Impact of the Updated 2018 American Society of Clinical Oncology/College of American Pathologists Guideline for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

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Context.—The updated American Society of Clinical Oncology/College of American Pathologists guideline for human epidermal growth factor receptor 2 (HER2) testing in breast cancer requires pathologists to re-evaluate HER2 status.

Objective.—To define HER2 status of breast cancer using immunohistochemistry and fluorescence in situ hybridization.

Design.—Diagnostic reports of invasive breast cancers made between 2014 and 2018 with HER2 immunohistochemistry and fluorescence in situ hybridization results were retrieved. HER2 status was re-defined using the updated recommendations.

Results.—Of 2514 tumors, 89.7% (2254 of 2514) suggested for fluorescence in situ hybridization assay were HER2 immunohistochemistry 2+. Approximately 8.9% (225 of 2514) and 1.4% (35 of 2514) of tumors were of immunohistochemistry 0/1+ and 3+, respectively. Based on the average HER2 copy number and HER2:CEP17 ratio, tumors were assigned into 5 groups, including 13.1% (330 of 2514) group 1 tumors, 2.1% (52 of 2514) group 2 tumors, 1.1% (27 of 2514) group 3 tumors, 7.0% (175 of 2514) group 4 tumors, and 76.8% (1930 of 2514) group 5 tumors. In combination with immunohistochemistry, all tumors in group 2 and group 4 changed HER2 status, from positive and equivocal into negative, respectively, while group 3 tumors remained positive. Comparative analyses of clinicopathologic features of tumors in different groups revealed that group 2 and 4 tumors displayed worse clinicopathologic features than those of group 5, while group 3 tumors shared similar clinicopathologic features to those of group 1.

Conclusions.—Following the updated guideline, HER2 status is clearly designated. Significant differences regarding clinical features were observed between tumors in different groups but they share the same HER2 status, suggesting further validation of the accuracy of this diagnostic approach is warranted.

Human epidermal growth factor receptor 2 (HER2) status can be revealed by HER2 gene status tested by in situ hybridization (ISH) assay or HER2 protein expression assessed by immunohistochemistry (IHC). Approximately 15% to 20% of breast cancers are estimated to be HER2 positive. Although HER2 positivity is associated with poor clinical outcomes, with the use of HER2-targeted therapy, patients with HER2-positive breast cancer have derived significant benefits in progression-free and overall survival. To ensure eligible patients for the targeted therapy and thereby avoid potential ineffectiveness, toxicity, and costly expense of HER2-targeted therapy, defining HER2 status of invasive breast cancers accurately has become a crucial mission to pathologists.

To ensure the accuracy of HER2 assessment, the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) first released the guideline recommendation for HER2 testing in the management of invasive breast cancer in 2007. Later in 2013, the determinations of HER2 status tested by both IHC and ISH were emended, and resulted in changes in the proportion of HER2 positivity reported by different studies, increasing from 2.6% to 6.7% or remaining constant in breast cancers, as well as the proportion of HER2 equivocal tumors, increasing 3.2% to 4.9% in many studies.

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Although HER2 IHC equivocal (2+1) cases are commonly reflex tested by ISH, afterward, HER2 fluorescence in situ hybridization (FISH) equivocal cases are still remaining following the 2013 guideline. A rigorous HER2 diagnostic approach for HER2 FISH equivocal cases (group 4), cases with HER2:chromosome enumeration probe 17 (CEP17) ratio 2.0 or more and average HER2 copy number less than 4.0/cell (group 2), and cases with HER2:CEP17 ratio less than 2.0 and average HER2 copy number 6.0/cell or more (group 3) is recommended in the updated 2018 ASCO/CAP guideline for HER2 testing in breast cancer, through a dual-probe ISH testing and concomitant IHC results to determine the most accurate HER2 status designation as positive or negative.2

Here, we aimed to review all the invasive breast cancers subjected to HER2 IHC, as well as HER2 FISH assay, to interpret HER2 status using the updated 2018 ASCO/CAP guideline recommendations for HER2 testing, and compare the updated HER2 status with that defined by the 2013 ASCO/CAP guideline. Furthermore, we compare clinicopathologic features of tumors that share the same HER2 status or the same average HER2 copy number.

METHODS

Patient Cohort

With the approval of the institutional review board and written patient informed consents, pathologic reports of invasive breast cancers diagnosed between January 2014 and August 2018 were retrieved from the archives of the Pathology Department at Fudan University Shanghai Cancer Center, Shanghai, China. Surgical resection specimens of invasive breast cancers with available HER2 status tested by IHC and FISH assay were selected. Biopsy specimens, tumors of consultation cases, and microinvasive carcinoma of the breast were excluded. All tumors were primary tumors. No primary tumors of patients who received neoadjuvant chemotherapy were included, and no recurrent tumors or metastatic tumors of patients who received adjuvant chemotherapy were included. Clinicopathologic features, including patient age, tumor size, tumor grade, histologic type, IHC results of estrogen receptor (ER), progestogen receptor (PR), HER2, Ki-67 expression, and HER2 FISH results, were retrieved.

IHC and FISH

Representative 4-μm thick formalin-fixed, paraffin-embedded tumor sections of all invasive breast cancers diagnosed in the authors’ institution were subjected to IHC with antibodies against ER (SP1, Roche Ventana), PR (IE2, Roche Ventana), HER2 (4B5, Roche Ventana), and Ki-67 (MB1, Roche Ventana). The staining procedure was carried out on a Ventana Benchmark automated immunostainer (Tucson, Arizona) using the standard streptavidin-biotin complex method with 3,3’-diaminobenzidine as the chromogen.10 Positive and negative controls of ER, PR, and HER2 were included in each slide run. The ER and HER2 IHC status were defined according to the ASCO/CAP guidelines.5,11 A cutoff of 20% or more was used to define high Ki-67 expression.12 As previously described,13 the clinicopathologic surrogate definitions of intrinsic molecular subtypes of breast cancer were used based on the results of ER, PR, HER2, and Ki-67 according to the definitions from the 2013 St Gallen International Breast Cancer Conference,12 as follows: luminal A (ER+, PR+, HER2−, and Ki-67 low), HER2− luminal B (ER+, PR−/+, HER2+, and Ki-67 high), HER2+ luminal B (ER+, PR−/−, HER2+, and any Ki-67 expression), HER2− overexpression (ER− and PR−, HER2+, and any Ki-67 expression), triple-negative (ER−, PR−, HER2−, and any Ki-67 expression). The Nottingham grading system was used to grade tumors.14

Afterward, FISH assay for HER2 amplification was suggested for patients with HER2 IHC 2+ results and performed according to patient willingness or clinicians’ suggestions. PathVysion HER2 DNA Probe Kit (Abbott Molecular, Abbott Park, Illinois) was used for HER2 FISH following the manufacturer’s instructions. Scoring was performed by 2 independent pathologists (QMB and XYZ) with expertise in HER2 FISH analysis, who recorded average HER2 copy number and CEP17 signals and determined HER2:CEP17 ratios.

According to HER2 FISH ratio and average HER2 copy number per tumor cell, HER2 FISH results were designated into the 5 following groups: group 1 (HER2:CEP17 ratio ≥ 2.0; average HER2 copy number ≥ 4.0/cell); group 2 (HER2:CEP17 ratio ≥ 2.0; average HER2 copy number < 4.0/cell); group 3 (HER2:CEP17 ratio < 2.0; average HER2 copy number ≥ 6.0/cell); group 4 (HER2:CEP17 ratio < 2.0; 4.0 ≤ average HER2 copy number < 6.0/cell); and group 5 (HER2:CEP17 ratio < 2.0; average HER2 copy number < 4.0/cell).

Per the 2013 ASCO/CAP guideline, groups 1, 2, and 3 were defined as HER2 positive, group 4 was equivocal, and group 5 was negative. Following the updated 2018 guideline,2 groups 1 and 5 remain unchanged, while only group 3 with concomitant HER2 IHC 2+/3+, and groups 2 and 4 with concomitant HER2 IHC 3+ are defined as HER2 positive.

Statistics

Comparisons of the frequencies of hormone receptor (HR, ER, and PR) status, Ki-67 expression, tumor grade, and tumor size between 2 groups were performed using Fisher exact test, and comparisons of the frequencies of molecular subtypes between 2 groups were performed using Pearson χ2 test. Comparisons of average HER2 copy number and CEP17 signals of each tumor in one group to another group were performed using Mann-Whitney U test. Two-tailed P values < .05 were considered statistically significant. Statistical analyses were performed with Stata 10.0 or GraphPad Prism v 7.0a.

RESULTS

Clinicopathologic Features of Study Cohort

A total of 2514 invasive breast cancers that met the selected criteria were included. Among all the patients, 8 were male patients. The median age at diagnosis was 51 years (range, 23–95 years), and the median tumor size was 2 cm (range, 0.3–12 cm). The majority of cases (93.6%, 2552 of 2514) were invasive ductal carcinomas of no special type, and 6.4% (162 of 2514) of tumors were of other histologic subtypes, including invasive lobular carcinoma, invasive micropapillary carcinoma, invasive papillary carcinoma, invasive muciucinous carcinoma, invasive cribriform carcinoma, invasive carcinoma with apocrine differentiation/neuroendocrine features, and mixed-type carcinoma.

All cases of invasive breast carcinoma were tested for ER, PR, HER2, and Ki-67 protein expression by IHC. HR was negative in 15.0% (376 of 2514) of tumors, and was positive in 85.0% (2138 of 2514) of tumors. Ki-67 expression was less than 20% in 30.9% (776 of 2514) of tumors, while 20% or more in 69.1% (1738 of 2514) of tumors. Following the Chinese breast cancer HER2 detection guideline, tumors with HER2 IHC 2+ expression are recommended for further FISH assay. Therefore, the majority of cases tested by FISH (89.7%, 2254 of 2514) were HER2 IHC 2+. Approximately 8.9% (225 of 2514) of HER2 IHC 0/+ tumors and 1.4% (35 of 2514) of HER2 IHC 3+ tumors subjected to HER2 FISH assay were performed upon patient requests or clinician suggestions. The average HER2 copy number of all cases was 3.4/cell (range, 0.9–49.0), and of CEP17 signals was 2.2/cell (range, 0.8–6.4). Expectedly, the average HER2 copy number of HER2 IHC 3+ tumors (median 11.3, range, 5.7–49.0) was significantly higher than those of HER2 2+ tumors (median 2.7, range, 0.9–25; Mann-Whitney U test, P < .001, updated 2018 ASCO/CAP guideline. Therefore, the majority of cases tested by FISH were included, and no recurrent tumors or metastatic tumors of patients who received adjuvant chemotherapy were included. Clinicopathologic features, including patient age, tumor size, tumor grade, histologic type, IHC results of estrogen receptor (ER), progestogen receptor (PR), HER2, Ki-67 expression, and HER2 FISH results, were retrieved.
Figure 1. Distribution of average HER2 copy number and CEP17 of invasive breast cancers with different HER2 IHC levels. A, Scatter plots indicate average HER2 copy number of tumors with HER2 IHC 0/1+, HER2 2+, and HER2 3+. B, Scatter plots indicate average CEP17 signals of tumors with HER2 IHC 0/1+, HER2 2+, and HER2 3+. The median is indicated by the long horizontal line. The first and third quartiles are indicated by top and bottom short horizontal lines, respectively. Abbreviations: CEP17, chromosome enumeration probe 17; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

Table 1. Human Epidermal Growth Factor Receptor 2 (HER2) Status of 2514 Cases of Breast Cancer According to the American Society of Clinical Oncology/College of American Pathologists 2013 and 2018 Guidelines

| Groups     | HER2 FISH Results | HER2 IHC Levels | Case Number (%) | 2013 Guideline | 2018 Guideline |
|------------|-------------------|----------------|-----------------|----------------|----------------|
| Group 1 (n = 330, 13.1%) | HER2:CEP17 ratio ≥ 2.0, average HER2 copy number ≥ 4.0/cell | 0/1+ | 0 (0) | All positive | All positive |
|            |                   | 2+            | 295 (89.4)      |                |                |
|            |                   | 3+            | 35 (10.6)       |                |                |
| Group 2 (n = 52, 2.1%) | HER2:CEP17 ratio ≥ 2.0, average HER2 copy number < 4.0/cell | 0/1+ | 0 (0) | All positive | Negative |
|            |                   | 2+            | 52 (100)        | Negative       | Negative       |
|            |                   | 3+            | 0 (0)           | Positive       | Positive       |
| Group 3 (n = 27, 1.1%) | HER2:CEP17 ratio < 2.0, average HER2 copy number ≥ 6.0/cell | 0/1+ | 0 (0) | All positive | Negative |
|            |                   | 2+            | 27 (100)        | Negative       | Negative       |
|            |                   | 3+            | 0 (0)           | Positive       | Positive       |
| Group 4 (n = 175, 7.0%) | HER2:CEP17 ratio < 2.0, 4.0 ≤ average HER2 copy number < 6.0/cell | 0/1+ | 11 (6.3) | All equivocal | Negative |
|            |                   | 2+            | 164 (93.7)      | Negative       | Negative       |
|            |                   | 3+            | 0 (0)           | Positive       | Positive       |
| Group 5 (n = 1930, 76.8%) | HER2:CEP17 ratio < 2.0, average HER2 copy number < 4.0/cell | 0/1+ | 214 (11.1) | All negative | All negative |
|            |                   | 2+            | 1716 (88.9)     | All negative   | All negative   |
|            |                   | 3+            | 0 (0)           | All negative   | All negative   |

Abbreviations: CEP17, chromosome enumeration probe 17; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.
levels assessed on the same tissue sample used for FISH assay.

**HER2 Status of Tumors in Group 2**

All tumors (n = 52) in group 2 had HER2 IHC 2+ expression. HER2 status of all cases was changed from positive to negative upon the updated 2018 guideline. The average HER2 copy number was 3.3/cell (range, 2.5–3.9), and of CEP17 signals was 1.5/cell (range, 1.1–1.8). Twelve of 52 (23.1%) tumors were HR negative (HR−), and the remaining 40 of 52 (76.9%) tumors were HR positive (HR+). Approximately 84.6% (44 of 52) of tumors displayed Ki-67 20% or more and the remaining 15.4% (8 of 52) of tumors had Ki-67 less than 20%. Due to the change of HER2 status, molecular subtypes of tumors in group 2 were subsequently changed. Approximately 23.1% (12 of 52) of tumors changed from HER2-overexpression into triple-negative subtype, and 63.5% (33 of 52) and 13.5% (7 of 52) of tumors changed from HER2+ luminal B into HER2− luminal B and luminal A subtypes, respectively (Figure 2, A). Histologic grade and tumor size are summarized in Table 2.

**HER2 FISH results and clinicopathologic features of tumors in group 2 and those of tumors in group 5 were compared, considering tumors in both groups had average HER2 copy number less than 4.0. The comparisons were restricted to HER2 IHC 2+ tumors, given that all the tumors in group 2 were HER2 2+. The average HER2 copy number of HER2 2+ tumors in group 5 (n = 1716) was 2.5/cell (range, 0.9–3.9), and of CEP17 signals was 1.5/cell (range, 1.1–1.8). Although tumors in both groups had average HER2 copy number less than 4.0, the average HER2 copy number of group 2 was significantly higher than that of group 5 (2.5/cell versus 2.0/cell; Mann-Whitney U test, P < .001, Figure 2, B). Average CEP17 signals of group 2 were significantly lower than group 5 (1.5/cell versus 2.0/cell; Mann-Whitney U test, P < .001, Figure 2, C) resulting in the different HER2 FISH ratios. Significant associations were observed between average HER2 copy number and Ki-67 expression for tumors in group 2 (Mann-Whitney U test, P < .001) as well as HER2 2+ tumors in group 5 (Mann-Whitney U test, P < .001). Comparisons of the 2 groups in terms of clinicopathologic features revealed that high Ki-67 expression and grade 3 were more frequently observed in group 2 tumors, as compared with those of group 5 tumors (Fisher exact test, P = .002 and P = .001, respectively, Table 2).

No significant differences were observed regarding HR status and tumor size (Table 2). Furthermore, we restricted the comparisons in HR+ tumors between the 2 groups. The frequencies of high Ki-67 expression and grade 3 in HR+ group 2 remained significantly higher than those in HR+ group 5 (Table 2).

**HER2 Status of Tumors in Group 3**

Group 3 had the least number of tumors among the 5 groups (n = 27). All tumors in group 3 were HER2 IHC 2−; therefore, HER2 status of all cases remained as HER2 positive. The average HER2 copy number was 7.1/cell (range, 6.0–9.9), and of CEP17 signals was 4.4/cell (range, 3.3–15.0). Approximately 85.2% (23 of 27) of tumors displayed Ki-67 20% or more, and 14.8% (4 of 27) of tumors had Ki-67 expression less than 20%. The majority of the tumors in this group were HR+ (81.5%, 22 of 27), and were classified as HER2+ luminal B subtype. The remaining tumors were HR−, thus classified as HER2-overexpression subtype (18.5%, 5 of 27). Histologic grade and tumor size are summarized in Table 3.

**HER2 FISH results and clinicopathologic features of tumors in group 3 were next compared with those tumors that also had average HER2 copy number 6.0 or more in group 1, which was named as “classic-amplified” tumors.7 Similarly, the comparisons were restricted to HER2 2+ tumors, given that all the tumors in group 3 were HER2 IHC 2+. The average HER2 copy number of HER2 2+ tumors in group 1 (n = 203) was 9.3/cell (range, 6–35), and of CEP17 signals was 2.5/cell (range, 1.5–5.4). HER2 2+ tumors in group 1 had significantly higher average HER2 copy number (9.3/cell versus 7.1/cell), but lower average CEP17 signals (2.5/cell versus 4.4/cell), when compared with those of tumors in group 3 (Mann-Whitney U test, both P < .001, Figure 3, A and B). No significant differences were observed...
between the 2 groups regarding HR status, Ki-67 expression, tumor grade, or size (Table 3).

**HER2 Status of Tumors in Group 4**

Group 4 tumors were the most common cases among the less common clinical scenarios (n = 175). The majority of these tumors (93.7%, 164 of 175) had HER2 IHC 2+ expression, and only 6.3% (11 of 175) of tumors were HER2 IHC 0/1+ (Table 1); therefore, upon the updated guideline, HER2 status of all tumors in group 4 changed from HER2 equivocal into negative. Of the tumors in group 4, average HER2 copy number was 4.7/cell (range, 4.0–5.8) and average CEP17 signals was 3.3/cell (range, 2.1–6.4). Although the average HER2 copy number and the average CEP17 signals of HER2 IHC 0/1+ tumors (median, 4.4) tended to be lower than that of HER2 IHC 2+ tumors (median, 4.7) the differences were not significant (Mann-Whitney U test, P = .07 and P = .21, respectively, Figure 4, A and B). In this group, 5.7% (10 of 175) of tumors were HR−, while the remaining were HR+ (94.3%, 165 of 175). Approximately 21.1% (37 of 175) of tumors displayed Ki-67 less than 20% and the remaining 78.9% (138 of 175) had Ki-67 20% or more. After reassigning HER2 equivocal as HER2 negative, group 4 consisted of 5.7% (10 of 175) triple-negative, 20.6% (36 of 175) luminal A, and 73.7% (129 of 175) luminal B subtypes (Table 4). Histologic grade and tumor size are summarized in Table 4. By using “5.0” as a cutoff value, tumors in group 4 were divided into “low-equivocal” (4.0 ≤ average HER2 copy number < 5.0, 66.9%, 117 of 175) and “high-equivocal” (5.0 ≤ average HER2 copy number < 6.0, 33.1%, 58 of 175) subgroups. No significant differences were observed regarding clinicopathologic features between “low-equivocal” and “high-equivocal” tumors (Table 5).

**HER2 FISH results and clinicopathologic features of tumors in group 4 were further compared with those of**

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### Table 2. Comparisons of Clinicopathologic Features Between Human Epidermal Growth Factor Receptor 2 (HER2) Immunohistochemistry (IHC) 2+ Tumors in Group 2 and Group 5

| IHC: HER2 2+; FISH: Average HER2 Copy Number < 4.0/Cell | Group 2 (n = 52), HER2:CEP17 Ratio ≥ 2.0 | Group 5 (n = 1716), HER2:CEP17 Ratio < 2.0 | P Value |
|----------------------------------------------------------|------------------------------------------|------------------------------------------|---------|
| HR status                                                |                                          |                                          | .06     |
| Positive                                                 | 40 (76.9%)                               | 1484 (86.5%)                             |         |
| Negative                                                 | 12 (23.1%)                               | 232 (13.5%)                              |         |
| Ki-67 expression                                         |                                          |                                          | .002    |
| <20%                                                     | 8 (15.4%)                                | 611 (35.6%)                              |         |
| ≥20%                                                     | 44 (84.6%)                               | 1105 (64.4%)                             |         |
| Molecular subtypes                                       |                                          |                                          | .006    |
| HER2-overexpression                                      | 0 (0%)                                   | 0 (0%)                                   |         |
| TNBC                                                     | 12 (23.1%)                               | 232 (13.5%)                              |         |
| Luminal A                                                | 7 (13.5%)                                | 565 (32.9%)                              |         |
| HER2− luminal B                                          | 33 (63.5%)                               | 919 (53.6%)                              |         |
| Tumor grade                                              |                                          |                                          | .001    |
| 1/2                                                      | 20 (39.2%)                               | 1004 (62.6%)                             |         |
| 3                                                        | 31 (60.8%)                               | 600 (37.4%)                              |         |
| Unavailable                                              | 1                                        | 112                                      |         |
| Tumor size                                               |                                          |                                          | .77     |
| <2 cm                                                    | 17 (32.7%)                               | 609 (35.9%)                              |         |
| ≥2 cm                                                    | 35 (67.3%)                               | 1088 (64.1%)                             |         |
| Unavailable                                              | 0                                        | 19                                       |         |

**Abbreviations:** CEP17, chromosome enumeration probe 17; FISH, fluorescence in situ hybridization; HR, hormone receptor; TNBC, triple-negative breast cancer.

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tumors that had average HER2 copy number 4.0 or more and less than 6.0 in group 1 (n = 93), which were previously named as “low-amplified” tumors.7 The average HER2 copy number of “low-amplified” tumors in group 1 was 5.0/cell (range, 4.0–5.9), and of CEP17 signals was 1.9/cell (range, 1.2–2.8). Although “low-amplified” tumors in group 1 and tumors in group 4 shared the same range of average HER2 copy number (4.0 ≤ average HER2 copy number < 6.0/cell), “low-amplified” tumors in group 1 had significantly higher average HER2 copy number than that of tumors in group 4 (5.0/cell versus 4.7/cell); the opposite result was observed for average CEP17 signals (1.9/cell versus 3.3/cell; Mann-

| Table 3. Comparisons of Clinicopathological Features Between Human Epidermal Growth Factor Receptor 2 (HER2) Immunohistochemistry (IHC) 2+ Tumors in Group 3 and HER2 2+ “Classic-Amplified” Tumors in Group 1 |
|---------------------------------------------------------------|
| **IHC: HER2 2+; FISH: Average HER2 Copy Number ≥ 6.0/Cell** |
| **Group 3 (n = 27),** | **Group 1 (n = 203),** | **P Value** |
| **HR status** | | |
| Positive (HER2+ luminal B) | 22 (81.5%) | 159 (78.3%) | .81 |
| Negative (HER2-overexpression) | 5 (18.5%) | 44 (21.7%) | |
| Ki-67 expression | | |
| <20% | 4 (14.8%) | 23 (11.3%) | .53 |
| ≥20% | 23 (85.2%) | 180 (88.7%) | |
| Tumor grade | | |
| 1/2 | 11 (44.0%) | 78 (42.2%) | >.99 |
| 3 | 14 (56.0%) | 107 (57.8%) | |
| Unavailable | 2 | 18 | |
| Tumor size | | |
| <2 cm | 5 (19.2%) | 68 (33.7%) | .18 |
| ≥2 cm | 21 (80.8%) | 134 (66.3%) | |
| Unavailable | 1 | 1 | |

Abbreviations: CEP17, chromosome enumeration probe 17; FISH, fluorescence in situ hybridization; HR, hormone receptor.

Figure 3. Comparative analysis of HER2 FISH results between HER2 2+ tumors in group 3 and HER2 2+ “classic-amplified” tumors group 1. A, Scatter plots indicate average HER2 copy number of HER2 2+ tumors in group 3 and HER2 2+ “classic-amplified” tumors group 1. B, Scatter plots indicate average CEP17 signals of tumors in HER2 2+ tumors in group 3 and HER2 2+ “classic-amplified” tumors group 1. The median is indicated by the long horizontal line. The first and third quartiles are indicated by top and bottom short horizontal lines, respectively. Abbreviations: CEP17, chromosome enumeration probe 17; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.
Figure 4. HER2 FISH results of tumors in group 4, and comparative analysis with those of tumors in groups 1 and 5. A, Scatter plots indicate average HER2 copy number of tumors with IHC HER2 0/1+ and HER2 2+. B, Scatter plots indicate average CEP17 signals of tumors with IHC HER2 0/1+ and HER2 2+. C, Scatter plots indicate average HER2 copy number of tumors in group 4 and “low-amplified” tumors in group 1. D, Scatter plots indicate average CEP17 signals of tumors in group 4 and “low-amplified” tumors in group 1. E, Scatter plots indicate average HER2 copy number of tumors in groups 4 and 5. F, Scatter plots indicate average CEP17 signals of tumors in groups 4 and 5. The median is indicated by the long horizontal line. The first and third quartiles are indicated by top and bottom short horizontal lines, respectively. Abbreviations: CEP17, chromosome enumeration probe 17; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.
### Table 4. Comparisons of Clinicopathologic Features Between Tumors in Group 4 and “Low-Amplified” Tumors in Group 1

| Feature                              | Group 4 (n = 175), HER2:CEP17 Ratio < 2.0 | Group 1 (n = 93), HER2:CEP17 Ratio ≥ 2.0 | P Value |
|--------------------------------------|------------------------------------------|------------------------------------------|---------|
| HR status                            |                                          |                                          | .008    |
| Positive                             | 165 (94.3%)                              | 78 (83.9%)                               |         |
| Negative                             | 10 (5.7%)                                | 15 (16.1%)                               |         |
| Ki-67 expression                     |                                          |                                          | .01     |
| <20%                                 | 37 (21.1%)                               | 8 (8.6%)                                 |         |
| ≥20%                                 | 138 (78.9%)                              | 85 (91.4%)                               |         |
| Molecular subtypes                   |                                          |                                          | <.001   |
| HER2-overexpression                  | 0 (0%)                                   | 15 (16.1%)                               |         |
| HER2+ luminal B                      | 0 (0%)                                   | 78 (83.9%)                               |         |
| TNBC                                 | 10 (5.7%)                                | 0 (0%)                                   |         |
| Luminal A                            | 36 (20.6%)                               | 0 (0%)                                   |         |
| HER2- luminal B                      | 129 (73.7%)                              | 0 (0%)                                   |         |
| Tumor grade                          |                                          |                                          | .60     |
| 1/2                                  | 85 (51.8%)                               | 44 (47.8%)                               |         |
| 3                                    | 79 (48.2%)                               | 48 (52.2%)                               |         |
| Tumor size                           |                                          |                                          | .89     |
| <2 cm                                | 49 (28.3%)                               | 25 (27.2%)                               |         |
| ≥2 cm                                | 124 (71.7%)                              | 67 (72.8%)                               |         |

Abbreviations: CEP17, chromosome enumeration probe 17; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; TNBC, triple-negative breast cancer.

### Table 5. Comparisons of Clinicopathologic Features Between “Low-Equivocal” and “High-Equivocal” Tumors in Group 4

| Feature                              | Low-Equivocal (66.9%, 117/175) | High-Equivocal (33.1%, 58/175) | P Value |
|--------------------------------------|---------------------------------|---------------------------------|---------|
| HR status                            |                                 |                                 | .30     |
| Positive                             | 112 (95.7%)                     | 53 (91.4%)                      |         |
| Negative                             | 5 (4.3%)                        | 5 (8.6%)                        |         |
| Ki-67 expression                     |                                 |                                 | .12     |
| <20%                                 | 29 (24.8%)                      | 8 (13.8%)                       |         |
| ≥20%                                 | 88 (75.2%)                      | 50 (86.2%)                      |         |
| Molecular subtypes                   |                                 |                                 | .18     |
| HER2-overexpression                  | 0 (0%)                          | 0                               |         |
| TNBC                                 | 5 (4.3%)                        | 5 (8.6%)                        |         |
| Luminal A                            | 28 (23.9%)                      | 8 (13.8%)                       |         |
| HER2+ luminal B                      | 84 (71.8%)                      | 45 (77.6%)                      |         |
| Tumor grade                          |                                 |                                 | >.99    |
| 1/2                                  | 56 (51.4%)                      | 29 (52.7%)                      |         |
| 3                                    | 53 (48.6%)                      | 26 (47.3%)                      |         |
| Tumor size                           |                                 |                                 | .48     |
| <2 cm                                | 35 (30.2%)                      | 14 (24.6%)                      |         |
| ≥2 cm                                | 81 (69.8%)                      | 43 (75.4%)                      |         |

Abbreviations: HER2, human epidermal growth factor receptor 2; HR, hormone receptor; TNBC, triple-negative breast cancer.
Whitney U test, both \( P < .001 \), Figure 4, C and D). Ballard et al \(^7\) reported “low-amplified” tumors in group 1 shared similar frequency of ER-positivity with group 4 tumors. Inconsistently, comparisons of the 2 groups regarding the clinicopathologic features revealed that HR negativity and high Ki-67 expression were more frequently observed in group 1 “low-amplified” tumors, as compared with those of group 4 tumors (Fisher exact test, \( P = .008 \) and \( P = .01 \), respectively, Table 4). No significant differences were observed between the 2 groups regarding tumor grade and tumor size (Table 4).

Finally, we investigated whether clinicopathologic features of tumors in group 4 would differ from those of all tumors in group 5 (\( n = 1930 \)), considering tumors in both groups were defined as HER2 negative. The average HER2 copy number of tumors in group 5 was 2.5/cell (range, 0.9–3.98), and of CEP17 signals was 2.0/cell (range, 0.8–5.9). As expected, tumors in group 4 had significantly higher average HER2 copy number (4.7/cell versus 2.5/cell), as well as average CEP17 signals (3.3/cell versus 2.0/cell) when compared with those of tumors in group 5 (Mann-Whitney U test, both \( P < .001 \), Figure 4, E and F). Although group 5 had a significantly higher frequency of triple-negative breast cancers than group 4, many clinicopathologic features that are indicative of worse outcome were observed in group 4, including high Ki-67 expression, grade 3 tumor, and tumor with larger size (\( \geq 2 \) cm; Fisher exact test, \( P < .001 \), \( P = .005 \), and \( P = .01 \), respectively, Table 6). The differences remained significant when we restricted the comparisons between HR\(^+\) group 4 (\( n = 165 \)) and HR\(^-\) group 5 (\( n = 1657 \); Fisher exact test, \( P < .001 \), \( P < .001 \), and \( P = .01 \), respectively; Table 6).

### DISCUSSION

Unresolved HER2 status of breast cancers has been a dilemma for pathologists and clinicians ever since the application of HER2 testing. It is crucial to define the
accuracy of HER2 status as the patients with HER2-positive tumors could derive significant benefits from HER2-targeted therapy. In the 2018 ASCO/CAP guideline, a rigorous diagnostic approach was recommended to determine HER2 status of tumors in groups 2, 3, and 4 by combining HER2ISH and IHC results, in order to define a most accurate HER2 status designation as positive or negative. In this study, we analyzed HER2 status of invasive breast cancers based on HER2 IHC and FISH results following the updated 2018 guideline. An increase of 9.0% (227 of 2514) in HER2-negative breast tumors, rectified from 2.1% (52 of 2514) HER2-positive and 7.0% (175 of 2514) HER2 equivocal tumors that were categorized by using the 2013 guideline, are defined. Comparative analyses revealed that HER2-negative tumors displaying the same range of average HER2 copy number could have different clinicopathologic features (group 4 versus group 1 “low amplified”), and the differences also exist when tumors shared the same HER2 status (group 2 versus group 5). Although HER2 status of tumors in group 4 had been considered as negative, significant differences in terms of clinicopathologic features were observed when compared with the HER2 negative tumors in group 5.

Murray et al16 recently applied the updated 2018 guideline to evaluate HER2 status of breast tumors with HER2 IHC equivocal results, and reported a 10.2% (107 of 1044) HER2 positivity in these tumors, relatively lower than the rate of HER2+ tumors among HER2 IHC 2+ tumors in our study (10.2% versus 14.2%, P = .001). A total 10.8% (113 of 1044) increase of HER2-negative tumors, derived from HER2 equivocal tumors in group 4 (3.6%, 38 of 1044) and HER2-positive tumors in group 2 (7.1%, 75 of 1044) as defined in the 2013 ASCO/CAP guideline, was observed. Xu et al15 defined a rather higher frequency of HER2 positivity among the HER2 IHC 2+ tumors (24.2%, 80 of 331), and a 13.3% (44 of 331) increase of HER2-negative tumors, all derived from tumors in group 4 with HER2 equivocal expression, while no tumors in group 2 were observed. Consistently, the 2 studies and ours (9.6%, 217 of 2254) all observed an increase of HER2-negative breast tumors following the updated 2018 guideline when compared with the 2013 guideline, suggesting a non-neglectable proportion of patients may not be eligible for HER2-targeted therapies.

Based on the evidence from The Breast Cancer International Research Group-006 and N9831 trials that patients with HER2 equivocal tumors (group 4) experienced similar overall survivals and disease-free survivals compared with patients with HER2-negative tumors and tumors in group 2 favored no benefit from trastuzumab,16 the Expert Panel of ASCO/CAP determined that tumors with HER2 IHC 2+ or 1+ in groups 2 and 4 should no longer be treated with HER2-targeted therapy, despite the limited sample sizes in these studies. Tumors in groups 2 and 4 with HER2 IHC 3+ are suggested to be considered as HER2+ in the updated 2018 guideline. In fact, no HER2 3+ tumors were observed among 52 (2.1%, 52 of 2514) group 2 tumors and 175 (7.0%, 175 of 2514) group 4 tumors in our study. Similarly, among 4331 breast cancers tested by HER2 FISH and IHC levels, Press et al15 reported that none of the 35 tumors in group 2 were HER2 3+, and only a sole case (0.75%) among 134 tumors in group 4 was HER2 3+. Another study also conducted by Press et al15 revealed that no tumor in group 2 (0%, 0 of 31) had HER2 3+, while 3 tumors (0.9%, 3 of 345) in group 4 had HER2 3+. Based on these observations, HER2 status of tumors in groups 2 and 4 with HER2 3+ should be determined with caution. Both HER2 FISH and IHC results are suggested to be reviewed by an additional pathologist and/or repeat testing if needed. However, there are too few patients in groups 2 and 4 reported in the literature to make a statistical conclusion about whether this population would benefit from HER2-targeted therapy, and about whether their response to HER2-targeted therapy would differ from HER2+ tumors in groups 1 and 3. Further investigation regarding patient survival and tumor response to HER2-targeted therapy of tumors in groups 2 and 4 is warranted to support the current HER2 status designation.

Tumors in groups 2, 4, and 5 were all HER2 negative, whereas clinicopathologic features of tumors in group 2 differed from those in group 5, and features of tumors in group 4 differed from those in group 5; the differences of Ki-67 expression and tumor grade existed regardless of HR tumor status. However, similar outcomes were reported between patients with tumors in groups 2 and 5, and between patients with tumors in groups 4 and 5.16 It is notable that more significant differences in terms of clinicopathologic features were observed between tumors in groups 4 and 5 when comparing tumors in group 4 with “low-amplified” tumors in group 1. Limited data suggest the benefits of HER2-targeted therapy in “low-amplified” tumors in group 1; further investigation about whether “low-amplified” tumors in group 1 with HER2 2+ expression should be defined as HER2+ or whether tumors in group 4 with HER2 2+ expression should be considered as HER2-negative is still warranted.

Consistent with recent studies, tumors in group 3 account for the minority of breast tumors tested by FISH assay.7,15 Previous studies7,18 have showed high frequencies of concurrent HER2 IHC 3+ in tumors of group 3; however, no such case was observed in our study. In addition, one study17 has revealed that the average HER2 copy number is positively correlated with HER2 IHC levels, regardless of its limited case number. Our study did not observe any significant correlation between average HER2 copy number and clinicopathologic features that are indicative of worse outcomes in group 3; however, it has been shown that tumors in group 3 share similar clinicopathologic features with tumors in group 1 regarding tumor grade and Ki-67 expression. Previous studies have suggested that average HER2 copy number 6 or more means HER2 gene amplification, and should be considered as HER2-positive regardless of HER2:CEP17 ratio.17,19,20 Approximately 13% to 77% of HER2 0/1+ tumors were previously reported in group 3 tumors.7,16,19 According to the updated guideline, HER2 status of these tumors should be defined as negative; further evidence is needed to support this suggestion.

The updated 2018 guideline leads to a clear designation of HER2 status of breast tumors, as positive or negative, by considering the concomitant HER2 IHC results. Overall, our study has shown a 9.0% (227 of 2514) increase of HER2 negativity following the updated guideline, originating from the redesignation of 7.0% (175 of 2514) HER2 equivocal tumors from group 4 and 2.1% (52 of 2514) HER2 2+ tumors from group 2. More importantly, the updated guideline eliminates the dilemma for oncologists when deciding on the use of HER2-targeted therapies at large, even though the cases only accounted for 7.0% (175 of 2514) in our study. In addition to updated HER2 status based on the 2018 guideline, our study analyzed the correlation between HER2 FISH results and HER2 IHC levels, and revealed the differences and similarities of clinicopathologic features
between one group and another. By using the combination of IHC and ISH results, HER2 status of the less common clinical scenarios could now be clearly designated, whereas, whether the diagnostic approach of HER2 status meets the practical application still requires further validation.

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