Abstract. The option to treat patients presenting with HER-2 overexpressed invasive breast carcinoma with Herceptin® requires quantitative determination of the HER-2 status. The aim of this study was to retrospectively evaluate the HER-2 mRNA expression levels using quantitative real-time RT-PCR (Q-RT-PCR) in tissue samples from 44 primary breast carcinomas and compare the results with immunohistochemistry (IHC). To determine the cut-off for altered mRNA expression, a normalized HER-2 expression value was determined for 20 normal breast tissue RNAs. Gene expression was categorized into three groups: normal expression (mRNAs<3); moderate overexpression (3≤mRNAs<10) and strong overexpression (mRNAs≤10). More than 38% (17/44) displayed strong overexpression, 25% (11/44) moderate and 36.3% (16/44) normal expression. Compared to IHC, only 7/44 cases were slightly discordant: strong mRNA overexpression/2+ protein staining (1 case), moderate overexpression/ 1+ (3 cases) and moderate overexpression/3+ (3 cases). These results show a high concordance rate (84%) between Q-RT-PCR and IHC (p<10^-4). We conclude that Q-RT-PCR is a useful complementary method for determination of the HER-2 status.

The HER-2 proto-oncogene, located on chromosome 17, encodes a membrane tyrosine kinase receptor often referred to as erb-B2, HER-2 or neu/p185, with structural homology to the human epidermal growth factor receptor (EGF-R). HER-2 plays a role in normal growth and development in a variety of embryonic epithelial tissues (1) and in the regulation of normal adult breast growth and development (2). HER-2 protein is overexpressed in 25 to 30% of breast carcinomas, usually as a result of gene amplification; this alteration is a major event in human breast carcinoma tumorigenesis and has been associated in many studies, but not all, with an adverse prognosis (3).

A positive HER-2 status may predict the likelihood of resistance to some conventional therapies, such as hormonal therapy, or chemotherapy with cyclophosphamide, methotrexate and 5-FU and probably the sensitivity to anthracycline dose intensification (4, 5). In addition to these prognostic and predictive values, the HER-2 receptor is a target for specific therapies, such as anti-HER-2 antibody, or gene therapy such as HER-2 antisense oligonucleotides (6). One example of this approach is the targeting of the HER-2 receptor on the surface of overexpressing tumor cells, using monoclonal antibodies such as trastuzumab (Herceptin®, ROCHE SA), a recombinant humanized anti-HER-2 monoclonal antibody that gave enhanced survival benefit in patients with metastatic breast cancer (7-9).

Consequently, these therapeutic advances require an accurate, sensitive and rapid method, available for routine diagnosis used to screen breast carcinoma patients for gene amplification/overexpression. In addition, the recent extension of the indication of trastuzumab therapy in breast carcinomas highlights the importance of quantitative tools to determine HER-2 status (10). Such a method should be simple, widely applicable and reproducible. Currently, no single assay is globally accepted as the gold standard for HER-2 testing. Two principal 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 immunohistochemistry (IHC), a method for the detection of gene expression at the protein level (11-14). Although there may be an interobserver variability in the interpretation of HER-2 IHC in difficult cases, there are
firm guidelines to assess an objective score for the HER-2 protein. Different levels of amplification and percentages of amplified tumors have been published, depending especially on the methodology used (15). FISH technology needs fluorescence microscopy, which is not performed in most routine diagnostic laboratories (16). Chromogenic in situ hybridization (CISH), an in situ hybridization method which enables detection of HER-2 gene copy with conventional peroxidase reaction, was recently developed as an alternative to FISH assays (17-19). However, neither FISH nor CISH assess gene expression, nor identify cases (approximately 3-10% of cases) in which the gene product is overexpressed in the absence of gene amplification (20-26). Such cases could benefit from Herceptin®, if detected by gene expression-based methods, and several studies have demonstrated a good correlation between mRNA concentrations yielded by Northern blotting or RT-PCR and protein overexpression assessed by Western blot, enzyme immunoassay (EIA) or IHC (26, 27).

In this study, 44 specimens of invasive breast carcinoma were examined using real-time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) to measure HER-2 overexpression at the mRNA level. Q-RT-PCR is a recently developed fluorescent technology based on the 5′-3′ exonuclease activity of the Taq polymerase used in combination with a fluorescent hybridization probe indicating PCR product accumulation. It has a high-throughput capacity because no post-PCR manipulations are required, contrary to the other quantitative PCR methods. In addition, it offers the advantage of a precise and reproducible RNA quantification with a large linear dynamic range (at least 4 orders of magnitude), meaning that samples do not need to contain equal starting amounts of RNAs. This technique is, therefore, suitable for analyzing small early-stage tumors or cytopunctured specimens.

Patients and Methods

Tissue samples from primary breast tumors excised from 44 women at the René Huguenin Center, France, were analyzed. The patients met the following criteria: mean age 61.7 years (range 39-83); primary unilateral non metastatic breast ductal carcinoma; no radiotherapy or chemotherapy before surgery; and full follow-up at the René Huguenin Center. The main prognostic factors are presented in Table I. In each case, both real-time Q-RT-PCR and IHC were performed independently. Two adjacent tumor samples were removed immediately following surgery: one was stored in liquid nitrogen until frozen samples by using the acid-phenol guanidinium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under ultraviolet light.

The theoretical and practical aspects of real-time Q-RT-PCR using TaqMan methodology and the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, USA) have been described in detail elsewhere (20). The efficiency (E) of PCR reaction, calculated as E = 101/∆m – 1, where m is the slope of the calibration curves, was higher than 90% for the RT-PCR-based ERBB2 and TBP mRNA assays. HER-2 gene expression was expressed relative to an endogenous RNA control (TBP gene coding for the TATA box-binding protein) and a calibrator (total RNAs from a normal human breast tissue sample). Experiments were performed with duplicates for each data-point. All samples with a CV of threshold cycle (Ct) value >1% were retested.

To determine the cut-off for altered HER-2 gene expression at the RNA level in breast cancer tissue, a normalized HER-2 expression value was determined for 20 normal breast tissue RNAs. As this value consistently fell between 0.5 and 1.7 [mean (0.95±standard deviation (0.37)], values of 3 (mean±5 SD) or more were considered to represent overexpression of the HER-2 gene in tumor RNA samples. Gene expression at the mRNA level was categorized into three groups: group 1, normal expression (mRNAs<3); group 2, moderate overexpression (3≤mRNAs<10); group 3, strong overexpression (mRNAs≥10).

Immunohistochemistry. HER-2 protein expression was detected in 44 fixed (30 with Bouin reagent and 14 with formalin), paraffin-embedded breast carcinomas with a rabbit polyclonal antibody (anti-human HER-2 oncoprotein, A0485, Dakocytomation, Trappes, France) and with a mouse monoclonal antibody (clone CB11, Novocastra, Antony, France). IHC expression was

<table>
<thead>
<tr>
<th>Feature</th>
<th>Number of patients (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>≤50 years old</td>
<td>5 (11.4)</td>
</tr>
<tr>
<td>&gt;50 years old</td>
<td>39 (88.6)</td>
</tr>
<tr>
<td><strong>Macrosopic tumor size</strong></td>
<td></td>
</tr>
<tr>
<td>≤30 mm</td>
<td>31 (70.4)</td>
</tr>
<tr>
<td>&gt;30 mm</td>
<td>13 (29.5)</td>
</tr>
<tr>
<td><strong>Histological grade (SBR)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (4.65)</td>
</tr>
<tr>
<td>2</td>
<td>12 (27.9)</td>
</tr>
<tr>
<td>3</td>
<td>29* (62.8)</td>
</tr>
<tr>
<td><strong>Estrogen receptor status</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>22 (50)</td>
</tr>
<tr>
<td>Negative</td>
<td>22 (50)</td>
</tr>
<tr>
<td><strong>Lymph node status</strong></td>
<td></td>
</tr>
<tr>
<td>Node-negative</td>
<td>14 (31.8)</td>
</tr>
<tr>
<td>Node-positive</td>
<td>30 (68.2)</td>
</tr>
</tbody>
</table>

*One case corresponded to a mucinous carcinoma and was not graded.

Quantitative real-time RT-PCR. Total RNA was extracted from frozen samples by using the acid-phenol guanidinium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under ultraviolet light.

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categorized into three groups using the Dako scoring system available at their Web site (http://www.dakocytomation.com): negative (0), weakly positive (1+), and strongly positive (2+); negative (score 0): no staining at all or membrane staining in less than 10% of the tumor cells; negative (score 1+): faint/barely perceptible partial membrane staining in more than 10% of the tumor cells; weakly positive (score 2+): weak to moderate staining of the entire membrane in more than 10% of the tumor cells; and strongly positive (score 3+): strong staining of the entire membrane in more than 10% of the tumor cells.

Results

Among the 44 consecutive tumor tissues assayed by Q-RT-PCR, 17 (38.6%) displayed strong HER-2 mRNA overexpression (group 3), 11 (25%) displayed moderate overexpression (group 2) and 16 (36.3%) displayed normal expression (group 1). HER-2 overexpression by IHC was similar with the two antibodies used, and then only the DAKO’s antibody was considered for analysis. Analysis of HER-2 overexpression by IHC of the 17 strong HER-2 mRNA overexpression samples showed strong staining (3+) in 16 cases, and the 16 normal expression mRNA samples displayed no protein staining (scored 0+ in 13 cases and 1+ in 3 cases). It is noteworthy that strong staining (3+) by IHC corresponded to mRNA expression levels ≥10-fold higher than that of normal breast tissues by real-time Q-RT-PCR.

In 7 cases, the findings were moderately discordant when IHC and Q-RT-PCR results were compared (16%, 95% CI 7-30). They included strong HER-2 mRNA overexpression/2+ protein staining (1 case), moderate mRNA overexpression/1+ protein staining (3 cases) and moderate mRNA overexpression/3+ protein staining (3 cases). There was no marked discordance between mRNA expression and protein staining.

Finally, 37 concordant cases were found in all the 44 cases investigated: strong HER-2 mRNA overexpression/3+ protein staining (16 cases); moderate mRNA overexpression/2+ protein staining (5 cases); and no mRNA expression/0, 1+ protein staining (16 cases). These results (Table II) indicate a high concordance rate (84%, 95% CI 70-93) between IHC and real-time Q-RT-PCR results (Fisher exact test \( p < 10^{-4} \)), and give the same therapeutic decision (91%, 95% CI 78 – 97).

Discussion

The main finding of this study is the validation of a new accurate, sensitive and rapid method, available for routine practice, to test HER-2 status in breast carcinomas. To date, there is only one report dealing with this technology in the assessment of HER-2 mRNA levels in invasive breast carcinomas (28). In their article, Mrhalova et al. found a fairly good correlation between mRNA and protein levels, especially for the high level protein overexpression, as well as normal protein expression as in other studies which compared mRNA to protein expression. Interestingly, in our study, we confirmed and extended their results by showing more concordant cases (only 16% moderately discordant cases, 7/44; no marked discordance). Discordant cases included: strong HER-2 mRNA overexpression/2+ protein staining (1 case), moderate mRNA overexpression/1+ protein staining (3 cases) and moderate mRNA overexpression/3+ protein staining (3 cases).

Several factors can explain these results. The high sensitivity of the current Q-RT-PCR assay allows for the detection of different levels of HER-2 expression in non-overexpressing samples by IHC. Furthermore, like other RT-PCR-based methods, it cannot avoid dilution artifacts inherent in the extraction of RNAs from tumor cells contained in heterogeneous tissue specimens (lymphoid cells, stroma cells, normal epithelial cells and intra-ductal carcinoma). To obtain a pure tumor cell population devoid of any contaminating cells, Lehmann et al. used laser-assisted microdissection under microscopic control, in conjunction with quantitative real-time PCR (29). In this way, they extracted only invasive carcinoma cells without contaminating bystander cells, such as stroma cells or intra-ductal carcinoma cells, and then increased the

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Table II. Comparison between RT-PCR and IHC in the detection of HER-2 expression in 44 breast carcinomas.

<table>
<thead>
<tr>
<th>IHC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.5</td>
</tr>
<tr>
<td>range</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>Group 1 (mRNAs&lt;3)</td>
<td>13</td>
</tr>
<tr>
<td>Group 2 (3≤mRNAs&lt;10)</td>
<td>0</td>
</tr>
<tr>
<td>Group 3 (mRNAs≥10)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
</tr>
<tr>
<td>0+</td>
<td>6</td>
</tr>
<tr>
<td>1+</td>
<td>6</td>
</tr>
<tr>
<td>2+</td>
<td>19</td>
</tr>
<tr>
<td>3+</td>
<td>19</td>
</tr>
<tr>
<td>mean</td>
<td>1.75</td>
</tr>
<tr>
<td>range</td>
<td>0.4-6.3</td>
</tr>
<tr>
<td>0.5-6.3</td>
<td>3.8</td>
</tr>
<tr>
<td>5.2-14</td>
<td>7.1-130</td>
</tr>
<tr>
<td>0.4-130</td>
<td>13.6</td>
</tr>
</tbody>
</table>
sensitivity of this method in cases with low levels of amplification. At least, a partial degradation of RNA could happen in some samples, and thus the results are underestimated by RT-PCR. It is important to note that among the cases scored 2+ in IHC (6 cases), one case showed mRNAs strong overexpression and could perhaps benefit from Herceptin®.

In contrast to Northern blotting, the current Q-RT-PCR requires small amounts of tissues and it is much less time-consuming and, therefore, could be suitable for routine diagnosis to complete IHC results. This method has several advantages over other RT-PCR-based quantitative assays. It is simple and rapid, suitable for large series, especially in the analysis of cytopunctured specimens for small early-stage tumor. The most important advantage is that it renders RNA quantification much more precise and reproducible, since it is based on Ct values rather than endpoint measurement of the amount of accumulated PCR products. In contrast, with the results obtained by FISH, CISH or by IHC, the current real-time Q-RT-PCR provides a continuum of levels of HER-2 expression (IHC, CISH or FISH have arbitrary thresholds of detection and the results are expressed as a binary scale: amplified/overexpressed versus non-amplified/non-overexpressed).

In conclusion, we have validated a complementary method to quantify mRNA HER-2 expression in breast carcinoma. IHC is still the most widely used method at present. It is rapid, requires fewer reagents and steps performed compared with FISH, CISH, PCR and RT-PCR (15, 17, 19, 30-33), especially for high level overexpression as well as for normal HER-2 status of breast carcinoma, when performed with the high control quality technique. However, borderline results (2+) should be interpreted with caution and a combination of several techniques, including Q-RT-PCR, should be utilized to improve the determination of the HER-2 status.

References


