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Original article

Interobserver reproducibility for HER2/neu immunohistochemistry: A comparison of reproducibility for the HercepTest™ and the 4B5 antibody clone

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ABSTRACT

Background: IHC results for HER2/neu vary with replicate testing using the same antibody clone and when alternate clones are utilized. A number of factors appear to be responsible for this variability, including fixation times, equipment utilized and training and experience of staff. A number of studies have documented interobserver variability for a single antibody clone but few have evaluated reproducibility between antibody clones and which clones demonstrate the highest degree of interobserver reproducibility.
Design: We studied a series of 93 cases stained by both the HercepTest™ and the 4B5 clone for interobserver reproducibility. Formalin-fixed, paraffin-embedded sections were stained by the immunohistochemical technique using the manufactures directions for both the HercepTest™ and the 4B5 clone. FISH testing was performed on formalin-fixed paraffin embedded sections according to the PathVysion HER-2 DNA probe kit instructions.
Results: Absolute agreement rate for Hercep was 85%. Absolute agreement for 4B5 was 69%. This difference was statistically significant ($p < 0.0001$). The chance-corrected agreement (weighted kappa) for the HercepTest™ was 79% and 71% for 4B5 ($p < 0.0001$). Absolute agreement between antibody clones was 58% with the chance corrected agreement being 51%. Absolute agreement of 4B5 with FISH was significantly greater than that of the HercepTest™ (54% vs 35%).
Conclusion: Agreement between evaluators was greater with the HercepTest. However, agreement with FISH results was superior for the 4B5 clone. Interobserver agreement was less than the 95% agreement threshold recommended by the ASCO/CAP guidelines for development of a new testing method for HER2 evaluation.

1. Introduction

Assessment of human epidermal growth factor receptor 2 (Her2) by either immunohistochemistry (IHC) or fluorescence in-situ hybridization (FISH) has become the standard of practice. Despite approximately two decades of HER2/neu testing in clinical practice both testing methods are associated with significant inaccuracy and lack of reproducibility [1–4]. The poor degree of reproducibility is seen both between observers using the same antibody clone, and between clones. Suboptimal agreement is seen between immunohistochemical (IHC) and fluorescence in-situ hybridization results (FISH). Complete agreement between observers was achieved in 48% of 46 cases studied by Hsu et al. utilizing the Dako HercepTest™ [5]. Lacroix-Triki et al. [6] reported excellent agreement in a series of 74 HER-2 immunostains for 0, 1+ and 3+ results, but 2+ scores showed poor agreement between observers [6]. Due to the published suboptimal agreement between observers, a number of quality assurance programs have been adopted and guidelines issued including those by the American Society of Clinical Oncologists (ASCO) and the College of American Pathologists (CAP) [1,7]. The CAP also offers proficiency testing programs to aid in quality improvement for both the technical and analytical components of HER2/neu testing. These guidelines along with additional training opportunities for pathologists appear to have improved interobserver reproducibility in some reports. Mamoon et al. [8] reported a 94% agreement for assessment of HER2/neu in a group receiving focused training, but only a 69% agreement in a group of pathologists not receiving such training.

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Al Haddabi et al. [9] showed that complete agreement for HER2 status between five pathologists evaluating immunohistochemical testing occurred in only 67% of cases, and agreement between three pathologists occurred in only 88% of cases despite following the ASCO/CAP 2007 guidelines. Agreement between antibody clones in the assessment of HER-2 overexpression in breast carcinoma has been variable [9–11]. In a study of 322 breast cancer cases, Powell et al. [11] reported an overall concordance between the CB11 and 4B5 clones of 84.7%. Concordance rates between IHC and FISH have also varied [11–13]. Powell et al. [11] reported a 89.5% concordance between IHC (4B5 clone) with FISH and an 81.2% concordance between CB11 and FISH. Kakar et al. [12] reported an overall concordance of 88% between IHC and FISH while Rhodes et al. [13] reported variable concordances for different antibodies using IHC with FISH. Review of the literature reveals that between 3 and 15% of breast cancers overexpress HER2/neu protein without evidence of gene amplification [11]. A variety of factors including antibody clone utilized, experience of observers and biological differences in protein overexpression and gene amplification may explain these discrepancies. A number of sources have been proposed for variability of results in HER-2 testing. These include fixation time, method of tissue processing, type of fixative, equipment utilized for staining, type of antigen retrieval, clone of antibody utilized and training and competency of staff [1].

A significant variable for the reproducibility of IHC HER-2 protein overexpression evaluation appears to be the individual evaluating the immunohistochemical slides [5]. Antibody clone utilized also appears to play a role. IHC staining using some clones appears more easily interpreted than staining by others [11]. We investigated a series of 93 breast carcinomas stained by two methods (HercepTest™ and 4B5 clone) to determine if interobserver agreement was superior in one method or the other. Additionally, we investigated whether or not one immunohistochemical method demonstrated superior correlation with FISH results. Herein we report the results of that study.

2. Materials and methods

2.1. Case selection

The surgical pathology files at the University of Utah were searched for all breast carcinoma cases with HercepTest™ results documented in the original biopsy report. Ninety-three cases with adequate material for repeat immunohistochemistry were selected and enriched for overrepresentation of equivocal (2+) HercepTest™ results. These 93 cases underwent repeat IHC testing with the 4B5 antibody, fluorescence in situ hybridization and repeat Hercep testing. Adequate testing was attainable for all 93 specimens with the 4B5 antibody and the FISH test. Adequate material for repeat Hercep testing was available in only 79 cases because the repeat Hercep testing was performed after all other testing had been performed. The original formalin-fixed, paraffin-embedded blocks were sectioned in the histology laboratory at ARUP. Following sectioning, the slides were de-identified according to the University of Utah IRB number 24487.

2.2. HER2/neu (4B5) immunohistochemistry

The PATHWAY HER-2/neu (4B5) immunohistochemistry was performed on a benchmark ultra-instrument in the CLIA certified immunohistochemistry laboratory of ARUP. Staining was performed according to the FDA approved PATHWAY anti-HER-2/neu rapid monoclonal antibody protocol (Table 1). Slides were enumerated by three board-certified anatomic pathologists (LJL,

Table 1
Methodology for Her2/neu testing using the 4B5 and HercepTest antibodies.

Protocol for 4B5	Protocol for HercepTest
Four-micron thick sections are cut from each sample and placed on plus slides.	Cut four-micron thick sections from each sample and place them on plus slides.
The slides are allowed to air dry at room temperature.	Air dry the slides at room temperature.
The slides are placed on the automated immunostainer. The program is started. (All steps performed on the Ventana BenchMark® XT autostainer are at 37 °C.)	Melt the slides in a 60 °C oven for 30 min.
The slides are deparaffinized with the EZ Prep solution. (Ventana Medical Systems.)	De-paraffinize the sections in 3 changes of xylene for 5 min each and hydrate in graded alcohols (100%, 95% and 70%), then placed in dH_2O.
The slides are treated with CC1 for 90 min.	Pre-treat the sections with HIER (heat induced epitope retrieval) in citrate buffer pH 6.0 in a water bath for 40 min at 100 °C (pre-warm buffer in water bath). Take coplin jar out of water bath and let cool at room temperature for an additional 20 min.
The primary pre-dilute antibody for Her2 is applied for 40 min.	Take the slides from the hot buffer and place in dH_2O.
The Avitin/Biotin blocking kit is applied. (Ventana Medical Systems.)	Place the slides on the automated immunostainer. Start the program. (Dako automated instrument, all staining steps are performed at room temperature)
The slides are detected with the IView DAB detection kit. (Ventana Medical Systems.)	Apply the peroxidase blocker for 10 min.
The slides are counterstained for 4 min with hematoxylin. (Ventana Medical Systems.)	Rinse slides with buffer.
The slides are removed from the autostainer and placed in a dH2O/DAWN mixture.	Apply the primary antibody for 30 min.
The slides are gently washed with the mixture to remove any coverslip oil applied by the automated instrument.	Rinse slides with buffer.
The slides are placed in Sodium Thiosulfate for 30 s to clear any Iodine.	Apply the polymer for 30 min.
The slides are dehydrated in graded alcohols (70%, 95% and 100%) and dipped 10 times each in 4 changes of xylene.	Rinse slides with buffer.
The slides are coverslipped and allowed to air dry.	Rinse slides with dH_2O.
	Apply the DAB for 10 min.
	Rinse slides with dH_2O.
	Rinse slides with buffer.
	Apply the counterstain of hematoxylin for 8 min.
	Rinse slides with buffer.
	Remove the slides from the autostainer.
	Dehydrate the slides in graded alcohols (70%, 95% and 100%) and dip them 10 times each in 4 changes of xylene.
	Coverslip the slides and allowed to air dry.