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ORIGINAL ARTICLE

Assessment of HER2 using the 2018 ASCO/CAP guideline update for invasive breast cancer: a critical look at cases classified as HER2 2+ by immunohistochemistry

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Abstract

In 2018, the American Society of Clinical Oncology/College of American Pathologists revised the criteria for HER2 immunohistochemistry (IHC) equivocal (2+) classification in their updated guideline. We reviewed invasive breast cancer specimens originally classified as equivocal (2+) under the 2018 guideline that underwent HER2 fluorescence in situ hybridization (FISH) testing from August 2018 to August 2019 at our Canadian reference hospital to investigate cases with ambiguous staining patterns between the 1+ and 2+ definitions. Demographics, pathologic features, and pre-analytic conditions were recorded. The H&E and corresponding HER2 IHC slides were reviewed to confirm tumor type and grade, and classify as HER2 indeterminate, 0, 1+, 2+, or "Intermediate" (staining features between the 1+ and 2+ classifications). FISH testing was performed on 289 cases and 273 met inclusion criteria. The FISH-amplified rate was 12.1%. Upon IHC review, 44.7% (122/273) of cases were reclassified as Intermediate. These cases had incomplete staining with moderate intensity (43/122, 35.3%) and/or <10% complete weak or moderate staining (102/122, 83.6%). Intermediate cases had a significantly lower frequency of amplified FISH results than 2+ cases ($p < 0.0001$), with only four (3.3%) FISH positive and two (1.6%) FISH heterogeneous. Our study highlights the ambiguity in the current guideline for classifying some HER2 IHC patterns. As the rate of gene amplification in these cases was low (4.9%), we recommend adhering to the 2018 HER2 2+ criteria for reflex FISH testing. However, cases with <10% moderate complete staining and certain heterogeneous patterns warrant special consideration. Further descriptive clarification of 1+ criteria is needed.

Keywords ASCO/CAP guidelines · HER2 testing · Invasive breast cancer · HER2 immunohistochemistry · Equivocal

Introduction

Human epidermal growth factor receptor 2 (HER2) is amplified or overexpressed in 15–20% of breast carcinomas and may confer eligibility for treatment with anti-HER2 targeted therapies [1–7]. In 2007, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) established guidelines for HER2 testing, which have been widely adopted internationally and have helped improve laboratory performance [8]. These guidelines recommend initial HER2 testing by immunohistochemistry (IHC), with staining patterns being grouped into four classifications: 0 and 1+ ("negative"), 2+ ("equivocal"), and 3+ ("positive") [8–10]. In most laboratories, equivocal cases (i.e., those classified as 2+ by IHC) undergo reflex testing by in situ hybridization (ISH) to assess the average *HER2* gene copy number and the ratio of *HER2* gene copy number to chromosome enumeration probe 17 (CEP17) [10]. Patients who have HER2 positive breast cancers by IHC or ISH may be eligible for HER2-targeted therapies.

The original ASCO/CAP HER2 guideline was updated in 2013 and 2018 based on a plethora of published evidence and pathologist feedback [11–15]. The 2013 IHC 2+ classification created some amount of confusion in the pathology community, with its definition given as "circumferential membrane

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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" [9]. The terms "circumferential" and "incomplete" were felt to be contradictory when referring to the same cell [16]. In the 2018 updated guideline, the definition was simplified to "weak to moderate complete membrane staining observed in >10% of tumor cells" [10]. The guideline also states that certain unusual staining patterns should also be considered 2+, including moderate to intense incomplete membrane staining in a basolateral pattern, and intense complete staining within ≤10% of tumor cells ("heterogeneous but very limited in extent"). In contrast, the IHC 1+ classification (negative) is defined as "incomplete membrane staining that is faint/barely perceptible and in >10% of tumor cells" [10].

Since the adoption of the 2018 ASCO/CAP HER2 guideline, we noted that a proportion of cases were difficult to classify as negative (1+) or equivocal (2+), having either incomplete staining of moderate intensity or <10% weak–moderate complete staining. Since an equivocal HER2 IHC result necessitates an expensive molecular reflex test (HER2 fluorescence in situ hybridization (FISH) is approximately $450 CAD), the published guideline should be followed closely to minimize the number of FISH tests required. In practice, however, cases with ambiguous staining patterns between the 1+ and 2+ classifications are likely to be reported as HER2 equivocal and undergo reflex FISH testing to rule out the possibility of *HER2* amplification.

This study investigates the HER2 IHC staining patterns and FISH results of a cohort of invasive breast carcinomas that were reported as equivocal (2+) by IHC. In an effort to contribute to further refinement of the HER2 IHC evaluation criteria in invasive breast cancer, we critically assessed each case for 2018 ASCO/CAP IHC classification criteria and/or other ambiguous staining patterns and correlated these patterns with FISH results.

Materials and methods

Sample collection

We identified all invasive breast cancer specimens that underwent HER2 FISH testing from August 2018 to August 2019 at our Canadian reference HER2 laboratory. Only cases that underwent reflex FISH for equivocal (2+) IHC were included; cases that had FISH testing for quality assurance purposes (reported IHC scores of 0/1+ or 3+), non-breast carcinomas, non-formalin fixative, or lack of confirmed invasive breast carcinoma on available slides were excluded. Primary tumor, resection post-neoadjuvant therapy, and metastases were included from both core biopsy and excisional specimens. Demographics, pathologic features, and pre-analytic conditions were recorded from the pathology report where available. This study was approved by the institutional Research Ethics Board.

Immunohistochemistry

All HER2 IHC and FISH testing was performed at the reference laboratory (accredited by Accreditation Canada). IHC was performed on 4-μm sections of routinely processed, formalin-fixed, paraffin-embedded tissue. Paraffin sections were cut and mounted on positively charged slides (Superfrost Plus Stain; Fisher Scientific, Pittsburgh, PA, USA).

IHC staining was performed with the Ventana Benchmark Ultra autostainer using HER2 clone 4B5 rabbit monoclonal antibody (predilute) and detected with heat-induced epitope retrieval (CC1 36 min) and Ultraview detection kit (all from Ventana Medical System Inc., Roche, Tucson, AZ, USA). On-slide external control tissues with negative, positive, and low-level HER2 amplified samples were utilized.

The hematoxylin and eosin (H&E) and corresponding HER2 IHC slides were reevaluated by two breast pathologists (PB, GB) to confirm tumor type, grade, and HER2 classification, and to assess HER2 staining patterns. For each case, the percentage of complete and incomplete staining with weak, moderate, and strong intensity was recorded, in addition to the presence or absence of basolateral or heterogeneous staining. Intensity of staining was determined by consensus between the two breast pathologists. Basolateral staining was defined as any amount of moderate-intense basolateral membranous reactivity. Heterogeneous staining was defined as distinct areas of differential staining of any intensity, interpreted as possibly related to different cancer clones (biclonal). If possible, HER2 status was classified according to the 2018 ASCO/CAP guideline; cases that had staining features between the 1+ and 2+ classifications were called "Intermediate." Criteria for the Intermediate IHC category were defined as any percentage of incomplete staining with moderate intensity (but without a basolateral pattern) and/or 1–9% complete staining with weak or moderate intensity. If <1% weak complete staining was the only aberrant staining pattern observed, we considered these HER2 IHC negative. FISH-amplified cases that were classified as IHC Intermediate underwent blinded second review of H&E and HER2 slides.

Fluorescence in situ hybridization testing

FISH testing was performed with a dual-probe assay (PathVysion HER2 DNA probe kit; Abbott Molecular, Abbott Park, IL, USA) containing a *HER2* locus-specific probe (LSI *HER2*) and a control probe specific for the pericentric region of chromosome 17 (D17Z1), following the manufacturer's instructions. The following information from dual-probe HER2 FISH reports was recorded for each

case: *HER2*/chromosome enumeration probe 17 (CEP17) ratio, mean *HER2* signals per cell, mean CEP17 signals per cell, and reported HER2 FISH results. Heterogeneous FISH results were treated as FISH amplified in all analyses. Results were interpreted and reported according to the 2018 ASCO/CAP guideline [10].

Technical issues

Pre-analytic technical issues were defined as cold ischemic time ≥60 min and/or >72 h formalin fixation [10]. Perceived analytic technical issues were recorded on slide review, defined as obscuring cytoplasmic staining, crush artefact, on-slide tissue that appeared poorly fixed, and edge effect. Three cases with obscuring cytoplasmic staining (IHC indeterminate) were excluded from all IHC staining analyses. For statistical analyses, technical issues were defined as the presence of a pre-analytic and/or analytic issue. Two cases had unknown cold ischemic times; however, based on the information available, they were inferred to not meet standard pre-analytic conditions.

Statistical analysis

We performed Pearson's chi-squared test on the FISH result data to determine whether Intermediate cases had a significantly lower frequency of amplified FISH results than 2+ cases. Cases with initial HER2 IHC classifications of 0 and 1+ were excluded from this analysis because none were FISH amplified. We conducted an identical chi-squared test on the technical issue data.

To identify which factors were most important for producing amplified FISH results, we fitted a logistic regression model to the FISH result data with a combination of seven fixed effects. Four of these fixed effects were continuous correlates (i.e., the percentages of the sample deemed weak complete, moderate complete, weak incomplete, and moderate incomplete) and three were binary correlates (i.e., presence/absence of basolateral staining, heterogeneous staining, and technical issues). We performed all analyses in R 3.6.1 using the lme4 package [17].

Results

FISH testing was performed on 289 cases during the study period, of which 273 met inclusion criteria from 263 patients. The majority of the patients were female (97.7%) and the median age at diagnosis was 63 years (range 29 to 89 years). Clinical and pathologic features are listed in Table 1. Ten patients had two specimens in the study. Seven of these patients had a breast core biopsy and corresponding excision. All but one of these had similar HER2 IHC and FISH results. One case was scored as Intermediate on the core biopsy and equivocal on the excision; both were FISH non-amplified. The remaining three patients had two specimens from different primary breast carcinomas.

Table 1 Clinicopathologic characteristics of 273 cases of invasive breast carcinoma from 263 patients. *NST* no special type, *ILC* invasive lobular carcinoma

Characteristic	*n*	Proportion
Gender		
Female	257	97.7%
Male	6	2.3%
Age		
Range	29 to 89 years	
Median	63 years	
Mean	61.9 years	
Specimen type		
Breast	250	91.6%
Needle biopsy	117	42.9%
Excision (less than total mastectomy)	73	26.7%
Total mastectomy	54	19.8%
Other (punch biopsy and reduction)	6	2.2%
Lymph node/other metastasis	23	8.4%
Biopsy	16	5.9%
Excision	7	2.6%
Cold ischemic/formalin fixation time		
Meets	221	81.0%
Does not meet	48	17.6%
Cannot be determined	4	1.5%
Tumor grade		
1	24	8.8%
2	156	57.1%
3	93	34.1%
Tumor histotype		
Invasive carcinoma of no special type	202	74.0%
Invasive carcinoma with other features	30	11.0%
ILC or lobular features	22	8.1%
Mixed ductal and lobular	9	3.3%
Other	10	3.7%

Pre-analytic parameters were not met in 48 cases, due to prolonged cold ischemic time ranging from 61 to 113 min (n = 17 of 48, or 35.4%; average 84.5 min) or prolonged formalin fixation time more than 72 h (25/48, 52.1%) or both (6/48, 12.5%). These 48 cases were reclassified as 2+ (20/116, 17.2%), Intermediate (26/122, 21.3%), or 1+ (2/28, 7.1%). The analytic issues of crush artefact and edge effect on small biopsies, and/or on-slide tissue that appeared poorly fixed were observed in 13.0% of cases.

Upon review of all 273 cases initially reported as IHC equivocal, 116 cases (42.5%) were confirmed as HER2 equivocal (2+). Thirty-two cases were reclassified as HER2 negative, with 28 IHC 1+ (10.3%) and four IHC 0 (1.5%). Three cases (1.1%) were reclassified as indeterminate by IHC due to dark cytoplasmic staining obscuring membranes. The remaining 122 cases (44.7%) were reclassified as Intermediate. HER2 Intermediate cases were equally split between breast core needle biopsies (55 cases) and breast excision/mastectomy specimens (55 cases); the remaining 12 HER2 Intermediate cases were from metastatic sites, of which eight were needle biopsies and four were surgical excisions. These Intermediate cases had incomplete staining with moderate intensity (43/122, 35.3%) and/or 1–9% complete staining with weak or moderate intensity (102/122, 83.6%). Of the cases with 1–9% complete staining, 71 cases had weak intensity, 22 cases had moderate intensity, and 9 cases had both weak and moderate intensity complete staining. Twenty-two cases had both moderate incomplete *and* 1–9% weak-moderate complete staining (22/122, 18.0%). Figure 1 shows examples of the various staining patterns encountered. In the whole study cohort, moderate incomplete staining (not in a basolateral pattern) was observed in 102 cases (102/270, 37.8%), of which 81 (30.0%) had ≥10% moderate incomplete staining.

The overall FISH-amplified rate was 12.1% (33/273). Cases classified as Intermediate had a significantly lower frequency of FISH amplification than those classified as 2+ by strict application of the 2018 ASCO/CAP HER2 guideline ($p < 0.0001$) (Fig. 2). The confirmed HER2 IHC equivocal (2+) cases had a FISH-amplified rate of 23.3% (27/116; 95% confidence interval (CI) = 15.6%, 31.0%), while the HER2 Intermediate cases had a FISH-amplified rate of 4.9% (6/122; 95% CI = 1.1%, 8.8%). None of the cases reclassified as HER2 negative by IHC was FISH amplified (0/32).

Table 2 describes the features of the six cases with Intermediate HER2 IHC staining that were amplified by FISH. The cases were from six different patients, all female and ranging in age from 47 to 74 years old. Four (3.3%) were FISH low amplified and two (1.6%) were FISH heterogeneous (i.e., biclonal with amplified and non-amplified clones). Three low amplified cases had a mosaic pattern of weak complete staining in 1–5% of cells. One low amplified case had 30% moderate incomplete staining, also in a mosaic pattern. These four cases had FISH scores showing homogenous tumor cell populations, all with mean HER2 counts around 5.0 and HER2/CEP17 ratios above 2.0. The remaining two cases had a heterogeneous clustered pattern of moderate complete (1–3%) and moderate incomplete (2–10%) staining, and this corresponded to distinct amplified clones (Table 2). On blind re-review of the HER2 IHC of these six cases, five had concordant results meeting our criteria for Intermediate. On first review of case 1, staining was recorded as 5% weak complete and 20% weak incomplete. On second review, staining was recorded as <1% weak complete and 10% weak incomplete, meeting our criteria for HER2 1+. Both times, the pathologists noted that the tissue appeared poorly fixed, despite documentation of adequate pre-analytic conditions.

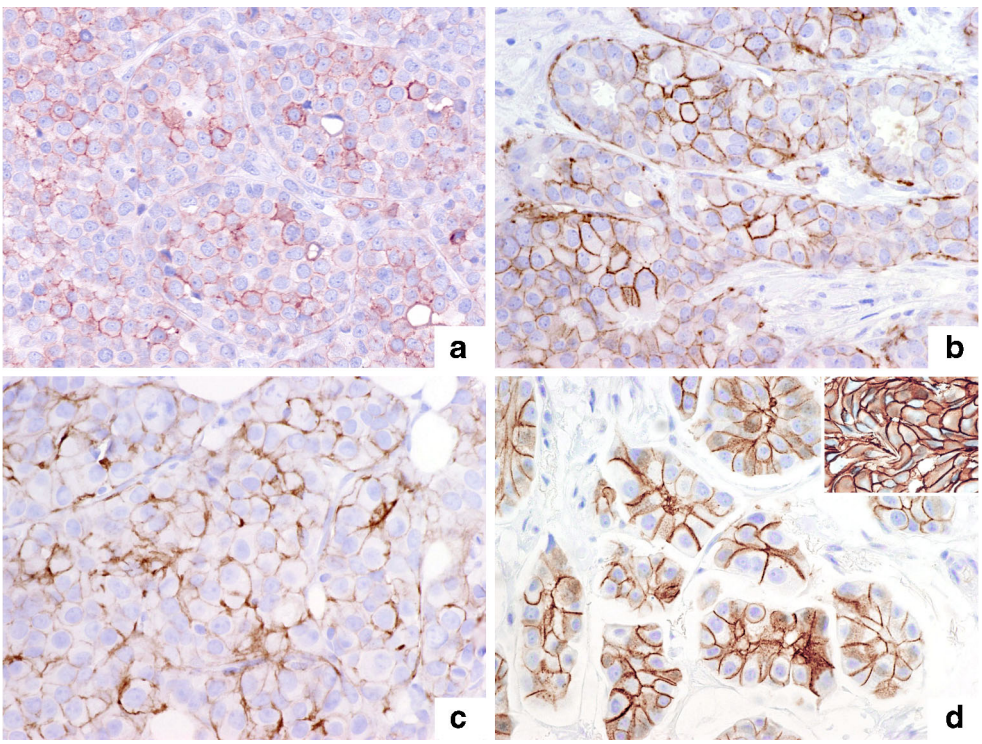

Fig. 1 Staining patterns and intensity for human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC). **a** Weak complete (<10% overall) and incomplete (Intermediate). **b** Moderate complete (<10% overall) and incomplete (Intermediate). **c** Moderate incomplete (Intermediate). **d** Moderate to intense basolateral (2+), inset: intense complete (3+). **a**–**d** Original magnification: ×400

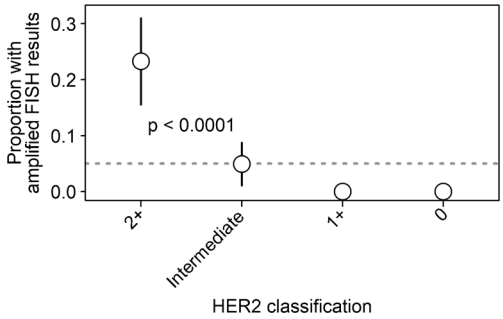

Fig. 2 Proportions of cases with amplified fluorescence in situ hybridization (FISH) results by HER2 classification. Error bars represent 95% confidence intervals, the dashed gray line denotes the accepted 5% discordance rate for validated HER2 testing methods [9], and the *p* value gives the outcome of Pearson's chi-squared test for the FISH result data from 2+ and Intermediate cases

Basolateral staining with moderate intensity was observed in 24 cases, all classified as HER2 equivocal (2+) on IHC, of which six were *HER2* amplified (6/24, 25.0%). Nine of 24 had micropapillary features and two of these were FISH amplified.

We observed heterogeneous staining in 35 cases, with 11 classified as HER2 equivocal and 24 HER2 Intermediate. None of these cases had intense membrane staining. The overall FISH-amplified rate in the cases with heterogeneous staining patterns was 20.0% (7/35), corresponding to five IHC equivocal cases (5/11, 45.5%) and two IHC Intermediate cases (2/24, 8.3%). Six of the seven *HER2* amplified cases with heterogeneous IHC patterns showed corresponding heterogeneous FISH amplification (Fig. 3).

Of the seven correlates we fitted to the FISH result data with the logistic regression model, only the percentage of moderate complete staining had a statistically clear, positive relationship with the probability of an amplified FISH result (Fig. 4). At 5% moderate complete staining, the mean probability of an amplified FISH result was 7.1% (95% CI = 2.4%, 19.2%); at 10% moderate complete staining, this probability increased slightly to 9.3% (95% CI = 3.3%, 23.5%); and at 50% moderate complete staining, this increased to 51.3% (95% CI = 23.8%, 78.0%). Weak complete staining also exhibited a positive relationship with FISH amplification, and the presence of basolateral (10.5%, 95% CI = 1.9%, 42.0%) and heterogeneous staining (17.1%, 95% CI = 6.0%, 40.1%) each increased the mean probability of an amplified FISH result relative to baseline (5.4%, 95% CI = 1.8%, 15.6%), but the uncertainty in these parameter estimates precluded statistical clarity (Fig. 4).

Intermediate cases had a higher rate of technical issues (37.7%, 95% CI = 29.1%, 46.3%) than 2+ cases (25.0%, 95% CI = 17.1%, 32.9%; $p = 0.048$).

Discussion

In this study of a cohort of 273 consecutive cases of invasive breast carcinoma originally reported as HER2 equivocal (2+), careful IHC review revealed that 44.7% of cases fell between the 1+ and 2+ classifications, of which less than 5% were *HER2* amplified by FISH. Our study draws attention to several related issues, including the uncertainty around classification of relatively common HER2 IHC staining patterns and the associated tendency to overcall cases as equivocal.

Few studies have addressed how to classify HER2 IHC with moderate incomplete staining. Yang et al. [18] evaluated HER2 IHC staining in over 2500 IHC 2+ breast cancers according to the 2013 ASCO/CAP guideline. They observed that 1579 cases (62.2%) had ≥10% moderate incomplete staining and that cases with ≤50% moderate incomplete staining had a FISH amplification rate of 8.8%, which was not significantly different from cases with weak incomplete staining. They also found that >50% moderate incomplete staining had a FISH amplification rate of 18.5%, which was not

Table 2 Details of the six cases in the HER2 immunohistochemistry (IHC) Intermediate category that were fluorescence in situ hybridization (FISH) positive or heterogeneous. *CI* cold ischemic time, *FF* formalin fixation time, *IDC* invasive ductal carcinoma, *PLC* pleomorphic lobular carcinoma, *CEP17* chromosome enumeration probe 17

Case	Tumor type and grade	FISH mean HER2|CEP17|ratio	Staining pattern	Heterogeneous staining and FISH	Technical issues	
					Pre-analytic	Analytic
1	IDC grade 2	5.0|1.4|3.5	Complete weak <10%*	N	N	Poor tissue fixation
2	IDC grade 3	5.0|1.4|3.67	Moderate incomplete	N	N	N
3	PLC grade 3	5.3|1.9|2.77	Complete weak <10%	N	Cannot be determined	Poor tissue fixation
4	IDC with focal micropapillary differentiation grade 3	5.1|2.2|2.28	Complete weak <10%	N	Y CI: 74 min FF: 96 h	N
5	IDC grade 3	15.1|2.3|6.7 and 2.0|2.7|0.7	Complete moderate <10%; moderate incomplete	Y	Y CI: 10 min FF: 96 h	N
6	IDC grade 3	11.1|2.9|3.8 and 2.5|2.7|0.9	Complete moderate <10%; moderate incomplete	Y	N	N

*On a second re-review, case 1 was classified as 1+

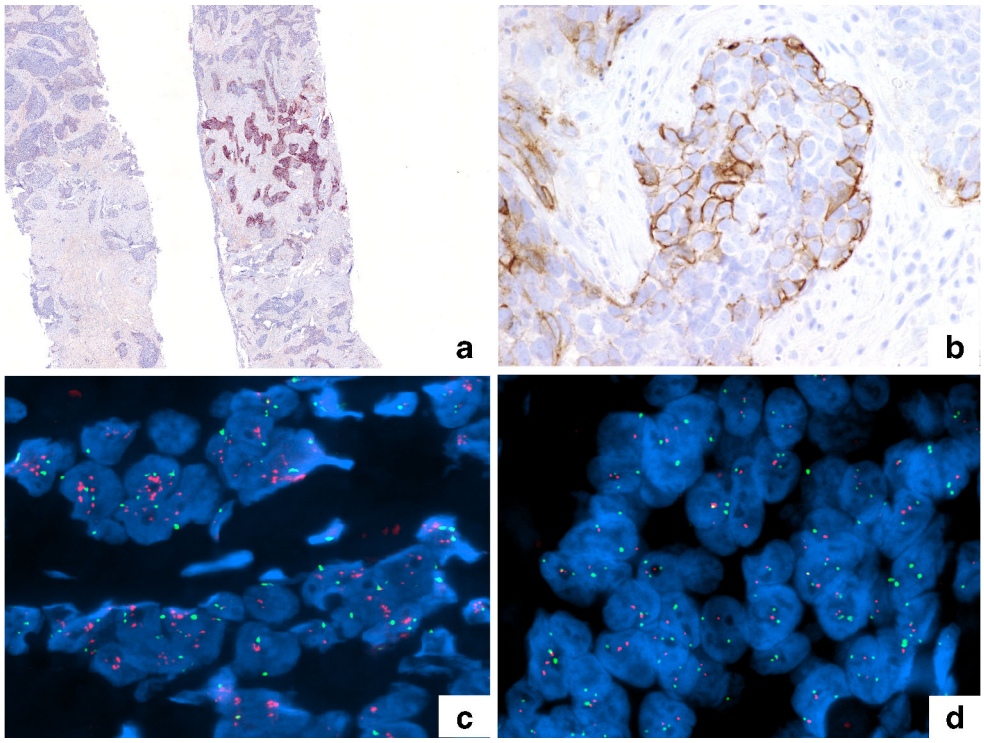

Fig. 3 Example of "Intermediate" HER2 immunohistochemistry in a biclonal heterogeneous pattern (**a**, **b**) with the corresponding heterogeneous pattern of FISH amplification (**c**, **d**). **a** Low magnification of a representative region of heterogeneous staining not meeting 2+ criteria (HER2 IHC overall scored as moderate complete (3%) and moderate incomplete (5%) membrane staining). **b** Higher magnification of a region of moderate incomplete and complete staining. **c** Amplified clone corresponding to the areas of moderate staining. **d** Non-amplified clone corresponding to regions lacking HER2 staining. **a** Original magnification: × 20; **b** original magnification: × 400; **c**, **d**, original magnification: × 1000

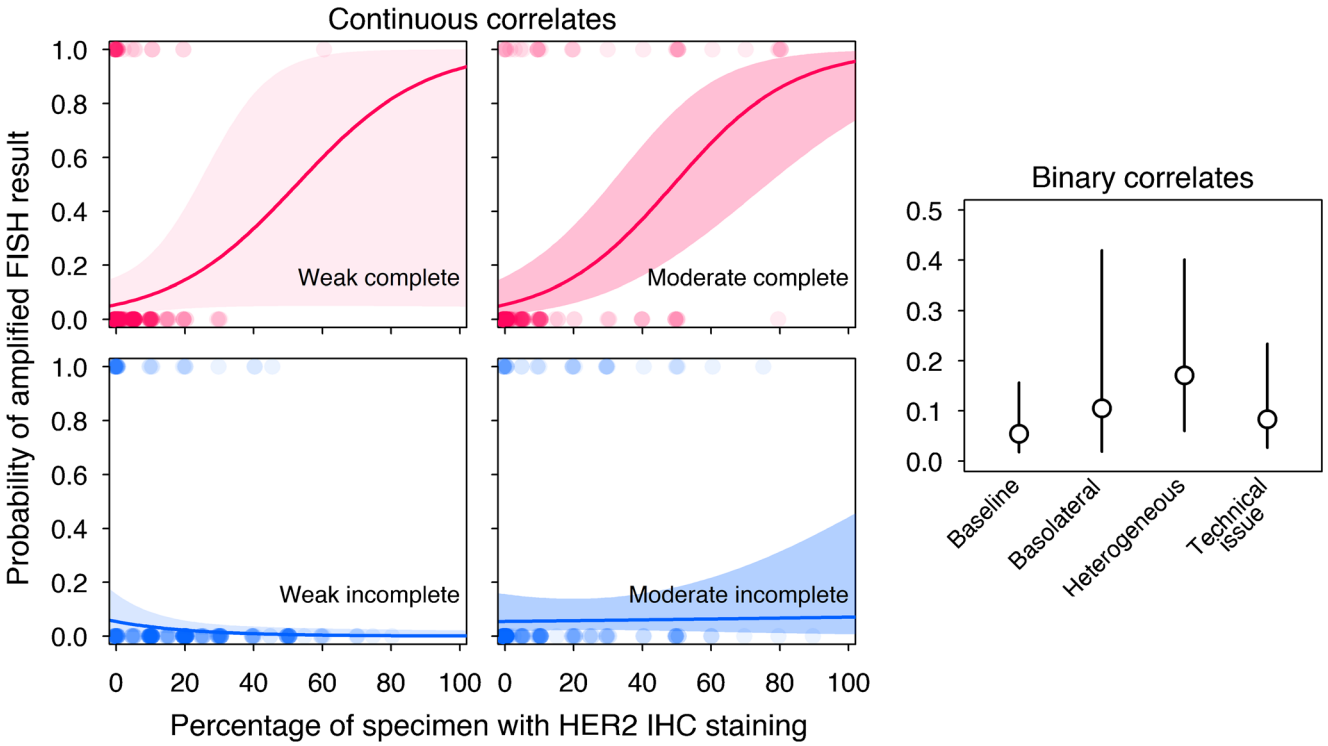

Fig. 4 Effect plots for each of the seven correlates (i.e., HER2 immunohistochemistry (IHC) staining patterns and technical issues) included in the logistic regression model fit to the fluorescence in situ hybridization (FISH) result data. The model predictions for each correlate are isolated such that the other correlates are equal to zero (for the continuous correlates) or not present (for the binary correlates). The probability of an amplified FISH result is visualized across the entire possible range of the four continuous correlates; for these four panels, the points describe the observed data (where 0 is a FISH-negative result and 1 is a FISH-amplified result), the lines denote the mean model predictions, and the shaded regions depict the 95% confidence intervals for the means. The rightmost panel presents the mean predictions and 95% confidence intervals for the three binary correlates, as well as the baseline (i.e., intercept) term from the logistic regression model

significantly different from cases with ≤85% complete staining. Although they did not describe whether cases had multiple staining patterns, it is noteworthy that such a high percentage of cases had a moderate incomplete staining pattern, which is not specifically addressed in the ASCO/CAP HER2 guideline. Yang et al. also found that an increase in the percentage of complete membrane staining was associated with *HER2* amplification. These results are complementary to those from our logistic regression model, which showed a positive relationship between percentage of moderate complete staining and probability of an amplified FISH result.

Weak complete staining in <10% of tumor cells was included in the HER2 IHC 1+ criteria in the 2007 ASCO/CAP guideline [8]. This parameter was removed in the 2013 guideline, and the reasoning for this was not specifically addressed [9]. Classification of <10% moderate complete staining has never been specifically mentioned in the guideline. Our logistic regression model results indicated that with 5% moderate complete staining, the mean probability of an amplified FISH result was 7.1% (95% CI = 2.4%, 19.2%). This supports considering whether any degree of moderate complete staining should be included in the 2+ criteria. Weak complete staining also exhibited a positive relationship with FISH amplification, but with a wide 95% confidence interval. It is not possible to determine whether this low statistical clarity is due to low sample size or if there is truly a lack of effect of weak complete staining on the probability of a positive FISH result.

The definition of heterogeneous staining warranting a 2+ classification in the ASCO/CAP guideline is limited to intense complete staining within ≤10% of tumor cells [10]. We observed 35 cases with heterogeneous staining of any intensity in a pattern suggestive of different cancer clones and found a 20.0% FISH-amplified rate (7/35). Of the seven heterogeneous cases with amplified FISH results, five were IHC 2+ (based on other criteria) and two were IHC Intermediate. Six of these seven cases were reported as FISH heterogeneous, with amplified and non-amplified clones, correlating to the IHC staining pattern. Heterogeneous staining increased the mean probability of an amplified FISH result in the logistic regression model, but the uncertainty around the mean estimate precluded statistical clarity. Based on our results, we suggest that caution should be taken when interpreting cases with any heterogeneous staining pattern, and if a biclonal pattern is felt to be a possibility, then reflex FISH testing should be considered even if IHC equivocal criteria are not specifically met. This would apply to both core biopsy and resection specimens.

Considering both pre-analytic and analytic factors, Intermediate cases had a borderline significantly higher rate of technical issues than 2+ cases. It is likely that the presence of technical issues in some Intermediate cases led to a HER2 IHC classification of 2+ in order to access FISH testing. The majority of pre-analytic technical issues in our cohort were only minor deviations, including two of the Intermediate IHC cases that were FISH amplified. As expected, the presence of technical issues did not show a relationship with probability of an amplified FISH result overall.

Our study highlights the subjectivity of estimating percentage and intensity of IHC staining at the low end of the spectrum, a problem encountered with other predictive markers such as estrogen receptor (ER) and programmed cell death ligand 1 (PD-L1) [19, 20]. Upon secondary, blinded re-review of the IHC, one of the six IHC Intermediate cases that was FISH amplified was reclassified as IHC 1+ (5% weak complete staining vs < 1% weak complete staining). Additionally, in our initial review of all IHC reported as 2+, we reclassified 11.7% as HER2 IHC negative. At our reference laboratory, the HER2 equivocal IHC rate (29%) for primary breast carcinomas during the study period is within the range of reported rates of equivocal staining in the literature (6.9 to 36.4%) [16, 21–25]. Our adherence to testing protocols, internal audits, and participation in multiple external proficiency testing schemes has ensured a consistent and accepted rate of IHC/ISH concordance and HER2 positivity (12.3%) in our reference laboratory [26, 27]. In this study, strict application of the ASCO/CAP guideline criteria was used by two breast pathologists to categorize cases. In routine practice, pathologists might select 2+ classification in ambiguous or even 1+ cases in order to access reflex FISH testing in certain clinical scenarios such as young patient age or hormone receptor negative phenotypes, or in cases with perceived on-slide analytic issues. Alternatively, some pathologists may routinely follow a process of exclusion and categorize all cases that do not meet 2+ criteria as 1+; these differing practices are likely reflected in the wide range of equivocal rates in the literature. Clarification of the guideline as well as a routine targeted second review of ambiguous potential HER2 2+ cases would likely help to improve interobserver agreement.

Recently, the concept of HER2-low breast carcinomas has emerged, referring to IHC 1+ and 2+ cases that are confirmed as ISH negative [28]. There is some evidence that tumors with low levels of HER2 expression but lacking *HER2* gene amplification benefit from novel anti-HER2 antibody-drug conjugates [29, 30]. This is the subject of ongoing clinical trials [31]. Since current HER2 reporting guidelines are designed to be dichotomous, with HER2 positive or negative results, re-evaluation of the HER2 assessment strategy will be needed if such treatments become mainstream. Our study utilizes the current binary definition of HER2 status and focuses on staining patterns that may be associated with gene amplification.

The ASCO/CAP guideline recommends ≥95% concordance between HER2 testing methods for validation [9]. A meta-analysis on HER2 concordance between IHC and FISH found that IHC 0/1+ cases were 96% concordant with FISH results [32]. Of our 122 HER2 Intermediate cases, only 3.3% were FISH positive and 1.6% were FISH heterogeneous.

Based on the accepted 5% discordance rate, it is likely appropriate to consider many of the cases in our Intermediate category as IHC 1+. These include the staining patterns of <10% weak complete and any percentage of moderate incomplete staining. Since two of our six FISH-amplified cases had heterogenous staining patterns not meeting ASCO/CAP criteria for 2+, this pattern, in addition to those with moderate complete staining that is less than 10%, may benefit from FISH testing. A larger sample size would be helpful to confirm our findings. We note that if we included cases with moderate complete staining in <10% and cases with a heterogeneous staining pattern (not meeting current 2+ criteria) within the existing 2+ classification, our overall FISH-amplified rate among the remaining Intermediate IHC cases would be reduced to 3.3%, on par with the IHC/ISH concordance rate for IHC 0/1+ cases in the literature [32]. If we had restricted reflex FISH testing to cases in this expanded 2+ category, we would have achieved a cost savings of $50,850 CAD, or a 41% reduction in spending on reagents and technician time during this study (based on $450 CAD per FISH test).

Our study demonstrates the interpretive ambiguity in the 2018 ASCO/CAP HER2 guideline with respect to HER2 IHC classification. A significant portion (44.7%) of cases have staining patterns between the 1+ and 2+ definitions, and overall, these cases have a low (4.9%) rate of *HER2* amplification. Based on our results, we recommend adhering to the 2018 HER2 2+ criteria in determining cases requiring FISH testing; however, we suggest considering reflex FISH testing for cases with <10% moderate complete staining or staining in a heterogeneous biclonal distribution. Further clarification in the guideline of these common IHC staining patterns, as well as weak complete staining in <10% and any amount of moderate incomplete staining, is needed to facilitate appropriate decision-making and resource allocation in breast cancer treatment.

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Authors' contributions V. Taylor, P. Barnes, and G. Bethune contributed to data collection, analysis, and manuscript writing/editing. S. Godwin contributed to figures, statistical analysis, and manuscript writing/editing. All authors approved the final version of the manuscript.

Data Availability Will be available upon request

Compliance with ethical standards This study was approved by the institutions' Research Ethics Board.

Conflict of interest The authors declare that they have no conflict of interest.

Code availability Not applicable

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