Simultaneous Analysis of HER2 Gene and Protein on a Single Slide Facilitates HER2 Testing of Breast and Gastric Carcinomas

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Abstract

This study sought to evaluate a new combined gene and protein detection platform in the context of HER2 evaluation in breast and gastric carcinomas. HER2 immunohistochemistry (IHC) and dual color in situ hybridization (Dual ISH) were combined on a single slide. Results were compared with conventional HER2 IHC and fluorescence ISH. Results from the gene and protein assay were reliable and highly reproducible for both breast and gastric carcinomas. Concordance was found between conventional HER2 IHC and ISH testing and the gene and protein assay in the same laboratory (>95% for Dual ISH; lower for IHC because of different antibody clones), between IHC and Dual ISH performed on the same slide (>92%), and in the gene and protein assays between laboratories (>96%). This cost- and time-effective method provides fast and definitive results (IHC confirmed by means of Dual ISH) to aid in rapid treatment decisions. It can also be applied to other gene and protein combinations.

HER2 testing is standard in breast carcinomas and has recently gained relevance in gastric carcinomas.1-3 Amplification of the HER2 gene (ERBB2 gene) and the corresponding overexpression of HER2 protein occur in approximately 15% to 25% of breast cancers.4 Recent studies indicate that HER2 is also overexpressed in approximately 15% to 25% of gastric cancers and is associated with aggressive tumor behavior and a poor clinical outcome.5 Trastuzumab, a humanized monoclonal antibody that binds to HER2 protein on cancer cells, has been shown to benefit patients with HER2-positive breast and gastric carcinomas.6-8 Determination of HER2 status in breast or gastric carcinomas is required to support treatment decisions by predicting responses to trastuzumab.

Immunohistochemistry (IHC) and either bright-field chromogenic in situ hybridization (CISH) or fluorescence in situ hybridization (FISH), performed on separate glass slides, are widely accepted standard-of-care methods to evaluate HER2 protein expression and gene status, respectively, in breast and, more recently, gastric cancers. Well-defined scoring algorithms exist for both of these methods in both breast and gastric cancer.1,3

In situ hybridization (ISH) measures the HER2 gene, with or without chromosome 17 (Chr17) copy number, using FISH or bright-field CISH detection methods, including silver staining (SISH) or dual-color staining (Dual ISH, Ventana Medical Systems, Tucson, AZ). HER2 gene detection was first established using FISH methodology, which is more time-consuming than IHC, requires a fluorescence microscope and additional training, and is less stable than IHC staining. Recently established CISH detection methods are also accurate indicators of HER2 gene amplification and overcome the disadvantages of the FISH technique. Several recent studies
demonstrated excellent concordance (>95%) between CISH (including SISH and Dual ISH) and FISH assays in determining the status of HER2 gene amplification in breast\textsuperscript{9-14} and gastric carcinomas,\textsuperscript{15} as required by HER2 testing guidelines.\textsuperscript{1-3}

All HER2 testing algorithms advocate the use of IHC and/or ISH.\textsuperscript{1-3} Frontline testing with IHC, followed by reflex testing of IHC 2+ cases with an ISH method, is the testing algorithm most commonly used around the world. However, some laboratories perform frontline ISH testing on breast cancer cases. The consensus guidelines, based on the Trastuzumab for Gastric Cancer trial, also indicate that frontline IHC testing, followed by reflex ISH testing on IHC 2+ cases, is the recommended method, in particular to avoid treating ISH-positive, IHC-negative patients with trastuzumab because this group did not respond in the trial.\textsuperscript{8} However, very focal and heterogeneous HER2 positivity is often seen in gastric cancer and can make it challenging to evaluate IHC and ISH results run separately. A recent publication on HER2 testing in gastric cancer suggests that the IHC1+/IHC2+ cases and samples with focal and intense membranous reactivity in fewer than 10% cells may also be retested with ISH methods.\textsuperscript{16} Similarly, breast cancer can display heterogeneity for HER2 expression and gene amplification. Furthermore, evaluating the 2 methods separately can be time consuming. Because IHC and ISH methods provide complementary information, a simultaneous assessment combining both methods on one slide would facilitate evaluation and enhance test efficiency. Tubbs and colleagues\textsuperscript{17} pioneered this technique and provided proof of principle in 2004. Two other previous studies further demonstrated the feasibility of this approach.\textsuperscript{18,19}

Downs-Kelly et al\textsuperscript{18} found good concordance between the CISH results obtained using a dual assay (IHC and CISH on the same slide) and the results of conventional FISH alone. Reisenbichler et al\textsuperscript{19} recently reported a dual assay (IHC and CISH on the same slide) and demonstrated concordance of the dual assay CISH results with the results of CISH when performed alone; however, this dual assay was associated with a reduced IHC signal in some cases. With the continued development of personalized health care and additional companion diagnostic markers, evaluation of other protein and gene combinations, beyond HER2 and Chr17, will become important. Simultaneous evaluation of such protein and gene combinations on a single glass slide offers an elegant detection method for such testing.

An automated gene and protein detection platform that combines IHC and dual-color ISH detection on a single slide and uses the established VENTANA BenchMark series slide-staining instrument (Ventana) is under development. This study examined the performance of the new combined gene and protein detection platform in the context of HER2 evaluation in breast and gastric cancer cases.

The aim of this study was to determine whether there was equivalence between the gene and protein detection platform and corresponding conventional single stains run separately, as per the study site’s usual clinical practice. The single-stain assays were performed on separate slides, and the gene and protein assay was conducted on serial sections of the same tissue microarrays (TMAs) independently at 2 laboratories (Institute of Pathology, Lucerne Cantonal Hospital, Lucerne, Switzerland and Ventana), thus making it possible to assess assay reproducibility.

**Materials and Methods**

**Study Design**

The new gene and protein detection platform combines PATHWAY anti-HER2/neu (4B5) rabbit monoclonal primary antibody (PATHWAY HER2 [4B5], Ventana) for IHC and the INFORM HER2 Dual ISH DNA probe cocktail assay (INFORM HER2 Dual ISH, Ventana) for ISH on a single slide. This gene and protein test was used for the analysis of HER2 in TMAs comprising breast and gastric carcinomas and normal tissue. Results were compared with conventional HER2 FISH (Zytovision, Bremerhaven, Germany) and HER2 IHC (clone CB11, Leica Biosystems, Buffalo Grove, IL).

**TMAs**

The TMAs were constructed from archival tissue samples of the Lucerne Institute of Pathology and comprised 82 breast carcinomas (28 FISH-positive, 2 borderline cases, and 20 cases with difficult previous FISH), 14 samples of normal breast tissue, 25 gastric carcinomas (6 FISH-positive), and 24 samples of normal gastric mucosa.

**IHC Alone**

HER2 IHC was performed using the CB11 mouse monoclonal antibody (dilution 1:40) on the VENTANA BenchMark XT System (CC1 standard; iView DAB Detection Kit). Scoring of the results was performed according to American Society of Clinical Oncology (ASCO) guidelines.\textsuperscript{3}

**FISH Alone**

FISH analysis was performed using the Zytolight HER2 probe set (Zytovision). After deparaffinization and rehydration, the slides were incubated in boiling citrate buffer (pH 6.0) for 15 minutes and then cooled in buffer at room temperature for 20 minutes. The sections were digested with pepsin (Leica Biosystems), followed by a 10-minute postfixation in 4% formalin. After dehydration in a series of increasing concentrations of ethanol (70%, 95%, and 100%) and air-drying, the HER2 probe set was added to the tissue.
The slides were co-denaturated for 10 minutes at 73°C (on a heating plate) and hybridized at 37°C overnight. Posthybridization washes were carried out in 1.5 SSC/0.1% Tween 20 at 73°C for 5 minutes. After drying of the slides, the tissue was covered with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). The results were scored following ASCO guidelines. For HER2 FISH, 60 nuclei were counted for signals from CEN 17 and HER2 using a Z1 AxioVision microscope (Zeiss, Jena, Germany) and corresponding filter sets.

**Gene and Protein Detection Platform**

The gene and protein detection platform combines HER2 IHC and HER2 Dual ISH (HER2 gene with Chr17 copy number) on a single section of formalin-fixed, paraffin-embedded human tissue on a VENTANA BenchMark XT series slide-staining instrument [Figure 1]. For detection of HER2 protein expression with IHC, the PATHWAY HER2 (4B5) antibody was used with the VENTANA ultraView DAB IHC detection kit. The HER2 gene and Chr17 centromere were detected using the INFORM HER2 Dual ISH DNA probe cocktail and visualized using the ultraView SISH DNP detection kit and ultraView Red ISH DIG detection kit. Dual ISH staining resulted in visualization via light microscopy in which HER2 appeared as discrete black signals (SISH) and Chr17 as red signals (Red ISH) in the nuclei.

IHC was always performed first and then followed by Dual ISH. The sequence of events on a single slide consisted of deparaffinization, pretreatment (cell conditioning 1), incubation with primary and secondary antibody (PATHWAY HER2 [4B5] and HRP-conjugated goat antirabbit antibody), IHC detection with the VENTANA ultraView DAB detection kit, pretreatment (VENTANA Cell Conditioning 2, protease, VENTANA HybClear Solution, denaturation), probe hybridization (simultaneous cohybridization of 2 probes, DIG and DNP) with HybClear applied simultaneously to enhance the reaction, stringency washes, silver and red ISH detection (detection of the DNP probe with the ultraView SISH detection [black dots], and detection of the DIG probe with the ultraView Red DIG detection), and counterstaining (hematoxylin II and bluing reagent to color the whole section in blue to improve the contrast). The run protocol was 15 hours on the VENTANA BenchMark XT system.

The PATHWAY HER2 (4B5) IHC, individually, is an approved product for determining HER2 status for breast cancer. The INFORM HER2 Dual ISH assay, individually, is an approved product for determination of HER2 status for both breast and gastric cancer. HybClear is a unique reagent specific to the VENTANA gene and protein detection platform that enables combination of both techniques on a single slide. All other reagents are already used for the single IHC assay or the Dual ISH assay. The VENTANA BenchMark series slide-staining instrument is also widely used in many laboratories.

**Evaluation of Slides**

The slides were scored according to guidelines for breast and gastric carcinomas. IHC was used to identify and select a target area with adequate staining, and then representative carcinoma cells that contain both HER2 (black signals) and Chr17 (red signals) were enumerated. In target areas that were genetically heterogeneous for HER2 copy numbers, only nuclei representing the population of invasive carcinoma nuclei with the highest average number of signals were counted. For breast carcinomas to be considered positive for HER2 protein overexpression (IHC), they had to meet threshold criteria for the intensity and pattern of membrane staining.
(3+ on a scale of 0 to 3+) and for the percentage of positive tumor cells (>30% according to ASCO guidelines). Staining had to localize to the cell membrane and be circumferential (complete). Staining of the cytoplasm could be present, but this staining was not included in the determination of positivity. For gastric carcinomas to be considered positive for HER2 protein overexpression, they had to meet threshold criteria for the intensity and pattern of membrane staining (3+ on a scale of 0 to 3+) and for the percentage of positive tumor cells (≥10% for a resection specimen or ≥5 cohesive cells for a biopsy specimen). Staining had to localize to the cell membrane but did not need to be completely circumferential (basolateral staining was regularly observed and considered for scoring). Staining of the cytoplasm and/or the nucleus could be present, but this staining was not included in the determination of positivity.

HER2 gene status was reported as a function of the ratio of the average number of HER2 gene copies to the average number of Chr17 copies in nuclei of cells within an invasive breast or gastric carcinoma. HER2 gene status was classified as nonamplified (HER2/Chr17 ratio <2.0) or amplified (HER2/Chr17 ratio ≥2.0). Once an adequate target area was identified, the scores for HER2 and Chr17 copy numbers present in 20 representative nuclei were scored. If the resulting HER2/Chr17 ratio fell within 1.8 to 2.2, an additional 20 nuclei were scored and the resulting ratio was calculated from the total of 40 nuclei.

Analysis of Results

IHC and ISH were compared separately between dual gene-protein staining and single assay staining methods. For IHC, specimens scored as 0 or 1+ were classified as negative and specimens scored as 2+ or 3+ were classified as positive. Gene status was classified as amplified if the SISH or FISH HER2/Chr17 ratio was 2 or more and classified as not amplified if the ratio was less than 2. In some cases, the ISH result was recorded as “cluster” or “focal positivity,” which were both classified as amplified; only 2 cores in the gastric tissue TMA were affected.

Agreement rates between assays or between laboratories were calculated with 95% confidence intervals (CIs).

Results

Validation and Technical Aspects of the Gene and Protein Detection Platform

When the gene and protein detection platform was used as recommended by Ventana, the ISH signal was excellent; however, the IHC signal was too weak. The IHC component of the assay was optimized by varying the incubation time of the pretreatment solution and antibody, which resulted in a signal comparable to that of IHC alone. Image 1 and Image 2. The dual assay described by Reisenbichler et al also resulted in weak IHC signals, which were not optimized, and in their assay IHC had to be performed after ISH. Furthermore, in their assay, the ISH signals appear to be reduced and were often missing in stromal cells. In contrast, the gene and protein detection platform always produced adequate HER2 signals in stromal cells, which served as internal controls.

Image 2 shows HER2 gene and protein detection in breast carcinomas with high IHC 3+ (Image 2A) and low IHC 1+ (Image 2B) levels of HER2 expression. The left panels show HER2 IHC alone (iView DAB with CB11 antibody), the middle panels show HER2 FISH alone, and the right panels show the gene and protein detection platform IHC (PATHWAY HER2 [4B5]) and Dual ISH on the same slide. Using the gene and protein detection platform, HER2 protein expression (brown staining), HER2 gene copies (black clusters), and Chr17 (red dots) were simultaneously revealed on a single slide.

Image 3 shows gene and protein detection in a gastric carcinoma with an intermediate IHC 2+ level of HER2 expression. Image 3A shows HER2 FISH alone, and Image 3B shows simultaneous IHC (4B5 antibody) and Dual ISH on the same slide. HER2 gene analysis revealed HER2 amplification with both FISH and Dual ISH. IHC of gastric carcinoma with 4B5 antibody often shows strong staining of neighboring normal gastric mucosa as an internal positive control; however, this is well known and always taken into account.

The gene and protein detection platform allows both gene and protein detection on 1 slide in 15 hours and is simple to implement in the laboratory using the VENTANA BenchMark XT system (or any VENTANA BenchMark series slide-staining instruments). The assay uses a standard slide-staining instrument, so it can be implemented and standardized across laboratories. Good results can even be obtained in difficult material (eg, varying fixation), demonstrating that the assay is robust even if there are technical issues with the samples.

Heterogeneity of tumor HER2 overexpression and gene amplification is common in gastric carcinomas compared with breast carcinomas. IHC and ISH together on the same slide enhance the ability to find areas of overexpression and amplification. IHC can be used to find an area of tumor with clear HER2 expression (brown staining), and HER2 gene amplification can be confirmed with Dual ISH. The guidelines for HER2 testing in gastric cancer state that precise scanning of tumors is important because of the heterogeneity of at least some advanced gastric carcinomas.

In our experience, Chr17 signals may sometimes be lost in tumor cell nuclei in cases of high HER2 amplification. Because this is only seen at high amplification and because
these cases also have a high IHC score, this is unlikely to cause false-negative results.

### Concordance Between Conventional HER2 Testing (IHC or FISH Alone) and the Gene and Protein Detection Platform in the Same Laboratory

HER2 gene detection results using the gene and protein detection platform (Dual ISH) agreed with conventional FISH results in 76 (96.2%) of 79 (95% CI = 89.4-98.7) and 41 (95.4%) of 43 (95% CI = 84.5-98.7) breast and gastric carcinomas, respectively. This high level of concordance is consistent with studies showing concordance between bright-field CISH and FISH assays in determining the status of HER2 gene amplification in breast and gastric carcinomas, and complies with the HER2 testing guidelines that require more than 95% concordance between assays. Discrepancies could be explained by intratumor heterogeneity and different sampling.

HER2 protein detection results using the gene and protein detection platform IHC (PATHWAY HER2 [4B5]) agreed with the single-staining IHC results (clone CB11 antibody) in 58 (76.3%) of 76 (95% CI = 65.6-84.5) and 36 (85.7%) of 42 (95% CI = 72.2-93.3) breast and gastric carcinomas, respectively. The poor concordance is caused by the
considerable cytoplasmic staining by CB11. We therefore performed an ex post comparison of HER2 protein detection using PATHWAY HER2 (4B5) for single-staining IHC with the VENTANA gene and protein detection platform IHC. Only 57 TMA dots were available for this analysis, which, however, showed complete agreement of IHC results from the single staining and the gene and protein detection platform (data not shown).

The IHC assay alone (clone CB11) that was used in this study is a well-established test. Monoclonal antibody clones CB11 and TAB250 and the polyclonal antiserum A0485 are the most widely used alternatives to the original HercepTest and to PATHWAY HER2 (4B5). The gene and protein detection platform used the PATHWAY HER2 (4B5) for HER2 IHC. The present study demonstrates that PATHWAY HER2 (4B5) is clearly superior to clone CB11. The accuracy of clone CB11 antibody for HER2 assessment is controversial. Recently Purdie et al reported excellent performance of clone CB11 on breast core biopsies with reliable prediction of HER2 amplification. In contrast, according to van der Vegt et al and Powell et al, clone 4B5 is more robust and correlates better than clone CB11 with FISH, a finding that is in agreement with our observations.

**Table 1**

Concordance Between Conventional HER2 Testing in Single Assays (IHC or FISH alone) and Dual Detection Using the GP Detection Platform in the Same Laboratory

<table>
<thead>
<tr>
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<th>Agreement Rate for ISH vs IHC</th>
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<tr>
<td></td>
<td>FISH Alone</td>
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<tr>
<td></td>
<td>No./Total (%)</td>
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<tr>
<td>Breast carcinoma (96 cases)</td>
<td></td>
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<tr>
<td>IHC alone (CB11)</td>
<td>59/77 (76.6)</td>
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<tr>
<td>IHC from GP test (4B5)</td>
<td>71/79 (89.9)</td>
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<tr>
<td>Gastric carcinoma (49 cases)</td>
<td></td>
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<tr>
<td>IHC alone (CB11)</td>
<td>32/38 (84.2)</td>
</tr>
<tr>
<td>IHC from GP test (4B5)</td>
<td>41/43 (95.4)</td>
</tr>
</tbody>
</table>

CI, confidence interval; FISH, fluorescence in situ hybridization; GP, gene and protein; IHC, immunohistochemistry; ISH, in situ hybridization; SISH, silver staining in situ hybridization.

* Assays were run at the Institute of Pathology, Lucerne Cantonal Hospital, Lucerne, Switzerland.
95.7% of breast and gastric carcinomas, respectively. Only a single breast case had an IHC (PATHWAY HER2 [4B5]) score of 0 but an ISH score of 2.1 with the gene and protein method. The concordance between HER2 GP IHC (PATHWAY HER2 [4B5]) and conventional FISH alone was also good: the results agreed in 89.9% and 95.4% of breast and gastric carcinomas, respectively. HER2 GP IHC (PATHWAY HER2 [4B5]) agreed with HER2 ISH gene status at higher rates than did the single-staining IHC (clone CB111 antibody); see Table 1.

Concordance of HER2 Testing Using the Gene and Protein Detection Platform Between 2 Different Laboratories

To assess reproducibility of the gene and protein detection platform, HER2 staining of the same TMAs was conducted at 2 independent laboratories (Lucerne and Tucson). Table 2 shows the agreement rates for gene and protein (PATHWAY HER2 4B5/INFORM HER2 Dual ISH) between the 2 laboratories. Both breast and gastric tissue with the dual gene and protein staining showed high concordance in both IHC and ISH between laboratories, with agreement rates higher than 96%. For Dual ISH, the agreement rates were 100.0% and 97.4% for breast and gastric carcinomas, respectively. For gene and protein IHC (clone 4B5 antibody), the agreement rates were 96.2% and 100% for breast and gastric carcinomas, respectively.

Discussion

Our results demonstrate that the gene and protein detection platform is highly reproducible between laboratories. Further testing of reproducibility (in Lucerne and Tucson) demonstrated good agreement of runs and slides (data not shown). Thus, the gene and protein detection platform is very robust, with reproducible staining and scoring, and shows very high concordance between independent laboratories. The small discrepancies observed between laboratories are probably due to differences in interpretation of heterogeneous samples.

In conclusion, the single-step, single-slide gene and protein detection platform, which uses standard automated slide-staining equipment (VENTANA BenchMark series slide-staining instruments), has provided reliable and highly reproducible results for both breast and gastric carcinomas in the context of HER2 and Chr17 assessment. It allows protein and gene detection on 1 slide in 15 hours and is simple to implement in the laboratory using a standard slide-staining instrument. Good results can even be obtained in difficult material, such as TMAs, demonstrating that the assay is robust even if there are technical issues with the samples. The method is both cost- and time-effective and provides definitive results (IHC confirmed by ISH) for HER2 status in a short time, which will aid in rapid treatment decisions.

Simultaneous IHC and ISH at the single-cell level allow direct cell-to-cell comparison of protein overexpression and gene amplification. In the context of HER2, this technique is particularly useful for assessment of equivocal IHC and borderline ISH results. It also enables detection of those rare cases with gene amplification and 0/I+ IHC results that would not normally undergo ISH testing but would still be eligible for trastuzumab therapy. The assay may become the preferred test for tumors with heterogeneous HER2 amplification (frequently seen in gastric carcinomas) and in cases with only a small focus of invasive tumor.

Finally, the new gene protein detection platform is an elegant technique to evaluate other gene and protein combinations as new companion diagnostic markers are developed to allow for personalized health care.

Table 2

<table>
<thead>
<tr>
<th>Agreement Rate Between 2 Different Laboratories*</th>
<th>No./Total (%)</th>
<th>(95% CI)</th>
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<tbody>
<tr>
<td>Breast carcinoma (96 cases)</td>
<td></td>
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<tr>
<td>GP SISH</td>
<td>79/79 (100.0)</td>
<td>(95.3-100.0)</td>
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<tr>
<td>GP IHC</td>
<td>75/76 (96.2)</td>
<td>(93.3-98.7)</td>
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<tr>
<td>Gastric carcinoma (49 cases)</td>
<td></td>
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<tr>
<td>GP SISH</td>
<td>37/38 (97.4)</td>
<td>(86.5-99.5)</td>
</tr>
<tr>
<td>GP IHC</td>
<td>38/38 (100.0)</td>
<td>(98.0-100.0)</td>
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* The 2 laboratories were at the Institute of Pathology, Lucerne Cantonal Hospital, Lucerne, Switzerland, and Ventana Medical Systems, Tucson, AZ.

References


From the 1Institute of Pathology, Lucerne Cantonal Hospital, Lucerne, Switzerland; 2Ventana Medical Systems, Tucson, AZ; 3Roche Diagnostics, Strasbourg, France; and 4Roche Tissue Diagnostics, Barcelona, Spain.

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