Immunocytochemistry using Liquid-based Cytology: A Tool in Hormone Receptor Analysis of Breast Cancer

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Abstract

**Context:** Efficacy of immunocytochemistry (ICC) in determining molecular biomarkers like estrogen receptor (ER), progesterone receptors (PRs), and human epidermal growth factor receptor-2 (HER2). **Aims:** To evaluate biomarkers using ICC in breast cancer as per American Society of Clinical Oncology/College of American Pathology (ASCO/CAP) guidelines. **Settings and Design:** The study was conducted over a period of 2 years from September 2012 to August 2014 and is the first such study in eastern India. **Materials and Methods:** Fine needle aspiration cytology (FNAC) was done for suspected cases of breast cancers and slides were prepared using ThinPrep (TP) technology of liquid-based cytology (LBC) for ICC and corresponding biopsy specimens were processed as formalin fixed paraffin embedded (FFPE) sections for comparison. Both the LBC slides and tissue sections were subjected to immunostaining for ER, PR, and HER2. **Statistical Analysis Used:** Statistical analysis done by Wilconxon Signed rank test on the SPSS program, Chicago, Illinois, USA. The results of ICC and IHC were compared by evaluation of sensitivity, specificity, kappa-value (k-value), positive predictive value (PPV), and negative predictive value (NPV). **Results:** The comparison of ICC with immunohistochemistry (IHC), ER, and PR showed very good correspondence rate, sensitivity, specificity, NPV, PPV, and agreement with k-value; whereas for HER2 the results were only good. **Conclusion:** ICC using LBC can be a useful tool in assessing biomarkers in advanced cases of breast cancer where surgery is not possible or cases where ASCO/CAP guidelines for management are not followed.

**Keywords:** Biomarkers, carcinoma, immunohistochemistry, ThinPrep

Introduction

Invasive breast cancer is the most common cancer in women. Since mid-1990s, the use of three basic molecular biomarkers ER, PR, and HER2 has revolutionized the approach to management and prognosis of breast cancers.[1] Although evaluation of the above molecular receptor status is routinely done on tissue sections by immunohistochemistry (IHC), they can also be assayed by immunocytochemistry (ICC). ICC is advantageous for patients undergoing preoperative chemotherapy and those for whom surgery is contraindicated because of advanced inoperable tumors, metastasis, recurrence, and malignant effusion.[2] Literature results reveal that commercially available LBC preparations give high quality specimens for immunostaining for ICC. LBC (TP) slides provide cells in monolayers, lack background staining, and give better spreading and visualization of cell morphology.[2,3] Re-determination of receptor status in disease progression, recurrence, and metastasis can be done with ease and rapidity using CC done on samples processed by TP cytosmears.[4] Though ICC characterization of breast carcinoma is recognized as an effective procedure, there are still very limited number of studies that compare the result obtained by FNAC with those of tissue sections. This study evaluates biomarkers using ICC in breast cancer as per ASCO/CAP guidelines and compares the same with IHC by statistical analysis. ICC using LBC can be a useful tool in assessing biomarkers in advanced cases of breast cancer where surgery was not possible or cases where ASCO/CAP guidelines for management are not followed.
Materials and Methods

The study was conducted in the Cytology Outpatient Department (OPD) in the period between September 2012 and August 2014 and is the first such study in eastern part of India.

Inclusion criteria of the study were i) patients newly diagnosed with invasive carcinoma of breast by FNAC and subsequently confirmed by biopsy and ii) availability of both LBC slides and corresponding FPPE tissue blocks of each patient for ICC and IHC.

Exclusion criteria of the study were i) patients lost to follow up after FNAC, ii) inadequate smears (<100 cells), iii) poor quality smear, and iv) uncooperative patients. FNAC was done in suspected breast carcinoma cases using a 22-gauge needle. Two passes were made to get adequate material. Alcohol fixed smears were prepared for routine diagnosis and were stained with hematoxylin and cosin (H and E) and Papanicolaou (PAP) stains. Cytologically, diagnosed cases of carcinoma breast were subjected to a second FNAC and processed by LBC technique for subsequent ICC. A total of three TP slides were prepared for ICC evaluation. For ICC, liquiPrep™ TP Kit (Cytyc Corporation, Marlborough, Massachusetts) was used. Smears were prepared by the basic steps of collection, concentration, and cellular encapsulation and adherence, where the slides were made from solution on poly-L-lysine coated slides. Collection consists of tissue aspiration and transferring the contents of the syringe by gently expelling into a collecting tube containing about 8 ml of preservative solution (denatured ethanol alcohol and water). Collection is followed by concentration in which specimen is centrifuged for 30 min and finally encapsulation and adherence using cellular base solution (alcohol and water).

Both LBC and tissue section slides were stained using similar protocol as per peroxidize-antiperoxidase technique of Biogenex with monoclonal antibodies (rabbit clone EP1 for ER, EP2 for PR, and EP3 for HER2) using antigen retrieval method, where unmasking of antigen sites in the smears was done by proteolytic enzyme digestion, heat mediated method, and mixed. Antigen retrieval solution was prepared using Tris Buffer at pH 9, which constitutes Tris 6.2 g, disodium EDTA 0.75 g, and deionized water 1,000 ml. Then wash buffer, phosphate buffer saline (PBS) was prepared using sodium dihydrogen phosphate 3.4 g, disodium hydrogen phosphate 12 g, sodium chloride 8.5 g, deionized water 100 ml. Preparation of hydrogen peroxide (H₂O₂) buffer was done using deionized water 97 ml and 6% H₂O₂ 15 ml. Antigen retrieval was performed in conventional microwave at 480 W for three cycles of 5 min each, 640 W for two cycles of 5 min each with antigen retrieval solution in heat resistant plastic container. It was allowed to cool down and washed buffer was poured into it for 2 min, repeated three to four times. Neutralization of tissue endogenous peroxide was done using peroxidase block for 12 min, washed in PBS buffer for 2 min four times, incubated in power block for 12 min, incubated in primary antibody for 2 min four times, incubated in superenhancer for 25 min, washed in PBS buffer for 2 min five times. Then supersensitivity label was added for 30 min, washed in PBS buffer for 2 min for five times, Diamino benzene (DAB) working solution was applied for 5 min (DAB chromogen two drops, DAB buffer 1 ml, 6% H₂O₂ one drop). Slides were rinsed in water for 5 min, counter stained with hematoxylin for 1 min; slides were rinsed in water for 5 min. Dehydration was done in graded alcohols 70%, 80%, and absolute for 5 min each. Then slides were dried and passed into two changes of xylene for 10 min each.

The ICC samples were considered adequate if at least 100 neoplastic cells were present on the TP slide.[2] Breast and endometrial tissue were taken as positive controls.

The IHC results of the corresponding tissue sections received in the form of core biopsy and modified radical mastectomy specimen (MRM) were processed as formalin fixed paraffin embedded (FFPE) sections for comparison. Five slides were prepared, two for H and E staining and three poly-L-lysine coated slides for IHC.

Scoring of the ICC slides was done by Allred scoring system (ASS) for nuclear markers ER and PR. It was interpreted as negative or positive based on total score calculated by summing up proportional score (PS) and intensity score (IS) as depicted in Table 1.

Scoring of the IHC slides were done by Quick scoring system (QSS) for nuclear markers ER and PR. It was interpreted as negative or positive based on total score calculated by summing up score of proportion (SP) and score of intensity (SI) as depicted in Table 2.

For HER2 of both ICC and IHC, scoring (0, 1+, 2+, 3+) was based on membrane staining pattern and interpreted as negative for scores 0 and 1+, positive for score 3+, and equivocal for score 2+ as depicted in Table 3.

### Table 1: Allred scoring system (ER, PR) for ICC

| Proportion score (PS) | % Positive cells | Intensity score (IS) | Intensity of positivity | Total score (TS): PS + IS | Interpretation |
|-----------------------|------------------|----------------------|------------------------|---------------------------|---------------|
| 0                     | 0                | 0                    | None                   | 0.2                       | Negative      |
| 1                     | >0 and ≤1        | 1                    | Weak                   | 3-8                       | Positive      |
| 2                     | >1 and ≤10       | 2                    | Intermediate           |                           |               |
| 3                     | >10 and ≤33      | 3                    | Strong                 |                           |               |
| 4                     | >33 and ≤66      |                      |                        |                           |               |
| 5                     | >66              |                      |                        |                           |               |
RESULTS

In an observational study of 60 patients, statistical analysis of the results for sensitivity, specificity, PPV, and NPV was done using Wilcoxon Signed rank test on the SPSS program, Chicago, Illinois, US. The degree of agreement between ICC and IHC was calculated by Cohen’s kappa test.

Of 60 cases 45 were evaluated as eight were lost to follow-up, four cases had poor quality smear and three showed inadequate cellularity. In ICC, of 45 cases, 27 (60%) were ER positive, 24 (53%) were PR positive, and 17 (38%) were HER2 positive [Figure 1]. The amount of ductal carcinoma in situ (DCIS) on the 45 FFPE tissue samples was also evaluated: 36 cases did not show any in situ component, whereas five cases had a DCIS <25% of the whole lesion; only four cases showed an in situ component >25% of the whole lesion. In IHC, ER was positive in 30 (67%) cases, PR positive in 26 (58%) cases, and HER2 positive in 14 (31%) cases. Total positive staining was higher in IHC compared to ICC for both ER and PR, but for HER2 staining with ICC showed more positive results [Table 4]. Positivity was based on ASS (ER, PR for ICC), QSS (ER, PR for IHC), and HER2 scoring system for both ICC and IHC.

The concordance rate (measure of agreement between two studies) for ER between ICC and IHC was 84%. Sensitivity for ICC was 83.3%, specificity was 86.7%, PPV was 92.6%, and NPV was 72.2% taking 95% confidence interval. Kappa value showing the agreement between the two methods was 0.81 and a P value less than 0.01 indicates a very good agreement (Cohen’s Kappa value of 0.81–1 is very good, 0.61–0.8 is good, 0.41–0.6 is moderate, 0.21–0.4 is fair, and <0.2 is poor agreement) between ICC and IHC [Table 5].

The concordance rate of PR of ICC to IHC was 91%. The sensitivity for ICC was 88.5%, specificity was 94.7%, PPV was 95.8%, and NPV was 85.7%. Cohen’s kappa value was 0.82 showing again a very good agreement, which is statistically significant [Table 5].

For HER2, concordance rate for ICC and IHC was 76%. The sensitivity was 71.4%, specificity was 77.4%, PPV was 58.8%, and NPV was 85.7%. Kappa value was 0.62, showing good agreement [Table 5]. High value of sensitivity, specificity, and concordance rules out the possibility to a greater extent of staining artifacts due to use ethanol-based preservative solution for LBC, which at times causes mechanical damage of biological membranes.

DISCUSSION

FNAC is a minimally invasive and cost-effective method for diagnosing cancer of breast and obtaining tumor cells for prognostic and predictive biomarker study. Thus, FNAC can easily provide material for assessment of ER, PR, and HER2 status. The evaluation of these markers is beneficial for therapeutic purposes. In advanced and inoperable cases of breast cancer, ER and PR status is important for making a decision about hormonal therapy. Also, HER2 status is important to make a decision about chemotherapy.

Table 2: Quick scoring system (QSS) for IHC, ER, and PR

| Score for proportion | Score for intensity |
|----------------------|---------------------|
| 0=no staining        | 0=no staining       |
| 1 ≥ 0 and ≤1% nuclei staining | 1=weak staining   |
| 2 ≥ 1 and ≤10% nuclei staining | 2=moderate staining |
| 3 ≥ 10 and ≤33% nuclei staining | 3=strong staining |
| 4 ≥ 33 and ≤66% nuclei staining |                   |
| 5 ≥ 66 and ≤100% nuclei staining |                   |

Maximum score=8 score ≤2 negative score >2 positive

Table 3: HER2 scoring system (ICC and IHC)

| Staining pattern                                      | Score | Assessment |
|------------------------------------------------------|-------|------------|
| No staining observed or incomplete, faint/barely perceptible membrane staining in ≤10% of invasive tumor cells | 0     | Negative   |
| Incomplete, faint/barely perceptible membrane staining in >10% of invasive tumor cells | 1+    | Negative   |
| 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 | 2+    | Equivocal  |
| Complete, intense, circumferential membrane staining in >10% of invasive tumor cells | 3+    | Positive   |

Table 4: Analysis of ER, PR, HER2 receptor expression

| Receptor type | IHC +ve | IHC −ve | Total |
|---------------|---------|---------|-------|
| ER            |         |         |       |
| ICC           | 25      | 2       | 27    |
| −ve           | 5       | 13      | 18    |
| Total         | 30      | 15      | 45    |
| PR            |         |         |       |
| ICC           | 23      | 1       | 24    |
| −ve           | 3       | 18      | 21    |
| Total         | 26      | 19      | 45    |
| HER2          |         |         |       |
| ICC           | 10      | 7       | 17    |
| −ve           | 4       | 24      | 28    |
| Total         | 14      | 31      | 45    |

Figure 1: ICC of breast (a, b) for ER and PR showing strong nuclear positivity (×400), (c) ICC for HER2 showing membrane positivity (×400)
breast carcinoma, FNAC can be an useful tool to obtain cells for diagnosis.\cite{5} Therefore, it is necessary to validate a reliable method for evaluation of baroreceptor status on alcohol-fixed samples and corresponding FFPE tissue samples, which is considered the gold standard as per ASCO/CAP guidelines.\cite{6,7}

In our study, we have used TP method to perform IHC reactions on FNAC material. It has been demonstrated that processing of FNAC samples for LBC is useful for standardization of cytological samples.\cite{8} Till date, few published receptors like hormone receptor (HR) status assessment in breast carcinoma by ICC using ThinPrep have shown excellent concordance rates for ER and PR, routine cytology and corresponding HP slides.\cite{9,10} In our study, a very good concordance rate (84% for ER, 91% for PR, and 76% for HER2 status) and an excellent consistency as determined by Cohen Kappa test for ER, PR status between TP and FFPE results [Table 5]. Few false positive results on TP (2 for ER and 1 for PR) could be ascribed to small number of neoplastic cells evaluated on FNAC specimens and to the very small cutoff of only 1% of positive tumor cells.

Overexpression of HER2 in breast cancers has become the standard of care and helps the clinician to select cases for Herceptin therapy. In some laboratories, use of HER2 testing is performed only on equivocal cases.\cite{3} Studies that have compared HER2 status in cytological material and in corresponding HP slides gave discordant results. They also found that HER2 detection on ethanol-fixed cell block preparation resulted in higher rate of equivocal and positive staining than that obtained by needle core biopsy samples.\cite{10} William et al. described only a moderate agreement with a k value of 0.571 for HER-2 expression between 34 ethanol-fixed cell blocks and corresponding tissue sections.\cite{11}

In their study, Beatty et al., demonstrated only good (k = 0.692) agreement between tissue IHC and FNA ICC results and concluded that HER2 expression on cytology preparations was insufficient for clinical use. Pegolo et al., described perfect (k = 1) agreement of HER2 evaluation between cytology and histology specimens, and concluded that FNA by TP is suitable for detection of HER2 overexpression in invasive breast cancer.\cite{12} In our study, we found a very good agreement for ER (k = 0.81) and PR (k = 0.82). For HER2 expression the results were good agreement with k = 0.62. Therefore, we can conclude that FNA processed by LBC method is reliable for detection of ER, PR, and HER2 status in carcinoma breast. American Cancer Society in its medical review on Treatment of Stage IV (Advanced) Breast Cancer indicates that cases where ASCO/CAP guidelines for management is not followed, ICC HRs in FNAC TP may be performed at regular intervals for management follow-up.\cite{13} Zhang et al. illustrated that the results of ICC HRs in FNAC TP specimens may be used instead, but HER2 assessment may not be reliable enough for clinical use; FISH testing is necessary in this setting.\cite{14}

Limitations of the current study are small sample size of only 45 cases and the method should be standardized by multicenter study and results should be reconfirmed.

In this study, attempt was made to use FNA as a method for tumor cell assessment of biomarkers like ER, PR, and HER2 using the process of LBC. Due to adequate cellularity, good preservative morphology and less background staining cytological assessment of ER and PR status using LBC allowed easy preparation of slides and showed very good concordance with IHC. ICC for HER2 results showed good agreement.

**Conclusion**

The results suggest that in cases where biopsy of surgical specimens are not available, cases where ASCO/CAP guidelines for management are not followed and in cases of recent metastasis; ICC is a better method for assessing ER, PR, and HER2 status and ICC can be used as reliable prognostic biomarker. It is suggested that, the study method and results may be reconfirmed by multicenter study.

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**Conflicts of interest**

There are no conflicts of interest.

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