Correction for chromosome-17 is critical for the determination of true Her-2/neu gene amplification status in breast cancer

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Abstract

Purpose: Trastuzumab is the cornerstone for treatment of women with HER2-overexpressing breast cancer, both in the adjuvant and in the metastatic settings. The accurate assessment of HER2 is, therefore, critical to identifying patients who may benefit from trastuzumab-based therapy. This project aimed to determine the optimal scoring method for fluorescence in situ hybridization (FISH) assay. Methods: FISH assay was done on 893 samples of breast cancer. Three scoring methods were evaluated: Her2/CEP17≥2, Her2>4, or Her2>6. Protein and gene expression were evaluated by immunohistochemistry (n = 584) and mRNA/assay/nucleic acid sequence–based amplification (NASBA; n = 90). Results: Samples were divided into five groups based on FISH results: disomic amplified and nonamplified, polysomic amplified, non-amplified, and discordant (10.8% of cases, mostly positive with Her2>4 scoring, but negative with the others). Her2/CEP17≥2 and Her2>6 scoring methods showed the best association (a) with regard to FISH scoring (κ = 0.906, P < 10⁻⁶) and (b) between FISH and immunohistochemistry (3+ as positive; κ > 0.650, P < 10⁻⁶) or NASBA (κ > 0.536, P < 10⁻⁶). Polysomy had an effect on Her2 copy number (P < 10⁻⁶), but had no effect on protein and mRNA content. Therefore, within the discordant subgroup, for which additive Her-2 gene copies are due to high polysomy, protein and mRNA levels were similar to those of the nonamplified samples. For this subgroup, the best concordance between FISH/immunohistochemistry/NASBA was observed with the Her2/CEP17 ratio and Her-2>6 scoring (68% and 58% perfect matches, respectively). No perfect matches were observed using the Her2>4 scoring method. Conclusion: Correction for chromosome-17 is the method of choice for clinical practice; Her-2>6, but not Her-2>4, could be used as an alternative. [Mol Cancer Ther 2006;5(10):2572–9]

Introduction

The Her-2/neu oncogene, located on chromosome-17, encodes a transmembrane-tyrosine kinase receptor protein belonging to the epidermal growth factor receptor family. Protein overexpression, identified in 20% to 30% of human breast cancer tumors, is due to gene amplification in >90% of cases. Overexpression and amplification have been linked to poor prognosis and response to therapy with the anti–HER-2-humanized monoclonal antibody, trastuzumab (Herceptin), in patients with advanced metastatic breast cancer, when used either as single agent (1, 2) or in combination with chemotherapy (3). Recently, trastuzumab used in sequence or in combination with adjuvant chemotherapy, showed an impressive improvement in disease-free survival (4–7) and overall survival (5) in women with HER-2-overexpressing early breast cancer enrolled in five randomized clinical trials. Therefore, laboratory assessment of Her-2/neu status has become a basic procedure for the appropriate management of patients with breast cancer (8).

Determining the best method to identify patients who should be treated by trastuzumab-based therapy has been a source of controversy. Indeed, immunohistochemistry, which relies on several antibodies (monoclonal and polyclonal) and detection systems, produces contradictory results. HercepTest (Dako), a more standardized immunohistochemical method, was developed to overcome or decrease this variability, although with sometimes disappointing results (9). These concerns regarding the accuracy of immunohistochemistry using standard formalin-fixed paraffin-embedded tissue sections (10) have stimulated the use of fluorescence in situ hybridization (FISH) assay. Moreover, several studies have shown that FISH is a better predictor of prognosis and is a better determinant of trastuzumab eligibility compared with immunohistochemistry (2, 11, 12), as it excludes the majority of 2+ cases, which seem to have a low probability of response to trastuzumab treatment (1, 3). As a result,
most laboratories either screen all cases by immunohistochemistry and triage-selected cases for FISH testing, or use FISH as the primary method for HER-2/neu testing. Therefore, the accurate assessment of Her-2/neu status based on gene amplification using FISH has become a critical factor in the management of patients with breast cancer.

Currently, there are two Food and Drug Administration–approved FISH assays with different definitions of Her-2/neu gene amplification: (a) the Ventana Inform test, which measures only Her-2/neu gene copies, and (b) the Vysis PathVysion test, which includes a chromosome-17 probe in a dual-color format. Tumors with an absolute Her-2 copy number of >4.0 per nucleus (Ventana), or with a ratio of ≥2.0 Her-2 gene copies to that of CEP17 (Vysis), are considered as amplified.

Several genomic situations can be observed (Fig. 1). For disomic samples, the situation is usually easy; samples will be amplified or nonamplified, whatever the FISH scoring method used. The situation is more complex for polysomic samples (chromosome-17 copy number alteration) with >4 Her-2 gene copies because in some cases, a FISH status discrepancy is observed depending on the scoring method. On the one hand, considering only the absolute number of Her-2 gene copies to define amplification might overestimate the presence of gene amplification because 15% of cases with >4 Her-2 gene copies present a Her-2/CEP17<2 ratio because of polysomy (13); on the other hand, the proportion of samples identified as Her-2/neu-amplified by the Her-2/CEP17 ratio might be underestimated because a small proportion of cases (≤3%) show increased numbers of Her-2 gene copies secondary to polysomy-17. It is conceivable that protein overexpression can result not only from an increased number of gene copies secondary to gene amplification, but also from concomitantly increased numbers of chromosome-17, with the consequence that patients whose tumors show these characteristics might also be potential candidates for trastuzumab treatment (13).

Today, there is no definite consensus about the optimal scoring system for assessing Her-2 gene status. Some investigators (2, 11, 12, 14–16) have found correlation for chromosome-17 to be critical for the determination of true gene amplification status as opposed to increased Her-2 gene copy number due to polysomy-17, whereas others (17, 18) believe that it is not required. Therefore, it is essential to establish a cutoff value or scoring system for Her-2 FISH amplification that takes into account this subset of patients with polysomy-17.

The main objective of our study was to determine the optimal FISH scoring method to detect Her-2/neu amplification in breast cancer tumors, with a special focus on tumors with polysomy-17. We examined how the use of dual-color versus single-color FISH scoring might affect the assignment of tumors to an amplified or nonamplified status and correlated these results with immunohistochemistry data using the CB11 antibody and real-time nucleic acid sequence–based amplification (NASBA) analysis for Her-2 mRNA.

**Materials and Methods**

**Patient Samples**

The breast cancer samples used were sent to the Pathology Department of the Bordet Institute for routine clinical FISH assessment (13). For 893 paraffin-embedded samples, FISH assay was done using the PathVysion assay (Vysis; ref. 13). For 584 of 893 samples, HER-2 expression was also evaluated by immunohistochemistry (CB11, Novocasta; ref. 13). For the NASBA assay, which was done in collaboration with BioMérieux (Marcy l’Etoile, France), frozen samples containing >70% of invasive tumor cells were collected from 90 of 893 patients. This study was approved by the ethics committee of the Bordet Institute.

**Criteria for FISH Positivity and Polysomy**

Samples with a Her-2/CEP17 ratio of ≥2.0 or with an absolute Her-2 copy number per nucleus of >4.0 or >6.0 were considered as amplified. This last scoring system was used for the chromogenic in situ hybridization assay. As described previously (12), the cutoff points for chromosome-17 copy number alterations were estimated for each group according to published standards (14, 19–23) adapted for truncated nuclei on paraffin sections. Specimens with chromosome-17 copies in the range of 1.5 to 2.25 signals/cell were defined as having disomy-17. The other cases were considered to have aneusomy-17: either hypodisomy-17 (<1.5 signal/cell) or polysomy (>2.25/cell).

**Duplex Real-time NASBA**

Total RNA was extracted from five 10-μm frozen sections/patient case using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Canada). RNA integrity and quantification were determined with the RNA 6000 Nano LabChip (Agilent, Germany).

Quantitative measurement of Her-2 mRNA was done with 50 ng of total RNA per duplex-NASBA reaction using cyclophilin B (PPIB) as the normalizing gene, as described previously (24). Forward and reverse primers for Her-2/neu and PPIB genes are: ERBB2-P1(T7) 5’aattctaatcactatacgagagagagGAGCCAGCGCAAGT-7’tagcactataaggagaagGAGCCAGCGCAAGTCTCTGTA3’, and 5’ cacccctactatacgagagagagCTGAGTCGAAATTGCCTGTCGAGCTGTTGCAGCTA3’.
Table 1. Association between FISH scoring systems

<table>
<thead>
<tr>
<th>Her-2 copy &gt;4</th>
<th>Her-2 copy &gt;6</th>
<th>Total no. of cases</th>
<th>Her-2 copy &gt;4</th>
<th>Ratio Her-2 / CEP17</th>
<th>Total no. of cases</th>
<th>Her-2 copy &gt;6</th>
<th>Ratio Her-2 / CEP17</th>
<th>Total no. of cases</th>
</tr>
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<td>13</td>
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<tr>
<td>Total no. of cases</td>
<td>83</td>
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<td>96</td>
<td>67</td>
<td>29</td>
<td>96</td>
<td>Negative</td>
<td>67</td>
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</tbody>
</table>

ERBB2-P25TCTTAGACCATGTCGGGAAAG3′, PPIB-P25(A3′) 5′aattctaaattctattagagaggAGGCTGTCTTGACTGACGACCCCTGTCTTGACTGAGCTG-A3′, PPIB-P25(A3′) 5′AGAGAGAAAGGATTGCGC3′ The 3′ antisense primers were elongated with a T7-promotor recognition sequence indicated in lowercase characters. Molecular beacons are FAM-cgatcgGGAGATGGTGCAGGGATGTGAGGAGAGAAAGGATTTGGCT3′ and ROX-cgatcgGATCCAGGGCAGACTCGcagatcg-Dabsyl for the Her-2/neu gene and PPIB gene. The stem sequences of the beacons are indicated in lowercase italic characters. GenBank accession numbers, MN_00448 for ERBB2 and M60857 for PPIB.

Statistical Analysis

The present work focused on determining the optimal cutoff to define Her-2/neu amplification and on its association with HER-2 expression at the transcriptional and translational levels. Moreover, the association between the various subgroups, defined on the basis of the FISH results and some clinicopathologic data were also evaluated. Standard descriptive analyses were conducted. The association between variables was assessed using the concordance coefficient \( \kappa \) (for binary variables), Mann-Whitney (for binary versus continuous variables) and \( \chi^2 \) tests. \( P < 0.05 \) was considered statistically significant. Statistical analyses were done using SPSS 11.5 (SPSS Inc., Chicago, IL).

Results

Polysomy-17 Is Responsible for Discordance in Her-2/neu Status as Defined by Different FISH Scoring Systems

The CEP17 and Her-2/neu gene copies, evaluated by FISH, were available for 893 samples (ref. 13; see Supplemental Data).4 For 96 of 893 cases (10.75%), Her-2/neu status was considered to be discordant according to the following criteria: (a) the Her-2/CEP17 ratio, (b) the Her-2 copy >4 scoring, and (c) the Her-2 copy >6 scoring (chromogenic in situ hybridization criteria).

As reported in Table 1, 78% (75 of 96) of the discordance was observed between Her-2>4 and the two other criteria, and 44% (42 of 96) between Her-2>6 scoring and the Her-2/CEP17 ratio. This explains why in the overall population, the best association between FISH scoring methods was observed between ratio Her-2/CEP17 and Her-2>6 scoring systems (\( \kappa = 0.906, P < 10^{-8} \)) compared with the association between Her-2>4 and the two other systems (\( \kappa = 0.832 \) with ratio Her-2/CEP17 and 0.833 with Her-2>6; \( P < 10^{-8} \)).

For most of the cases, Her-2/neu status discrepancy was due to aneusomy-17: 71 of 96 (74%) presented polysomy, and 4 of 96 (4%) hypodisomy. As hypodisomic cases were <10% (80 of 893) of the total population, and the reported discordant hypodisomic cases represented <0.5% (4 of 893), hypodisomic samples were not considered for the subsequent analyses.

According to CEP17 and Her-2/neu copy number, samples were divided into five major groups: amplified polysomic, amplified disomic, nonamplified polysomic, nonamplified disomic groups, and discordant polysomic. The comparison of these groups revealed that polysomy-17 had an effect on Her-2 copy number, as gene copy number was statistically higher in polysomic versus disomic groups (\( P < 10^{-6} \)). Additionally, a statistically higher CEP17 copy number was observed in polysomic amplified versus polysomic nonamplified samples (\( P = 0.005 \)). In the discordant polysomic subgroup, which presented the highest CEP17 copy number (all \( P < 3 \times 10^{-5} \)), polysomy by itself accounted for a statistically higher Her-2/neu gene copy number when compared with the nonamplified polysomic subgroup (\( P < 10^{-6} \); Table 2).

Association Between Immunohistochemistry and the Three FISH Scoring Methods

In order to determine the optimal scoring methods to evaluate Her-2/neu amplification status, we assessed the association between FISH status, according to the three scoring systems, and immunohistochemistry data, which was available for 584 cases. This assessment is particularly relevant, as the receptor is the target of trastuzumab.

No statistically significant difference could be observed between amplified polysomic and disomic subgroups or between nonamplified polysomic and disomic subgroups in terms of HER-2 protein expression (0, 1+, 2+ versus 3+; Table 2). Importantly, there was no statistically significant difference observed between polysomic nonamplified and discordant samples (\( P = 0.21 \)). Therefore, additional Her-2 gene copies induced by polysomy mainly in the discordant cases (compared with the nonamplified polysomic subgroup; \( P < 10^{-6} \)) were not associated with HER-2 overexpression. Nevertheless, in 3 of 161 IHC3+ samples, HER-2 overexpression (as confirmed by Herceptest) was

4 Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org).
due to polysomy-17. It is noteworthy that for these cases, the CEP17 copy number exceeded 10 copies per cell, which resulted in more than 19 Her-2 gene copies/cell as confirmed by the chromogenic in situ hybridization assay (data not shown).

When considering HER-2 IHC3+ as positive, the $\kappa$ association between immunohistochemistry and FISH data dichotomized according to the three scoring systems was 0.689 using Her-2>6, 0.650 using the Her-2/CEP17 ratio and 0.556 using Her-2>4 (all $P < 10^{-6}$). Concordances between immunohistochemistry and FISH data, according to the three scoring systems, are summarized in Table 3 for the discordant polysomic subgroup. With Her-2>4 FISH scoring, 42 samples classified as amplified were not scored as IHC3+. In contrast, with the two other scoring systems, only one to four samples classified as nonamplified were scored as IHC3+. Therefore, concordance between FISH and immunohistochemistry was observed in 10.6% of samples using Her-2>4 scoring, in 87% of samples using Her-2>6 scoring, and in 81% of samples using the Her-2/CEP17 ratio.

**Association Between mRNA Expression and the Three FISH Scoring Methods**

To determine which FISH scoring system conferred the best concordance between Her-2/neu gene amplification and gene expression, Her-2 mRNA content was assessed by mRNA/assay/NASBA for 90 cases from which frozen samples were available. Samples were distributed into four groups: 24 samples considered as disomic amplified, 22 as polysomic amplified, 21 as polysomic nonamplified, and 23 as discordant polysomic (according to FISH criteria). As expected, we observed a good association between mRNA and protein expression, the latter being defined as positive when IHC3+ ($Z = -4.126, P = < 10^{-4}$). By using a cutoff of >0.078 (ratio Her-2/PPIB) for NASBA as previously published (25), the $\kappa$ association between immunohistochemistry and NASBA assay was 0.465 ($P < 10^{-4}$).

In terms of Her-2 mRNA content, we observed no statistically significant difference between amplified polysomic and disomic groups, whereas there was a statistically significant difference between amplified groups and the nonamplified polysomic subgroup (both $P < 4 \times 10^{-5}$). Interestingly, we found no statistical difference between polysomic nonamplified and discordant cases. Therefore, additional Her-2 gene copies induced by polysomy in the discordant cases were not associated with higher gene expression.

The Her-2/CEP17 ratio scoring ($Z = -5.376$) and the Her-2>6 definition ($Z = -5.392$), when compared with the Her-2>4 ($Z = -3.992$) criterion (all $P < 7 \times 10^{-5}$), provided the best association between NASBA assay and FISH. Using a cutoff of >0.078 (ratio Her-2/PPIB) for NASBA, the $\kappa$ associations between FISH and NASBA were 0.564 for Her-2>6, 0.536 for ratio Her-2/CEP17, and 0.325 for Her-2>4 (all $P < 3 \times 10^{-5}$). Concordance between the three FISH scoring methods and NASBA for the subgroup of

Table 2. Her-2 and CEP17 copy number as well as HER-2 protein expression in the five subgroups

<table>
<thead>
<tr>
<th>Biological markers</th>
<th>Subgroups</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Amplified disomic</td>
</tr>
<tr>
<td>Her-2 copy number (median)</td>
<td>14.75</td>
</tr>
<tr>
<td>CEP-17 copy number (median)</td>
<td>1.94</td>
</tr>
<tr>
<td>HER-2 immunohistochemistry</td>
<td>1.85%</td>
</tr>
<tr>
<td>2+</td>
<td>25.8%</td>
</tr>
<tr>
<td>3+</td>
<td>72.35%</td>
</tr>
</tbody>
</table>

Table 3. Association between immunohistochemistry or NASBA and the three FISH scoring methods

<table>
<thead>
<tr>
<th>HER-2 immunohistochemistry</th>
<th>Her-2 copy &gt;4</th>
<th>Total no. of cases</th>
<th>Her-2 copy &gt;6</th>
<th>Total no. of cases</th>
<th>Ratio Her-2/CEP17</th>
<th>Total no. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 1+, 2+</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>42</td>
<td>43</td>
<td>38</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>3+</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total no. of cases</td>
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<td>47</td>
<td>39</td>
<td>8</td>
<td>47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NASBA</th>
<th>Her-2 copy &gt;4</th>
<th>Total no. of cases</th>
<th>Her-2 copy &gt;6</th>
<th>Total no. of cases</th>
<th>Ratio Her-2/CEP17</th>
<th>Total no. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0</td>
<td>19</td>
<td>19</td>
<td>16</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Total no. of cases</td>
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<td>23</td>
<td>19</td>
<td>4</td>
<td>23</td>
</tr>
</tbody>
</table>
discordant breast cancer is summarized in Table 3. With the Her-2>4 FISH scoring, 19 patients classified as amplified by FISH did not overexpress mRNA; this number decreased to three and one patient(s) with the two other scoring systems. In contrast, three and four patients with high mRNA levels (all IHC2+) were classified as nonamplified by FISH using the Her-2>6 and the Her-2/CEP17 ratio criteria. Therefore, 17%, 74%, and 83% of samples were concordant according to NASBA and the Her-2>4, Her-2>6, and Her-2/CEP17 ratio scoring systems, respectively.

Perfect concordance between FISH, immunohistochemistry, and NASBA was observed in 68% of cases with the Her-2/CEP17 ratio criteria, in 58% with the Her-2>6 criteria, and in 0% with the Her-2>4 criteria. Therefore, correction for chromosome-17 seemed to be the optimal scoring system for FISH amplification evaluation. Her-2>6 scoring could be used as an alternative criterion, even if among the four of five samples with conflicting status according to Her-2>6 or ratio Her-2/CEP17 criteria, no protein (IHC<3+) and no mRNA expression (except one) was detected. The Her-2>4 criterion seemed to be the worst scoring system (Fig. 2). We observed that the minimal cutoff for absolute Her-2 copy number must be more than six copies in order to obtain a good association between FISH and NASBA or immunohistochemistry, the association with immunohistochemistry or NASBA being increased by 1.5 with a cutoff of 6 rather than 4.

### Biological Characteristics of the Five Subgroups Defined Previously According to the Three FISH Scoring Methods

For most of the 584 samples for which immunohistochemistry data are presented, other clinicopathologic variables such as Ki-67, estrogen receptor status, as well as tumor size, histologic grade, and nodal status were also available (Table 4). Regarding tumor proliferation, a statistically higher percentage of highly proliferating tumors (Ki-67>25%) were observed in amplified versus nonamplified samples ($P = 0.03$). Nevertheless, when only polysomic cases were considered, the proportion of highly proliferating tumors was statistically higher in the non-amplified polysomic subgroup compared with the non-amplified disomic subgroup ($P = 0.03$), a proportion similar to the amplified polysomic and disomic subgroups. The percentage of ER-positive tumors was not statistically different between polysomic and disomic subgroups, but it was lower in amplified groups compared with the nonamplified groups ($P = 0.004$). Interestingly, discordant cases presented a percentage of ER-positive tumors similar to that of nonamplified groups. No statistically significant difference was observed regarding tumor size, histologic grade, and nodal status.

According to the report of Nielsen et al. (39), which suggested a molecular classification of breast carcinomas based on immunohistochemical markers, our study

![Figure 2](mct.aacrjournals.org)
population was divided into three subgroups: a basal-like subgroup (ER−, Her-2 FISH−) with 67 of 436 (15%) samples, a “HER-2 overexpressing” subgroup (ER+/−, Her-2 FISH+) with 180 of 436 (41%) samples, and a “luminal-like” subgroup (ER+, Her-2 FISH−) with 143 of 384 (33%) samples. Forty-six out of 436 samples (10.5%) could not be classified because of discordant FISH status. Among these 46 samples, the 23 ER-negative samples would be classified as either “basal-like” or HER-2-overexpressing subgroups, two subgroups of poor prognosis. However, the 23 ER-positive samples (23 of 436, 5.3% of the total population) would be classified as either Her-2-overexpressing with Her-2>4 FISH scoring or luminal-like, a subgroup of potential better clinical outcome, with the two other FISH scoring system.

### Discussion

To determine the optimal FISH scoring system to select patients that might benefit from a trastuzumab-based treatment, three criteria for FISH positivity were considered, and the ensuing status was then compared with immunohistochemistry and mRNA/NASBA data, two techniques evaluating HER-2 expression status at a different level (translation and transcription, respectively) and whose results seemed to be significantly associated. Similar to previous studies (26, 27), we found a good concordance between Her-2/CEP17≥2 ratio and Her-2>4 FISH scoring methods, for disomic and low polysomic cases, as well as for cases with increased Her-2 gene copies consecutive to gene amplification. Problematic cases, discrepant for FISH scoring result, are samples with no gene amplification but increased Her-2 gene copies secondary to high polysomy-17 (>3 copies per cell) in agreement with Lal et al. (27). Here, these samples corresponded to 10% of the patients for which trastuzumab would be given based on Her-2>4 criterion, but not on the Her-2/CEP17 ratio criterion.

We clearly observed that polysomy-17 had an effect on Her-2 copy number. On the one hand, a statistically higher Her-2 gene copy number was found in polysomic groups, but on the other hand, the polysomic amplified group presented a higher number of CEP17 copy number compared with the polysomic nonamplified group, as previously reported (20, 28). The challenge was to determine whether additional Her-2 gene copies consecutive to polysomy-17 might be sufficient to induce HER-2 protein overexpression, the target of trastuzumab, with the consequence being that these patients might be candidates for trastuzumab treatment. As previously reported (15, 20, 29, 30), we clearly showed that, in the absence of Her-2 gene amplification, polysomy-17 resulted in a modest increase of Her-2/neu gene copies and, consequently, a rare increase of HER-2 protein and mRNA content, except for a very small number of cases with more than 10 copies of CEP17 per cell. As a consequence, correction for chromosome-17 seems to be critical for the determination of true Her-2 gene status in breast cancer. These results were corroborated by the fact that the best association between FISH and immunohistochemistry, as reported by other studies (27, 29), as well as between FISH and NASBA, seemed to be with Her-2/CEP17 ratio criteria. Wang et al. (29) reported that FISH assay using the Her-2/CEP17 criterion achieved higher concordance with image analyzer–assisted immunohistochemical quantification than did the Her-2>4 criterion. Lal et al. (27) observed that more immunohistochemistry-negative tumors were FISH-positive with the Her-2>4 criterion. Therefore, the Her-2/CEP17 ratio, which considered most of these discordant cases as nonamplified, seemed to be the better scoring system to reflect the real biology of the tumor.

As an alternative to the Her-2/CEP17 ratio, absolute Her-2 gene copy >6 criterion could be used as a good scoring system. A recent report recommended changing the cutoff mean Her-2 signal number to ≥6 for the Ventana-Inform assay to account for the discrepant cases due to polysomy-17 (31). We effectively confirmed that the minimum number of Her-2 gene copies necessary to obtain a good correlation between FISH, immunohistochemistry, and NASBA must be ≥6. This scoring system might also be useful mainly for cases with more than 10 CEP17 copies per cell and IHC3+. This hypothesis must be confirmed on a higher number of samples.

The comparison of the biological characteristics showed that discordant polysomic samples were biologically similar to polysomic nonamplified samples. Indeed, like

<table>
<thead>
<tr>
<th>Biological markers</th>
<th>Amplified disomic</th>
<th>Nonamplified disomic</th>
<th>Amplified polysomic</th>
<th>Nonamplified polysomic</th>
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<tr>
<td>Ki-67 &gt; 25% (%)</td>
<td>34/76 (44.7)</td>
<td>29/106 (27.36)</td>
<td>31/67 (46.27)</td>
<td>24/53 (45.3)</td>
<td>11/33 (33.3)</td>
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<td>ER ≥ 2 (%)</td>
<td>41/76 (53.94)</td>
<td>91/121 (75.2)</td>
<td>46/76 (60.5)</td>
<td>36/54 (66.6)</td>
<td>28/34 (82.35)</td>
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<td>Size &gt; 2 cm (%)</td>
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<td>21/57 (36.8)</td>
<td>16/43 (37.2)</td>
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<td>Grade 3 (%)</td>
<td>50/81 (61.73)</td>
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<td>43/63 (68.25)</td>
<td>29/53 (54.7)</td>
<td>13/27 (48)</td>
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<td>67/72 (93)</td>
<td>51/57 (89.5)</td>
<td>28/32 (87.5)</td>
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<td>&gt;4 positives nodes (%)</td>
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<td>31/49 (62.26)</td>
<td>23/37 (62.16)</td>
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</tbody>
</table>

### Table 4. Biological characteristics of the five subgroups
others (28, 32), we observed that Her-2 amplification was associated with a lower proportion of estrogen receptors, as opposed to the nonamplified and discordant samples. We also reported that tumoral genomic alterations, either amplification or chromosome-17 polysomy, were associated with proliferation, although, no correlation was observed with the histologic grade, probably due to the high number of cases with unknown status. Our results were in agreement with a recent report from Fridlyand et al. (33), which also showed a correlation between chromosomal level instability as assessed by comparative genomic hybridization and grading. They discriminated three breast tumor subtypes according to their genomewide DNA copy number profiles. At the low end of chromosomal level instability were ER-positive tumors, which were exclusively of moderate or well-differentiated grade and whose patients did not recur. At the other extreme of chromosomal instability were tumors with many low-level copy number aberrations, which were ER negative, of high grade, and associated with significantly worse outcome compared with the other groups. The third group comprised both ER-positive and -negative tumors and was characterized by the presence of low level gains and losses and recurrent amplifications (33).

Recent DNA microarray profiling studies on breast tumors have identified distinct subtypes of breast carcinoma associated with different clinical outcomes (34–37). On the basis of these studies, immunohistochemical classification of breast carcinomas has been proposed (38), wherein Her-2 status evaluation is critical for all subgroup assignments. Consequently, apart from the identification of patients that might benefit from trastuzumab-based treatment, the definition of FISH scoring system is important for the classification of breast carcinomas in a subgroup of poor or good clinical outcome. Among the discordant samples according to FISH criteria, samples identified as HER-2-expressing with Her-2:4 scoring would be classified as basal-like or luminal-like depending on the ER status and according to the two other criteria. Therefore, a sample with a potentially good clinical prognosis (luminal-like) could be classified in a subgroup of poor prognosis with all the therapeutic consequences that it may imply.

Three recently published randomized adjuvant trastuzumab trials have considered both immunohistochemistry and/or FISH to assess HER-2 protein overexpression and/or gene amplification. As the effectiveness of a targeted therapy depends on the identification of potentially responsive patients and to avoid lack of agreement among studies, patients should be registered based on HER-2 expression evaluation in reference laboratories or by FISH. Nevertheless, even with the use of FISH, response rates may differ according to the FISH criteria used. The definition of a unique FISH scoring system is therefore crucial for the comparison of response rate among studies, and correlation between the scoring system and treatment efficacy must be assessed in trastuzumab-treated patients in order to determine which scoring system accounts for the best response rate. Moreover, with the advent of the significantly positive first results of these trials, it has become crucial to limit the number of false-positive cases, as trastuzumab is associated with the risk of severe cardiotoxicity. By limiting this number, the use of the Her-2/CEP17 ratio would effectively identify potentially responsive patients and limit the number of patients exposed to potential toxic effects without any treatment benefit.

To conclude, we showed that the correction for chromosome-17 is the optimal scoring system for FISH amplification evaluation. Alternatively, an absolute Her-2 gene copy number of >6 could be used. Our results suggest that the CEP17 polysomic subset of carcinoma without Her-2/neu gene amplification behaves similarly to nonamplified tumors. Nevertheless, this hypothesis still needs to be validated in trastuzumab-treated patients.

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Correction for chromosome-17 is critical for the determination of true *Her-2/neu* gene amplification status in breast cancer

Lissandra Dal Lago, Virginie Durbecq, Christine Desmedt, et al.

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