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Research Paper

A decision tree-based prediction model for fluorescence *in situ* hybridization *HER2* gene status in HER2 immunohistochemistry-2+ breast cancers: a 2538-case multicenter study on consecutive surgical specimens

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Abstract

Objective: To investigate the proportion of *HER2* gene amplifications and the association between the HER2-IHC-staining pattern and gene status in IHC-2+ breast cancers according to 2013 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines.

Methods: We retrospectively analyzed and re-evaluated the IHC-staining pattern of 2538 IHC-2+ surgical specimens of breast cancer from November 2014 to October 2015 in 12 institutions. All cases used for building a prediction model of *HER2* gene amplification according to the IHC-staining pattern and were randomly divided into a training set (n = 1914) or validation set (n = 624).

Results: The overall *HER2* fluorescence *in situ* hybridization (FISH) amplification, non-amplification and equivocation rates in HER2 IHC-2+ cases were 17.8%, 76.2% and 6.0%, respectively. In the training set, cases that had ≤ 10% of cells with intense, complete and circumferential membrane staining or had > 85% of cells with complete membrane staining of any staining intensity tended to be *HER2* gene amplified (77.0% and 60.5%, respectively). And cases with weak and incomplete membrane staining had the lowest amplification rate of 6.1%. The prediction model was constructed based on IHC-staining pattern in the training set and validated using a validation set. The positive and negative prediction values were 51.6% and 79.2%, respectively, in the validation set. Moreover, the *HER2* copy number per cell was much higher in cases with

amplification-associated staining patterns (7.84 and 8.75) than in cases with non-amplification-associated staining patterns (2.97 to 4.41, $P < 0.05$).

Conclusions: In HER2 IHC-2+ breast cancers, the staining pattern is associated with the *HER2* gene status. This finding is compatible with recommendations of 2013 ASCO/CAP guidelines.

Key words: Breast Cancer, HER2, Immunohistochemistry, Fluorescence *in situ* hybridization

Background

Human epidermal growth factor receptor-2 (HER2; also named HER2/neu or ERBB2) is localized to chromosome 17q21 and encodes a transmembrane tyrosine kinase receptor [1]. Since HER2 was discovered in 1984 [2], and its prognostic value in breast cancers was reported in 1987 [3], this gene has been thoroughly studied and implicated in the diagnosis, therapy and prognosis of breast cancers. After the detection of HER2 overexpression and/or amplification, breast cancer patients are recommended to receive HER2-targeted therapy, such as trastuzumab and pertuzumab. Several studies have confirmed that HER2 expression has an inverse relationship with prognosis [1, 4]. However, only HER2-positive breast cancers can benefit from HER2-targeted therapy, and patients who receive targeted therapy show higher disease-free survival and overall survival than those who do not [5, 6].

Accurate evaluation of the HER2 status is mainly based on immunohistochemistry (IHC) and/or fluorescence *in situ* hybridization (FISH) by pathologists. Through IHC assays, the expression levels of HER2 protein are classified into 0, 1+, 2+ and 3+ based on American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines [7, 8]. Breast cancers with HER2 expression levels of 0 and 1+ are HER2-negative, 2+ are HER2-equivocal and 3+ are HER2-positive. Based on ASCO/CAP 2007 guidelines [7], HER2 IHC-2+ breast cancers comprise up to 17% of newly diagnosed cases [9, 10]. However, with the update to the ASCO/CAP 2013 guidelines [8], the cut-off value between HER2 IHC-2+ and HER2 IHC-3+ tumors is down to 10%, which suggests that more breast cancers are HER2 IHC-3+. Additionally, the guidelines define > 10% of tumor cells with an incomplete, weak membrane or moderate staining as HER2 IHC-2+, which leads to a higher proportion of HER2 IHC-2+ tumors [11].

Approximately 90% HER2 overexpression breast cancers are caused by gene amplification [12]. FISH directly reflects *HER2* gene copy numbers, and is thus considered the "golden standard". However, it is more time-consuming and more expensive than IHC method. Thus, FISH is typically used for HER2-IHC-2+ breast cancers to identify gene-amplified cases. And the change of cut-off value in *HER2/CEP-17* ratio, which is 2.0 in ASCO/CAP 2013 guidelines [8] and 2.2 in 2007 guidelines [7], is likely to lead to a higher proportion of gene-amplified cases in HER2 IHC-2+ tumors [13].

Moreover, along with the increased incidence rate of breast cancer in China and other areas worldwide [14, 15], the number of HER2 IHC-2+ tumors will also increase. Although there is an increasing need for FISH and the complexity of the IHC staining patterns in HER2 IHC-2+ cases, which differ in intensity (weak, moderate or intense), integrity (complete or incomplete) and percentage, little is known about the relationship between FISH results and different IHC staining patterns in HER2 IHC-2+ cases.

To investigate the proportion of *HER2* gene amplifications and the association between the HER2-IHC-staining pattern and gene amplification in IHC-2+ breast cancers, we designed a multicenter study including over 2,000 HER2 IHC-2+ consecutive surgical specimens based on ASCO/CAP 2013 guidelines [8].

Materials and Methods

1. Patient population

This retrospective, multicenter study included patients who received routine surgical excision of breast invasive ductal carcinoma between November 2014 and October 2015 from 12 institutions in China. The revised guideline for HER2 detection for Chinese breast cancers according to ASCO/CAP 2013 guidelines [8] was published in April 2014, and it was immediately accepted and applied in daily work nationwide. Thus, we selected this time period to begin our study. The pathology departments in these institutions passed "Pathology Quality Control Centre (PQCC)" of National Health and Family Planning Commission certification. And external quality assessment system of HER2-IHC in breast cancers is a major project of the PQCC which lasts for about seven years, aimed to assess the consistency and accuracy of HER2-IHC staining and interpretation in different pathology departments. All breast cancers included in this study were HER2 IHC-2+ cases, and the gene status was detected through FISH. The definition of HER2 IHC-2+ cases is based on the ASCO/CAP 2013 guidelines [8]. By the

way, we excluded invasive micropapillary carcinoma in this study because of its unique staining pattern [16, 17]. The study profile is illustrated in Figure 1.

2. IHC and FISH

All participating institutions used Ventana anti-HER2/neu (4B5, rabbit monoclonal antibody, Ventana Medical Systems) for HER2-IHC staining. The cases included in this study were re-evaluated on the percentage of staining intensity and membrane integrity by two experienced pathologists. The re-evaluation of percentage in the IHC staining model was focused on invasive ductal carcinoma with higher intensity in the entire slide. Different staining intensities with complete or incomplete membrane integrity are shown in Figure 2.

Dual-color *HER2* FISH probe kits were used in the present study, and all kits were certificated by the China Food and Drug Administration (CFDA), Food and Drug Administration (FDA) and/or Council of Europe (CE). If there was *HER2* genetic heterogeneity, then the tumor regions with higher *HER2/CEP17* ratios and/or *HER2* copy numbers were considered in the final interpretation.

3. Statistical analyses

The re-evaluation of the IHC staining pattern was primarily based on zones with higher intensity. To reveal the association between the staining pattern and the FISH test results, we used a decision tree-based prediction model, which can automatically calculate and classify characteristics with specific endpoints. To generate a more precise prediction model, we added more cases into the training set. Subsequently, all cases in the present study were randomly divided into training sets (n = 1914) and validation sets (n = 624). The ratio of the number of cases in the training set to that in the validation set was 3:1, and the prediction model was calculated based on training set data. Then, we used this prediction model in the validation group. According to ASCO/CAP 2013 guidelines [8], tumor cells within 10% intense, complete and circumferential membrane staining were also classified as HER2 IHC-2+ cases. We regarded these special tumors as having an independent staining pattern. Based on results from a decision tree-based prediction model, we divided all cases into six different categories according to different staining patterns, and differences in the HER2 copy numbers among these categories were calculated by Student-Newman-Keuls (SNK). Continuous variables are presented as the means ± standard deviation (SD). The two-sided significance level was set at $P < 0.05$.

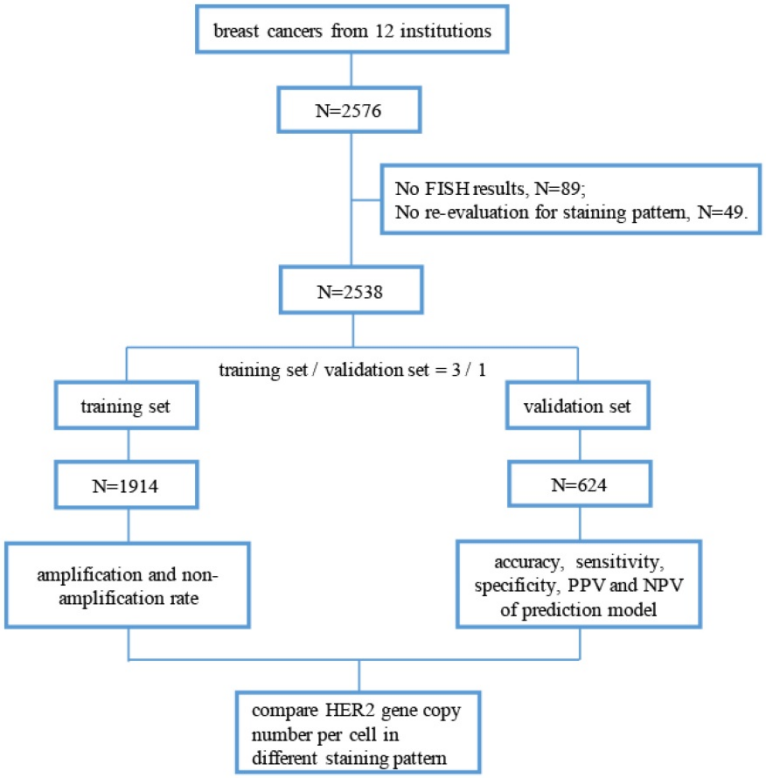

Figure 1. Flow chart of this study. (FISH, fluorescence *in situ* hybridization; PPV, positive prediction value; NPV, negative prediction value).

Result

1. Profile of HER2 IHC-2+ staining pattern

After excluding cases with no re-evaluation data of staining pattern or FISH results, 2538 HER2 IHC-2+ breast cancer cases were included in the present study. The overall *HER2* FISH amplification rate in participating institutions was 17.8% (ranging from 9.0% to 37.0%). Moreover, the *HER2* FISH equivocation rate was 6.0% (ranging from 2.6% to 10.6%), and the non-amplification rate was 76.2% (ranging from 59.8% to 88.4%). An overview of the re-evaluation of the IHC staining pattern is shown in Table 1. A total of 103 (4.1%) cases had complete and circumferential membrane staining that was intense and within 10% of the cells. In addition, 604 (23.8%) cases showed complete membrane staining, which had weak or moderate staining intensities. However, more cases (72.1%) had incomplete membrane staining. For staining intensity, 856 (33.8%) cases with weak staining and 1579 (62.2%) cases with moderate staining were observed. The distribution of the staining pattern in the training and validation sets is also shown in Table 1, and the differences in the

membrane integrity, staining intensity and percentage between the two sets were not statistically significant ($P > 0.05$). The different staining patterns of HER2 IHC-2+ cases were shown in Figure 2.

2. Prediction of FISH results in the training and validation sets

In the training set, we used a decision-tree method to model, in which the staining pattern was associated with the FISH results. As shown in Figure 3, the cases in the training set were divided into six groups according to membrane integrity, staining intensity and percentage. In this prediction model, no group was associated with equivocal *HER2* FISH cases. However, four of the six groups were associated with *HER2* FISH non-amplification cases, and two of the six groups were associated with amplification cases. In particular, cases with ≤ 10% of cells with intense, complete and circumferential membrane staining tended to show amplification results, and 57 of the 74 (77.0%) cases in this pattern were detected to have *HER2* amplification. Moreover, cases that had > 85% cells with complete membrane staining had 60.5% (49/81) amplification rates.

Except for above 155 cases, remaining 1759 cases were classified into non-amplification groups. The non-amplification rate in these four nodes ranged from 67.8% to 89.6%. Although cases with weak and incomplete membrane staining had the highest non-amplification rate of 89.6%, 34 of the 557 (6.1%) cases had amplification FISH results. Among these 34 cases, 23 cases had *HER2* copy numbers ≥ 6.0, and 11 cases had *HER2/CEP-17* ratios ≥ 2.0 but *HER2* copy numbers < 6.0.

Next, we applied this model to the validation set. The predicted and actual FISH results are shown in Table 2. In this set, the accuracy, sensitivity, specificity, and positive and negative prediction values were 76.4, 30.2, 94.5, 51.6 and 79.2%, respectively.

Table 1. Profile of immunohistochemistry (IHC) staining pattern in all cases and cases in the training/validation set.

		Weak (n, %)		Moderate (n, %)		Strong (n, %)	Total (n, 100%)
		10-50%	50%-100%	10-50%	50%-100%	0-10%	
All Case	complete	60 (11.5)	54 (16.2)	159 (23.2)	331 (37.0)	103 (100)	707 (27.9)
	incomplete	462 (88.5)	280 (83.8)	526 (76.8)	563 (63.0)	--	1831 (72.1)
	Total	522 (20.6)	334 (13.2)	685 (27.0)	894 (35.2)	103 (4.1)	2538 (100)
Training Set	complete	45 (11.6)	36 (14.5)	111 (20.9)	249 (37.1)	73 (100)	514 (26.9)
	incomplete	344 (88.4)	213 (85.5)	421 (79.1)	422 (62.9)	--	1400 (73.1)
	Total	389 (20.3)	249 (13.1)	532 (27.8)	671 (35.1)	73 (3.8)	1914 (100)
Validation Set	complete	15 (11.3)	18 (21.2)	48 (31.4)	82 (36.8)	30 (100)	193 (30.9)
	incomplete	118 (88.7)	67 (78.8)	105 (68.6)	141 (63.2)	--	431 (69.1)
	Total	133 (21.3)	85 (13.6)	153 (24.5)	223 (35.7)	30 (4.8)	624 (100)

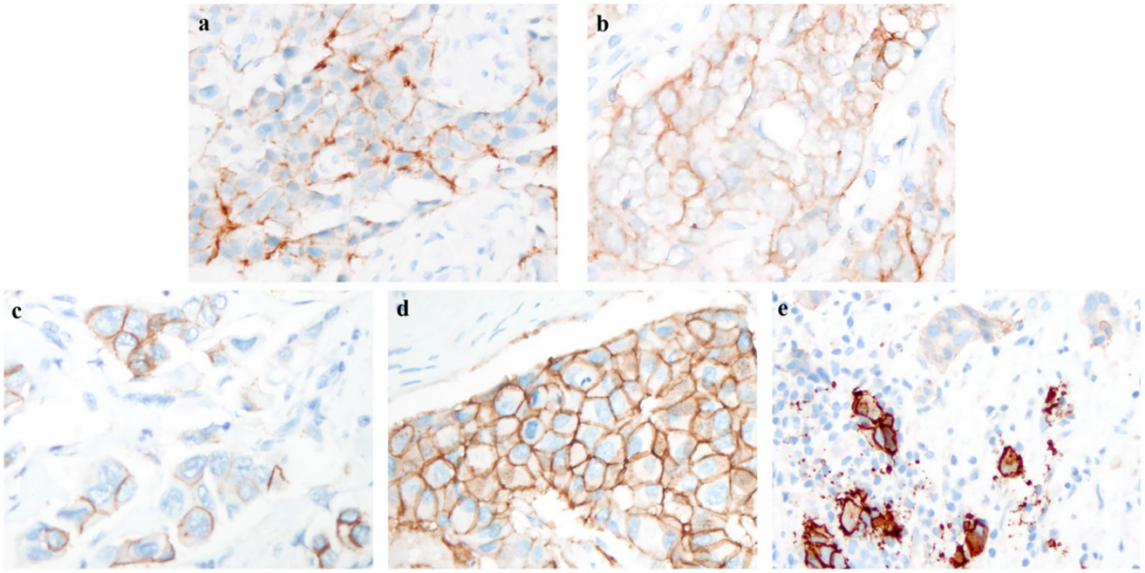

Figure 2. Example of different intensity and integrity in immunohistochemistry (IHC) staining pattern. a. Weak and incomplete membrane staining; b. weak and complete membrane staining; c. moderate and incomplete membrane staining; d. moderate and complete membrane staining; e. intense, complete and circumferential membrane staining. (a-e, original magnification: ×400).

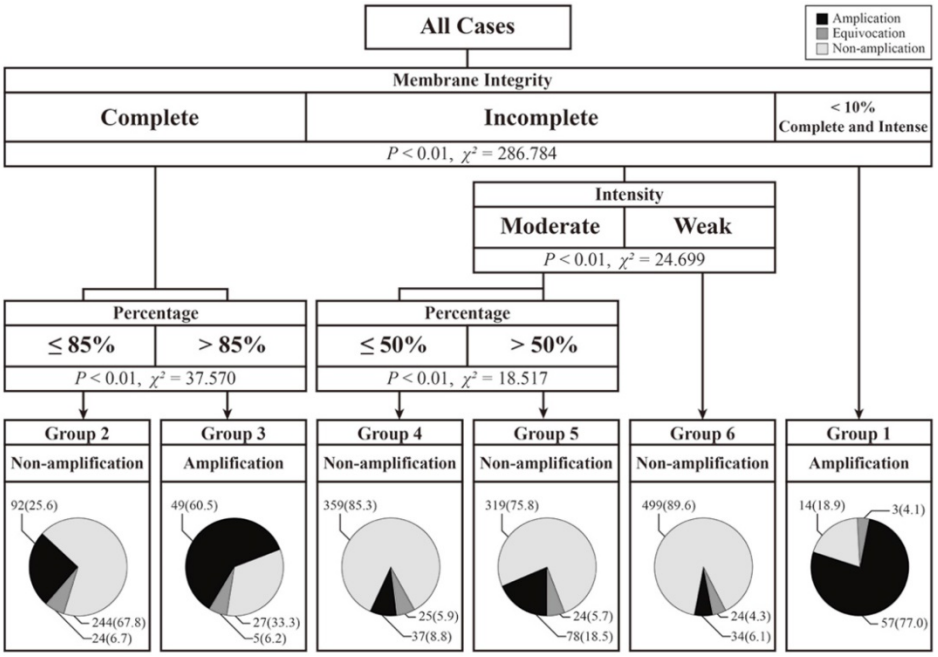

Figure 3. Decision-tree based prediction model of *HER2* gene status and details on the number and ratio of different fluorescence in situ hybridization (FISH) results.

Table 2. Predicted and actual fluorescence *in situ* hybridization (FISH) results.

Actual FISH Results	Predicted FISH Results			Total
	Non-amplification	Equivocation	Amplification	
Non-amplification	445	0	26	471
Equivocation	43	0	4	47
Amplification	74	0	32	106
Total	562	0	62	624

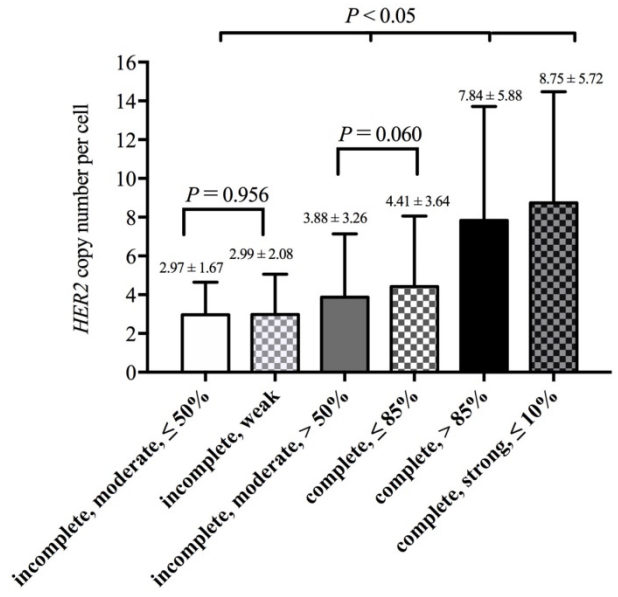

Figure 4. *HER2* gene copy number per cell in different staining patterns

3. *HER2* copy number per cell in different staining patterns

Based on results from the decision-tree method, 2538 cases could be divided into six groups according to membrane integrity, staining intensity and percentage. The lowest *HER2* copy number per cell (2.97 ± 1.67) was observed in cases that had ≤ 50% of cells with moderate and incomplete membrane staining. Cases that had ≤ 10% of cells with intense, complete and circumferential membrane staining had the highest *HER2* copy numbers per cell (8.75 ± 5.72). The means and SD of the *HER2* copy number per cell in these groups are shown in Figure 4.

Then, we used SNK to calculate the differences among these groups (Figure 4). There was no significant difference between "incomplete, moderate, ≤ 50%" and "incomplete, weak" ($P = 0.956$), and there was also no difference between "incomplete, moderate, > 50%" and "complete, ≤ 85%" ($P = 0.060$). The four groups above could be observed as two separate categories, and two groups in one category showed no differences in the *HER2* copy number per cell. Thus, these six groups could be divided into four categories. For *HER2* copy number per cell, there were significant differences between every two categories ($P < 0.05$, Figure 4).

Discussion

After ASCO/CAP 2013 guidelines were published, several studies had reported amplification rates of IHC-2+ cases ranging from 15.1% to 30.6% based on this revised guideline [18-21]. However, these studies were single-center and did not focus on IHC-2+ cases. Thus, the new cut-off value of HER2-IHC and FISH test in ASCO/CAP 2013 guidelines demonstrates a need for more large-scale and multi-center studies focusing on the HER2

IHC-2+ proportion in breast cancers and FISH-amplification rate in HER2 IHC-2+ breast cancers. Herein, we conducted a multicenter, large-scale study on HER2 IHC-2+ consecutive surgical specimens. Institutions participating in this study were distributed in China, and each facility was a region representative. Overall, the *HER2* amplification rate was 17.8%, ranging from 9.6% to 37.0%. Ten of the twelve (83.3%) institutions had amplification rates ranging from 10% to 30%.

There are few studies on the amplification rates in different staining patterns for HER2 IHC-2+ cases. Onguru et al. [19] reported the amplification rates in different percentages of moderate, complete/ incomplete membrane staining. For 124 IHC-2+ cases in their study, the amplification rate in tumors, which had > 50% of cells with moderate complete/ incomplete membrane staining, was 54.5% (12/22). This value was much higher than that observed (26.2%, 234/893) in the validation set in this study. Except for differences in amplification rates, the previous study showed that the amplification rates increased with increasing staining percentage (<10%, 10–50% and >50%) from 14.3% and 25.4% to 54.5% in the same staining intensity. The same phenomenon was also observed in the present study. With the same integrity and/or intensity, higher percentages had higher amplification rates (Figure 3; Group 2 vs. Group 3, 25.6% vs. 60.5%; Group 4 vs. Group 5, 8.8% vs. 18.5%). These facts show that cases with a higher proportion of stained cells are more likely to have *HER2* gene amplification.

In this study, we constructed a prediction model based on staining patterns for gene amplification. And every cut-off value was produced automatically. From the prediction model, membrane integrity should be considered first. In cases that had cells with complete membrane staining, percentage was more important than staining intensity, and the higher percentage (> 85%) tended to be gene amplified. In contrast, cases containing cells with incomplete membrane staining were associated with non-amplified FISH test results, regardless of staining intensity and percentage. For all cases in this study, the *HER2* gene amplification rate in tumors with complete membrane staining was 37.3% (264/707), which was much higher than that for tumors with incomplete membrane staining (10.3%; 189/1831).

In the training set, the highest amplification rate (77.0%) was in cases that had ≤ 10% of cells with intense, complete and circumferential membrane staining (Group 1). The lowest amplification rate (6.1%) was observed in cases with incomplete and weak staining (Group 6). Moreover, these cases had the highest non-amplification rate (89.6%). We found that the staining pattern in cases with the highest amplification rate was close to the definition of IHC-3+, which had cells with intense, complete and circumferential membrane staining [8]. Instead, the staining pattern in cases with the lowest amplification rate was closer to the definition of IHC-1+, indicating cells with faint/barely and incomplete membrane staining [8]. Some studies reported amplification rates in IHC-3+ cases ranging from 83.3% to 92.9% [18, 20, 21] and in IHC-1+ cases ranging from 2.9% to 4.1% [18, 20]. In the 2013 revised guidelines [8], the evaluation of the IHC staining model was changed along with the cut-off value of FISH. However, based on either the 2007 guidelines [7] or the 2013 revised guidelines [8], there was discordance between IHC and FISH in IHC-1+ and 3+ cases [18, 20, 22, 23]. This phenomenon suggests that HER2 protein overexpression can occur without gene amplification and vice versa [24]. The possible reason for this finding is that breast cancers with mutations in the *HER2* gene may be IHC-negative but gene-amplified [25, 26].

From the above findings, we found that the HER2 IHC staining model was associated with the FISH test results to some extent. In the 2013 revised guidelines [8], the *HER2* gene status was determined by not only the *HER2/CEP17* ratio but also the *HER2* copy number per cell. Lim et al. [20] reported an increase in the *HER2* copy number per cell from IHC-negative to IHC-positive cases, and the *CEP17* copy numbers showed no significant changes among different IHC groups. Although the *CEP17* copy numbers were more stable among different breast cancers, it could be useful to estimate aneuploidy and make FISH test results more reliable. Thus, we used the FISH test results to generate a prediction model, and we compared the mean *HER2* copy number per cell among decision-tree-based staining groups. Cases that had over 85% cells with complete membrane staining (Group 3) or had ≤ 10% cells with intense, complete and circumferential membrane staining (Group 1) were predicted to be HER2 gene-amplified. Those cases also had a higher *HER2* copy number per cell than that of other cases, which were predicted as non-amplification cases ($P < 0.05$). As expected, the Group 1 with the highest amplification rate had the highest *HER2* copy number per cell, and the Group 6 and Group 4 with lower amplification rates had lower *HER2* copy numbers per cell.

In conclusion, we conducted a multicenter study to evaluate *HER2* gene status in HER2 IHC-2+ breast cancers and constructed a decision-tree model to evaluate the relationship between the IHC staining pattern and the FISH results. These results show that the IHC staining pattern is also associated with *HER2*

gene status in HER2 IHC-2+ breast cancers. And it is compatible with recommendations of 2013 ASCO/CAP guidelines. If there were amplified FISH test results in cases with weak and incomplete staining pattern or non-amplified FISH test results in cases with ≤ 10% cells with intense, complete and circumferential membrane staining, pathologists should check IHC and FISH test results over again. Moreover, with increasing amplification rate in each staining pattern, the *HER2* copy number per cell also increased.

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Competing Interests

The authors have declared that no competing interest exists.

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