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ORIGINAL ARTICLE

Histologic analysis according to *HER2* gene status in HER2 2 + invasive breast cancer: a study of 280 cases comparing ASCO/CAP 2013 and 2018 guideline recommendations

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Abstract

The American Society of Clinical Oncology and College of American Pathologists guidelines for HER2 testing in breast cancer (BC) have been updated with more stringent criteria regarding immunohistochemistry (IHC) 2 + interpretation. The aim of our study was to determine HER2 status in IHC 2 + cases based on 2013 and 2018 guidelines and to investigate specific histologic characteristics that might predict HER2 status in tumors with equivocal IHC staining. Two hundred eighty BC cases reported as IHC 2 + and 24 cases reported as non-IHC 2 + were reviewed with 12 histologic characteristics. Of the IHC 2 + cases based on 2013 guideline, 21% were reclassified to IHC 1 + when applying the 2018 guidelines. Consequently, it led to an 8% increase of *HER2* amplification rate in 2018 IHC 2 + group. Seven characteristics were significantly associated with prediction of *HER2* amplification in IHC 2 + BCs, including high tumor-infiltrating lymphocytes (TILs), distinct cellular membrane, no apical snout, large nuclear size, nuclear size variation, high nuclear grade, and tubule formation < 10%. Using these criteria, the presence of four or more characteristics significantly indicates *HER2* amplification. Moreover, four characteristics among them, including high TILs, distinct cellular membrane, nuclear size variation, and high nuclear grade, were also associated with *HER2* amplification in non-IHC 2 + cases, demonstrating their predictive value as complements to IHC. In conclusion, we provide specific morphologic features that will improve pathologist performance in identifying more HER2-positive BCs. We further suggest an algorithm for trastuzumab therapy decisions using a combination of histomorphologic evaluation and the updated 2018 guidelines.

Keywords HER2 · Equivocal · Immunohistochemistry · FISH · ASCO/CAP guidelines

Introduction

The human epidermal growth factor receptor 2 gene (*HER2/neu* or *HER2*) encodes a transmembrane receptor tyrosine kinase protein that is overexpressed in 10–30% of invasive breast cancers (BCs). HER2 overexpression occurs primarily through amplification of wild-type *HER2* gene and is associated with poor disease-free survival and resistance to certain chemotherapeutic agents [6, 19]; however, HER2 overexpression is also predictive of response to HER2-targeted therapies including trastuzumab (Herceptin; Roche, Basel, Switzerland) [12]. In the neoadjuvant setting, adding trastuzumab to chemotherapy has improved pathologic complete remission (pCR) rates up to 78% in HER2-positive BCs [5, 23]. Recent data from a randomized phase III trial (NSBAP B-47) confirmed the lack of benefit from adjuvant trastuzumab for patients whose tumors lack gene

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amplification and are immunohistochemistry (IHC) 1 + or 2 + for HER2 [7, 8]. Consequently, *HER2* gene amplification assessed by in situ hybridization (ISH) or protein overexpression assessed by IHC remains the primary predictor of responsiveness to HER2-targeted therapies and is essential for personalized treatment in BC.

The American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) have periodically issued detailed guidelines and updates for conducting and interpreting HER2 testing, which may include IHC and/or ISH, to standardize the performance and reliability of HER2 testing across laboratories [28–30]. IHC is used as a screening method to determine the level of HER2 protein expression in BCs, and the results are generally expressed in a four-scale scoring system ranging from 0 to 3 + [28]. Tumors scored as IHC 2 + are considered HER2-equivocal and should be further tested with a validated assay for *HER2* gene amplification, such as fluorescence in situ hybridization (FISH), before considering trastuzumab therapy. Determination of *HER2* gene status by FISH can either be done by assessing the mean number of *HER2* copies or by calculating the mean *HER2/CEP17* ratio in a population of tumor nuclei, where *CEP17* represents the number of chromosomes 17.

IHC 2 + BC has been defined in the 2013 ASCO/CAP guidelines as invasive BC showing "circumferential membrane staining that is incomplete and/or weak/moderate and within > 10% of tumor cells, or complete and circumferential membrane staining that is intense and within ≤ 10% of tumor cells." However, many pathologists expressed concern that the terms "circumferential" and "incomplete" were confusing, could not be reconciled when used together in IHC interpretation of HER2 expression, and could lead to many IHC 1 + (HER2-negative) tumors being called IHC 2 + (HER2-equivocal) and submitted for reflex testing [18, 27]. Based on subsequently published works [1, 16, 17, 21], an update to the 2013 ASCO/CAP guidelines has recently been published [30]. In the updated 2018 guidelines, the revised definition of IHC 2 + is invasive BC with "weak to moderate complete membrane staining observed in > 10% of tumor cells." Consequently, "incomplete circumferential membrane staining within > 10% of tumor cells" or "complete circumferential membrane staining that is intense and within ≤ 10% of tumor cells" referred to an unusual pattern that did not need to be specified in the main portion of the guideline algorithm. The consecutive ISH testing algorithm for IHC 2 + BC has also been revised. The diagnostic approach includes more rigorous interpretation criteria for less common ISH patterns, described as ISH groups 2 to 4, and requires concomitant IHC review.

Tumor morphology has an important role in both the previous and updated ASCO/CAP guidelines, with recommendations included for situations in which histopathologic features suggest possible discordance with HER2 testing. For example, an alternate HER2 test should be considered when grade 1 tumors have an initial HER2-positive result. Similarly, if an initial HER2 test on a core needle biopsy is negative, a new HER2 test should be considered on the excision for grade 3 tumors. Even in this molecular era, histology maintains a fundamental role in identifying tumor subtypes with particular clinical behavior.

In this study, we report our results of HER2 final classification with definite FISH diagnosis interpreted according to the updated ASCO/CAP guidelines for IHC 2 + BCs and compare these classifications with the expected results using 2013 guidelines. Another aim of the study is to investigate the specific histologic features that lead to accurate diagnosis of HER2 status with regard to both 2013 and 2018 guidelines.

Materials and methods

Case selection

A total of 426 BC cases previously reported as HER2 IHC 2 + according to 2013 ASCO/CAP guidelines during 2004 and 2010 were retrieved from the computerized records system at Samsung Medical Center, Seoul, Korea. Of these, 280 cases were available for review of both IHC and FISH. Besides the IHC 2 + group, 24 cases of IHC 0, 1 +, or 3 + BC with consecutive FISH analysis were collected irrespective of IHC results to compare histologic characteristics with those of the IHC 2 + group. Twenty-four cases consisted of three cases of IHC 0, nine cases of IHC 1 +, and 12 cases of IHC 3 + BC. Clinicopathologic parameters including age, histologic type, HER2-targeted therapy, recurrence, follow-up status, and follow-up period were obtained by thorough review of clinical records. Study protocols including case selection, slide review, and collection of clinical parameters were approved by the Samsung Medical Center Institutional Review Board (No. 2019–03-034).

Histologic characteristics

Based on the results of prior studies [4, 11, 15] and the personal pathology experience of authors, 12 histologic characteristics were reviewed, including (1) nuclear grade (high vs non-high), (2) nuclear size (≥ 2 × size of benign ductal epithelial cell nuclei vs < 2), (3) nuclear size variation (present vs absent), (4) nucleoli (conspicuous vs inconspicuous), (5) cellular membrane (distinct vs indistinct), (6) mitosis (≥ 8/10HPFs vs < 8), (7) necrosis (present vs absent), (8) tubule formation (≥ 10% of tumor area vs < 10%), (9) apical snout (present vs absent), (10) micropapillary features (present vs absent), (11) tumor-infiltrating lymphocytes (TILs) (≥ 50% vs < 50%), and (12) extensive intraductal component

(EIC) (positive vs negative) (Figs. 1 and 2). Two expert breast pathologists (E.Y.C. and H.W.H.) evaluated each criterion, and any discrepancies were resolved by consensual agreement.

HER2 IHC

Formalin-fixed, paraffin-embedded 4-μm-thick sections of tissue were used for IHC staining. HER2 IHC was performed using a Ventana automated platform (Benchmark ULTRA; Ventana, Tuscon, AZ, USA). IHC assay for HER2 (rabbit monoclonal antibody, clone 4B5) was carried out by following the manufacturer's instructions and using appropriate controls. Immunohistochemical reactivity was determined at the time of diagnosis by visual estimation of intensity and completeness of membrane staining in invasive tumor cells as well as the percentage of positive cells. The results were interpreted separately according to the 2013 and 2018 ASCO/CAP guidelines.

HER2 FISH

Formalin-fixed, paraffin-embedded 4-μm-thick sections of tissue were used for FISH testing. A pathologist evaluated hematoxylin and eosin (H&E)–stained sections to label the invasive cancer. FISH analyses were performed using dual probe *HER2/CEP17* assays (PathVysion Probe Kit; Abbott Molecular Inc., Des Plaines, IL, USA). Fluorescence hybridization signals were analyzed and captured under a fluorescence microscope (Zeiss Axioskop, Obercochen, Germany) using filter sets recommended by Vysis (4′,6-diamidino-2-phenylindole [DAPI]/Spectrum Orange dual bandpass, DAPI/Spectrum Green dual bandpass). *HER2* and *CEP17* signals were manually counted, at least non-overlapping 20 tumor cell nuclei

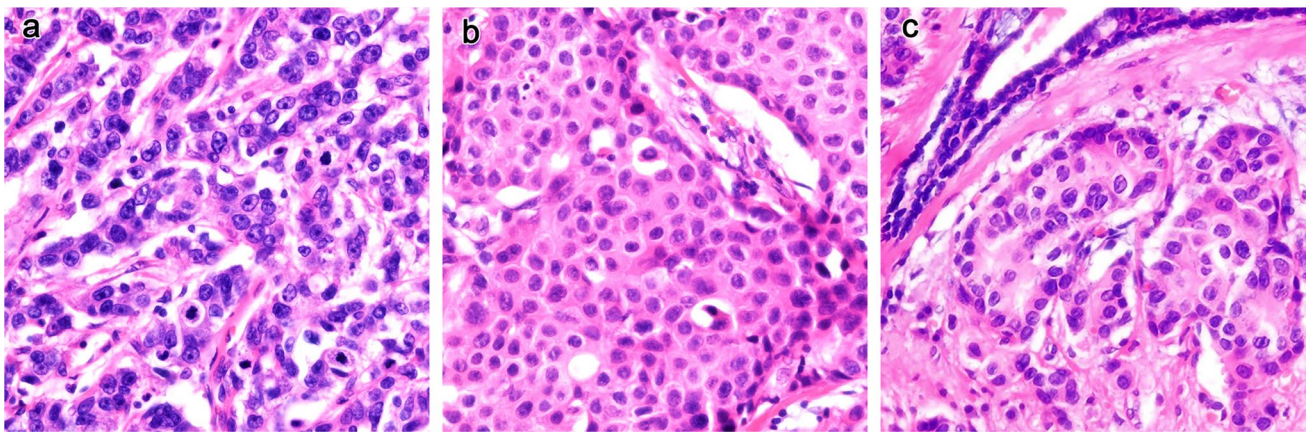

Fig. 1 Histologic characteristics of HER2 2+breast cancers including **a** high-grade tumor cells with indistinct cellular membrane, **b** distinct cellular membrane, and **c** nuclear size ≥ 2 × size of adjacent benign ductal epithelial cell nuclei are shown in these examples (hematoxylin and eosin stains, magnification × 400)

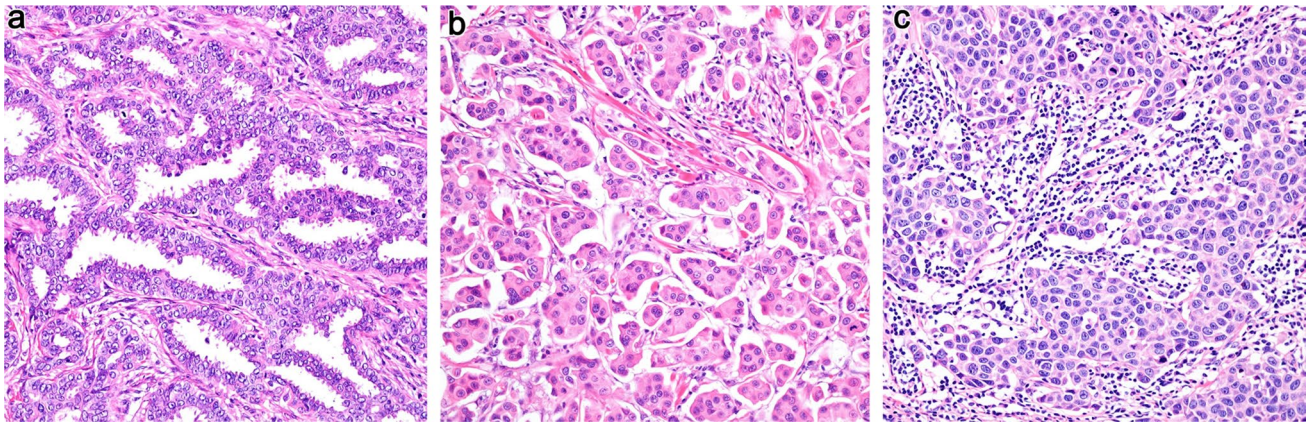

Fig. 2 Representative images of histologic characteristics including **a** tubule formation with apical snouts, **b** micropapillary features, and **c** high tumor infiltrating lymphocytes (hematoxylin and eosin stains, magnification × 400)

were individually scored, and independent *HER2/CEP17* ratios were calculated.

Statistical analysis

Statistical analyses were performed with SPSS Ver.25 software (SPSS Inc., Chicago, IL, USA). The incidence of each histologic characteristic was evaluated for its predictive value on HER2 status by performing univariable Fischer's exact *T*-test on the obtained data. The reliable histologic indicators of amplification were then selected, and the number of histologic indicators was calculated for each patient with *HER2*-amplified and unamplified conditions. Statistical analysis for the selected variables of interest was performed with Pearson's chi-square test. Optimal cutoff value of number of histologic characteristics for predicting *HER2* amplification was obtained from receiver-operating characteristic (ROC) curve analysis, and the area under the ROC curve (AUC) was calculated. All statistical tests were two-sided and were regarded as statistically significant when the *p*-value was less than 0.05.

Results

HER2 IHC and FISH results interpreted according to 2013 and 2018 ASCO/CAP guidelines

HER2 FISH was performed on HER2 IHC 2 + BCs, and final HER2 classification was reported according to the 2013 ASCO/CAP guidelines. Review of IHC and FISH was available for 280 patients in the 2013 IHC 2 + group. The median age was 48 years (range: 25–75 years), and 91% ($n = 255$) were invasive carcinoma of no special type (invasive ductal carcinoma) (Table 1). Of all 280 cases, 87 (31%) were *HER2*-amplified and 193 (69%) were unamplified by final FISH results. Distribution of mean *HER2* copy number per nucleus and mean *HER2/CEP17* ratio per nucleus in the study population of 280 IHC 2 + cases is shown in Table 1. Clinically, 79 patients (28%) received trastuzumab therapy.

For each case, HER2 IHC was reclassified using the 2018 guidelines for comparison. Classification of the same 280 cases using 2018 ASCO/CAP guidelines resulted in 221 cases of IHC 2 + BCs and 59 cases of IHC 1 + . Consequently, use of the 2018 guidelines versus 2013 guidelines has led to classification change of about 21.1% of cases, namely a decrease in IHC 2 + cases. Consecutive FISH

Table 1 Clinicopathologic characteristics in accordance with 2013 and 2018 ASCO/CAP guidelines

Variables	2013 IHC 2 + *n* (%)			2013 non-IHC 2 + *n* (%)	
	Total ($n = 280$)	2018 IHC 2 + ($n = 221$)	2018 IHC 1 + ($n = 59$)	IHC 0, 1 + ($n = 12$)	IHC 3 + ($n = 12$)
Histologic type					
Ductal	255 (91.1)	205 (92.8)	50 (84.7)	10 (83.4)	12 (100.0)
Lobular	6 (2.1)	2 (0.9)	4 (6.8)	1 (8.3)	0 (0.0)
Mixed	3 (1.1)	2 (0.9)	1 (1.7)	0 (0.0)	0 (0.0)
Micropapillary	12 (4.3)	11 (5.0)	1 (1.7)	1 (8.3)	0 (0.0)
Special types	4 (1.4)	1 (0.4)	3 ((5.1)	0 (0.0)	0 (0.0)
HER2 FISH					
Positive	87 (31.1)	86 (38.9)	1 (1.7)	0 (0.0)	12 (100.0)
Negative	193 (68.9)	135 (61.1)	58 (98.3)	12 (100.0)	0 (0.0)
HER2/CEP17 ratio					
Mean	3.04	3.47	1.42	1.18	4.37
HER2 copy number					
Mean	7.31	8.03	3.22	2.54	9.78
Trastuzumab therapy					
Yes	79 (28.2)	78 (35.3)	1 (1.7)	0 (0.0)	12 (100.0)
No	201 (71.8)	143 (64.7)	58 (98.3)	12 (100.0)	0 (0.0)
Recurrence					
Yes	76 (27.1)	58 (26.2)	18 (30.5)	2 (16.7)	2 (16.7)
No	204 (72.9)	163 (73.8)	41 (69.5)	10 (83.3)	10 (83.3)

FISH, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; *IHC*, immunohistochemistry

reclassification showed 39% ($n=86$) FISH-positive *HER2*-amplified rate depending on the 2018 guidelines.

The median age of the 2013 non-IHC 2 + group ($n=24$) was 45 years (range: 28–59 years), and 92% ($n=22$) of cases were invasive carcinoma of no special type (invasive ductal carcinoma) (Table 1). After applying the 2018 guidelines, all cases remained their original classification of IHC and FISH in accordance with 2013 guidelines.

Histologic predictors of HER2 status in IHC 2 + BCs

The prevalence of 12 different histologic characteristics and their predictive sensitivity and specificity identified in 2013 *HER2*-amplified or unamplified BCs are shown in Table 2. By using univariable analysis, seven of the 12 histologic characteristics were significantly associated with *HER2* amplification: nuclear grade ($p<0.001$), nuclear size ($p<0.001$), nuclear size variation ($p<0.001$), cellular membrane ($p<0.001$), tubule formation ($p<0.001$), apical snout ($p<0.001$), and TILs ($p<0.001$). Five histologic characteristics were not significantly associated with *HER2* amplification (nucleoli [$p=0.067$], mitosis [$p=0.391$], necrosis [$p=0.066$], micropapillary features [$p=0.622$], and EIC [$p=0.433$]). Statistical analysis of histologic features was also performed with 221 re-defined IHC 2 + BCs according to 2018 guidelines (Table 3). Of the 12 histologic characteristics, eight were significantly associated with *HER2* amplification while four were not (mitosis [$p=0.152$], necrosis [$p=0.199$], micropapillary features [$p=0.525$], and EIC [$p=0.544$]). In addition to the seven aforementioned characteristics, presence of conspicuous nucleoli was also a significant predictor of *HER2* amplification according to the updated guidelines.

For further comprehensive prediction of HER2 status, the most helpful characteristics were assessed based on greatest odds of prediction. When odds ratios were calculated for the remaining seven or eight characteristics, all were > 1.00 (range: 1.94–14.17), indicating increased odds of prediction if that specific characteristic was identified in a tumor. TILs had the highest odds ratio (OR 9.32; 95% CI 4.49–19.36).

Table 4 lists the probabilities of *HER2* amplification for various combinations of the seven variables. To determine an analytically optimal cutoff point with highest discriminatory power for *HER2* amplification, ROC curves were used (Fig. 3), and accuracy was measured by the AUC. A cutoff value of four produced the highest accuracy; sensitivity and specificity were 82.6 and 86.7%, respectively (Table 5). At a cutoff value of four, 71 of 86 patients with amplified group, 18 of 135 patients with unamplified group were distinguished as *HER2*-amplified. It was thus clear that using four or more key histologic criteria significantly demonstrated cases with *HER2* amplification ($p<0.001$). Applying 2018 ASCO/CAP guidelines resulted in 98.9% sensitivity, 30.1% specificity, and 51.4% diagnostic accuracy in our study (Table 6). Using ≥ 4 key histologic criteria additionally showed significantly higher specificity (86.7%) and accuracy (85.1%) compared with applying 2018 guidelines only, whereas it resulted in slightly lower sensitivity (82.6%).

Table 2 Frequency and predictive value of assessed histologic features in 2013 IHC 2 + breast cancers

Characteristic	HER2 FISH (+) ($n=87$)		HER2 FISH (-) ($n=193$)		*p* value	Odds ratio	95% CI	Sensitivity (%)	Specificity (%)
	No	%	No	%					
High nuclear grade	52	60	56	29	< 0.001	3.69	2.24–6.07	59.7	71.3
Large nuclear size	69	79	87	45	< 0.001	4.67	2.70–8.05	79.1	55.2
Nuclear size variation: present	58	67	58	30	< 0.001	4.63	2.80–7.67	66.7	69.9
Conspicuous nucleoli	40	46	68	35	0.067			45.8	65.4
Distinct cellular membrane	51	59	31	16	< 0.001	7.47	4.25–13.13	59.0	83.8
Mitosis ≥ 8/10HPFs	37	42	69	36	0.391			41.7	64.0
Necrosis: present	38	44	60	31	0.066			43.9	69.0
Tubule formation < 10%	71	82	108	56	< 0.001	3.58	2.08–6.17	81.9	44.1
Apical snout: absent	83	95	140	73	< 0.001	7.32	3.13–17.08	95.1	27.2
Micropapillary features: absent	81	93	183	95	0.622			92.8	5.4
TILs ≥ 50%	39	45	15	8	< 0.001	9.32	4.49–19.36	44.8	92.0
EIC: negative	54	62	131	68	0.433			62.3	32.5

CI, confidence interval; *EIC*, extensive intraductal component; *FISH*, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; *HPFs*, high power fields; *IHC*, immunohistochemistry; *TILs*, tumor-infiltrating lymphocytes

Table 3 Frequency and predictive value of assessed histologic features in 2018 IHC 2 + breast cancers

Characteristic	HER2 FISH (+) ($n=86$)		HER2 FISH (-) ($n=135$)		p value	Odds ratio	95% CI	Sensitivity (%)	Specificity (%)
	No	%	No	%					
High nuclear grade	52	60	30	22	< 0.001	5.44	2.89–10.24	59.9	78.5
Large nuclear size	68	79	59	44	< 0.001	4.64	2.49–8.67	78.8	55.6
Nuclear size variation: present	57	66	32	24	< 0.001	6.18	3.32–11.52	66.2	75.9
Conspicuous nucleoli	40	45	41	30	0.032	1.94	1.08–3.46	45.8	69.6
Distinct cellular membrane	51	59	18	13	< 0.001	9.71	4.62–20.39	58.5	87.3
Mitosis ≥ 8/10HPFs	36	42	43	32	0.152			41.5	68.4
Necrosis: present	38	44	41	30	0.199			43.8	70.2
Tubule formation < 10%	70	81	81	60	< 0.001	3.04	1.64–5.64	81.7	40.5
Apical snout: absent	82	95	97	72	< 0.001	7.44	3.01–18.40	95.1	27.8
Micropapillary features: absent	77	90	120	89	0.525			92.7	8.0
TILs ≥ 50%	39	46	8	6	< 0.001	14.17	4.88–41.10	45.5	94.4
EIC: negative	54	63	90	67	0.544			62.5	32.9

CI, confidence interval; *EIC*, extensive intraductal component; *FISH*, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; *HPFs*, high power fields; *IHC*, immunohistochemistry; *TILs*, tumor-infiltrating lymphocytes

Table 4 Incidence of key histologic criteria in *HER2*-amplified and unamplified groups

No. of characteristics present	*HER2* amplification (+) ($n=86$)	*HER2* amplification (-) ($n=135$)	Probability of amplification (%)
7	11	0	100.0
6	20	3	87.0
5	22	7	75.9
4	18	8	69.2
3	6	26	18.8
2	6	30	16.7
1	3	28	9.7
0	0	33	0.0

HER2, human epidermal growth factor receptor 2

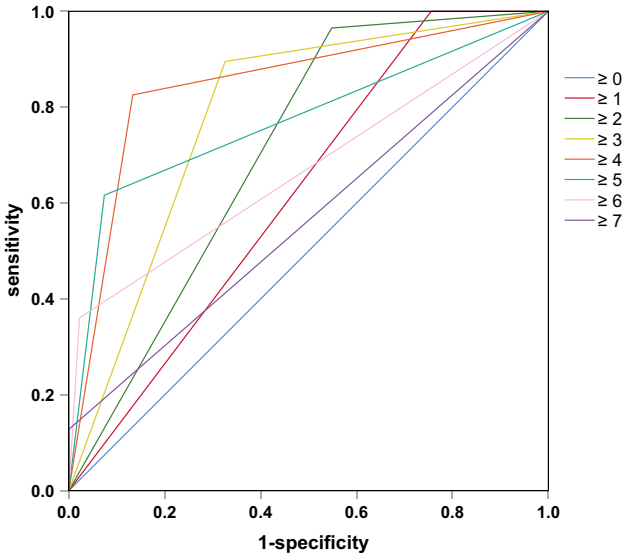

Fig. 3 ROC curves for predicting *HER2* amplification compared by number of histologic characteristics present shows optimal cutoff value of four that gives 82.6% sensitivity and 86.7% specificity with an AUC of 0.85 (95% confidence interval, 0.79–0.90; $p < 0.001$)

Predictive efficacy of histologic criteria in non-IHC 2 + BCs

Additional statistical analyses for histologic characteristics were also performed in the 2013 non-IHC 2 + group for validating their predictive efficacy. High nuclear grade, nuclear size variation, distinct cellular membrane, and high TILs were significantly associated with *HER2* amplification in this group. Tubule formation and apical snouts were not available for evaluation as there was no amplified case showing those features. No obvious correlations were observed in nuclear size, prominent nucleoli, high mitotic rate, or necrosis. Here, 59 cases reclassified as IHC 1 + when applying 2018 ASCO/CAP guidelines were combined, and a total of 83 cases were re-evaluated for 12 histologic predictors. The strong predictive value of four aforementioned characteristics, including high nuclear grade, nuclear size variation, distinct cellular membrane, and high TILs, was also demonstrated in this analysis. Moreover, tubule formation < 10% and no apical snout were also significantly associated with *HER2* amplification.

Table 5 Results of receiver-operating characteristics (ROC) curve analyses for optimal cutoff value

	Cutoff	Sensitivity (%)	Specificity (%)	AUC (95% CI)
No. of characteristics present	≥ 0	100.0	0.0	0.50 (0.42–0.58)
	≥ 1	100.0	24.4	0.62 (0.55–0.70)
	≥ 2	96.5	45.2	0.71 (0.64–0.78)
	≥ 3	89.5	67.4	0.79 (0.72–0.85)
	≥ 4	82.6	86.7	0.85 (0.79–0.90)
	≥ 5	61.6	92.6	0.77 (0.70–0.84)
	≥ 6	36.0	97.8	0.67 (0.59–0.75)
	≥ 7	12.8	100.0	0.56 (0.48–0.64)

AUC, area under the curve; *CI*, confidence interval

Table 6 Comparison of diagnostic efficacy of 2018 ASCO/CAP guidelines and histologic criteria

	Applying 2018 ASCO/CAP guidelines	Applying 2018 ASCO/CAP guidelines + ≥ 4 key histologic criteria
Sensitivity, %	98.9	82.6
Specificity, %	30.1	86.7
Accuracy, %	51.4	85.1

Discussion

The 2018 updated ASCO/CAP guideline recommendations include major changes in HER2 classification, including IHC 2 + tumors re-defined to include "weak to moderate complete membrane staining observed in > 10% of tumor cells." The majority of HER2 testing commences with IHC screening and refers 2 + equivocal results for FISH testing. Therefore, it is important to know how the updated guidelines change the final HER2 classification in IHC 2 + group of BC patients.

IHC 2 + cases in BC are very controversial in the literature. The frequency of IHC 2 + has been reported to be 26 to 39.5% [3, 13]. Interpretation of IHC 2 + score is subject to high intra- and inter-observer variability compared to 0/1 + and 3 + categories. The wide range and very high rates of the 2 + category in BCs in previous studies may support this observation [2, 9, 24, 31]. Therefore, identification of specific and reproducible morphologic characteristics should improve pathologist performance in identifying more HER2-positive BCs among IHC 2 + cases.

According to current ASCO/CAP guidelines, there is no need for complementary FISH testing of IHC 0 or 1 + BCs. However, data on FISH results in IHC 0/1 + cases show a wide range of *HER2* amplification rates from 0.8 to 14% [10, 14, 20, 25]. As therapy targeted against HER2 has significantly improved prognosis in patients with *HER2*-amplified BC, identification of a subset of patients most likely to benefit from targeted therapy in the IHC 0/1 + group is a high priority.

In this retrospective review, 280 IHC 2 + tumors were analyzed to identify which pathologic tumor factor might predict HER2 status in tumors showing equivocal HER2 staining. Because the updated ASCO/CAP guidelines have moved to more stringent criteria regarding HER2 IHC interpretation than previous 2013 guidelines, our data was analyzed by considering the different criteria defining a *HER2*-amplified tumor. We sought to ascertain whether significant differences exist between the guidelines.

The utility of several well-established and some less well-appreciated histologic characteristics in identifying *HER2*-amplified BCs was assessed. Consistent with findings in previous studies [4, 11, 15], poorly differentiated or high-grade morphologic features were a strong predictive marker for *HER2* amplification. We defined seven histologic characteristics that are statistically significant in separating *HER2*-amplified from unamplified BCs. These seven histologic features were validated as persistent predictors for *HER2* amplification in accordance with 2018 guidelines. Of note, for every 1-point increase in the number of key histologic characteristics identified per case, there was an incremental increase in the odds of amplification. The presence of four or more criteria could significantly indicate *HER2* amplification. Therefore, our results provide specific and reproducible morphologic characteristics for separating *HER2*-amplified and unamplified groups among IHC 2 + cases. Other studies have investigated histopathologic characteristics predicting HER2 status, but to the best of our knowledge, this is the first study to provide specific and detailed morphologic features easily assessed in routine practice. While predictive impact is important, if they are only rarely present in amplified samples, then their helpfulness in accurate diagnosis becomes questionable. Hence, the prevalence of helpful characteristics is even more critical. This is exemplified by our data of helpful features present in over 45% of cases.

Secondly, the predictive impact of histologic features in the non-IHC 2 + group was validated. The results confirmed that four novel histologic features, including high nuclear

grade, nuclear size variation, distinct nuclear membrane, and high TILs, could predict *HER2* amplification status irrespective of HER2 IHC results. Therefore, our findings suggest that not only IHC 2 + but also IHC 0 or 1 + should be considered for confirmative FISH testing when certain histologic features are shown on H&E evaluation. Taken together, our data suggest that a proper algorithm for trastuzumab therapy decisions in cases scored IHC 2 + should consider FISH (with respect to ASCO/CAP guidelines) with a combination of histologic features and IHC results.

Lastly, HER2 IHC, FISH results, and final HER2 status of 280 cases were reviewed to compare 2013 with 2018 guidelines. Our data have shown that implementation of the 2018 ASCO/CAP guidelines has changed IHC categorization in 59 of 280 (21%) cases. In our comparison, use of the 2018 updated guidelines has led to an 8% increase of *HER2* amplification rate in the IHC 2 + group. Therefore, our study suggests that the updated guidelines could reduce the false-positive rate and unnecessary FISH tests, which are costly and time consuming. This will consequently increase the efficacy of selecting a subset of patients that will benefit from further FISH testing. We further suggest the use of 2018 guidelines in combination with histologic evaluation for four or more key morphologic criteria to significantly improve diagnostic accuracy and specificity.

Of the 59 cases reclassified as IHC 1 +, only 1 case demonstrated *HER2* amplification by FISH. Review of this discordant case revealed that it had focal micropapillary features. Micropapillary carcinoma is a specific type of BC characterized by cuboidal to columnar cells with finely granular or dense eosinophilic cytoplasm that forms morule-like clusters surrounded by clear spaces [22, 26]. The 2018 guidelines suggest that micropapillary carcinoma with HER2 IHC staining that is intense but incomplete (basolateral or U-shaped) could be considered 1 + and may actually be *HER2*-amplified by FISH. Thus, it is recommended that pathologists consider reporting these specimens as equivocal (2 +) and perform an alternative testing methodology. As this pattern of incomplete expression is also present in some cases of micropapillary carcinoma associated with *HER2* amplification, our study provides additional support for the ASCO/CAP recommendation that an alternate testing methodology be considered in cases of micropapillary carcinoma with intense but incomplete expression of HER2. Small sample size of BCs with micropapillary features is a limitation in this study. Future study using a larger sample size might identify important variables with statistical significance to determine HER2 status in micropapillary carcinoma.

In conclusion, this study reveals specific histologic criteria that are helpful in identifying *HER2* amplification and are applicable by both very experienced and less experienced pathologists. Additionally, our study showed that use of 2018 ASCO/CAP guidelines has improved diagnostic accuracy of HER2 status within the equivocal immunostaining group. Hence, a combination of key histologic characteristics and 2018 ASCO/CAP guidelines can potentially maximize identification of *HER2* amplification not only in IHC 2 + BCs, but also in the IHC 0/1 + group.

Author contribution **Hye Won Hwang**: conceptualization, investigation, methodology, formal analysis, writing—original draft, and writing—review and editing. **Soon Auck Hong**: data curation, investigation, methodology, formal analysis, validation, visualization. **Seok Jin Nam**: data curation, investigation, validation. **Seok Won Kim**: data curation, investigation, validation. **Jeong Eon Lee**: data curation, investigation, validation. **Jong-Han Yu**: data curation, investigation, validation. **Se Kyung Lee**: data curation, investigation, validation. **Soo Youn Cho**: conceptualization, investigation, methodology, formal analysis, writing—original draft, and writing—review and editing. **Eun Yoon Cho**: conceptualization, investigation, methodology, formal analysis, writing—original draft, and writing—review and editing.

Data availability All data relevant to the study are included in the article.

Code availability Not applicable.

Declarations

Ethics approval This study was approved by the institutions' Research Ethics Board.

Conflict of interest The authors declare no competing interests.

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