

Original Article

Immunohistochemical Detection of Estrogen and Progesterone Receptor and HER2 Expression in Breast Carcinomas: Comparison of Cell Block and Tissue Block Preparations

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Abstract: Fine needle aspiration (FNA) is a rapid tool for detection of breast carcinomas. Evaluation of estrogen and progesterone receptors (ER, PR) and HER2 expression by immunohistochemistry (IHC) are routinely performed in breast carcinomas. Formalin fixation of tissue for a minimum of 6 hours, and for HER2 not more than 48 hours is the current recommended practice. In this retrospective study, we compared ER, PR and HER2 expression in breast carcinomas using archival ethanol-fixed FNA cell block with formalin fixed resection tissue block preparations. 34 archival breast carcinoma FNA cell blocks of primary origin with subsequent resection tissue blocks were identified retrospectively. All 34 cases were diagnosed as invasive ductal carcinoma. Cases with neoadjuvant or adjuvant chemotherapy were excluded. Cell blocks were initially fixed in 50% ethanol (4-12 hrs), followed by formalin fixation (minimum 6 hrs). Tissue blocks were formalin-fixed within 4-8 hrs for 6-48 hrs. ER, PR, and HER2 IHC results on the cell blocks and tissue blocks were compared. Alcohol fixed cell block samples for detection of ER and PR by IHC show good agreement with tissue block samples and are therefore a reliable method (weighted Kappa of 0.773 and 0.785, respectively) to triage patients for hormonal treatment. However, HER2 results show only moderate agreement with a weighted kappa of 0.571. The increase in discrepant results may be due to ethanol fixation which results in false positive increased HER2 expression. These results demonstrate the importance of adherence to the College of American Pathologists/ASCO guidelines for HER2 IHC.

Key Words: ER, PR, HER2, breast carcinoma, fine needle aspiration, cell block, formalin fixation, alcohol fixation, immunohistochemistry

Introduction

Breast cancer is a major health concern and a leading cause of death among women [1]. The lifetime risk of developing breast cancer in women is 13% and 210,000 new cases are diagnosed each year in the United States [1]. With so many lives affected, identification of biomarkers plays a paramount role in the treatment, management, and prognosis of breast cancer.

Determination of hormonal status is an important primary assessment at the time of a breast cancer diagnosis. Estrogen receptor expression is present in about 70% of breast cancer cases [2]. Although clinical experience

has shown that estrogen (ER) and progesterone (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 [2]. 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 [2].

Determination of human epidermal receptor protein-2 (c-erbB-2; HER2/neu) status is also important; as it is known to be a prognostic as well as predictive marker in both node-negative and node-positive patients [2, 4, 5,

7]. 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 non-neoplastic epithelia, including breast duct epithelium, and is over-expressed in 10-20% of primary breast cancers [2-7]. When the protein is over-expressed, tyrosine kinase is constitutively activated, resulting in mitogenic transduction and poor prognosis [3, 8, 9]. HER2/neu status not only predicts poor outcome, but predicts sensitivity to anthracycline-based chemotherapy regimens, and resistance to cytoxan and tamoxifen therapies in the setting of ER-positive breast cancers [2, 10]. Just as breast cancers expressing ER and PR receptors are responsive to Tamoxifen, breast cancers with HER2 over-expression are candidates for treatment with Trastuzumab (Herceptin), a humanized monoclonal anti-HER2/neu antibody [3]. Recent studies have shown that adjuvant Trastuzumab can reduce the risk of recurrence by one half, and mortality by one third, in early-stage breast cancer patients [2, 10].

Because of these prognostic implications, the need for accurate and precise assessment of ER, PR, and HER2/neu expression in breast cancer is critical in the determination of patients appropriate for treatment with these drugs [3, 8, 9]. Immunohistochemistry (IHC) is the most commonly used method of testing for ER, PR, and HER2/neu status [3]. However, several factors can affect the ER, PR, and HER2/neu IHC, including tissue fixation, choice of antibody, and the determination of criteria for positive results [2]. This retrospective study set out to evaluate one such factor - tissue fixation and the use of ethanol versus neutral buffered formalin - the recommended fixative of the College of American Pathologists/American Society of Clinical Oncology (CAP/ASCO).

Materials and Methods

Case Selection

Thirty-four archival primary breast carcinoma cases with both fine needle aspiration cell blocks (CB) and subsequent resection/tissue blocks (TB) were identified retrospectively within a 3 year period (July 2001 to September 2004) at our institution. All 34 cases were diagnosed as invasive ductal carcinoma.

Cases with neoadjuvant or adjuvant chemotherapy were excluded from this study. This study was approved by the Emory University School of Medicine Institutional Review Board (IRB 08271).

Immunohistochemistry and Scoring for ER, PR and HER2

CB samples were initially fixed in 50% ethanol (4-12hrs), followed by fixation in 10% neutral-buffered formalin (minimum 6 hrs). TB were formalin-fixed within 4-8 hrs for 6-48 hrs (minimum 6 hrs). IHC was performed, following epitope retrieval, with a polymer based detection system (Envision plus, Dako, Carpinteria, CA) using mouse monoclonal antibodies for ER and PR (Dako, Carpinteria, CA), ER (1D5; 1:50), PR (PgR636; 1:400), and Herceptin kit (HercepTest, Dako, Carpinteria, CA) according to the manufacturer's instructions. For ER and PR, antigen retrieval is performed as follows: sections were deparaffinized and rehydrated with deionized water. They were then heated in citrate buffer (pH 6.0), using an electric pressure cooker for 3 minutes at 12-15 pounds per square inch (PSI) approximately at 120°C, and cooled for 10 minutes prior to immunostaining. All slides were loaded on an automated system (DAKO Autostainer) and exposed to 3% hydrogen peroxide for 5 minutes, incubated with primary antibody for 30 minutes, with labeled polymer (Envision® + dual link) for 30 minutes, 3,3'-diaminobenzidine (DAB) as a chromogen for 5 minutes, and hematoxylin as counterstain for 5 minutes. These incubations were performed at room temperature. Between incubations sections were washed with Tris-buffered saline (TBS). Cover-slipping was performed using the Tissue-Tek SCA (Sakura Finetek USA, Inc, Torrance, CA) coverslipper. 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. Nuclear staining in more than 10% of tumor cells was considered positive for ER and PR.

Antigen retrieval for HER2/neu using HercepTest is 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 are rinsed in the wash buffer, and later soaked in the buffer for 5-20 minutes prior to staining. The slides

Table 1 Correlation of ER, PR and HER2 expression by IHC between CT and TB samples

	Positive agreement (%)	Negative agreement (%)	Discrepancy (%)	Kappa	Correlation (Spearman)
ER	90.4	86.7	5.80%	0.773	0.76 (<0.0001)
PR	93.9	94.7	8.8%	0.785	0.79 (<0.0001)
HER2/neu	73.3	81	15.6%	0.571	0.56 (<0.002)

Positive: >10% for ER and PR; 3+ for HER2/neu. Negative: <10% for ER and PR; 0 or 1+ for HER2/neu

are loaded onto the autostainer using the HercepTest program™ as described in the manufacturers' insert. In the autostainer, the slides are 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 minutes, rinsed twice and finally immersed in 200 µL substrate chromogen solution (DAB) for 10 minutes. The slides are counterstained with hematoxylin, and finally coverslipped. HER2 results were 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 were considered negative and cases scored as 2+ equivocal, and 3+ were considered positive, evaluated on 4x and 10x magnifications.

Statistical Analysis

ER, PR and HER2 IHC performed on CB and TB samples were compared. Statistical analyses, including positive and negative agreement and discrepancy rate were calculated using the Spearman rank correlation analysis (ρ) and Cohen kappa (κ) test of agreement. Kappa values above 0.6 correlated with good agreement, 0.4-0.6 was considered moderate agreement, whereas kappa results below 0.4 corresponded to fair, below 0.2 reflected poor agreements.

Results

Positive and negative agreements of ER, PR and HER2 expression by IHC on CB and TB samples are described in **Table 1**. It was noted that fifteen of thirty-four total cases (44.1%) had HER2/neu IHC discrepant results between

FNA ethanol-fixed CB and formalin-fixed TB samples (**Table 2**). Of those 15 cases, 7 (46.7%) had either equivocal HER2/neu staining (4 cases) or positive HER2/neu staining (3 cases) by ethanol-fixed cell block IHC but were negative by formalin-fixed TB IHC. 2 of the 15 cases were either equivocal for HER2/neu IHC (1 case) or negative for HER2/neu IHC (1 case) by ethanol-fixed cell block, while the formalin-fixed tissue blocks were positive for HER2/neu IHC. One ethanol-fixed cell block was positive for HER2/neu IHC, while the formalin-fixed tissue block was equivocal for HER2/neu IHC. Three ethanol fixed cell blocks were negative for HER2/neu IHC, while the formalin-fixed tissue blocks were equivocal for HER2/neu IHC.

Overall, nine of fifteen cases (60%) were either equivocal for HER2/neu IHC (4 cases) or positive for HER2/neu IHC (5 cases) by ethanol-fixed CB samples, while being either negative for HER2/neu IHC (7 cases) or equivocal for HER2/neu IHC (1 case) by formalin-fixed TB samples (**Table 2**).

Discussion

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 and is routinely performed in the laboratory [8]. However, recent studies have brought to light significant variability in the IHC assessment of HER2/neu expression [4, 11, 12]. It is estimated that 20% of current HER2/neu testing may be inaccurate [6]. This variation may be related to multiple pre-analytic and analytic factors such as types of fixatives, time of fixation, primary antibody used, and variation in scoring stained slides (scoring is reported as negative (0/1+), equivocal (2+), or positive (3+) [4, 5, 13, 14].

With such variability, accurate HER2/neu results are critical in identifying patients for

Table 2 Discrepancy of HER2/neu IHC results between ethanol-fixed CB and formalin-fixed TB samples

Ethanol-fixed CB (N)	Formalin-fixed TB (N)
Negative (5)	2+ (3) 2-3+ (1) 3+ (1)
2+ (5)	Negative (4) 3+ (1)
3+ (4)	Negative (3) 2+ (1)

whom this therapy is appropriate as treatment because Trastuzumab treatment is not without risks [2, 11]. 1.4% of patients receiving Trastuzumab as a single agent developed cardiotoxic side effects and patients receiving Trastuzumab with paclitaxel or anthracyclines developed cardiotoxic side effects at 13% and 27%, respectively [2]. In addition to the possible side effects, Trastuzumab is costly with a treatment expense of approximately \$100,000 [6].

It is these reports of variability along with possible drug side effects and drug expense that prompted the recent recommendations of the American Society of Clinical Oncology/College of American Pathologist (ASCO/CAP) group and the ad hoc committee on improved standardization for IHC [15]. Recommendations from the above governing bodies dictated not only fixation time, but also specific fixative type essential for obtaining accurate results [15]. According to the recommendations, breast specimens should be fixed for a minimum of 6 hours and a maximum of 48 hours duration [2, 6, 10]. Minimum and maximum fixation times are important as delayed fixation results in increased proteolytic degradation which may cause irreversible weak or absent staining [14]. Formalin fixation times that are too short cause a mixture of formaldehyde, and ethanol fixation results in tissues showing intense staining of the center or of the periphery of the histologic section of interest [14].

The other recommendation from the ASCO/CAP meeting involved usage of 10% neutral-buffered formalin as the mandated fixative. Although formaldehyde was discovered in 1859, it wasn't until 1892 while it was being evaluated as an antiseptic that the observation of its skin firming/hardening

quality was noted [18]. Hence the use of formaldehyde in histology began [17, 18]. Through the years, "alternative fixatives" such as ethanol have come about; however, formalin still offers a stable and cost-effective medium to conduct immunohistochemistry [2, 14-17]. Ethanol fixatives initially seemed acceptable; however, drawbacks such as flammability, and the recent recognition of it being a "detrimental" fixative for certain antibodies, including hormone receptors such as HER-2/neu were noted [15].

The detrimental effect of ethanol as a fixative was further explored in the current study in which we 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.

The results of this study are consistent with the literature findings, confirming the inconsistency and variation that occurs in ethanol-fixed tissues with HER2/neu IHC. Alcohol fixed FNA CB samples are reliable to triage patients for hormonal treatment, but they may not be appropriate for management of breast cancer patients with Trastuzumab. This study further proves the value of formalin-fixed tissues in the hormonal assessment of breast cancer by IHC and the importance of adherence to the ASCO/CAP guidelines for HER2/neu IHC.

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