Quantitative PCR and HER2 Testing in Breast Cancer
A Technical and Cost-Effectiveness Analysis

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Abstract
We performed a technical and cost-effectiveness analysis of quantitative reverse transcription–polymerase chain reaction (Q-RT-PCR) for the assessment of HER2 in breast cancer. We evaluated 44 frozen and 55 formalin-fixed paraffin-embedded (FFPE) breast carcinoma specimens by Q-RT-PCR, immunohistochemical analysis, and fluorescent in situ hybridization (FISH). Immunohistochemical and FISH analyses were performed on individual slides and on tissue microarray. Costs of techniques were calculated to study 1 case and 10 or 40 cases. Q-RT-PCR provided reliable data in frozen and FFPE specimens, which were significantly correlated. HER2 messenger RNA levels were significantly stratified in agreement with immunohistochemical data ($P < .05$). There was complete concordance between Q-RT-PCR and immunohistochemical results for negative and strongly positive (3+) cases. The intermediate immunohistochemical group (2+), including FISH+ and FISH− cancers, could also be stratified by Q-RT-PCR. Cost analysis documented the advantage of Q-RT-PCR in all US Food and Drug Administration–approved assays. Our data support the use of Q-RT-PCR for testing breast cancer specimens to select patients for HER2 inhibitory therapy.

Recently, novel targeted therapies have been introduced in clinical oncology with significant benefits in terms of survival and quality of life, such as imatinib for leukemia and trastuzumab for breast cancer. Trastuzumab prolongs survival not only in patients with diffuse metastatic disease but also in patients with operable HER2+ cancer in the adjuvant setting. The number of patients that can be treated with trastuzumab in the United States is approximately 15,000 per year with a predicted cost of $750 million per year.1

Trastuzumab therapy can be used as a general paradigm for successful targeted therapy: patients have to be selected based on HER2 gene amplification and the consequent activation of the pathway for which the humanized monoclonal antibody–based inhibitory therapy was developed. Because positive HER2 status determines the indication for trastuzumab therapy, it is important to use simple, accurate, widely applicable, and reproducible methods to screen tumors for gene amplification and/or overexpression.

Two methods are used and recommended by all current national testing guidelines: fluorescent in situ hybridization (FISH), a quantitative method for detection of gene amplification, and immunohistochemical analysis for the detection of protein expression.2,3 Currently, however, no single assay is accepted as the “gold standard” for HER2 testing. FISH and immunohistochemical analysis are microscopic techniques and require intensive and time-consuming interpretation. Moreover, these techniques are difficult to standardize across laboratories and are subject to interobserver variability.4,5

Molecular techniques based on the quantitative evaluation of HER2 messenger RNA (mRNA) have been proposed. The first method has been to detect HER2 gene amplification with polymerase chain reaction (PCR) that uses end point
Specificity validation of the probes used in Q-RT-PCR was performed on fresh or snap-frozen samples, which is material not available in most hospitals and laboratories.

Recent data suggest that Q-RT-PCR can be performed using archival, formalin-fixed, paraffin-embedded (FFPE) tissue samples. We performed a comparative study on frozen and FFPE tissue samples using immunohistochemical analysis, FISH, and Q-RT-PCR to establish the effectiveness of HER2 mRNA quantitative analysis in readily available clinical material. Specifically, the objectives of our investigation included validation of Q-RT-PCR for FFPE tissue samples and comparison of Q-RT-PCR with immunohistochemical analysis and FISH.

We realized the overall economic and medical implications of HER2 testing to optimize therapy for breast cancer patients and, therefore, decided to analyze the total testing costs of the aforementioned techniques. It has been proposed that HER2 testing using tissue microarray (TMA) may provide satisfactory results with a cost reduction by a factor of 7. Therefore, we constructed a TMA including all cases in our series and performed immunohistochemical and FISH techniques using individual and TMA slides. The TMA evaluation was included to provide a scenario applicable to high-volume laboratory testing. Our objective was to determine the cost-effectiveness of Q-RT-PCR compared with the other alternative and established techniques.

Materials and Methods

Samples, Immunohistochemical Staining, and FISH

Samples from a consecutive series of 44 breast carcinomas from January to June 2006 were immediately preserved in RNA later (Ambion, Austin, TX) and stored at -80°C until tested. Immunohistochemical staining for estrogen receptor (ER) and progesterone receptor (PR), HER2 (clone A0485, DAKO, Glostrup, Denmark), and Ki-67 were routinely performed on FFPE samples. Time of fixation in 10% buffered formalin, pH 7.2, ranged from 16 to 36 hours. To evaluate cases with different ranges of HER2 mRNA value, we selected another 55 cases of breast cancers with different HER2 immunohistochemical scores: 26 cases with score 2+, 21 with score 3+, and 8 with score 0 to 1+. This series was used also to assess Q-RT-PCR reliability on the suboptimal quality RNA that is usually obtained from FFPE material. Specificity validation of the probes used in Q-RT-PCR was based on the quantification of HER2 mRNA levels in 2 cell lines, MCF-7 and SKBR3. Optimal RNA was extracted from fresh cell cultures. As reported previously, HER2 mRNA levels were overexpressed in the SKBR3 cell line compared with MCF-7.

The mean age of patients was 62.6 years (range, 30-92 years). There were 89 infiltrating ductal carcinomas, 8 infiltrating lobular carcinomas, 1 infiltrating papillary carcinoma, and 1 apocrine carcinoma. The Elston-modified Scarff-Bloom-Richardson grades in these cases were as follows: grade 1, 20 (20%); grade 2, 68 (69%); and grade 3, 11 (11%); 82 cases (83%) were ER+/PR+, whereas 17 (17%) were ER-/PR-.

A representative tumor block from each case was chosen to construct TMA blocks to reevaluate HER2 status by immunohistochemical analysis and FISH. Four tumor areas were selected from each block. For TMA construction, a semiautomated, computer-assisted microarrayer was used (Galileo TMA CK3500; Integrated Biosystems Engineering, Milan, Italy). Staining was evaluated using the 0 to 3+ DAKO scoring system. FISH was performed with the PathVysion HER2 assay (Abbott Molecular, Des Plaines, IL) according to the manufacturer’s instructions. Cases were scored as amplified when the final HER2/chromosome 17 signal ratio was 2 or more. All cases had HER2 evaluation performed by immunohistochemical analysis and FISH on individual slides and TMA slides.

RNA Extraction and Q-RT-PCR

The extraction of total RNA from FFPE samples (three 10-μm sections) was performed as described by Bijwaard et al. Total RNA from frozen tissue was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). RNAs were reverse transcribed using the High-Capacity cDNA Archive Kit (500 ng of total RNA in a final volume of 100 µL; Applied Biosystems, Foster City, CA).

HER2 mRNA levels were measured by Q-RT-PCR based on the TaqMan method in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Q-RT-PCR data analysis was performed with geNorm software (http://medgen.ugent.be/~jvdesomp/genorm), with the following 3 housekeeping genes: hydroxymethylbilane synthase (HMBS), TATA box binding protein (TBP), and succinate dehydrogenase complex, subunit A (SDHA). Our results are expressed as relative levels of HER2 mRNA referred to a calibrator sample, the MCF-7 cell line, chosen to represent 1× expression of this gene. All analyzed tumors expressed n-fold HER2 mRNA relative to the calibrator. For quantification of 3 internal control samples, we used Assay-on-Demand FAM-MGB–labeled probes (Applied Biosystems). The assay identification numbers are Hs00609297_m1 (HMBS), Hs00427620_m1 (TBP), and Hs00188166_m1 (SDHA). Primers and probes...
for HER2 mRNA, designed using Primer Express software (Applied Biosystems), and thermal cycling conditions have been previously reported. Ampliplex sizes were 64 base pairs (bp) for HMBS, 70 bp for SDHA, 91 bp for TBP, and 119 bp for HER2. Amplification reactions were performed with TaqMan Universal PCR master mix (Applied Biosystems), using 5 µL of cDNA in a final volume of 25 µL. HMBS, TBP, and SDHA primers and probes were added to the reaction mixture according to the manufacturer’s directions, and the HER2 primers and probe were present at 0.3 and 0.1 µmol/L, respectively.

Statistical Analysis

The Kruskal-Wallis, Mann-Whitney U, and \( \chi^2 \) tests and Pearson correlation were used for statistical analyses. A \( P \) value of less than .05 was considered to indicate statistical significance.

Cost Analysis

We determined the cost of each procedure by recording all reagent costs related to immunohistochemical analysis with HercepTest (DAKO) and anti–HER2/neu (A0485); immunohistochemical analysis with TMA; FISH; FISH with TMA; and Q-RT-PCR. Then we performed a time-per-test analysis for each assay type, considering separately the technical components and the analytic components. In the TMA cost analysis, we included the construction cost of the TMA, based on a semiautomated, computer-assisted platform. Indirect and marginal costs were not considered. We calculated the single specimen testing costs and costs for batches of 10 and 40 cases. The latter are more relevant to large institutions or referral pathology laboratories. To ensure the reproducibility of results, we performed cost analysis separately in our two institutions. Results displayed less than 5% variation between the two centers and are reported as the mean value for all tests.

Results

The first unselected, consecutive series of 44 breast cancers evaluated by immunohistochemical analysis with the HercepTest had 35 cases (80%) with a score of 0 or 1+, 5 (11%) with a 2+ score, and 4 (9%) with a 3+ score. Q-RT-PCR evaluation of mean ± SD HER2 mRNA expression in these 3 groups was as follows: 12 ± 19 in cases with an HER2 score of 0 or 1+, 44 ± 66 in cases with an HER2 score of 2+, and 176 ± 51 in cases with an HER2 score of 3+. The difference was statistically significant (\( P < .05 \); Kruskal-Wallis test).

To validate Q-RT-PCR analysis in archival tissue samples, HER2 mRNA expression was assessed in 17 paired frozen and FFPE breast carcinomas. The Pearson coefficient of correlation was 0.96.

The second series included 55 selected FFPE breast carcinomas that were evaluated by immunohistochemical analysis, FISH, and Q-RT-PCR. Immunohistochemical analysis showed strong HER2 expression (3+) in 21 cases (38%), moderate expression (2+) in 26 cases (47%) and negative or weak staining (0/1+) in the remaining 8 cases (15%). FISH analysis documented HER2 gene amplification in 30 cases (55%).

HER2 mRNA values measured by Q-RT-PCR were subdivided into groups of normal, moderate, and high expression based on their immunoreactivity score. HER2 mRNA levels of the 8 breast carcinomas with negative immunohistochemical results (0/1+) ranged from 0.6 to 1.2 (mean ± SD, 0.9 ± 0.2); therefore, we considered values greater than 1.3 (mean ± 2 SD) indicative of elevated HER2 mRNA expression. To identify among these cases the carcinomas with moderate and strong expression, we analyzed the breast carcinomas with 2+ immunohistochemical scores. HER2 mRNA values of 2+ carcinomas ranged from 0.5 to 13.3 (mean ± SD, 3.82 ± 3.1); therefore, the upper cutoff level of moderate overexpression was set at 10.0 (mean ± 2 SD), and cases with HER2 levels higher than 10 were considered high-expression cancers. Indeed, in the 3+ immunohistochemical score group, HER2 mRNA values ranged from 3.4 to 49.8 (mean ± SD, 19.4 ± 13.6). The HER2 mRNA levels of the 3 groups of breast carcinomas were significantly different (\( P < .05 \); Kruskal-Wallis test).
Comparative results for Q-RT-PCR and immunohistochemical analysis are shown in Table 1 and for Q-RT-PCR and FISH in Table 2. All immunohistochemically negative carcinomas showed no HER2 gene amplification and had normal HER2 mRNA levels. All immunohistochemically 3+ breast carcinomas had HER2 gene amplification and moderate to strong mRNA levels. In particular, 15 (71%) of 21 cases had strong levels of RNA, and 6 (29%) of 21 had moderate levels. The immunohistochemically 2+ tumors displayed the largest spectrum of mRNA expression, including 2 FISH– carcinomas with normal mRNA levels and 2 FISH+ cancers with high mRNA levels. Finally, among the 22 cases with moderate mRNA levels, there were 7 with HER2 gene amplification, whereas 15 were FISH−.

Overall comparison between immunohistochemical analysis and Q-RT-PCR, including normal (0/1+ and mRNA ≤ 1.3), moderate expression (2+ and 1.3 < mRNA < 10), and high expression (3+ and mRNA ≥ 10), displayed a significant correlation (percentage of agreement, 82%; \( P < .05, \chi^2 \) test).

We further analyzed the mRNA expression levels in relation to the status of the HER2 gene in the 2+ immunohistochemical group, which included the highest number of discordant cases. There were 17 FISH− tumors with mean ± SD mRNA levels of 2.4 ± 1.35 (range, 0.5-6.6) and 9 FISH+ carcinomas with mean ± SD HER2 mRNA levels of 6.5 ± 3.7 (range, 3.6-13.3). Although there was partial overlap, the difference in the distribution of HER2 mRNA levels was significant (\( P < .05, \) Mann-Whitney test) Figure 2.

Cost Analysis of HER2 Assays

Examples of immunohistochemical and FISH analyses performed on TMA for cost analysis are shown in Image 1, and the overall results are detailed in Table 3. There was complete concordance in HER2 evaluation by comparing results obtained using individual slides and TMA slides for HercepTest and FISH analysis. Considering the commonly applied testing condition (1 case per slide for immunohistochemical analysis and FISH), immunohistochemical

### Table 1

<table>
<thead>
<tr>
<th>Expression</th>
<th>0/1+ (n = 8)</th>
<th>2+ (n = 26)</th>
<th>3+ (n = 21)</th>
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<tr>
<td>Normal (mRNA ≤ 1.3; n = 10)</td>
<td>8 (80)</td>
<td>2 (20)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Moderate (1.3 &lt; mRNA &lt; 10; n = 28)</td>
<td>0 (0)</td>
<td>22 (79)</td>
<td>6 (21)</td>
</tr>
<tr>
<td>Strong (mRNA ≥ 10; n = 17)</td>
<td>0 (0)</td>
<td>2 (12)</td>
<td>15 (88)</td>
</tr>
</tbody>
</table>

mRNA, messenger RNA.

* Data are given as number (percentage).

### Table 2

<table>
<thead>
<tr>
<th>Expression</th>
<th>FISH− (n = 25)</th>
<th>FISH+ (n = 30)</th>
</tr>
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<tr>
<td>Normal (mRNA ≤ 1.3; n = 10)</td>
<td>10 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Moderate (1.3 &lt; mRNA &lt; 10; n = 28)</td>
<td>15 (64)</td>
<td>13 (46)</td>
</tr>
<tr>
<td>Strong (mRNA ≥ 10; n = 17)</td>
<td>0 (0)</td>
<td>17 (100)</td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization; mRNA, messenger RNA.

* Data are given as number (percentage).
analysis with off-the-shelf antibodies is always the least expensive assay. Q-RT-PCR, however, is less expensive than the other US Food and Drug Administration–approved assays evaluated (HerceptTest and FISH). Notably, even the inclusion of TMA testing, which significantly reduces the cost of expensive reagents, does not cancel the cost-effectiveness of Q-RT-PCR testing.

Discussion

Our study documents that Q-RT-PCR is a rapid, sensitive, reliable, and cost-effective method to evaluate HER2 status in frozen and FFPE breast cancer specimens, which

<table>
<thead>
<tr>
<th>Assay Costs*</th>
<th>No. of Cases</th>
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<tr>
<td></td>
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<tr>
<td>HerceptTest</td>
<td>106</td>
</tr>
<tr>
<td>TMA-HerceptTest</td>
<td>ND</td>
</tr>
<tr>
<td>HER2/neu antibody</td>
<td>69</td>
</tr>
<tr>
<td>TMA-HER2/neu antibody</td>
<td>ND</td>
</tr>
<tr>
<td>FISH</td>
<td>457</td>
</tr>
<tr>
<td>TMA-FISH</td>
<td>ND</td>
</tr>
<tr>
<td>Real-time Q-RT-PCR</td>
<td>89</td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization; ND, not done; Q-RT-PCR, quantitative reverse transcription–polymerase chain reaction; TMA, tissue microarray.

* Data are given as cost in euros. The cost of personnel includes technicians, biologists (for Q-RT-PCR), and pathologists (for immunohistochemical analysis and FISH). HerceptTest Kit (DAKO, Glostrup, Denmark); HER2/neu antibody, polyclonal rabbit antihuman c-erbB-2 oncoprotein A0485 (DAKO). See the “Materials and Methods” section for additional details.
could be applied to routine clinical practice. HER2+ breast cancers require proper classification because the survival of patients can be significantly improved by trastuzumab when combined with traditional chemotherapy and hormonal therapy.\textsuperscript{21,22} Consequently, the evaluation of HER2 status has a pivotal role in selecting patients eligible for this therapy. Ideally, robust, sensitive, and cost-effective techniques should be used to support optimal patient classification.

However, there is substantial scientific documentation supporting the variability of HER2 evaluation among pathology laboratories: substantial rates of false-positive and false-negative results are reported, similar to the disagreements in assessing hormone receptors and tumor grades.\textsuperscript{23} Recently, Masood\textsuperscript{24} reported that the variability in the results of prognostic and predictive factors is a long-standing issue, with 20\% and 26\% error rates in ER and HER2 testing, respectively.

Some progress has been achieved, as demonstrated by HER2 testing with FISH technology by the results of the United Kingdom–based pilot National External Quality Assurance Scheme.\textsuperscript{25} Overall, however, the variability in these types of tests persists, which impacts patient safety and can contribute to an increase in medical costs. The morphologic evaluation of tissues with immunohistochemical analysis and FISH will always require a certain level of subjective decisions, which are very difficult to overcome, with major interobserver variability.

Q-RT-PCR is now considered the gold standard for mRNA quantitative evaluation, and its application to HER2 status evaluation could contribute to method standardization and reduce reports of variability. Q-RT-PCR is a true quantitative technique, it is assessable according to Clinical and Laboratory Standards Institute guidelines, and it is highly sensitive and specific because primers and hybridization probes are sequence specific. Therefore, we applied this technique to fresh frozen and FFPE breast cancer specimens and performed a comparative cost evaluation.

First, we validated Q-RT-PCR by analyzing HER2 mRNA levels of SKBR3 and MCF-7 cell lines, with results in agreement with previous reports.\textsuperscript{15,20} We then verified results with 2 independent, although partially overlapping, series of tumors and compared the data with those from more established morphologic techniques. Finally, we constructed a TMA from our series of breast cancers. TMA technology allowed us to analyze HER2 gene amplification and protein expression by FISH and immunohistochemical analysis on 2 consecutive sections with only 2 separate laboratory runs.

Zhang et al\textsuperscript{26} demonstrated that a single 0.6-mm tissue core per case ensures full representation of the entire tissue block, with no sample bias for identifying relevant biomarkers in breast cancer. The same authors and, more recently, Bhargava et al\textsuperscript{27} demonstrated that the concordance between TMA and full sections using FISH analysis was 99\%. To avoid potential limitation of this technique and misinterpretation in cases with tumor heterogeneity, in our study, we selected 4 tissue cores for each block, as reported by others.\textsuperscript{14,28}

Taken together, the results of Q-RT-PCR analysis for HER2 testing seem promising and could provide even more clinically important information. However, some criticism of the application of this technique has already appeared. The first problem relates to the suboptimal quality of nucleic acids extracted from FFPE samples owing to formalin. Nucleic acid degradation also depends on the presence of tissue enzymes and the duration and temperature of storage.\textsuperscript{29} These problems can be overcome by modern techniques, and it has been demonstrated that it is possible to measure mRNA levels and gene expression using FFPE tissue samples as a source of RNA, despite the fact that RNA can be present in fragments shorter than 300 bases.\textsuperscript{12,30,31} Recent studies performed on FFPE specimens have identified expression profiles that are prognostic, predictive, or both for patients with breast cancer.\textsuperscript{32,33} Indeed, the large number of FFPE samples that are present in pathology archives, associated with clinical records, represent an important source of information for patients who underwent operation before the approval of trastuzumab, which could now be used for recurrences.

Another possible limitation of Q-RT-PCR testing relates to the dilution of the tumor cell component with stromal, inflammatory, and normal epithelial cells because this dilution could lead to an underestimate of the HER2 mRNA levels of the tumor. This phenomenon requires mandatory histologic examination of all samples before RNA extraction. In selected cases, the problem could be avoided by enriching the sample with the target cells of interest using, eg, laser-assisted microdissection.\textsuperscript{13} This technique has been improved significantly, and it can be envisioned that a microdissection laboratory could easily provide the appropriate specimens for Q-RT-PCR analysis. In our series, we used only specimens in which tumor cells constituted at least 75\% of the sample, and, when the cutoff was not reached, the blocks were dissected with a safety blade to achieve the desired percentage of tumor cells. This very simple selection has been successfully used by others.\textsuperscript{11,34} These solutions cannot be easily applied to microinfiltrating cancers present in the context of prevalent in situ ductal carcinoma: FISH and immunohistochemical analysis remain the tests of choice for these relatively rare cases.

The last important criticism of Q-RT-PCR testing relates to the identification of chromosome 17 polysomy: dual-color FISH testing can easily discriminate this event from HER2 gene amplification. Chromosome 17 polysomy has been reported in cases with borderline or low HER2 protein concentrations in the absence of gene amplification.\textsuperscript{35,36} Moreover, Wang et al\textsuperscript{37} showed that chromosome 17 polysomy alone may not significantly contribute to HER2 gene copy number and HER2 protein overexpression. The most
important clinical question, however, is whether patients with chromosome 17 polysomy/FISH–immunohistochemical analysis positive profiles could benefit from trastuzumab therapy. Q-RT-PCR could provide additional support to the transcriptional activity of the gene and identify patients who might benefit from therapy. There are not enough data to draw a definitive conclusion on this issue.

One of the advantages of Q-RT-PCR could relate to the HER2 nature of quantitative continuous variables, which, once correlated with the clinical data and response to therapy, may provide additional clues on the best ways to treat patients.

Finally, our cost analysis showed that Q-RT-PCR is highly cost-effective. Even the use of TMA for FISH and immunohistochemical analysis, which markedly reduces the cost of reagents, does not eliminate the cost advantage of Q-RT-PCR technology. It is noteworthy that immunohistochemical analysis and Q-RT-PCR cost 157.27 euros for each case vs 525.68 euros of immunohistochemical analysis combined with FISH. This financial consideration would favor the use of Q-RT-PCR instead of FISH for HER2 evaluation. But it is conceivable that, given the cost advantages of Q-RT-PCR, a standardized test could be introduced to the market with the appropriate supervisory authority approval, and, thus, Q-RT-PCR could become the test of choice to evaluate HER2 status in breast cancer. To achieve this result, however, it will be necessary to validate Q-RT-PCR by multicenter trial, with the inclusion of several testing laboratories and the analysis of responses to inhibitory therapy.

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