Clinical Laboratory Assays for HER-2/neu Amplification and Overexpression

Quality Assurance, Standardization, and Proficiency Testing

Cell Markers and Cytogenetics Committees, College of American Pathologists

Objective.—To present and contrast the results of immunohistochemistry and fluorescence in situ hybridization (FISH) proficiency testing surveys for HER-2/neu, as conducted by the Cell Markers and Cytogenetics Committees of the College of American Pathologists.

Design.—During the past 2 years, unstained sections from invasive breast carcinomas have been used for both immunohistochemistry and interphase FISH proficiency surveys. In most instances, the same cases were used for both the Cell Markers and Cytogenetics surveys. Additional data regarding interpretative variability for immunohistochemistry surveys have also been captured.

Results.—The number of laboratories performing FISH for HER-2/neu testing doubled during the 2-year period. The results of FISH testing have been remarkably concordant for laboratories submitting results. In contrast, the results of immunohistochemistry testing continue to highlight substantial variability among laboratories evaluating the same cases. The data show that this variability is at least in part due to variability in interpretation among observers, as well as inherent differences between immunohistochemistry and FISH techniques.

Conclusions.—College of American Pathologists proficiency survey programs provide useful information about clinical testing for HER-2/neu amplification/overexpression.

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expression. The College of American Pathologists (CAP) surveys have addressed this problem, and we review these data here.

**ESSENTIAL ELEMENTS OF INTRAMURAL HER-2/neu QUALITY ASSURANCE**

The approach to introduction of an immunohistochemical or FISH assay for HER-2/neu in the clinical laboratory should be essentially the same as that for any other immunohistochemistry- or FISH-detected analyte. We refer readers to published guidelines for validating and establishing new FISH and immunohistologic assays. 49-52

As a reasonable approach, 30 invasive breast carcinomas are selected for technical validation. These cases would typically come from intramural pathology specimens submitted to other laboratories for which archival HER-2/neu results are known. Case selection should parallel the frequency of positive cases expected—approximately 10 of the cases would be known-positive cases, and the remainder would be known-negative cases, based on archival results. Laboratories should be blinded to the archival results when preparing and reviewing the immunostained or FISH slides. The initial results should be reproducible in at least 2 more experiments. Laboratories are certified by probe vendors for FISH and must follow prescribed protocols with controls. The exchange of cases with another institution can also help to evaluate the laboratory's performance and may benefit both parties. The clinical laboratory should subscribe to a regular proficiency survey such as that provided by the CAP on an ongoing basis, with a thorough review of the survey results. Clinical testing can be offered when the laboratory director has written and approved a procedure formatted for the National Committee for Clinical Laboratory Standards. Laboratories should identify appropriate charges and Current Procedural Terminology codes to ensure proper billing.

**INTERLABORATORY QUALITY ASSURANCE AND EDUCATIONAL PROGRAMS**

Although the CAP Cell Markers Committee distributed a case of invasive breast carcinoma in 1999 (1999-MK-B-04) for assessment of HER-2/neu overexpression, it was not until the year 2000 that survey participants were asked to score all HER-2/neu immunohistochemistry results using the Food and Drug Administration (FDA)-approved scoring system for the HercepTest. It is thus difficult to draw any significant conclusions from the results of the 1999 and earlier surveys, since cases were simply scored as positive or negative on a semiquantitative quartile basis.

The CAP currently provides 2 proficiency surveys for HER-2/neu testing in breast cancer. The Cell Markers Committee selects and distributes cases for HER-2/neu immunohistochemistry surveys twice annually, and the Cytogenetics Committee in conjunction with the Molecular Pathology Committee provides 2 cases once annually for an interphase FISH survey. These survey programs are coordinated through the Molecular Pathology Committee, and if possible, the same pathologic material is used for both the immunohistochemistry (MK) and FISH (CYH) surveys (Table 1).

The first FISH survey was conducted in August/September 2000 with the distribution of specimens 2000-CYH-01 and 2000-CYH-02, and a total of 35 laboratories participated. The 2000-CYH-01 specimen was not amplified by FISH and had a mean HER-2/neu copy number of 1.9 and a HER-2/chromosome 17 ratio of 0.4; the 2000-CYH-02 specimen was amplified by FISH and had a mean HER-2/neu copy number of 9.7 and a HER-2/chromosome 17 ratio of 3.7. Remarkably, the concordance was 100% for the 35 participants.

The second HER-2/neu FISH survey was conducted in September 2001 with the distribution of specimens CYH-2001-01 and 02. These 2 cases have not yet been distributed in the immunohistochemistry (MK) surveys. A total of 63 laboratories participated in 2001, a substantial increase from the 35 participants in the year 2000. All laboratories submitting results for both the 2001-CYH-01 and 2001-CYH-02 specimens submitted correct results. However, not all of the 63 participants submitted results: curiously, only 49 of 63 (78%) submitted results for the 2001-CYH-01 specimen (a case in which HER-2/neu was not amplified), but 58 of 63 (92%) provided results for the 2001-CYH-02 specimen (a case in which HER-2/neu was amplified). The reason(s) for absent responses in general, and the fact that fully 22% of participants did not provide a result for the FISH-nonamplified case in particular, is unknown. Questions intended to capture such information were not asked as part of either the CYH or the MK HER-2/neu surveys.

Most FISH survey participants enumerated signals for 60 to 99 nuclei. The level of concordance observed for the FISH surveys may reflect the nonambiguous and quantitative nature of FISH as compared with immunohistochemistry.

The immunohistochemistry survey results for the shared cases had much greater variability, particularly for the FISH-negative case (Table 2). Specimen CYH-01 from 2000 CYH-A (2001-MK-01) was not amplified by digoxigenin-based indirect FISH, biotin-based FISH, and direct-label FISH. It should also be noted that the FISH-nonamplified case selected for 2001-MK-01 was also specifically assayed for HER-2/neu messenger RNA by autoradiographic RNA-RNA in situ hybridization (courtesy of Mark Stoler, MD, University of Virginia Health Sciences Center, Charlottesville, Va), and there was no enhanced message expression relative to actin messenger RNA expression in the tumor cells.

Other information pertaining to the nonamplified 2001-MK-01/2000-CYH-01 case is of interest. Of participants reporting results, the most common vendors for HER-2/neu antibodies were DAKO (209 laboratories) and Ventana (117 laboratories). Of the 415 laboratories reporting results, 72.3% reported the tumor to be nonimmunoreactive; however, 27.7% reported the specimen to be immunoreactive. One hundred eighteen of the participants then scored the immunoreactivity using the FDA-approved guidelines for interpretation of HercepTest results. By these interpretative criteria, 37 participants (8.9% of laboratories reporting results) scored the specimens as 2+ or

<table>
<thead>
<tr>
<th>Table 1. Two Specimen Pairs Used in CAP Surveys*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH (CYH)</td>
</tr>
<tr>
<td>2000-CYH-01</td>
</tr>
<tr>
<td>2000-CYH-02</td>
</tr>
</tbody>
</table>

* CAP indicates College of American Pathologists; FISH, fluorescence in situ hybridization.
Table 2. Immunohistochemistry (MK) Results for the Shared MK and CYH Survey Specimens*

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Immunohistochemistry (MK) Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2000-CYH-02 (amplified by FISH) (N = 371 participants)</td>
<td>19 (5.1)</td>
</tr>
<tr>
<td>2000-CYH-01 (not amplified by FISH) (N = 381 participants)</td>
<td>275 (72.2)</td>
</tr>
</tbody>
</table>

* Values are No. (%) of participants reporting individual scores. FISH indicates fluorescence in situ hybridization.

Table 3. Vendors for Anti-HER-2/neu Antibodies Used by Participants in the CAP Cell Markers Immunohistochemistry Survey*

<table>
<thead>
<tr>
<th>Vendor</th>
<th>Immunohistochemistry Score+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2+</td>
</tr>
<tr>
<td>BioGenex</td>
<td>3</td>
</tr>
<tr>
<td>DAKO</td>
<td>9</td>
</tr>
<tr>
<td>DAKO+</td>
<td>5</td>
</tr>
<tr>
<td>Ventana</td>
<td>6</td>
</tr>
<tr>
<td>Zymed</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
</tr>
<tr>
<td>Not given</td>
<td>2</td>
</tr>
<tr>
<td>Invalid</td>
<td>1</td>
</tr>
</tbody>
</table>

* Values are No. of participants reporting individual scores. CAP indicates College of American Pathologists.
† Immunohistochemistry results were scored on a scale of 0 to 3+. Only results for scores of 2+ and 3+ are shown.
‡ Specifically using HercepTest.

INTEROBSERVER REPRODUCIBILITY AND CLINICAL OUTCOMES PUBLISHED LITERATURE

Published studies of interobserver reliability of HER-2/neu assessments and peer-reviewed publications relating clinical outcomes to the results of clinical laboratory HER-2/neu assays are limited. Persons et al52 described the interinstitutional reproducibility of FISH results from 5 institutions, using sections from 4 tumors. Of the cases selected for the study, 1 case had no amplification, 2 had low-level amplification, and 1 had high-level amplification based on results obtained with the PathVysion (Vysis) FISH assay. The FISH results were highly reproducible on 3 different assay days among 5 different institutions. Kakar et al33 have reported an 88% concordance between the results of immunohistochemistry and FISH assays, emphasizing a much higher correlation for 3+ cases. They also correlated the intensity of the immunostaining pattern and the results of FISH with survival (Table 5) and found that survival was best correlated with 3+ immunohistochemistry results and FISH results. Seidman et al53 reported on HER-2/neu status in 95 patients with metastatic breast cancer who were treated weekly with trastuzumab and paclitaxel, 88 of whom were fully evaluable. In this phase II study, clinical efficacy was most strongly correlated with the results of interphase FISH and the results of immunohistochemistry for CB11 and TAB250 (Table 6).

THE FUTURE OF HER-2/neu QUALITY ASSURANCE

The CAP is committed to the distribution of meaningful and illuminating survey specimen results. We will continue to provide survey material distributed from the same cases used for immunohistochemistry and FISH. The number of cases evaluated in this manner is of necessity small, but the number of observations gleaned from the program has yielded much useful data. These survey results are initial findings. The FISH assay for detection of HER-2/neu amplification/overexpression is a new methodology. Further comparison is warranted. In the final analysis, variability between methods and among laboratories performing HER-2/neu testing will never be clearly and fully understood until sufficient clinical outcomes studies have been published. The testing algorithms used in the clinical laboratory for HER-2/neu continue to evolve. The rate of false-positive immunohistochemistry results in the 2+ range may stabilize over time.

The survey program will evolve and adapt to the development of new technology. Pathologists may ultimately prefer a bright field–based chromogenic in situ hybridization (CISH) or autometallographic in situ hybridization assay that can be evaluated in a nonquantitative fashion.
Photomicrographs distributed to participants as part of the 2000-MK-B Survey. The referees' consensus and participants' results are summarized in Table 4. All photomicrographs were images of HercepTest preparations made using diaminobenzidine as the chromogen and hematoxylin as the counterstain (original magnification ×200).
Table 4. Grading of Survey Photomicrographs in the CAP MK Survey Supplemental Exercise*

<table>
<thead>
<tr>
<th>Photomicrograph</th>
<th>Referees’ Score</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Her 2-01</td>
<td>1+</td>
<td>25</td>
<td>352</td>
<td></td>
<td></td>
<td>477</td>
</tr>
<tr>
<td>Her 2-02</td>
<td>2+</td>
<td>0</td>
<td>60</td>
<td></td>
<td></td>
<td>60</td>
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<tr>
<td>Her 2-03</td>
<td>3+</td>
<td>8</td>
<td>0</td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Her 2-04</td>
<td>1+</td>
<td>92</td>
<td>350</td>
<td></td>
<td></td>
<td>442</td>
</tr>
<tr>
<td>Her 2-05</td>
<td>2+§</td>
<td>27</td>
<td>166</td>
<td></td>
<td></td>
<td>194</td>
</tr>
<tr>
<td>Her 2-06</td>
<td>2+</td>
<td>7</td>
<td>133</td>
<td></td>
<td></td>
<td>140</td>
</tr>
</tbody>
</table>

* CAP indicates College of American Pathologists.
† Values are No. of interpretations for individual scores.
§ One dissenter gave a score of 0.

Table 5. Correlation of the Results of Immunohistochemistry and FISH With Survival*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Score*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunohistochemistry</td>
<td>3+</td>
<td>.02</td>
</tr>
<tr>
<td>FISH</td>
<td>+</td>
<td>.03</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>2+</td>
<td>.7</td>
</tr>
</tbody>
</table>

* Adapted from Kakar et al.† FISH indicates fluorescence in situ hybridization.
† Results of immunohistochemistry were scored on a scale of 0 to 3+; results of FISH were scored as + or −.

Table 6. Correlation of the Overall Response to Trastuzumab and Paclitaxel Therapy With HER-2/neu Assay Results*

<table>
<thead>
<tr>
<th>Assay</th>
<th>No. Responding/No. Assessed (%)</th>
<th>No. Responding/No. Assessed (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH</td>
<td>17/39 (44)</td>
<td>30/40 (75)</td>
<td>.004</td>
</tr>
<tr>
<td>HercepTest</td>
<td>18/39 (46)</td>
<td>35/51 (69)</td>
<td>.03</td>
</tr>
<tr>
<td>Pab1</td>
<td>11/27 (41)</td>
<td>42/63 (67)</td>
<td>.02</td>
</tr>
<tr>
<td>CB11</td>
<td>22/49 (45)</td>
<td>31/41 (76)</td>
<td>.003</td>
</tr>
<tr>
<td>TAB250</td>
<td>25/58 (43)</td>
<td>29/36 (81)</td>
<td>.001</td>
</tr>
</tbody>
</table>

* Adapted from Seidman et al.‡ FISH indicates fluorescence in situ hybridization.
‡ Assessment of automated image analysis of immunohistochemical HER-2/neu staining,§ and potentially of bright-field chromogenic or autometallographic preparations, may also become important survey programs. As these methods are developed, reproducibility is established, staining protocols are standardized, and thorough validation is completed, the CAP will provide a survey venue to assess CISH HER-2/neu testing as the discipline matures.

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References


