Human Epidermal Growth Factor Receptor 2 Testing: Where Are We?

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See accompanying editorial on page 4293 and articles on pages 4300 and 4307

In this issue of Journal of Clinical Oncology, Baehner et al have reported from a Kaiser Permanente case-control study that there is a high degree of concordance (97%) between quantitative reverse transcription polymerase chain reaction (qRT-PCR) and central laboratory fluorescent in situ hybridization (FISH) assessment of HER2 status. This provides us the opportunity to examine the status of human epidermal growth factor receptor 2 (HER2) testing today.

For the most part, cancer is a genetic disease, and successful treatment of breast cancer is particularly dependent on a number of complex factors, including detection of the tumor early in the course of development, accurate assessment of the right biomarker, and the biology of the underlying disease. Amplification and overexpression of the HER2/ERBB2 oncogenes are observed in 15% to 25% of invasive breast cancers. HER2-positive tumors define a clinically important breast cancer subgroup that is generally associated with poor prognosis and variable response to conventional systemic cytotoxic therapy. HER2 testing is routinely performed in patients with a new diagnosis of invasive breast cancer. Accurate testing to identify HER2 status for patients with breast cancer who can benefit from anti-HER2 treatment (eg, trastuzumab, lapatinib) is a clinical and economic necessity (Fig 1). As a consequence, issues relating to accurate and reliable laboratory assessment of HER2 status in patients with breast cancer are a matter of significant concern to patients, pathologists, and oncologists.

![Figure 1](image-url)
Amplification is the primary mechanism of HER2 overexpression. Gene amplification was initially detected by Southern hybridization in frozen tumor specimens and was subsequently found to correlate with overexpression at the mRNA and protein level. Initial trials of trastuzumab in breast cancer enrolled patients after central testing by a specific immunohistochemistry (IHC) clinical trials assay, which identified staining patterns for HER2 as negative (0 and 1+) or positive (2+ and 3+). For randomized adjuvant trials of trastuzumab, testing algorithms for HER2 (for anti-HER2 treatment) were subjectively developed, consisting of either IHC testing with confirmatory FISH testing if IHC 2+ or reliance on FISH testing alone to detect gene amplification ratios of 2.0 or greater. Patients with breast cancer with evidence of amplification of HER2 by FISH and/or overexpression by IHC (3+) were considered appropriate for anti-HER2–related clinical trials.

In early 2000, a huge discordance (>27%) of IHC data was observed between outside (community) laboratories versus a central reference laboratory. Whereas community laboratories rapidly developed the capacity to assay HER2 overexpression, their experience with the test was relatively low. Retrospective analyses suggested that only patients with IHC 3+ staining and/or HER2 gene amplification by FISH benefited from trastuzumab treatment. Clinical benefit from trastuzumab in combination with chemotherapy was observed to be greater in patients with IHC 3+ metastatic breast cancer than in patients with IHC 2+ (H0648g trial). To date, two different histochemical assay methods and one FISH assay method have been approved by the US Food and Drug Administration for the selection of patients for trastuzumab therapy. These assay methods are significantly correlated with one another in most published comparisons. Initially, overall concordance between IHC and FISH was 82%, and recent data has shown discordance between HER2 gene amplification and HER2 protein overexpression at approximately 4% (among 1,503 patients screened centrally; LabCorp; Burlington, NC). This improvement over time may be a result of the development of and adherence to American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines regarding HER2 testing. Considering their (IHC+vFISH) relative cost, turnaround time, and a growing body of data demonstrating high concordance between the two methods (in 0, 1+, and 3+ scores by IHC), IHC has emerged as a method of choice for regular screening; whereas FISH is used as a confirmatory test particularly in equivocal IHC cases (2+).

Even with the ASCO/CAP guideline, concerns remain about the precision and accuracy of HER2 testing. Anderson et al reported that concordance between FISH results performed at a reference laboratory and IHC tests performed locally were 66% (IHC 2+; not amplified by FISH) and 73% (IHC 3+; amplified by FISH). As late as October 2009, Viale mentioned that there is significant discordance of IHC data for both estrogen receptor (ER) determination (16.6% false negative) and HER2 determination (14% false-positive results rate in 2009; down from 27% in 2002). Differences in results have the potential to lead to inappropriate treatment decisions. Indeed, an internal study at United Healthcare showed that 12% of patients receiving trastuzumab therapy did not have HER2 amplification/overexpression.

Again the question posed by Viale—“Is it prognostically more important whether less percentage of tumor cells (for example 11%) with +3 immunoreactivity or 99% of tumor cells with +2 immuno-reactivity?”—remains unresolved. Although IHC was the original method of assessment for HER2 status, now IHC alone cannot be recommended for determining anti-HER2 treatment. IHC results may vary substantially as a result of multiple factors, including time to fixation, duration of fixation, time of processing, antigen retrieval, staining procedure, and staining interpretation.

FISH is a semi-quantitative approach based on the average ratio of HER2 signals to chromosome 17 centromere (CEP17) signals in non-overlapping interphase nuclei of the lesion. It has been reported that the response rate of trastuzumab monotherapy is higher with high signal ratio of HER2/CEP17 in breast cancer. According to the recent ASCO/CAP guidelines for HER2 testing, a tumor is considered positive for HER2 gene amplification when the HER2/CEP17 ratio is greater than 2.2, negative when less than 1.8, and equivocal if the ratio is between 1.8 and 2.2. The need to use CEP17 as reference for HER2 evaluation is supported by the fact that an increased HER2 gene copy number as a result of chromosome polysomy may not bear the same clinical significance as HER2 amplification (see Baehner et al regarding this issue). Oncologists began treating patients with FISH scores greater than 2.0, rationalizing that these patients were “close enough” to the threshold. Recently, Dowsett et al demonstrated in the Herceptin Adjuvant (HERA) clinical trial that the signal ratio of HER2/CEP17 amplification does not influence prognosis or benefit from adjuvant trastuzumab in patients previously treated with adjuvant chemotherapy. Similarly, Paik et al also found no significant association between HER2 gene copy number and benefit from trastuzumab therapy. Even patients with normal gene copy numbers seemed to benefit from treatment.

However, Arnold et al reported that there was a correlation between the level of HER2 amplification and response to trastuzumab in the neoadjuvant setting. FISH directly measures the HER2 DNA, but some fixatives, chemicals, or heat may interfere with the FISH assay. However, an internal control (one positive and one negative control tissue being processed together with the tissue of interest in the same staining batch but on different tissue slides) is used in each assay to rule out false-positive or false-negative results. FISH is more dependent on good tissue quality and the use of an appropriate fixative. As a result, FISH is also more robust against false-positive or false-negative results given that different tissue preparation methods are unlikely to affect the HER2/CEP17 ratio (either FISH works and provides correct results, or it does not work and no false results can be produced), whereas tissue preservation has large effects on IHC results.

Of yet greater challenge is the exact definition of the responding population based on molecular pathway, taking into account that data from a phase II monotherapy trial with trastuzumab showed response in women who have IHC 3+/FISH-negative status (HER2 assessment from central reference laboratory) and noting that the precise level of threshold of HER2 amplification/overexpression in relation to benefit from trastuzumab has recently been brought into doubt by data from the National Surgical Adjuvant Breast and Bowel Project (NSABP) –B31 trial, which indicate that the subgroup of 174 patients conventionally considered as having HER2-negative disease (centrally tested IHC and FISH negative) seemed to benefit from trastuzumab. A similar analysis in 103 patients in the North Central Cancer Treatment Group trial also showed a trend toward benefit of trastuzumab, but this was not statistically significant. It has also been suggested that women whose breast cancers lack HER2 gene amplification but have chromosome 17 polysomy may be responsive to HER2-directed...
therapy in the metastatic setting.\textsuperscript{35} Using robust data from a phase III trial of patients with \textit{HER2}-FISH negative or \textit{HER2}-untested metastatic breast cancer in which patients were randomly selected to receive paclitaxel along with \textit{HER2}-driven therapy, Press et al\textsuperscript{16} demonstrated that chromosome 17 polysomy in patients with nonamplified \textit{HER2} was not associated with improved outcome of chemotherapy when lapatinib was added as a \textit{HER2}-targeted treatment. These findings argued against a role for chromosome 17 polysomy as a mechanism of response to \textit{HER2}-directed therapy in this patient population.\textsuperscript{36}

Recently, bright field in situ hybridization techniques, such as chromogenic in situ hybridization (CISH) and silver-enhanced in situ hybridization—which combine features of immunohistochemical analysis and FISH—have been introduced to determine \textit{HER2} status. Several investigators have examined the prognostic value of using CISH (either standard CISH for \textit{HER2} probe or dual-color CISH for \textit{HER2} and CEP17 probe) to determine \textit{HER2} status.\textsuperscript{37–40} There has been a great deal of controversy about how CISH should be analyzed and what the cutoff should be.\textsuperscript{41} At this time, however, reagents that have been prepared according to good manufacturing practice standards are not available for this purpose. Furthermore, US Food and Drug Administration–approved reagents of analyze-specific reagents class are not available for bright-field HER2 genotyping.

In this issue of \textit{Journal of Clinical Oncology}, Baehner et al\textsuperscript{1} have demonstrated a high degree of concordance (97\%) between qRT-PCR and central laboratory FISH assessment of \textit{HER2} status. Their results (using \textit{OncoType Dx}) show a higher percentage of equivocal samples as determined by qRT-PCR than FISH (15\% vs 2\%). This is probably not a result of qRT-PCR, but it may be a result of the particular technology of \textit{OncoType Dx} they have used.

According to the Genomic Health Web site,\textsuperscript{42} \textit{OncoType Dx} has been used since 2004. To date, there are not much data available for clinical trials using this methodology for particular gene identification. The large Trial Assigning Individualized Options for Treatment (TAILORx) clinical trial (National Cancer Institute; Bethesda, MD) was launched on the basis of identification of genes by \textit{OncoType Dx}. This trial is almost fully accrued, but trial results are not anticipated until 2013.\textsuperscript{43}

qRT-PCR is a comparatively newer approach to detect \textit{HER2}/\textit{Neu} gene amplification in breast cancer. Several investigators demonstrated that concordance between FISH and qRT-PCR was 92\% to 94\%, and their data suggest that qRT-PCR is the better methodology (Table 1), especially for equivocal samples (IHC 2+).\textsuperscript{45–47} Again, qRT-PCR does not register single-cell expression, which is important for therapeutic decision making. Ductal carcinoma in situ may cause false results with qRT-PCR if no appropriate tissue dissection is used. In addition, small \textit{HER2}-positive foci might be diluted with qRT-PCR assays though they might be detected in 20 cell counts with FISH assay. Roepman\textsuperscript{48} recently demonstrated that microarray-based readout (mRNA determination) of ER, progesterone receptor, and \textit{HER2} show high concordance with IHC (ER, 93\%; progesterone receptor, 83\%; and \textit{HER2}, 96\%).

On the basis of the results of those studies,\textsuperscript{45–47} the argument in favor of choosing qRT-PCR instead of FISH for equivocal IHC 2+ samples are that \textit{HER2} gene amplification is highly associated with mRNA overexpression;\textsuperscript{49} determination of mRNA by using qRT-PCR is highly matched with DNA amplification by FISH;\textsuperscript{45} qRT-PCR offers quantitative “numerical values,” whereas FISH data are semiquantitative and require “trained eyes”; and qRT-PCR is high-throughput technology, whereas FISH is time-consuming.\textsuperscript{47}

Despite the apparent advantages, one possible limitation of qRT-PCR relates to the tumor cell components included in any analysis of stromal, inflammatory, and normal epithelial cells, possibly leading to a dilution that could result in an underestimation of \textit{HER2} mRNA levels of the tumor. This phenomenon may require mandatory histopathologic evaluation of all samples before RNA extraction. Cutoffs will have to be defined, and the influences of kits/reagents, test platforms, and software all meticulously and systematically examined and verified. On the basis of previous experience, we should not expect standardization to be easier for qRT-PCR simply because it yields a numerical result. Standardization with IHC and FISH after some 10 years of study and debate remains elusive. Limited amounts of DNA, impurities in samples and reagents, and minimal variations in assay technique can result in a steep decrease in the percentage of reliability. The development and application of this technique to the clinic will, as a first step, require the validation of qRT-PCR testing through a multicenter clinical trial, using several testing laboratories and including the analysis of clinical response to anti-\textit{HER2} therapy.

\textbf{AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST}
The author(s) indicated no potential conflicts of interest.

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\textbf{REFERENCES}

\begin{table}
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\hline
 & IHC & FISH & qRT-PCR \\
\hline
Identifies protein overexpression & Uses gene amplification & Analyzes mRNA determination \\
\hline
Is subjective & Is semiquantitative & Is quantitative \\
\hline
Needs experienced eyes & Needs experienced eyes & Uses numerical values with statistics \\
\hline
Is fast & Is slow & Is fast \\
\hline
Is inexpensive & Is expensive & Is moderately priced \\
\hline
Is easy to administer & Is difficult to administer & Is difficult to administer \\
\hline
Has a high false-negative/false-positive rate & Is accurate (may give false-negative results in case of polysomy) & Is accurate (following a lot of standardization) \\
\hline
\end{tabular}
\caption{Relative Comparison of IHC and FISH With qRT-PCR}
\end{table}
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