Research article
Chromogenic in situ hybridization (CISH): a novel alternative in screening archival breast cancer tissue samples for HER-2/neu status
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Received: 23 Apr 2004 Revisions requested: 1 Jun 2004 Revisions received: 23 Jun 2004 Accepted: 29 Jun 2004 Published: 29 Jul 2004

Breast Cancer Res 2004, 6:R593-R600 (DOI 10.1186/bcr915)
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Abstract

Background Chromogenic in situ hybridization (CISH) is emerging as a practical, cost-effective, and valid alternative to fluorescent in situ hybridization in testing for gene alteration, especially in centers primarily working with immunohistochemistry (IHC).

Methods We assessed Her-2/neu alteration using CISH on formalin-fixed paraffin-embedded primary invasive ductal carcinoma tumors in which IHC (CB11 antibody) had previously been performed, and we compared the results with IHC. The 160 selected cases were equally stratified randomly into the four IHC categories (scores of 0, 1+, 2+, and 3+). We also compared age at diagnosis and tumor histologic grade with IHC and CISH Her-2/neu.

Results We were able to perform and evaluate CISH successfully on all cases. The agreement between 3+ IHC and CISH-amplified cases as well as between all IHC and CISH Her-2/neu negative cases was 100%, and the concordance on all positive cases was 72.50%, with an overall agreement of 86.25%. All the discordant cases had 2+ IHC scores. Although we noted Her-2/neu positivity more in premenopausal women, the age at diagnosis was not significantly associated with IHC or CISH results. Similarly, although the small group of well-differentiated tumors was apparently Her-2/neu negative in both tests, no significant association was noted between any tumor histologic grade and either IHC or CISH results.

Conclusions CISH is easily integrated into routine testing in our laboratory. It is a necessary adjunct in determining the subset of non-amplified IHC-positive invasive tumors that will not benefit from trastuzumab therapy. Those cases with 2+ IHC results will be triaged and subjected to CISH. Her-2/neu testing should be done on all breast cancer cases regardless of age at presentation and tumor histologic grade.

Keywords: breast cancer, chromogenic in situ hybridization, fluorescence in situ hybridization, Her-2/neu, immunohistochemistry

Introduction

The Her-2/neu proto-oncogene, also known as c-erbB-2, is a member of the type I growth factor receptor gene family and is located in the long arm of chromosome 17 (17q12-21.32) [1]. It encodes a 185 kDa cytoplasmic membrane glycoprotein involved in tyrosine kinase signal transduction for epithelial cell proliferation, including the breast epithelium [2].

In 20–30% of breast carcinomas, Her-2/neu status is altered, and this is manifested either as amplification of the gene or overexpression of the protein product [3]. Such alteration has been associated with poor prognosis and with resistance to conventional adjuvant chemotherapy and tamoxifen, regardless of the nodal or hormone receptor status [4-8]. Moreover, patients with breast carcinomas with amplified or overexpressed Her-2/neu can benefit from anthracycline-based regimens as well as trastuzumab (Herceptin), a recombinant humanized monoclonal antibody against the Her-2/neu protein [9].

Tumor Her2/neu is generally assessed as protein overexpression by using immunohistochemistry (IHC), and patients with tumors that either have 2+ or 3+ results with this method become good candidates for treatment with trastuzumab.

CISH = chromogenic in situ hybridization; FISH = fluorescence in situ hybridization; IHC = immunohistochemistry; PBS = phosphate-buffered saline; SLMC = St Luke’s Medical Center.
However, studies indicate that Her2/neu determined as gene amplification provides better prognostic information and is associated with a better response to trastuzumab [10-12]. A subset of patients with tumors having 2+ IHC results were found to show no response to the drug, whereas all those having gene amplification responded favorably. Nevertheless, a negative (0 or 1+) or a 3+ Her-2/neu IHC correlates well with a negative or positive Her-2/neu gene amplification, respectively.

Her-2/neu gene amplification is primarily detected by in situ hybridization and uses fluorescence (FISH) to detect the signals. This method is both cumbersome and expensive and needs a fluorescence microscope, appropriate filters, and a sophisticated camera; it is therefore not practical as a screening tool. Chromogenic in situ hybridization (CISH) is a recently introduced method, and although it makes use of the in situ hybridization technology of FISH, it also takes advantage of the chromogenic signal detection of IHC that can be detected with the ordinary light microscope and costs one-quarter as much as FISH. CISH is potentially able to detect Her-2/neu gene amplification and to minimize, if not eliminate, the false positive fraction with the IHC procedure. Here we report an evaluation of the CISH assay in St Luke's Medical Center (SLMC), Philippines.

Methods
Inclusion criteria
This study focused on in-patient female breast cancer tissue samples with final histopathologic diagnosis of invasive ductal carcinoma of no special type, with archival paraffin blocks, and with prior Her-2/neu alteration determined by IHC from 1 January 2000 to 31 December 2001 in our laboratory.

Method
IHC was previously determined with CB11 antibody (Zymed Laboratories) on breast tumor samples fixed in buffered formalin and embedded in paraffin. Only one pathologist reviewed the Her-2/neu results of all IHC cases in the period covered, reassessing them in accordance with the US Food and Drug Administration-approved Her-2/neu IHC scoring guidelines [10]. Immunoreactivity was defined as negative with a score of 0 (no staining in all cells or very weak membrane staining in less than 10% of the tumor cells) or 1+ (weak perceptible partial membrane staining in more than 10% of the tumor cells), and was defined as positive with a score of 2+ (weak to moderate complete membrane staining in more than 10% of the tumor cells) or 3+ (strong complete membrane staining in more than 10% of the tumor cells). Areas with intraductal carcinoma were excluded from the evaluation.

Her-2/neu gene amplification was then analyzed with CISH on cases selected through stratified random sampling into the four IHC categories. Each category contained an equal number of samples (40 samples in each IHC category), for a total of 160 samples.

The CISH assays were run by one technologist in the Histopathology Section of the Institute of Pathology following the protocol of Zymed. Thereafter, CISH results were read by the same pathologist who was blinded to the previous IHC results. CISH was assessed on invasive ductal carcinoma areas only; foci of intraductal carcinoma were excluded from the analysis of results. Table 1 shows the recommended CISH scoring by Zymed.

In addition, the patients' ages were arbitrarily grouped as either premenopausal (less than 50 years) or postmenopausal (50 years or more). The histologic grades of the invasive tumor foci were assessed in the IHC and CISH slides by using the modified Bloom–Richardson classification [13].

CISH procedure for formalin-fixed paraffin-embedded breast cancer tissue samples
In brief, tissues 4–5 µm thick were mounted on Histogrip-treated microscope slides, dried at 37°C, and baked for 2–4 hours at 60°C. The slides were deparaffinized for 15 min three times in xylene at room temperature (22–27°C) and washed for 2 min three times in 100% ethanol at room temperature.

The slides were microwaved in SPOT-Light Tissue Heat Pretreatment Buffer for 10 min at 92°C and washed for 3 min twice in phosphate-buffered saline (PBS). They were covered with 100 µl SPOT-Light Tissue Pretreatment Enzyme for 10 min at 37°C and washed for 2 min three times in PBS at room temperature. The slides were then dehydrated in 70%, 85%, 95%, and 100% ethanol for 2 hours at 60°C. The slides were deparaffinized for 15 min three times in xylene at room temperature (22–27°C) and washed for 2 min three times in 100% ethanol at room temperature.

Denatured probe (15 µl) was added to the center of each sample and covered with a 24 mm × 32 mm coverslip, the edges of which were sealed with thin layer of rubber cement to prevent the evaporation of probe solution during incubation. The slides were denatured at 94°C for 3 min and placed in a dark humidity box for 16–24 hours at 37°C.

After removal of the rubber cement and coverslip, the slides were immersed in 0.5 × SCC buffer in a Coplin jar for 5 min at 75°C. They were then washed for 2 min three times in PBS-Tween 20 buffer at RT.

The slides were submerged in peroxidase quenching solution and then washed for 2 min three times with PBS, after
which endogenous biotin blocking was performed with Reagent A (100 µl of CAS Block).

Using Zymed’s SPOT-Light Detection Kit, 100 µl each of fluorescein isothiocyanate-labelled sheep anti-digoxigenin, horseradish peroxidase-labelled goat anti-fluorescein isothiocyanate, and diaminobenzidine chromogen were sequentially added to the slides, with three 2 min rinses with PBS-Tween between the addition of reagents.

The slides were counterstained with 150 µl of Gill-2 hematoxylin and incubated for 3 min. They were then dehydrated with a graded series of alcohol, cleared in xylene, and mounted with a coverslip.

Statistics
All CISH data were compared with the previous IHC results with the use of descriptive statistics and the χ² test for association. Similarly, age and tumor histologic grade were correlated with the IHC and CISH Her-2/neu with the same statistical methods. P < 0.05 was considered statistically significant.

Results
There were a total of 681 breast cancer Her-2/neu determinations in 2000–2001, 245 of which (35.98%) were in-patient cases. Of the in-patient cases, 104 (42.45%) had positive Her-2/neu overexpression. One hundred and sixty in-patient cases were randomly selected for the study. The ages ranged from 24 to 77 years, with a median of 51 and a mean of 51.11 (standard deviation 10.86). Seventy-four of the (46.25%) patients were less than 50 years of age.

All 160 tests were successfully performed with the Zymed protocol for CISH. At least two detectable amplicons per tumor nucleus were observed, and these served as the internal control. In addition, a highly amplified positive control was run simultaneously with each test.

CISH results were evaluated with the light microscope at low-power and high-power magnification. Diaminobenzidine background staining was variably noted, but this was not sufficient to interfere with the interpretation of the CISH signals. Amplified cases showed at least six signals per nucleus; distinct clustering of amplicons was also obtained. Those cases reported as having no amplification or low amplification were not verified further for chromosome 17 aneuploidy.

Table 2 shows the CISH assay outcomes of the 160 cases in relation to the IHC category and tumor histologic grades. Of the 160 cases, 80 were IHC positive and 80 were IHC negative. With the CISH assay, 58 (36.25%) of the 160 cases showed Her2/neu gene amplification. Of the 58 amplified cases, 17 (29.31%) showed low amplification and 41 (70.69%) showed high amplification. Forty of the 58 CISH-amplified cases had 3+ IHC results, and 18 had 2+ results. Most of the tumors were of histologic grade 2 (70%), followed by grade 3 (27.5%), then grade 1 (2.5%).

Table 3 shows the correlation of CISH and IHC in 160 breast tumor samples. There was a statistically significant difference (P = 0.000) in the percentage of those without amplification between IHC-negative (100%) and IHC-positive (28%) cases; only positive IHC tended to have amplification. There were 22 IHC-positive cases that subsequently showed no gene amplification with CISH, all of which had 2+ IHC scores.

The agreement between 3+ IHC and CISH-amplified cases was 100%. The concordance between IHC and CISH on all negative cases was 100%, whereas the concordance on all positive cases was 72.50%, with an overall agreement of 86.25%.

Table 4 shows the association between age stratum (menopausal status), tumor histologic grade, IHC, and CISH. Many more patients less than 50 years old (premenopausal) seemed to be Her-2/neu positive and amplified, whereas more patients 50 years old and older (postmenopausal) seemed to be Her-2/neu negative and non-amplified. However, these associations were not statistically significant (P = 0.156).

Similarly, no significant association was noted between age and histologic grade (P = 0.658).

Table 5 shows the association between results of Her2/neu alteration with the histologic grades of the tumor. All histologic grade 1 cases (of which there were only four) did

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| Table 1 Evaluation of Her-2/neu gene status using chromogenic in situ hybridization |
|----------------------------------------|----------------|
| Amplification | Her-2/neu gene status |
|----------------|----------------------|
| High-level | >10 copies or large cluster of amplicon per nucleus in >50% of cancer cells |
| Low-level | 6–10 copies or small cluster of amplicon per nucleus in >50% of cancer cells |
| None | 1–5 copies per nucleus of cancer cells |

aVerify gene amplification or chromosome 17 aneuploidy with SPOT-Light Chromosome 17 Centromeric Probe. bIf 3–5 copies per nucleus, verify chromosomal aneuploidy with SPOT-Light Chromosome 17 Centromeric Probe.
Table 2

Breast tumor samples and their corresponding immunohistochemistry (IHC), chromogenic in situ hybridization (CISH) results, and tumor histologic grade

| IHC score | No. of tests | CISH amplification | Histologic grade |
|-----------|--------------|--------------------|------------------|
|           |              | Negative Low High  | 1 2 3           |
| 0         | 40           | 40 0 0             | 1 33 6          |
| 1+        | 40           | 40 0 0             | 3 28 9          |
| 2+        | 40           | 22 8 10            | 0 24 16         |
| 3+        | 40           | 0 9 31             | 0 27 13         |
| Total     | 160          | 102 17 41          | 4 112 44        |

Table 3

Correlation of chromogenic in situ hybridization and immunohistochemistry in archival breast tumor samples

| Chromogenic in situ hybridization | Immunohistochemistry | Total |
|-----------------------------------|----------------------|-------|
|                                   | Negative (0 and 1+)  | Positive (2+ and 3+) |       |
| No amplification                  | 80                   | 22    | 102   |
| With amplification                | 0                    | 58    | 58    |
| Total                             | 80                   | 80    | 160   |

Table 4

Comparison of age strata with tumor histologic grade, immunohistochemistry and chromogenic in situ hybridization results

| Parameter                      | Age <50 | Age ≥ 50 | Total |
|--------------------------------|---------|----------|-------|
| Immunohistochemistry           |         |          |       |
| Negative (0 and 1+)            | 33 (41.25%) | 47 (58.75%) | 80    |
| Positive (2+ and 3+)           | 41 (51.25%) | 39 (48.75%) | 80    |
| Total                          | 74      | 86       | 160   |
| Chromogenic in situ hybridization |       |          |       |
| No amplification               | 43 (42.16%) | 59 (57.84%) | 102   |
| With amplification             | 31 (53.44%) | 27 (46.55%) | 58    |
| Total                          | 74      | 86       | 160   |
| Histologic grade               |         |          |       |
| 1                              | 0 (0.00%) | 4 (100.00%) | 4     |
| 2                              | 53 (47.32%) | 59 (52.68%) | 112   |
| 3                              | 21 (47.73%) | 23 (52.23%) | 44    |
| Total                          | 74      | 86       | 160   |
not overexpress or amplify Her2/neu. No significant association was noted between histologic grade and IHC scores. Similarly, no association was observed between histologic grade and CISH results.

**Discussion**

Her-2/neu status can be detected by analyzing the number of gene copies by Southern blotting, PCR, or FISH, or the amount of expressed protein can be ascertained by Western blotting, enzyme-linked immunosorbent assay, or IHC. The two most widely used methods at present are IHC and FISH because they are able to evaluate Her-2/neu in formalin-fixed paraffin-embedded archival tissues.

Most Her-2/neu studies have been performed by IHC, which detects overexpression of the Her-2/neu protein product on the cell membrane of tumor cells. This study used the CB11 monoclonal antibody, which, like the standardized polyclonal HercepTest, is directed against the intracytoplasmic domain of the Her2/neu molecule [11,14].

IHC is widely accessible and easy to perform at a reasonable cost. However, this semi-quantitative procedure is beset by technical artifacts, sensitivity differences between different antibodies, and subjective interpretation, resulting in interobserver variability between pathologists [15]. Studies reveal the oversensitivity of US Food and Drug Administration-approved HercepTest, with a false positive rate as high as 50% [10,11]. Thomson and colleagues concluded that in cases with an IHC stain intensity of 1+ or 2+, the interobserver agreement is poor and the predictive value is unsatisfactory for clinical use; they recommended additional testing, preferably with FISH [16].

FISH is a fairly objective and quantitative procedure in detecting Her-2/neu gene amplification on the nuclei of tumor cells. The drawbacks of this procedure are its steep cost (10 times that of IHC), sophistication, the need for a fluorescence microscope, the temporary signal (it requires a special camera), and its inability to detect and assess tumor histomorphology.

Her-2/neu overexpression detected by IHC is highly correlated with gene amplification detected by FISH (as high as 98% concordance) [17]. However, 3–15% of breast carcinomas show moderate Her-2/neu membrane staining without evidence of amplification. They most often represent highly sensitive nonspecific staining (false positive) and, rarely, either enhanced mRNA transcription in the absence of amplification or gene amplification that is below the detection level of in situ hybridization methods [11,18,19].

Kakar and colleagues concluded that IHC is appropriate for the initial Her-2/neu assessment, but patients whose tumors scored less than 3+ (particularly those interpreted as 2+) would benefit from FISH to assess Her-2/neu status more accurately and to avoid inaccurate prognostication and inappropriate treatment [20].

CISH is a recent advancement in Her-2/neu detection. It uses a robust unique-sequence probe developed for in situ hybridization and is a promising practical alternative to FISH. After the first CISH study by Tanner and colleagues [21], seven other reports favorably validated CISH results [22-28]. The concordance between CISH and FISH ranged from 85% [25] to as high as 100% [23,24]. All these reports noted the advantages of CISH over FISH: it requires an ordinary microscope; the method is less cum-

### Table 5

| Histologic grade | Immunohistochemistry Her2/neu (%) | Chromogenic in situ hybridization Her2/neu (%) |
|------------------|---------------------------------|-----------------------------------------------|
|                  | Negative (0 and 1+) 2+ 3+       | No amplification With amplification Low High |
| 1                | 4 (5.00) 0 (0.00) 0 (0.00)      | 4 (3.92) 0 (0.00) 0 (0.00)                     |
| 2                | 61 (76.25) 24 (60) 27 (67.50)   | 75 (73.53) 12 (70.59) 25 (60.98)               |
| 3                | 15 (18.75) 16 (40) 13 (32.50)   | 23 (22.55) 5 (29.41) 16 (39.02)               |
bersome and more economical; the signal intensity is permanent; and pathologists are familiar with IHC signals and are able to correlate findings with the underlying tumor histomorphology. CISH allows observers to select fields of invasive ductal carcinoma, avoiding foci of intraductal carcinoma for which Her-2/ neu has a different clinical significance. CISH is a specific, sensitive, and easily applicable method for the detection of Her-2/ neu gene amplification, and it can be used together with IHC for the evaluation of patients with breast carcinoma [22]. Ross and colleagues recently reviewed the current status of Her-2/neu testing, and they concluded that CISH is a more convenient gene-based technique that is ‘waiting in the wings’ [29].

In this present study, the concordance between 3+ IHC and CISH-amplified cases was 100% (40 of 40), denoting all gene amplified cases to be overexpressing the Her-2/ neu protein. In contrast, the agreement on 2+ IHC and CISH-amplified cases was only 45%, which is lower than previously reported (93%) [26]. The 13.75% (22 of 160) IHC-positive/CISH non-amplified tumors in this study, all of which had 2+ IHC scores, is higher than the 6% false positive result by Zhao and colleagues [24], although comparable to the 17% result (IHC CB11 versus FISH) obtained by Tubbs and colleagues [11]. Apparently, we find that the 2+ IHC score is equivocal when compared with CISH. This subset might benefit from further CISH reflex testing. All negative IHC cases were CISH non-amplified, showing complete agreement of all negative results. The 86.25% overall concordance between IHC (using CB11 antibody) and CISH in this study is lower than the previous reports (94–96%) [24,30]. We concur with Sapino and colleagues that gene amplification analysis can be avoided in all 0, 1+, and 3+ IHC cases because results are predictable from IHC and are completely concordant with CISH [30]. The utility of CISH is therefore in further testing Her-2/neu alteration on equivocal cases in IHC, which remains the primary screening method. The combined IHC–CISH algorithm provides an economical and comprehensive Her-2/neu data to guide clinicians in mapping treatment options.

The low-amplified CISH category (6–10 signals) was the most difficult to interpret, requiring an accurate enumeration of gene copy. Signal clustering, more probably a result of intrachromosomal amplification of homogeneously staining regions, was immediately apparent in highly amplified cases and was easily evaluated [23,31].

A test for chromosome 17 aneuploidy was not performed on non-amplified and low-amplified CISH assays, because recent studies showed that it makes the analysis more costly and time consuming without adding relevant data, apart from reporting the recommended number of nuclear amplicons and acting as another form of internal control for the hybridization reaction [11,18,21-24]. In addition, although the manufacturer (Zymed) suggested that chromosome 17 aneuploidy be tested on all gene-amplified cases, we concur with Vera-Roman and colleagues [28], who correctly pointed out that because most polysomy results fall within the gray area of three to five signals, setting the threshold of cutoff signal points to a high of six or more (as in this study) virtually eliminates the polysomy variable.

This study did not observe IHC-negative CISH-amplified tumors. This can probably be explained by the fact that this was a single-institution study likely to have uniformly fixed and processed tumor tissue paraffin blocks as well as a uniform antigen retrieval technique, both of which make high-quality IHC easier. Zhao and colleagues reported occasional IHC-negative CISH-amplified cases, and they considered that these represent a small undetermined percentage that amplified Her-2/neu without overexpression [24]. The same study and other FISH studies also noted rare 3+ IHC tumors that were non-amplified (false positive) [11,24,30]. The reasons offered for the IHC–CISH discrepancies are similar to those between IHC and FISH, as previously stated in the present report.

The causes of testing failure and false negativity offered by previous CISH studies were an absence of tumor on the section, an inability to score owing to high background, a low signal intensity despite repeated trials, an absence of signals from the internal control, and the use of alcohol–formalin–acetic acid fixative [22-24,26]. It was further noted that heat pretreatment and digestion with pepsin are the most critical procedures for optimized CISH performance, and that successful rates of CISH were low when 20-year-old tissue blocks were used [24].

Sauer and colleagues [32] correlated IHC and FISH results with clinical prognosis. They noted that IHC/FISH-positive cases as well as FISH-positive-only cases had the same prognosis regarding survival, whereas IHC-positive-only cases had a prognosis similar to that of IHC/FISH-negative tumors. In contrast, IHC-negative/FISH-positive cases had a survival probability similar to that of IHC/FISH-positive cases. The same authors concluded that if only IHC were used as a primary tool, IHC-negative/FISH-positive cases would be missed; those women would not have the benefit of trastuzumab and their survival would be adversely affected. In contrast, IHC-positive-only tumors seem to belong to a better prognostic group, and failure to detect them would probably not have a negative effect on survival [32].

Because clinical outcome studies have yet to address the utility of the low-amplified and high-amplified CISH results, especially the uncertain significance of the low-amplified group, these categories are still in flux. This limitation not-
withstanding, there is still value in reporting such amplified groups separately at present, pending whatever clinical associations will be obtained in the future. Intraobserver and interobserver variability on reporting low-amplified and high-amplified CISH results were not included in this study, although the pathologist who interpreted the CISH assays consistently applied the criteria set by the manufacturers.

Breast cancer in premenopausal women has a different biopathologic profile from that in older patients, with a predominance of unfavorable prognostic parameters, including a higher frequency of grade 3 tumors and more frequent Her2/neu overexpression [33]. Young age serves as a surrogate measure of adverse prognostic profiles [34]. This study failed to establish the association between menopausal status, which was arbitrarily set at 50 years and above, and Her2/neu alteration.

Her2/neu amplification was directly correlated with grade and histologic type of breast cancer [27,30,35]. Sapino and colleagues suggested that, on the basis of tumor grade and histologic type, the pathologist should be able to indicate to the oncologist which breast cancer requires Her2/neu analyses [30]. However, while none of the four well-differentiated tumors showed Her2/neu amplification or overexpression, no significant correlation was gathered in this study in terms of Her2/neu status and histologic grade; we therefore still subscribe to testing Her2/neu on all newly diagnosed breast cancers. Overall, age distribution of breast cancer patients and tumor histologic grade did not significantly predict IHC and CISH Her2/neu status.

Conclusion

The study has successfully evaluated Her2/neu CISH as a molecular biology procedure that is easily integrated into routine testing in our laboratory. CISH is a promising, practical alternative to FISH that can be used in conjunction with IHC, which remains the first screening procedure of choice. IHC is easy to perform, relatively inexpensive, and able to detect a majority of breast cancer patients whose tumors have negative (0 or 1+) or positive (3+) Her2/neu status, all three of which have complete concordance with CISH.

In the SLMC laboratory setup, Her2/neu CISH can be done directly at the request of the physician. Because discrepancies between the two Her-2/neu tests were observed in the 2+ IHC category, cases with 2+ IHC results will be triaged and subjected to CISH before trastuzumab therapy. This IHC–CISH test stratification not only identifies the IHC false-positive subset that will not benefit from trastuzumab but also keeps testing costs for Her2/neu status at a reasonable minimum.

Her2/neu testing has achieved a standard-of-practice status in the USA [28], and it should be performed on all breast cancer cases regardless of age at presentation and tumor histologic grade.

Competing interests

None declared.

Acknowledgements

This study was made possible by the support and funding of the Institutional Review Board (IRB) of SLMC. We thank Mr Warlito B Vallesjos RMT for his technical assistance in the CISH assays. We also thank Glenwood Technologies International, Inc., for the CISH probes and kits of Zymed.

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