Interlaboratory Comparison of HER-2 Oncogene Amplification as Detected by Chromogenic and Fluorescence in situ Hybridization

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ABSTRACT

Purpose: Chromogenic in situ hybridization (CISH) is a new modification of the fluorescence in situ hybridization (FISH) technique for detection of oncogene amplification in archival tumor samples. In CISH, the oncogene probe is detected using a peroxidase reaction, allowing use of transmitted light microscopy. We compared detection of HER-2/neu amplification by CISH with a Food and Drug Administration-approved two-color FISH test in an interlaboratory setting.

Experimental Design: Formalin-fixed paraffin-embedded tumor samples from 197 breast cancers were analyzed for HER-2 amplification by CISH. Two-color FISH (PathVysion) CISH of 17 centromere was done if the observer considered it necessary to ascertain amplification status in tumors with borderline HER-2 CISH copy numbers.

Results: Paired CISH/FISH results were available from 192 (97%) of 197 cases, no clear difference in success rates of either method was observed. Centromere 17 CISH was considered necessary in seven tumors. CISH and two-color FISH results were concordant in 180 cases (93.8%). There were 92 and 88 tumors found HER-2 amplified and nonamplified, respectively, by both methods. Eight tumors were amplified by CISH but not by FISH, and four tumors exhibited the opposite condition (kappa coefficient 0.875). In 7 of 12 cases differences between the two methods could have related to a lack of CISH chromosome 17 information. The remaining cases were explained by difficult histology (ductal carcinoma in situ, poor representativity, dense lymphocytic infiltration, or intratumoral heterogeneity).

Conclusions: These results indicate that CISH could provide an accurate and practical alternative to FISH for clinical diagnosis of HER-2/neu oncogene amplification in archival formalin-fixed breast cancer samples.

INTRODUCTION

Amplification of the HER-2 oncogene and concomitant overexpression of the HER-2 protein are currently implicated as important biomarkers in breast carcinoma in predicting response to trastuzumab, which is an antibody drug targeting the HER-2 protein (1). HER-2 may also have a role in predicting a more favorable response to anthracycline-based adjuvant chemotherapy (2, 3) and resistance to tamoxifen (4), and as a prognostic factor associated with poor overall survival of patients, irrespective of therapies used (5).

The majority of HER-2 studies have been carried out using HER-2 protein immunohistochemistry, the analytical validity of which has been criticized in several studies (6, 7, 8). The main problems of this method relate to the ability of the antibodies to detect HER-2 protein overexpression in tumor samples, which have been fixed in different formalin-based fixatives for variable lengths of time. Another major source of variability is the subjectivity of the interpretation of staining results (8). In contrast with immunohistochemistry, fluorescence in situ hybridization (FISH) detects the gene copies, the increased number of HER-2 amplification (2, 3) and resistance to tamoxifen (4), and as a prognostic factor associated with poor overall survival of patients, irrespective of therapies used (5).

Evaluation of FISH requires a modern epifluorescence microscope equipped with high magnification oil immersion objectives (×60–100) and special multi-bandpass fluorescence filters. CISH is evaluated with light microscopy, which is more familiar to most pathologists. However, it is general practice to evaluate histology with an adjacent H&E-stained section when performing both FISH and CISH analysis because morphological assessment is central to the accurate diagnosis of breast cancers.

We have recently introduced chromogenic in situ hybridization (CISH), which is similar to FISH, except that the HER-2 DNA probe is detected using a peroxidase reaction instead of a fluorescent dye (14, 15). The hybridization result can be viewed
by an ordinary transmitted light microscope. In this study, we report an interlaboratory comparison of CISH with FISH in the detection of HER-2 amplification in a large set of archival formalin-fixed paraffin-embedded breast cancers.

**MATERIALS AND METHODS**

**Patients and Tumors.** Archival formalin-fixed paraffin-embedded tissue sections from 197 breast cancers were selected for this study. Approximately one-half of the samples (98 cases) were newly diagnosed cases analyzed for HER-2 by CISH in clinical diagnostics at the Laboratory Cancer Biology, University of Tampere. The other half of the tumors (99 cases) came from the University of Glasgow from material previously characterized for HER-2 amplification by FISH (Vysis PathVysion, Abbott Laboratories, Glasgow, Scotland) and p185HER2 overexpression (12). Because these samples were preselected from previously analyzed breast tumors, it is not possible to derive information on the relative success rates of FISH and CISH from this study. To maximize the statistical power of the study, the cases were selected to contain ~50% of tumors with and without HER-2 amplification. The tumors used were previously successfully analyzed by either FISH or CISH (50% each) and were selected from the authors’ databases only with a criterion “amplified” or “not amplified,” ignoring the copy number ratios. This approach was chosen to ensure that the material was representative for the cases showing borderline copy number ratios.

**Fluorescence in situ Hybridization.** All of the FISH analyses were performed in Glasgow by A. F., A. D. W., and J. M. S. B. Approximately 50% of samples were scored by two observers with 99% agreement (see Ref. 12 for details). A PathVysion detection kit was used for FISH analysis after pretreatment using the VP2000 tissue processor (Vysis) and the Vysis tissue pretreatment protocols with minor modifications. Tissue digestion was assessed and samples were re-digested as Vysis tissue pretreatment protocols with minor modifications. The sections were deparaffinized, and incubated in 0.1 M Tris–HCl (pH 7.0) in a temperature-controlled microwave oven (at 92°C for 10 min, followed by cooling down for 20 min at room temperature). After a wash with PBS, enzymatic digestion was carried out by applying 100 µl of digestion enzyme to the slides for 10 to 15 min at room temperature (Digest-All III solution; Zymed Inc., South San Francisco, CA). The slides were then washed with PBS and dehydrated with graded ethanol. A ready-to-use digoxigenin-labeled HER-2/neu DNA probe (Zymed; consisting of two contiguous BAC clones) was applied to the slides, which were covered with 18 × 18 mm coverslips (10 µl of probe mixture per slide). The sections were denatured on a thermal plate (94°C for 3 min), and hybridization was carried out overnight at 37°C. After hybridization, the slides were washed with 0.5× SSC (5 min at 75°C), followed by three washes in PBS (at room temperature). The HER-2/neu probe was detected by sequential incubations with mouse anti-digoxigenin (diluted 1:300; Roche Biochemicals, Mannheim, Germany), antimouse-peroxidase polymer (Powervision+; Immunovision Inc., Daly City, CA) and diamobenzidine chromogen according to the manufacturer’s protocol. The tissue sections were lightly counterstained with hematoxylin and were embedded. A positive and a negative control sample (tumors with and without HER-2 amplification in FISH) were included in every hybridization batch.

The CISH sections were evaluated using a 40× dry objective. A nonamplified gene copy number was defined as one to five signals per nucleus. Amplification was defined as six or more signals per nucleus in more than 50% of cancer cells, or when a large gene copy cluster was seen. In tumors with borderline copy number count, the observer requested that CISH of an adjacent section be hybridized with a chromosome 17 centromere probe (Zymed) for comparison. All CISH hybridizations were evaluated with observers blinded to the result of the FISH assay and vice versa.

**Statistics.** Comparison of CISH with FISH was estimated using the kappa test. The analytical performance of CISH was also estimated with specificity, sensitivity, and negative and positive predictive value.

**RESULTS**

**Performance of FISH and CISH.** CISH was successful in 194 (98.5%) samples attempted, and FISH in 195 (99.0%) of 197 cases. Repeated analysis was needed in less than 10 tumors. CISH was successful in 96 (97%) of 99 FISH-selected samples (Vysis/Abbott, PathVysion, Glasgow), whereas FISH was successful in 96 (98%) of 98 samples selected for this study according to the CISH result obtained in routine diagnostics in Tampere. Thus, paired FISH and CISH results were achieved for 192 (97%) of 197 samples selected for study.

In CISH, gene copies visualized by CISH were clearly distinguishable using a ×40 objective in tissue sections, which were counterstained with hematoxylin. Amplified gene copies presented typically as large peroxidase-positive intranuclear gene copy clusters (Fig. 1A), or as numerous individual peroxidase-positive small signals (Fig. 1B). Tumors with no amplification of HER-2 showed typically one to two spots per nucleus (when diploid), or three to five spots in chromosomally aneuploid cases. The microscopic evaluation of CISH was highly reproducible as evidenced by the excellent agreement obtained between two observers (Table 1). The observers’ classification of CISH scores was in agreement in 186 tumors, with disagreement of diagnosis observed only in 6 tumors (Table 1). CISH of 17 centromere was considered necessary to ascertain the amplification status only in seven tumors.

**Comparison of CISH and FISH Results.** Of the 192 tumors with results from both CISH and FISH, 92 tumors were HER-2 amplified by both techniques and an additional 88 showed no evidence of amplification by either method (Table 2). Only eight tumors (4.2%) were identified as amplified by CISH but not by FISH, and four tumors (2.1%) amplified by FISH showed no evidence of amplification by CISH. The kappa coefficient for the inter assay agreement was 0.875. Using the Food and Drug Administration (FDA)-approved FISH test (Vy-
As the gold standard, the specificity of CISH was 92.0% and the sensitivity was 95.7% (positive predictive value, 91.7%; negative predictive value, 95.8%).

To study the impact of the chromosome 17 centromere correction (done in FISH, but considered necessary to be done in CISH only in seven tumors), we compared CISH data also with the mean copy number counts of HER-2 by FISH (i.e., ignoring the 17 centromere counts), using the same cut-point (six copies per cell) in both assays. The classification (amplification/no amplification) was changed in FISH in 13 tumors, but the concordance between CISH and 1-color FISH remained as good as that with 2-color FISH (Table 2).

Chromosome 17 centromere evaluation was considered necessary to distinguish between gene amplification and aneuploidy in only seven cases by CISH (HER-2 copy number by CISH, range 4.3–5.8, and by FISH, range 2.6–6.1 copies per cell). In addition to those seven cases, we enumerated the CISH gene copies in all of the tumors that were interpreted as having HER-2 amplification but showed no gene copy clusters (n = 24, 13.0% of evaluable cases), because copy numbers in cases with gene clusters could not be enumerated. The mean copy number counts between CISH (range, 4.3–14.2) and FISH (range, 3.5–14.8) were very similar, the correlation coefficient being 0.75 (P < 0.0001). In 81 cases (88% of all of the tumors amplified by CISH) in which gene copy clusters were found by CISH and dot counting was not possible, the average HER-2 copy number count by FISH was 14.6 (SD, 6.1; range, 1.9–32). In 87 cases in which CISH was considered negative, without scoring of signals, the mean HER-2 copy number by FISH was 2.79 (range, 1.23–14.70; median, 2.42).

A detailed characterization of those tumors with discordant CISH versus FISH results is shown in Table 3. In 7 of 12 cases (tumors 4–10) differences between the two methods were related to a lack of chromosome 17 information in CISH. Two of these cases (cases 6 and 7) had elevated copy numbers of HER-2 by both methods but were interpreted as not amplified by FISH because of high centromere counts. In five cases (cases 4, 5, 8, 9, and 10), discrepancies were caused by either different cutoffs (tumors 4 and 5) or by the scoring of adjacent signals as HER-2 clusters by CISH. In each case, these discrepancies were related to chromosome 17 copy numbers. Difficult tumor histology (ductal carcinoma in situ with scanty tumor cells or intratumoral heterogeneity) could explain the discordance in five tumors (tumors 1, 2, 3, 11, and 12).

Table 2 Concordance between HER-2 amplification status by two-color FISH and CISH in 192 breast cancers

<table>
<thead>
<tr>
<th>HER-2 FISH (PathVysion)</th>
<th>CISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification (&lt;6 copies/cell)</td>
<td>88</td>
</tr>
<tr>
<td>Amplification (≥6 copies or a gene copy cluster)</td>
<td>4</td>
</tr>
</tbody>
</table>

- FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization.
- PathVysion, Abbott Laboratories, Glasgow, Scotland.
- Kappa coefficient, 0.875 (0.84–0.91).
- Kappa coefficient, 0.854 (0.817–0.891).
CISH and FISH Assessment of HER-2 Amplification

Although centromere correction is considered to be gold standard in FDA-approved FISH test, in cases 6 and 7 it would have resulted in a classification of no amplification despite 3+ immunohistochemistry staining (with CB11 antibody; data not shown). We have previously interpreted these as FISH false negatives (12). Conversely, cases 1 and 2 would have been interpreted as discordant between CISH and immunohistochemistry, but concordant between FISH and immunohistochemistry.

**DISCUSSION**

Accurate determination of HER-2 status is of critical importance in the identification of patients for treatment with the novel molecularly targeted therapy Herceptin. Previous evidence has shown that in terms of patient outcome in the pivotal clinical trials (1, 13, 17–19), identification of cases with high HER-2 expression (12, 18, 19) and patient survival (11), molecular based FISH analysis is a better predictor of Herceptin responsiveness than immunohistochemical techniques. FISH has been shown to be reproducible (11, 20), accurate, and precise (12, 18) as a clinical diagnostic assay. Although FISH has been widely used in research for a number of years (18, 21, 22), this technology has been applied in clinical diagnostic laboratories only during the past 6–8 years and represents a different and novel challenge for the pathologist (18, 20, 23, 24). The requirement for molecular analysis coupled with the need to perform fluorescence microscopy has meant that FISH testing is performing fluorescence microscopy has meant that FISH testing is not yet widely available in many routine pathology laboratories. Although it is becoming increasingly clear that FISH is a relatively simple molecular test to perform on a technical level, it is also apparent that an alternative that did not require fluorescence microscopy with its inherent shortcomings would be desirable.

The recent development of CISH-based assays provides an attractive alternative to FISH, but the assays, to date, have not been directly compared with FDA-approved two-color FISH in an unbiased interlaboratory setting. The CISH method used in the present study has been improved from the one of our initial report (14). The probe detection was performed with unconjugated anti-digoxigenin antibody and an antimouse peroxidase polymer, which increases the intensity of the hybridization signal when compared with the previously used antibody-based detection system. The new detection system allows better visualization of the nonamplified gene copies, thereby improving the scoring of CISH.

The success rates of FISH and CISH were high in this material, indicating that both methods worked well in formalin-fixed paraffin-embedded samples. Most importantly, there was excellent agreement between CISH and FISH (kappa coefficient, 0.875). When the HER-2:chromosome 17 ratio of FDA-approved FISH tests was used as the “gold standard,” the CISH assay predicted the FISH result correctly in 93.8% of cases with a positive predictive value 91.7% and a negative predictive value 95.8%.

At present, no large interlaboratory studies of FISH have been published, which makes it impossible to estimate whether an interlaboratory comparison of two FISH assays would have resulted in any higher concordance than the present results between CISH and FISH. According to a recent report (25), small interlaboratory comparisons of FISH have resulted in a similar ~90–95% concordance. Therefore, we, here, present evidence that supports the use of CISH as a possible alternative to FISH in the diagnosis of HER-2 gene amplification.

We have previously defined the cutpoint for HER-2 amplification as six or more gene copies per cell, or as the presence of a gene copy cluster (14, 15). The present study confirms our previous findings and indicates that the use of this cutpoint in single-color FISH would actually lead to an almost identical classification of tumors when using the HER-2:17 centromere copy number ratio of two-color FISH. This is most likely because the tumors with borderline copy number counts seem to form only a small minority of clinical breast cancers (12).

A detailed characterization of the discordant tumors indicated several possible explanations, the most obvious one re-

### Table 3  Characterization of the 12 tumors discrepant between HER-2 oncogene FISH* and CISH

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Mean HER-2 copy number by FISH</th>
<th>HER-2:17cen ratio by FISH</th>
<th>CISH (ampl status, mean copy number)</th>
<th>Possible explanation for discrepancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.7</td>
<td>7.7</td>
<td>No ampl</td>
<td>CISH false negative; scanty cancer cells</td>
</tr>
<tr>
<td>2</td>
<td>8.7</td>
<td>4.7</td>
<td>No ampl</td>
<td>CISH false negative; scanty cancer cells</td>
</tr>
<tr>
<td>3</td>
<td>7.6</td>
<td>5.0</td>
<td>No ampl</td>
<td>Focal amplification: tumor heterogeneous for HER-2 (small part amplified)</td>
</tr>
<tr>
<td>4</td>
<td>5.2</td>
<td>2.4</td>
<td>No ampl; mean, 4.8</td>
<td>Chr17 related discrepancy; Chr 17 = 2.0 copies</td>
</tr>
<tr>
<td>5</td>
<td>6.9</td>
<td>1.9</td>
<td>Amplified; mean, 8.8</td>
<td>Chr 17 related discrepancy; Chr17 = 3.7 copies; abundant intratumoral lymphocytic infiltration</td>
</tr>
<tr>
<td>6</td>
<td>11.2</td>
<td>1.1</td>
<td>Amplified; mean, 14.2</td>
<td>FISH false negative: amplified centromere? Chr17 = 9.7 copies</td>
</tr>
<tr>
<td>7</td>
<td>11.8</td>
<td>1.3</td>
<td>Amplified, clusters</td>
<td>FISH false negative: amplified centromere? Chr 17 = 10.5 copies</td>
</tr>
<tr>
<td>8</td>
<td>7.7</td>
<td>1.4</td>
<td>Amplified, clusters</td>
<td>Chr17 related discrepancy; Chr17 = 5.5 copies</td>
</tr>
<tr>
<td>9</td>
<td>5.4</td>
<td>1.0</td>
<td>Amplified, clusters</td>
<td>Chr 17 related discrepancy; Chr17 = 5.0 copies</td>
</tr>
<tr>
<td>10</td>
<td>8.3</td>
<td>1.7</td>
<td>Amplified, clusters</td>
<td>Chr 17 related discrepancy; Chr17 = 4.9 copies; abundant lymphocytic infiltration</td>
</tr>
<tr>
<td>11</td>
<td>2.5</td>
<td>1.5</td>
<td>Amplified, clusters</td>
<td>Scanty cancer cells (DCIS)</td>
</tr>
<tr>
<td>12</td>
<td>1.9</td>
<td>1.1</td>
<td>Amplified, clusters</td>
<td>Scanty cancer cells (DCIS)</td>
</tr>
</tbody>
</table>

* FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; cen, centromere; ampl, amplification; Chr, chromosome; DCIS, ductal carcinoma in situ.

Amplification if ratio > 2.0.
lated to chromosome 17 centromere counts (a possible explanation in 7 of 12 cases). In the remaining five cases, four showed heterogeneity of amplification or poorly representative tumor histology (ductal carcinoma in situ, abundant lymphocytic infiltration, scanty tumor cells). Although only seven CISH cases were selected for chromosome 17 hybridization prospectively, our results suggest that even a higher concordance with FISH might have been achieved if chromosome 17 CISH were applied in parallel with HER-2 CISH.

However, we also show quite clearly that chromosome 17 is not required in a vast majority of HER-2-positive or -negative cases to accurately diagnose HER-2 amplification. Therefore, there remains a question as to how many CISH cases might require confirmatory chromosome 17 measurements. On the basis on the present data, we are unable to give a clear answer to this question, and it is likely to vary from observer to observer. However, we can observe, firstly, that, in at least 14 cases (the seven selected for chromosome 17 centromere and 7 discordant cases), chromosome 17 CISH would have been advantageous (= ~7% of all cases). We further observed that 43 cases showed HER-2 FISH copy numbers within the range of samples selected for chromosome 17 by CISH (21.2%). We consider it likely that the true number of cases that require confirmatory chromosome 17 CISH will lie between these values, possibly closer to the lower threshold. The need for an extra centromere CISH, in some cases, as well as the possibility of performing multiprobe FISH assays (like 17 centromere, HER-2, and topoisomerase IIA) are potential advantages of FISH over CISH. Conversely CISH may be combined with other, immunohistochemical markers (estrogen and progesterone receptors, and so forth). The present study shows that CISH has accuracy comparable to FISH for HER-2 diagnosis, and, as with any technique, the advantages of CISH (e.g., light microscopy, archivability of slides, use of other markers, and so forth) should be weighed against the disadvantages (e.g., no simultaneous centromere counts and the lack of multiprobe hybridizations) for each diagnostic application under consideration. The appropriate method is, thus, likely to vary for different applications.

The high interobserver agreement for CISH found in the present study indicates that the microscopic evaluation of CISH is reproducible and straightforward when carefully applied. Similar evidence is also available for FISH, as documented previously (12). The main difference in the microscopic of FISH and CISH is that, in cases of high-level amplification, which form a vast majority of the HER-2 positive tumors, CISH shows the signal as a typical peroxidase positive “cluster,” which is a peroxidase reaction precipitate merged over the location of numerous closely located gene copies. The gene copies forming a peroxidase-positive cluster cannot be enumerated, but based on the size of the cluster and its typical irregular shape, it is easy to distinguish as a sign of gene amplification. We have previously documented (12, 20–27) interobserver agreements in diverse FISH-based assays of ~10%. Our data, which show a higher degree of concordance between CISH and FISH, would also suggest that, contrary to our expectation, there is no detrimental impact on the discrimination of results relating to the use of fluorescence microscopy for FISH versus light microscopy for CISH. Despite the prejudice that light microscopy might be more appropriate for morphological discrimination of cases, we have not seen this to be a major issue. It should be noted in this context, however, that material was preselected for successful hybridization and that both laboratories are highly experienced in the relevant methodologies.

The evidence presented here forms part of a growing literature that shows concordance between FISH and CISH results (27, 28, 30). Both assays have specific advantages, and additional studies are under way to further develop both systems. It is, therefore, likely that both CISH and FISH will become more widely applied, in different laboratories, for the determination of HER-2 status. Both are robust simple assays that accurately reflect HER-2 gene status.

Most countries have recommended a two-step testing system for the determination of HER-2 status, with FISH used to confirm only 2+ staining (31, 32). However, in the light of growing evidence for the value of HER-2 gene testing (to date largely using FISH) to predict response to a range of therapies and Herceptin in particular, we believe there is a wider role for gene-based testing using either CISH or FISH in clinical diagnostics. The Finnish recommendation is to use CISH or FISH as a confirmatory test for all 2+ and 3+ immunohistochemical HER-2 staining results (32, 33). This protocol has the advantage that it also detects false positive 3+ staining, which may comprise up to 10% of all 3+ HER-2 results (12). Moreover, confirming both 2+ and 3+ results by CISH/FISH omits the need to distinguish between 2+ and 3+ immunostaining, which is known to be difficult and subjective. In the future, molecular testing may expand to include the 0/1+ cases also, in which a small proportion of HER-2 positives (~3%) have also been observed (12). Evidence is growing that, in terms of both patient care and health economics, this may represent the optimal approach to HER-2 testing.

In conclusion, we have shown that, in terms of both feasibility and accuracy, CISH (as performed here) can provide an alternative for the detection of HER-2 gene amplification by FISH, currently considered as the gold standard in the prediction of response to Herceptin therapy in metastatic breast cancer. On the basis of our evidence, we conclude that CISH is highly concordant with the FDA-approved FISH test (Vysis/Abbott PathVysion) and that both tests can be used in large multilaboratory studies to further validate gene testing for HER-2 status in clinical diagnostics. The lack of data showing CISH testing and survival in pivotal clinical Herceptin trials should be addressed in the future.

REFERENCES
Assessment of HER-2 Amplification

Interlaboratory Comparison of HER-2 Oncogene Amplification as Detected by Chromogenic and Fluorescence in situ Hybridization

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