%, respectively, while specificity was 92.85%, 96.43% and 94.44%, respectively.¹¹

Kimambo *et al* conducted a study on evaluation of Estrogen Receptor Immunohistochemistry on cell blocks from Breast cancer patients and found out that overall ER IHC concordance was 90.3% and positive concordance was 87.9% ($\kappa = 0.81$, $P = .69$).¹²

Francis *et al* conducted another study on Hormone Receptors and Human Epidermal Growth Factor (HER2) Expression in Fine Needle Aspirates from Metastatic Breast Carcinoma and its role in patient management and found out that ER, PR, and HER2 by IHC in cell blocks of metastatic lymph nodes were reliable. Change in receptor (34.2%) and HER2 status (21.9%) was documented.¹³

Pinto *et al* reviewed the literature on published articles regarding the use of ICC in FNAB samples of breast and concluded that both diagnostic and theranostic markers may be performed in all types of cytological material.¹⁴

Salama *et al* conducted a study on the digital validation of breast biomarkers (ER, PR, AR, and HER2) in cytology specimens using three different scanners and reached the conclusion that digital images are reliable for breast IHC assessment in CB and offer similar reproducibility to microscope reads.¹⁵

There was a study conducted in Kerala, India which showed, in case of ER there was a moderate agreement between ICC and IHC ($\kappa = .428$, $P = 0.005$) and no agreement was seen in case of PR ($\kappa = .073$, $P = 0.625$).¹⁶

Nair *et al* also drew the conclusion that immunocytochemistry (ICC) using ER done on FNAC samples is of high diagnostic accuracy.¹⁷

Pareja *et al* did a similar study in metastatic breast carcinoma and found out that ER, PR and HER2 concordance rates between Primary Breast Carcinoma and Metastatic Breast Carcinoma Cell Blocks are similar to those reported in paired surgical specimens.¹⁸

CONCLUSION

In conclusion, our study shows that when compared to IHC, there is 82.5% diagnostic accuracy of ICC for ER. For PR and HER2 diagnostic accuracy of ICC is 87.5% and 77.5% respectively. Hence, ICC done on alcohol fixed smears shows high concordance with IHC done on CNB and may be routinely performed in all suspected cases of breast cancer.

Funding Declaration: There was no Funding.

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