Evaluation of Dual Immunohistochemistry and Chromogenic In Situ Hybridization for HER2 on a Single Section

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Abstract

The evaluation of HER2 status in invasive breast carcinoma can be performed by multiple methods. We assessed the feasibility of performing 2 of these, chromogenic in situ hybridization (CISH) and immunohistochemical staining, on single tissue sections of breast carcinoma. During assay development, sequential performance of immunohistochemical staining after CISH resulted in weaker HER2 expression than that obtained when immunohistochemical staining was performed alone; this was ameliorated by increased antibody incubation time. Performance of both techniques in a combined/hybrid protocol resulted in HER2 protein expression and gene signals identical to those produced by the individual techniques performed alone. Prospective validation of these dual staining protocols in 31 cases of breast carcinoma resulted in 100% concordance with results of CISH when performed alone, but was still associated with a reduced immunohistochemical signal in some cases. Although further testing is needed, we conclude that performance of both immunohistochemical staining and CISH on a single section is possible and could allow for direct “cell-by-cell” comparison of HER2 signals and potentially offer a more economical and real-time method for ongoing validation of HER2 testing.

Protein overexpression and/or gene amplification of the human epidermal growth factor receptor-2 (HER2) is seen in approximately 20% of breast carcinomas1-3 and is associated with significant prognostic and therapeutic implications. Multiple techniques are available for the evaluation of HER2 status in invasive breast carcinoma, the most common of which are immunohistochemical analysis and fluorescence in situ hybridization (FISH), each with distinct advantages and disadvantages.2,4 Another method increasing in popularity is chromogenic ISH (CISH),5,6 whereby gene amplification is evaluated using conventional bright-field microscopy, a distinct advantage over FISH.

HER2 status determines patient eligibility for particular adjuvant or neoadjuvant therapeutic regimens that can include trastuzumab or other similar receptor kinase inhibitors. Given the cost and potential side effects of such agents, accurate evaluation of HER2 is essential for correctly identifying patients who may benefit from such therapy and excluding patients who would not and might also have serious side effects from such unnecessary treatment. Accordingly, the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) have recently established guidelines for assessing HER2 status7 to help improve the accuracy and consistency of HER2 testing. The ASCO/CAP recommendations have been shown to improve the specificity of HER2 immunohistochemical staining8 and decrease interobserver variability in its interpretation.9

Several ASCO/CAP guidelines require that some cases need to be tested by both immunohistochemical staining and ISH. These obviously include cases found to be equivocal for protein overexpression or amplification that need to be tested by an alternative method. Dual immunohistochemical and ISH testing is also performed for initial and ongoing validation of
laboratory testing to maintain the required 95% concordance between the 2 tests. Moreover, there are certain laboratories that perform dual testing on additional nonequivocal cases as part of quality control/assurance\textsuperscript{10} and/or because some clinicians require both before deciding on further management.\textsuperscript{11} This is obviously confounded by the fact that it is still unclear which assay (immunohistochemical staining or ISH) is superior to the other in predicting benefit from anti-HER2 therapy.\textsuperscript{7}

Given the aforementioned reasons to test for protein overexpression and gene amplification of HER2, it might be beneficial to simultaneously test for these on 1 section. In fact, 2 prior studies have previously found this to be feasible\textsuperscript{12,13} and, using different methods, found good concordance with results of FISH. To the best of our knowledge, however, there are no studies that directly compare results of both immunohistochemical analysis and CISH when each is used alone with the results of using them in combination on a single section. As is the case for immunohistochemical antibody cocktails,\textsuperscript{14} we believe this is a fundamental approach in characterization of any new test that attempts to combine 2 or more individual tests.

Accordingly, the goal of our study was the performance of both immunohistochemical analysis and CISH for HER2 on a single section and compare the results with those obtained using both immunohistochemical analysis and CISH performed individually. Furthermore, in an attempt to limit the inherent bias associated with retrospective studies, a prospective approach was used for validation.

**Materials and Methods**

**Assay Development**

We selected 4-μm sections of formalin-fixed, paraffin-embedded tissue from 2 invasive breast carcinomas, previously known to be positive or negative for HER2 overexpression and amplification, to be used for assay development. These cases were first tested using immunohistochemical staining and CISH separately, after which the staining protocols were adjusted to allow for dual testing for protein expression and gene amplification on the same section. The separate and dual protocols used for staining are described subsequently.

**Immunohistochemical Studies**

Immunohistochemical staining was carried out using a HER2 antibody (clone SP3, dilution 1:400; ThermoFisher, Kalamazoo, MI) and an automated immunostainer (Autostainer 720, Lab Vision, Fremont, CA). Following deparaffinization, heat-induced antigen retrieval (citrate buffer pH 6.0 for 20 minutes at 95°C), and blocking of endogenous peroxidase with hydrogen peroxidase for 10 minutes and ultra block (ThermoFisher) for 5 minutes, sections were incubated for 30 minutes with the HER2 antibody. The horseradish peroxidase (HRP) probe was applied for 10 minutes, followed by the HRP polymer detection kit (Biocare Medical, Concord, CA) for 10 minutes. The reaction was visualized by incubation with diaminobenzidine (DAB) substrate-chromogen solution for 5 minutes. Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.

**Chromogenic In Situ Hybridization**

CISH was performed using manufacturer-provided protocols and reagents (Invitrogen SPOT-Light kit, Invitrogen, Carlsbad, CA) as follows: Following deparaffinization, the tissue was placed in the heat pretreatment solution (reagent A) for 15 minutes at 98°C. After rinsing with distilled water (dH\textsubscript{2}O), the enzyme pretreatment reagent (reagent B) was added for 5 minutes at room temperature followed by an additional dH\textsubscript{2}O wash. Dehydration in ethanol was followed by addition of the HER2 probe (reagent C) with overnight hybridization at 37°C. Sections were then incubated in saline sodium citrate buffer (reagent D) for 5 minutes at 70°C. After rinsing in dH\textsubscript{2}O, 3% hydrogen peroxide was added for 10 minutes followed by CAS-Block (reagent F) for 10 minutes at room temperature. Sections were then incubated with mouse antidigoxigenin antibody for 30 minutes. After application of the antimouse HRP polymer conjugate for 30 minutes, the reaction was visualized by incubation with DAB substrate-chromogen solution for 30 minutes. Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.

**Sequential Protocol: Immunohistochemical Studies Followed by CISH**

Following the immunohistochemical protocol described, sections were rehydrated, and the CISH protocol described was performed, beginning with antigen retrieval until the end of the protocol.

**Sequential Protocol: CISH Followed by Immunohistochemical Studies**

Following the CISH protocol described, sections were rehydrated, and the immunohistochemical protocol described was performed, beginning with antigen retrieval until the end of the protocol. After evaluation of preliminary findings (see the “Results” section), incubation with the HER2 antibody was increased to 45 minutes.

**Combined/Hybrid Protocol**

The CISH protocol was performed according to manufacturer-provided protocols but interrupted just before incubation with DAB. Slides were then transferred to the automated immunostainer where the immunohistochemical protocol was carried out as described for immunohistochemical studies alone (antigen retrieval, peroxidase block, HER2 antibody application,
polymer detection, and visualized by incubation with DAB substrate-chromogen solution). Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.

**Assay Validation**

The previously described immunohistochemical, CISH, sequential (CISH followed by immunohistochemical analysis), and combined/hybrid protocols were performed on a HER2 CAP tissue microarray (TMA; 2009 HER2-01 A, mailing module B) that included 10 cases and prospectively on 31 sequential cases of invasive breast carcinoma diagnosed at our institution.

**Interpretation of Staining**

Evaluation of HER2 protein expression (0-3+) and gene amplification (negative, equivocal, or positive) was performed according to the ASCO/CAP guidelines.\(^7\) Results of gene amplification by CISH are derived from evaluating a minimum of 30 tumor cell nuclei. In cases with equivocal results, an additional 30 cells were counted. Results of staining using the different protocols were compared using agreement rates and by calculation of weighted \(\kappa\) values.

**Regulatory Approval**

The study was approved by the University of Alabama at Birmingham Institutional Review Board.

**Results**

**Assay Development**

**CISH and Immunohistochemical Studies Alone**

In the HER2\(^+\) case, immunohistochemical staining showed intense membranous staining of the majority of invasive tumor cells (3+ score) Image 1A, whereas CISH displayed small signal clusters within the tumor nuclei Image 1B. The
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Immunohistochemical staining alone. Increasing the antibody incubation to 45 minutes (vs 30 minutes) seemed to correct this as it resulted in a 3+ membranous staining pattern (Image 1E), practically identical to that seen using immunohistochemical staining alone.

Combined CISH/Immunohistochemical Studies

The combined protocol resulted in a pattern of staining that was identical to that seen using immunohistochemical staining and CISH when performed individually (Image 1F).

Assay Validation in 10 TMA Cases

CISH Alone

CISH performed alone displayed amplification in 2 cases, and 8 cases were nonamplified. This finding was 100% concordant with the correct responses provided in the CAP survey results.

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Sequential CISH Followed by Immunohistochemical Studies

The sequential protocol demonstrated CISH results identical to those seen when CISH was performed alone. The 2 cases showing amplification by CISH also showed continuous membranous staining (3+) Image 2L, which was also concordant with the results of the CAP survey.

Assay Validation in 31 Prospective Cases

CISH and Immunohistochemical Studies Alone

CISH performed alone displayed HER2 amplification in 6 cases Image 3L with the remaining 25 nonamplified Image 4L. Immunohistochemical analysis performed alone resulted in 4 cases positive for HER2 overexpression (3+), 6 equivocal (2+), and 21 negative (0 or 1+).

Sequential CISH Followed by Immunohistochemical Studies

Compared with separately performed CISH, the sequential protocol demonstrated identical gene amplification results in all cases (100% agreement). Results of immunohistochemical staining using this protocol showed agreement with the original separately performed immunohistochemical staining in 78% of cases (weighted $\kappa = 0.610$), with a reduced immunohistochemical score in 6 cases (Image 3, case 27) and a higher immunohistochemical score in 1 case Image 1A.

Combined CISH/Immunohistochemical Studies

Compared with separately performed CISH, the combined protocol demonstrated identical gene amplification results in all except 1 originally nonamplified case that became equivocal (97% agreement; weighted $\kappa = 0.974$). Results of immunohistochemical staining using this protocol showed agreement with the original separately performed immunohistochemical staining in 71% of cases (weighted $\kappa = 0.467$), with a reduced immunohistochemical score in 8 cases and a stronger immunohistochemical score in 1 case Image 1B.

Discussion

In this study, we have clearly shown that using a “sequential” or “combined” protocol to perform HER2 immunohistochemical analysis and HER2 CISH on a single section can produce results similar to those using each method individually. These dual protocols produced excellent concordance with separately performed CISH but were associated with a reduced immunohistochemical signal in some cases resulting in suboptimal concordance with separately performed immunohistochemical staining. Although additional studies are still needed, such dual immunohistochemical/CISH protocols could allow for the evaluation of HER2 protein expression and gene amplification on a single slide, which would be advantageous for small invasive tumors, equivocal cases, and validation purposes.

Inconsistency with immunohistochemical staining for HER2 is not uncommon, and variability in the sensitivity of commercially available antibodies and susceptibility of tissue to processing variables are only some explanations for the occasional unpredictability of HER2 by immunohistochemical analysis.15,16 The ASCO/CAP guidelines were established to reduce such variations in performing and evaluating HER2. The recommended algorithm includes the use of immunohistochemical analysis and FISH.7 Despite the
Assay validation. Two prospectively evaluated cases of breast carcinoma that were positive for HER2 overexpression and gene amplification. Notice the reduced HER2 immunohistochemical score in case 27. Sequence protocol, immunohistochemical analysis after chromogenic in situ hybridization (×600).

ASSAY validation. Two prospectively evaluated cases of breast carcinoma that were negative for HER2 overexpression and gene amplification. Sequence protocol, immunohistochemical analysis after chromogenic in situ hybridization (×600).
Two studies have addressed combining HER2 immunohistochemical staining and ISH on a single section of breast carcinoma.12,13 On a TMA of 94 invasive breast carcinomas, Downs-Kelly et al12 combined conventional immunohistochemical staining with a silver-based bright-field ISH method (SISH) and compared the results with those produced by immunohistochemical staining and FISH when performed alone. Although the authors found an excellent correlation between the HER2 gene copy numbers determined by SISH and FISH, there were 4 discrepant cases (including 1 that would currently be considered equivocal per the ASCO/CAP guidelines) in which amplification was detected by one method but not the other (96% concordance rate). There was also excellent correlation between immunohistochemical staining combined with SISH and immunohistochemical staining alone; however, no concordance rate was reported, and an analysis of discrepant cases was not performed. The second study, by Ni et al,13 used full-face sections (81 cases) and a TMA (53 cases) to combine immunohistochemical staining and CISH and compare the results with those obtained by immunohistochemical staining and FISH when performed alone. Although the authors found an excellent correlation between the HER2 gene copy numbers determined by SISH and FISH, there were 4 discrepant cases (including 1 that would currently be considered equivocal per the ASCO/CAP guidelines) in which amplification was detected by one method but not the other (96% concordance rate). There was also excellent correlation between immunohistochemical staining combined with SISH and immunohistochemical staining alone; however, no concordance rate was reported, and an analysis of discrepant cases was not performed. The second study, by Ni et al,13 used full-face sections (81 cases) and a TMA (53 cases) to combine immunohistochemical staining and CISH and compare the results with those obtained by FISH. There was also excellent correlation between the HER2 gene copy numbers determined by CISH and FISH, with more than 90% concordance between CISH and FISH results with all of the discordant cases secondary to low-level amplification (currently considered equivocal) and/or chromosome 17 aneusomy. The latter study did not perform any comparison to separately performed immunohistochemical studies.

Both of the aforementioned studies clearly document the feasibility of combining immunohistochemical staining and ISH for HER2 on a single section. Nevertheless, one has to note that although the results of the combined assays were
compared with FISH performed on the same sections and, as such, are sufficient for validation of a “new” HER2 test per ASCO/CAP guidelines, neither of these studies specifically compared the results of each component of the combined immunohistochemical/ISH assay with those of separately performed immunohistochemical studies and ISH (on the same sections). Such an approach is necessary to characterize the effect of modifying the staining protocols to accommodate both tests. Accordingly, we designed the current study to specifically address this, to better evaluate the concordance of the immunohistochemical component with separately performed immunohistochemical staining, and to minimize the potential bias associated with retrospective studies.

One significant finding of the current study was that the sequential protocol of CISH after immunohistochemical analysis did not produce any gene signals. Previous studies,21-25 combining immunohistochemical analysis and ISH to evaluate protein and messenger RNA simultaneously, have produced variable results, often with a reduced RNA signal when ISH is performed after immunohistochemical analysis.22,23 Apart from the issue of RNA degradation due to ribonucleases, Chiu et al23 hypothesized that chromogens, such as DAB, used during the final immunohistochemical step could be responsible for messenger RNA signal loss. Similarly, it is quite plausible that free oxygen radicals generated during the peroxidase/DAB reaction irreversibly damaged the DNA and prevented probe hybridization in our study, resulting in absence of HER2 gene signals. Supporting this is that an alkaline phosphatase immunohistochemical detection method, used with or before ISH in the studies by Downs-Kelly et al12 and Ni et al,13 did not seem to impair hybridization. We believe that there could be other potential explanations, especially because we were also unable to detect HER2 gene signals in this sequential protocol when an alkaline phosphatase detection method was used instead of HRP (data not shown).

Although the reverse sequential protocol (immunohistochemical staining after CISH), as well as the combined protocol, clearly allowed for visualization of protein and gene signals with excellent concordance in HER2 amplification status, they were both associated with reduced HER2 protein expression signal in some cases. It is interesting that Downs-Kelly et al12 also found that the correlation between the immunohistochemical results in their dual-staining technique was lower than that of ISH; however, no details were provided about discrepant cases. It is likely that this reduction is secondary to membrane digestion by protease, a necessary step for probe penetration. We were able to correct this during assay development in our study by increasing the antibody incubation time from 30 minutes to 45 minutes; however, this issue resurfaced again in the prospective series (Figure 1), which may be related to the potential day-to-day variation in testing induced by the prospective nature of the study (compared with retrospective studies in which cases are more likely to be batched together for testing). One of our future goals is to test cases (including the discrepant ones in this study) with yet a longer antibody incubation period (60 minutes) and again compare the results with those obtained by immunohistochemical testing alone.

Another technical issue that needs to be mentioned was the occasional loss of tissue from the sections secondary to the stringent preparation conditions necessary for dual CISH and immunohistochemical analysis, including protease digestion and duplicate heat pretreatment. To the best of our knowledge, there are no data regarding the frequency of this with dual staining in the literature, but loss of tissue necessitated repeated testing in a few of our cases (<15%; a rate close to our repetition rate for CISH alone due to loss of tissue, overdigestion, hybridization failure, etc).

Finally, a shortcoming of this study that warrants discussion is that it included a relatively small proportion of HER2-amplified and HER2-equivocal cases, which is a reflection of the prospective nature of the study (and both proportions being similar to larger series). A future goal is to specifically analyze a larger number of cases, especially cases equivocal for HER2 protein overexpression and/or gene amplification, to better determine the role of dual immunohistochemical studies/CISH, if any, for the routine evaluation of HER2 status in breast carcinoma. One should note, however, that an equivocal or negative HER2 immunohistochemical score by itself would not affect patient management when gene amplification status is known because either HER2 protein overexpression or gene amplification is a sufficient trigger for trastuzumab (or similar) treatment. In fact, review of our results would clearly show that the reduced HER2 protein signal with dual staining seen in some of our cases would not have changed the clinical management of these cases.

We have confirmed that the performance of HER2 immunohistochemical and CISH studies on a single section is feasible and, although associated with reduced HER2 protein expression in some cases, results in excellent concordance with separately performed CISH. Such dual immunohistochemical/CISH staining techniques, as described herein, could provide pathologists with the opportunity for simultaneous evaluation of HER2 protein overexpression and gene amplification at the single cell level and may be particularly useful in cases with only a small focus of invasive tumor, in cases with equivocal results using only a single method, and/or in cases that are heterogeneous for HER2 amplification. Finally, such dual staining could potentially offer more economical and real-time methods for validation of HER2 testing.
in breast carcinoma. Clearly, further studies are needed to determine the usefulness of such methods.

References


