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IMMUNOHISTOCHEMICAL EVALUATION OF ESTROGEN AND PROGESTERONE RECEPTORS AND HER2 EXPRESSION USING NOVEL RABBIT MONOCLONAL ANTIBODIES ON CELL BLOCKS AND TISSUE BLOCKS SECTIONS OF BREAST CARCINOMA

Pathology

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ABSTRACT

Introduction: Fine needle aspiration (FNA) is a good modality of choice for early detection of breast carcinomas. Assessment of estrogen and progesterone receptors (ER, PR) and HER2 expression by Novel Rabbit monoclonal antibodies SP1, SP2 and SP3 immunocytochemistry (ICC) have higher sensitivity than routinely used FDA-approved mouse monoclonal and polyclonal antibodies performed on cellblocks from FNA of breast carcinoma as earlier reported in the studies.

Materials and Methods: In this prospective study, we compared ER, PR and HER2 expression on cellblocks in breast carcinomas using formalin-fixed FNA samples with formalin fixed resection tissue block sections.

Results: 70 fresh FNA cell blocks from breast carcinoma of primary and metastatic with subsequent resection tissue blocks were identified. All 70 cases were diagnosed as invasive ductal carcinoma on FNA. Few studies on breast carcinomas using Novel Rabbit monoclonal antibodies have determined the validity of such testing using cellblocks with correlation of tissue bocks. This study revealed 100% correlation for positive and negative Her2/neu results between the cellblocks and tissue blocks sections. For ER testing the results showed 95.65% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 96.42% negative predictive value (NPV). For PR testing the results showed 90.5% sensitivity, 100% specificity, 100% PPV, and 93.5% NPV respectively.

In conclusion: Immunocytochemistry for ER,PR and Her2/neu using rabbit monoclonal antibodies performed on formalin-fixed, paraffin-embedded cell blocks prepared from fresh FNA is most reliable methods in predicting the expression of these markers when correlated with immunohistochemistry (IHC) performed on the corresponding tumor tissue sections.

KEYWORDS

ER; PR; Her2; rabbit monoclonal antibodies; formalin fixation, cell blocks and tissue blocks

INTRODUCTION

Breast cancer is the commonest malignancy in the women. It can also arise at an early age and is the most common cause of cancer related mortality. Breast cancinoma has been studied intensively by researchers interested in either its management or find out new prognostic and predictive factors to reduce mortality. Cytologic cellblocks for evaluation of hormonal receptors(ER and PR) and Her2 assessment have been found to have excellent correlation with the tissue sections counterparts in the breast cancer. The prognostic and therapeutic value of the female hormone receptor status in patients with breast cancer is well established[1,2]. These therapeutic advances in breast cancer patients are having better survival with some showing late recurrences.

SP1(ER) rabbit monoclonal antibody (RabMAb) is reported to have 5.6-6.6% higher sensitivity than ID5 when determining ER status of the breast carcinoma on paraffin embedded tissue. Heat treatment is not required when we use the SP1 RabMAb because of its has higher affinity (eight times more) to target epitopes.

SP2 (PR), a rabbit monoclonal antibody that recognizes both PR (A) and PR (B) forms, has a higher affinity (12 times) when compared with the standered commonly used PR antibody (clone 1A6), but with comparable reactivity in detecting PR in tissue sections.

SP3, a rabbit monoclonal antibody that recognizes the extracellular domain of the HER2 receptor, is a most reliable candidate to evaluate the expression of HER2 in breast cancer cases. Her2neu is an independent prognostic and predictive marker in the breast carcinoma. Her2 positive breast cancer have a more aggressive course of disease with increased chance for recurrence, distant metastasis, and shorter survival in the women. Her2 status is also predictive of response to treatment with the humanized monoclonal antibody Herceptin (Transtuzumab), which has been shown to improve survival time in females with Her2 positive tumors[3-7]. It is considered that almost one third of invasive breast carcinomas overexpress the HER-2/neu protein, so the use of the anti-HER-2/neu monoclonal antibody Herceptin (trastuzumab) to block the protein has become important in the management of and in prolonging the survival for patients with metastatic breast cancer. The effectiveness of this therapy is dependent on accurately evaluating the HER-2 status in these tumors, which can be done either by studying the expression of HER-2 protein by immunocytochemistry (ICC) or by evaluating HER-2 gene amplification by fluorescent in situ hybridization (FISH).

Determination of hormonal status is an also important primary assessment at the time of a breast cancer diagnosis. Earlier studies showed estrogen receptor expression was present in about 70% of breast cancer cases. Although clinical experience has shown that ER and PR receptors are weak prognostic markers for outcome, they are, however, strong biomarkers of response to tamoxifen treatment, and PR status is known to be associated with disease-free and overall survival. Patients with ER-positive/PR-positive tumors have a better prognosis than patients with ER-positive/PR-negative tumors,who have a better prognosis than patients with ER-negative/PR-negative tumors [8,9,10].

In this study, to establish the usefulness of performing hormone receptors and Her2 testing on cell block material, we have studied ICC results ER and PR, and Her2 using novel rabbit monoclonal antibodies performed on formalin-fixed, paraffin embedded cell blocks from FNA and compared them to results of IHC performed on the excised original tumor tissue sections.

Materials and Methods

Seventy cases with cellblocks with adequate cellularity from cytologic specimens obtained from patients with known primary (70 patients) with metastatic breast carcinoma and had been evaluated for the

expression of ER, PR, and Her2/neu rabbit monoclonal antibodies by ICC were selected from the computerized HIS records at our institution. The seventy cases included 70 FNA specimens (70 breast primaries in which 15 out of 70 cases showed lymph node axillary masses). Cellblocks from FNA specimen details are outlined in **Table 1.** The Cellblocks result were compared to tissue blocks result obtained on the original tumor tissue core biopsy or resection tissue using immunohistochemistry (IHC).

Cell Blocks Preparation

Cell blocks were prepared from fresh FNA samples of 70 consecutive breast carcinoma cases using direct formalin fix and processed as biopsy specimen with following criterias **Inclusion criteria :** Female patients of any age group with clinical, mammographic and cytologically diagnosed cases of breast carcinoma will be included in the study after their consent taken. **Exclusion criteria :** Cases with neoadjuvant or adjuvant chemotherapy will be excluded from study.

Immunocytochemistry ER, PR and HER2/neu

a) ER and PR expression

Immunocytochemistry for ER and PR Formalin-fixed, paraffin-embedded sections of CB and tissue were stained for ER and PR using the primary mono- clonal antibodies SP1 at dilution 1: 100 for ER and SP2 at dilution 1:200 for PR from labvision (Envision, Carpentaria, CA) according to manufacturer specification. Stains were evaluated by the signing pathologists as positive or negative. Cellblocks from FNA samples was fixed in 10% neutral-buffered formalin (minimum 6hrs). immunocytochemistry was performed, following epitope retrieval, with a polymer based detection system (Envision plus) using Rabbit monoclonal antibodies SP1 and SP2 (Envision).

b) Her2neu expression

Formalin-fixed, paraffin-embedded sections of CB and tissue were stained for Her2neu using the primary monoclonal antibody SP3, TAB250 (Zymed, San Francisco, CA) according to manufacturer specification and as previously detailed in Wixom et al. Her2neu (SP3; dilution 1:100). 3-5 micro sections was deparaffinized and rehydrated with deionized water. Antigen retrieval for HER2/neu using SP3 rabbit monoclonal antibodies (HercepTest) was performed by immersing and incubating the slides in 10mmol/L citrate buffer in a calibrated water bath (95–99°C) for 40 (±1) minutes. After decanting the epitope retrieval solution, the sections were rinsed in the wash buffer, and later soaked in the buffer for 5–20 minutes prior to staining, the slides were rinsed, followed by 200 µL peroxidase-blocking reagent for 5 minutes, followed by rinsing and then placed in 200 µL primary anti-HER2 protein (or negative control reagent) for 30-60 minutes, rinsed twice and finally immersed in 200 µL substrate chromogen solution for 10 minutes. The slides were counter stained with hematoxylin, and finally coverslipped. Positive controls of known positive tissues (endometrium and breast) and negative controls with primary antibody replaced with TBS are run with the patient study slides.

HER2 results will be determined based on the maximum area of staining intensity, according to the package insert and ASCO/CAP guidelines as follows: strong circumferential membranous staining in > 30% of invasive carcinoma cells = **3+**; moderate, circumferential membranous staining in ≥ 10% of invasive tumor cells or **3+** in ≤ 30% of cells = **2+**; weak and incomplete membranous staining in invasive tumor cells= **1+**, no staining= **0**. Tumors with **0** and **1+** staining will considered negative and cases scored as **2+** equivocal, and **3+** will be considered positive, will be evaluated on 4× and 10× magnifications.

Results

The source of the cell blocks and the results of immunocytochemical staining of hormone recepters and HER2 Neu on cellblocks and tissue blocks are summarized in **Table 1.** All cytologically diagnosed cases of invasive ductal carcinomas studied with immunocytochemistry of ER,PR and HER2/neu on cellblocks and evaluated with correspondings tissue blocks of breast carcinoma cases. Positive controls of known positive tissues (breast) and negative controls with primary antibody replaced with TBS are run with the patient/study slides.

Immunostaining interpretation on cellblocks from FNA

Nuclear immunostaining in more than 10% of tumor cells was considered positive for ER and PR recepters. In our study, ER was positive in 32(figures 1a, 1b, 1c & 1d) and negative in 38 cell blocks. Its corresponding tissue blocks showed positive in 33 and negative in 37 cases. PR was positive in 29(figures 2a, 2b) and negative in 41 cell blocks. Its correspondings tissue blocks showed positive immunostaining in 31 and negative in 39 cases. ER+, PR+ and Her 2Neu+ positive immunostaining were seen in 6 out of 70 cases. ER + and

Table 1: Comparative description of Immunocytochemistry ER, PR and HER2Neu expression on cellblocks and tissue blocks from Breast Carcinomas(n=70)

Rabbit Monoclonal Antibodies	Cell blocks (n=70)		Tissue blocks (n=70)	
	Positive	Negative	Positive	Negative
SP1 (ER)	32	38	33	37
SP2 (PR)	29	41	31	39
SP3 (Her2Neu)	25	45	25	45

PR+ positive immunocytochemistry (ICC) were seen in 18 out of 70 cases. PR+ and Her2Neu+ positive ICC was seen in 9 out of 70 cases. ER + and Her2Neu+ positive ICC was seen in 8 of 40 cases. ER -,PR- and Her2Neu- negative ICC (Triple Negative) were seen in 16 out of 70 cases.

Table 2: Correlation of Her2/neu, Immunocytochemistry(ICC) Stain Scores for Cell Blocks and Tissue Blocks (n=70)

Her2Neu (SP3) Immunostaining	No. of Cases (n=70)	Cellblocks ICC Scores	Tissue blocks IHC Scores
25 Positive Cellblocks	20	3+	3+
	5	2+	2+
45 Negative Cellblocks	3	1+	1+
	42	0	0

HER2/neu results was determined based on the maximum areas of staining intensity, according to **ASCO/CAP guidelines** as follows-

Strong circumferential membranous staining in > 30% of invasive carcinoma cells as 3+ seen in 20 of 70 cases(figures 3a, 3b, 3c & 3d), **Moderate** (≥ 10 < 30% cells) as 2+ in 5 out of 70cases(figure 2d), **Weak** (<10 Cells) as 1+ in 3 of 70cases(figure 2c) and **No staining** as 0 were seen in 45 of 70 Cases. Tumors with 0 and 1+ immunostaining considered as **negative.** Cases scored as 2+ **equivocal**, and 3+ considered as **positive**, evaluated on 4× and 10× magnifications. Positive and negative agreements of ER, PR and HER2 expression by ICC on CB and TB samples alongwith correlation results for Her2/neu by IHC on CB with tissue blocks are also described in (**Table 1 and 2).**

Discussion

Breast cancer is a major health concern and a leading cause of death among women. The prognostic and predictive value of Her2/neu and the hormone receptors in patient with primary or metastatic breast cancer is essential for a favorable outcome of patient treatment.

The lifetime risks of developing breast cancers in women is 13% and 210,000 new cases are diagnosed each year in the United States [11]. With so many lives affected, identification of biomarkers plays a paramount role in the treatment, management, and prognosis of breast cancer. It is reported that one in 22 women in India is likely suffer from breast cancers during her life time. Fine needle aspiration (FNA) is a rapid, less invasive, and accurate diagnostic tool for the detection of breast carcinomas.

IHC is the recommended primary screening tool for hormonal assessment in breast cancers with important prognostic and therapeutic implications and is routinely performed in the laboratory. In this prospective study, we studied ER, PR and HER2 expression in breast carcinomas using FNA cell block with formalin fixed resection tissue block preparations. All 70 cases were diagnosed as invasive ductal carcinoma on FNAC with cellblocks and histopathology and correlated with ICC findings.

Hanley et al [12] and **Williams et al** [13] reported their findings on CB in comparison to needle cores and tissue blocks, concluded that Her2 testing on CB is not reliable but ER and PR showed good concordance; however, their cell blocks were fixed in 50% ethanol.

HER2/neu is a transmembrane glycoprotein in the epidermal growth factor receptor family with tyrosine kinase activity. It is expressed at low levels in normal nonneoplastic epithelium, including breast duct epithelium, and is over-expressed in 10-20% in primary breast cancers [14,15]. When the protein is over-expressed, tyrosine kinase is constitutively activated, resulting in mitogenic transduction and poor prognosis[16,17].

Table 3: Comparing the ER, PR and Her2Neu Expression on the cellblocks of breast cancers from Previous studies

Previous Studies		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic Accuracy	Total No. of Cases
Ahmed Shabaik et al (2010)	ER	85.7	100	100	85.7	85	45
	PR	80	100	100	88.8	82	
	HER	100	100	100	100	100	
Present study (2014)	ER	96	100	100	96.5	97	70
	PR	95	100	100	95.5	96	
	HER	100	100	100	100	100	

It is demonstrated that the rabbit monoclonal antibody provides an excellent quality of staining, a simplification of the immunocytochemistry protocol, high sensibility and specificity, saving time and cost. Recently developed SP1 rabbit monoclonal antibody,on other hand, recognize the ER alpha C-terminal portion and is reported to have 5.6-6.6% higher sensitivity than ID5 on tissue section without heat pretreatment due to higher affinity to target epitopes. The RMab SP2 antibody is targeted against the C-terminal region of the PR alpha subunit as well[18].

Previous comparative study of Rabbit monoclonal antibodies (RMab-ER,PR and HER2/neu) against FDA–approved Mab (FMab-ER,PR, Dako) and HercepTest (HerFDA) in the 52 breast carcinoma cellblocks sections using visual and image quantification has shown in literature. Fluorescent in situ hybridization (FISH) was used as reference standered for HER2. Reanalyzed with an automated cellular imaging system (ACIS III, Dako). Frequency of ER(38.5% vs.36.5% for visual; 55.85% vs 57.7for image)and PR(28.8% vs36.58 for visual ; 50% vs 51.9% for image, and concordance (overall agreement is 71.2% and 75% for visual and image ER; and 84.6% and 59.6 % for visual and image PR) were similar for both FMab and RMab, respectively. Overall agreement is 53.8% vs77.1% for visual and image detection, respectively. Using HerFDA and RMab is poor to moderate for Her2. For Her2 overexpression, RMab proved to be superior [12] to HerFDA and showed excellent agreement with FISH results with both quantitative detection methods.

The detrimental effect of ethanol as a fixative was further explored in the previous study in which they compared ER, PR and HER2/neu expression of breast carcinomas using archival ethanol fixed FNA CB with formalin fixed resection TB preparations. Ethanol-fixed tissues have greater over-expression of HER2/neu immunostaining and therefore yield false positive results. 90.4 % and 93.9% positive agreement (weighted Kappa of 0.773 and 0.785) was obtained for ER and PR stained ethanol-fixed CB and formalin-fixed TB samples, respectively. However, only a moderate positive agreement of 73.3% (weighted Kappa of 0.571) was obtained between ethanol-fixed and formalin-fixed tissues with HER2/neu IHC. The increase in discrepant results in HER2/neu stained sections is most likely due to ethanol fixation which results in increased false positive HER2/neu expression since all other factors including fixation time, choice of antibody, and HER2/neu reporting thresholds were controlled [13]

Ahmed Shabaik(2010) et al [19] reported and using tissue ER IHC results as the gold standard, cell block ICC showed 85.7% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 85.7% negative predictive value (NPV).Using tissue PR IHC results as the gold standard, cell block ICC showed 80% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 88.8% negative redictive value (NPV)

Occasionally cell blocks may be acellular or specimens are not sufficient to prepare cell blocks. Studies of immunocytochemical analysis of ER, PR, and Her2/neu performed on cytology direct smears, touch prep, cytospin prep, and liquid-based prep with different fixation methods and with different antibodies have shown conflicting results particularly for Her2/neu[20-22].

In a reviewing about the prevailing recommendations and contemporary practices of breast fine-needle aspiration biopsy they cautioned against the use of cytology smears and cytospins for ancillary testing unless the laboratory has specific protocols for immunohistochemistry on cytologic material and they recommended to use cell blocks as they are analogous to surgical pathology material[23].

A total of 134 cases of breast carcinoma were identified with both FNA cell blocks (fixed in 10% formalin) and corresponding available tissue blocks and ER, PR, and HER2 were characterized in both specimens. Concordance for ER expression evaluated on a cell block compared with the corresponding tissue block was 96.2%. Concordance for PR expression was 77.5%. Overall agreement of HER2 FISH testing between cell blocks and tissue blocks was 96.7%. For both cell blocks and tissue blocks, HER2 expression by immunohistochemistry demonstrated ≥98% positive and negative concordance with the FISH reference method.[24].

Our study in 70 cases showed and using tissue blocks ER IHC results as the gold standard, cell block ICC showed 95.65% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 96.42% negative predictive value (NPV). Using tissue blocks PR IHC results as the gold standard, cell block ICC showed 90.5% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 93.5% negative redictive value (NPV). Her2Neu on cellblocks showed 100% sensitivity,100% specificity,100% PPV and 100 % NPV **(Table 3)**.

In conclusion our study indicated that ICC for ER , PR and Her2/ neu performed on formalin fixed and paraffin embedded cell blocks prepared from fresh FNA is an accurate and reliable in predicting the expression of these markers when correlated with immunohistochemistry performed on the corresponding tumor tissue block sections.

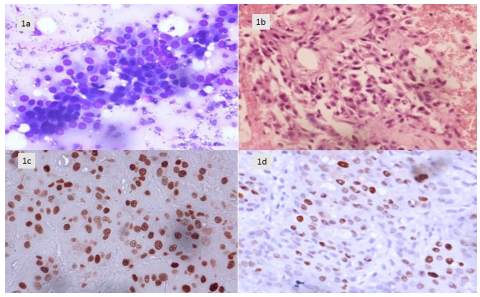

Figure 1a: FNA smear from left breast Lump (IDC) showed cluster of malignant ductal epitrhelial cells, **1b :** cellblock: Sheets of malignant ductal epithelial cells (h&e, 40x magnification), **1c:** ER immunostaning showed strong nuclear positivity on cellblock, **1d:** ER immunostaning showed strong nuclear positivity on tissue block.

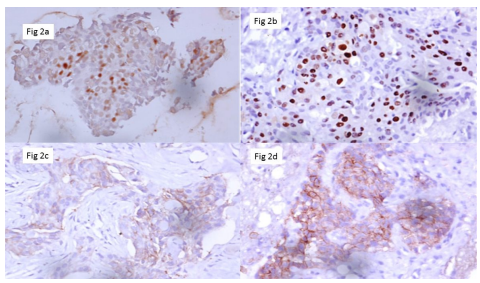

Figure 2a: PR immunostaning showed strong nuclear positivity on cellblock of breast carcinoma, **2b:** PR immunostaning showed strong nuclear positivity on tissue block, **2c:** Her2 immunostaining showed 1+ (weak) staining intensity, **2d:** Her2 immunostaining showed 2+ (moderate, incomplete) membranous staining intensity on cellblocks

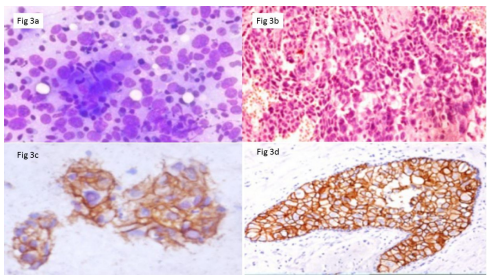

Figure 3a: FNA smear from right breast lump (IDC) showed clusters of malignant ductal epitrhelial cells, **3b :** cellblock: sheets of malignant ductal epithelial cells (h&e, 40x magnification), **3c:** Her2 neu immunostaining showed complete strong cytoplasmic membranous positivity on cellblock, **3d:** Her2 neu immunostaining showed complete strong cytoplasmic membranous positivity on tissue blocks

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