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Gene protein detection platform—a comparison of a new human epidermal growth factor receptor 2 assay with conventional immunohistochemistry and fluorescence in situ hybridization platforms

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are widely used semiquantitative assays for selecting breast cancer patients for HER2 antibody therapy. However, both techniques have been shown to have disadvantages. Our aim was to test a recent automated technique of combined IHC and brightfield dual in situ hybridization—gene protein detection platform (GPDP)—in breast cancer HER2 protein, gene, and chromosome 17 centromere status evaluations, comparing the results in accordance to the American Society of Clinical Oncology/College of American Pathologists recommendations for HER2 testing in breast cancer from both 2007 and 2013. The GPDP technique performance was evaluated on 52 consecutive whole slide invasive breast cancer cases with HER2 IHC 2/3+ scoring results. Applying in turns the American Society of Clinical Oncology/College of American Pathologists recommendations for HER2 testing in breast cancer from 2007 and 2013 to both FISH and GPDP DISH assays, the HER2 gene amplification results showed 100% concordance among amplified/nonamplified cases, but there was a shift in 4 cases toward positive from equivocal results and toward equivocal from negative results. This might be related to the emphasis on the average HER2 copy number in the 2013 criteria. HER2 expression by IVD market IHC kit (Pathway®) has a strong correlation with GPDP HER2 protein, including a full concordance for all cases scored as 3+ and a reduction from 2+ to 1+ in 7 cases corresponding to nonamplified cases. Gene protein detection platform HER2 protein "solo" could have spared the need for 7 FISH studies. In addition, the platform offered advantages on interpretation reassurance including selecting areas for counting gene signals paralleled with protein IHC expression, on heterogeneity detection, interpretation time, technical time, and tissue expense.
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1. Introduction

Invasive breast cancer is the most frequent carcinoma in women and a principal cause of death from cancer among women globally [1,2]. Substantial improvements in survival have been recorded in Western countries in the last 4 decades, as a result of the combined effect of population screening, improved surgical techniques, and adjuvant treatment [2,3].

Besides the clinical and radiologic examinations, 4 predictive biomarkers are generally being evaluated on biopsies and on the surgically resected breast tumors [4,5]. The results of these diagnostics have a major impact on the classification of the lesion and on decisions for the sequential treatment [1,4,6]. For example, tumors that express the estrogen receptor α are considered eligible for endocrine therapy [7-10]. The proportion of Ki-67–positive cells identify tumors with high proliferation [5,10-12] rate and sensitivity to chemotherapy [13,14]. The human epidermal growth factor receptor 2 (HER2) encoded by the *ERBB2* gene (often referred to as the "*HER2* gene"), is a plasma membrane receptor tyrosine kinase with important functions during cell growth and differentiation [15]. In approximately 10% of breast cancers, *ERBB2* is amplified [16], causing overexpression of HER2 [17]. Overexpression of HER2 has been shown to play a role in sustaining multiple cancer pathways, including self-sufficiency in growth signals, sustained angiogenesis, increased cell division, and accelerated invasion [18-22]. HER2-positive breast cancer was earlier correlated to poor outcome. However, the development of HER2-targeted therapies has dramatically improved outcome for this group of patients. Inhibition of HER2 membrane signaling in breast cancer cells through therapy with humanized anti-HER2 antibodies such as trastuzumab or with small molecule inhibitors of HER2 tyrosine kinase activity has been shown to

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improve outcomes for women with both primary and metastatic *HER2*-amplified tumors [23-27]. However, this therapy is only recommended for patients with *HER2*-amplified tumors according to strict testing guidelines [28]. Because of unclear benefits and risk of substantial side effects, patients with negative or equivocal test scores are not recommended HER2-targeted therapy [29-31]. Consequently, accurate testing for *HER2* amplification or overexpression is essential [1].

Determination of HER2 status by immunohistochemistry (IHC) is relatively simple, rapid, and inexpensive, and pathologists have an established familiarity with IHC techniques and reagents. Immunohistochemistry HER2 status evaluations using formalin-fixed, paraffin-embedded tissues is, however, influenced by the tissue handling, fixation, and processing, which can greatly affect immunoreactivity of tissue antigens [28,32]. In addition, the interpretation of IHC is inherently subjective and qualitative, which leads to observer variability and affects the accuracy of results using this technique, and although computerized image analysis may reduce the interobserver variability, it cannot address the preanalytic variability owing to delay to fixation time, tissue fixation, and processing [23,28,33].

The fluorescence in situ hybridization (FISH) technique to determine *HER2* status uses a fluorescent tag-labeled DNA probe specific for the *HER2* gene. Some assays also use a fluorescent tag-labeled chromosome 17 centromere–specific enumeration probe (CEP17). These probes are hybridized to tissue sections under high-stringency conditions, and the *HER2* gene amplification status is assessed by enumeration of *HER2* gene copy signals (and chromosome 17 centromere signals). Fluorescence in situ hybridization offers some advantages: The DNA is relatively more insensitive to tissue handling or variation in fixative type or fixation time, and the scoring is quantitative with gene copy number being individually assessed. Fluorescence in situ hybridization is, therefore, by many authors considered more accurate in the determination of *HER2* status relative to IHC, especially in ambiguous cases [34-36]. On the other hand, FISH is usually a 2-day protocol, the signals fade with storage, specialized equipment is required (ie, fluorescence microscope and dark room), it has increased reagent expense relative to IHC and requires more pathologic expertise [33], and concerns have been raised on the consistency in cases with genomic heterogeneity and loss of chromosome enumeration probe or gene signals [28,33,37].

Well-established testing guidelines can be applied to the use of both IHC and FISH [10,28], and several HER2 test kits using IHC and in situ hybridization (ISH) have been approved by the Food and Drug Administration to date [38]. This includes methods of dual-color in situ hybridization (DISH) [28,39,40] in which HER2 status is typically determined by detecting *HER2* gene copies with silver ISH and chromosome 17 copies with chromogenic red ISH. This gives the pathologists the benefit of counting copies of chromosome 17 and *HER2* genes on the same slide in light microscopes that permit concurrent analysis of tissue morphology. In addition, the subtractive DISH probes reduce nonspecific hybridization caused by repetitive DNA sequences, and the staining is permanent and allows for tissue to be archived [39,41].

In this study, we measure the concordance of a recently developed automated combination of the IHC and the DISH techniques for HER2 determination in whole slide breast cancer specimens, which previously has been showing promising results in a tissue microarray (TMA) study [42]—the gene protein detection platform (GPDP) (Ventana Medical Systems, Tucson, AZ) with 2 well-established conventional IHC and FISH platforms for HER2 testing.

2. Materials and methods

The GPDP performance was evaluated in 52 consecutive invasive breast cancer resection specimens with HER2 IHC scores of 2+ and 3+ after approval from the local ethics committee at Karolinska Institutet, Stockholm, Sweden. New slides were prepared from archived paraffin blocks (registered at the Department of Pathology and Cytology, Karolinska University Hospital, during 2011 and 2012) and were processed in parallel for hematoxylin and eosin, IHC, FISH, and GPDP.

2.1. Immunohistochemistry platform

The Ventana (Ventana Medical Systems) PATHWAY anti-HER2/neu (clone 4B5) rabbit monoclonal antibody is a rabbit monoclonal antibody directed against the internal domain of the c-erb-2 oncoprotein (HER2). The antibody is identified by biotinylated secondary antimouse immunoglobulin G antibodies decorated with horseradish peroxidase (HRP). The site of HER2 is recognized by light microscopic identification of a brownish-red diaminobenzidine reaction product deposited by the action of the HRP [33].

2.2. Fluorescence in situ hybridization platform

The Abbott/Vysis (Abbott/Vysis, Inc, Downers Grove, IL) PathVysion HER2 DNA FISH Probe Kit is a dual-probe assay, using both a fluorescent tag-labeled DNA probe specific for the HER2 gene and a fluorescent tag-labeled CEP17. The DNA-associated proteins are removed before probe hybridization with proteinase digestion of all proteins. Subsequently, the double-stranded DNA of the tissue is denatured by heating to separate the DNA strands. A DNA probe directly labeled with a fluorescent tag is then incubated with the tissue section under high-stringency conditions. The HER2 probe binds specifically to the genomic *HER2* sequence and remains bound to this site after high-stringency washes to permit enumeration of the number of *HER2* gene copies in each nucleus [33].

2.3. Gene protein detection platform

The GPDP (Ventana Medical Systems) combines the PATHWAY HER-2/neu rabbit monoclonal antibody IHC (performed with iView DAB 3,3′-diaminobenzidine detection kit on a BenchMark XT automated staining system) and Ventana INFORM HER2 DISH DNA Probe Assay for *HER2* and CEP17 quantitation (performed with *HER2* and CEP17 probes labeled with 2,4-dinitrophenyl [DNP] and digoxigenin [DIG], respectively, on a BenchMark XT automated staining system). The DISH technique involves a number of steps: Initially, the DNP-labeled probe binds to the genomic *HER2* target; a monoclonal rabbit anti-DNP linker antibody binds to the DNP hapten; the site of this primary antibody is recognized by a second antibody, a goat antirabbit antibody that is labeled with an HRP multimer; silver reagents are added to the tissue section, resulting in the deposition of metal nanoparticles at the site of the HRP, which allow visualization of the ISH signal. The CEP17 detection kit uses a mouse antidigoxigenin and a goat antimouse labeled with alkaline phosphatase, allowing visualization of a red signal.

Before the 52 whole slide evaluations were performed, a TMA containing 30 cores of invasive breast cancer cases (registered at the Department of Pathology and Cytology, Karolinska University Hospital, during 2010 and 2011) was built to optimize the GPDP protocol. This TMA contained 10 negative controls (IHC score 0), 10 positive cases (IHC score 3+), and 10 equivocal cases (IHC score 2+) (Fig. 1). The cores had a diameter of 1 mm and contained both neoplastic epithelial and surrounding stromal tissue. Briefly, some assay procedure steps were altered such as cell conditioning time, antibody incubation time, avidin/biotin block, protease time, hybridization temperature, stringency temperature, silver chromogen time, and red chromogen time to improve both ISH signals, reduce silver background, enhance IHC staining, and control morphology quality (Fig. 2). We then proceeded with the scoring of whole slide specimens reported in detail below.

An experienced pathologist and a trained resident evaluated all material independently. Results were reported in accordance to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendations for HER2 testing in breast cancer from

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