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PRECLINICAL STUDY

HER2 testing in breast cancers: comparison of assays and interpretation using ASCO/CAP 2013 and 2018 guidelines

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Abstract

Purpose HER2 overexpression and gene amplification are routinely tested by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), respectively. In addition, HER2 mRNA expression is also tested by the Oncotype DX assay. Discordance between laboratories among the different assays remains a problem. To improve the routine HER2 reporting, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) updated their guidelines in 2018. Our study will compare concordance of HER2 status by IHC and FISH using ASCO/CAP 2013 and 2018 guidelines with Oncotype DX.

Methods We retrospectively reviewed 657 estrogen receptor positive primary breast cancer cases with available Oncotype DX tests between 2011 and 2018. Medical records were reviewed for HER2 results by IHC, FISH, and Oncotype DX. The HER2 results by different assays and between 2013 and 2018 guidelines were compared.

Results Of the 657 cases, 280 were tested by IHC, FISH, and Oncotype DX. HER2-equivocal cases by IHC 2013 guidelines were all negative (67/67, 100%) by FISH 2018 guidelines and by Oncotype DX. HER2-equivocal cases by FISH 2013 guidelines were all negative (16/16, 100%) by FISH 2018 guidelines, while 15/16 (93.8%) negative and 1/16 (6.2%) equivocal by Oncotype DX. The HER2-equivocal and HER2-negative groups were similar in age, gender, histology, grade, and Ki67 score.

Conclusions HER2 concordance was highest between Oncotype DX (99.6%) and FISH per 2018 guidelines. This suggests that the ASCO/CAP 2018 guidelines improved the accurate stratification of HER2-equivocal cases.

Keywords *HER2* amplification · HER2 overexpression · FISH · ASCO/CAP guidelines · Breast cancer · Oncotype DX

Introduction

The prognostic and predictive markers in breast carcinoma include hormone receptors and the status of HER2 protein overexpression and/or *HER2* gene amplification. HER2-positive breast cancers represent approximately 15–20% of invasive breast carcinoma, and HER2 positivity is associated with an aggressive clinical course and poor outcomes independent of other factors such as tumor size, grade, or hormonal status. The amplification/overexpression of HER2 also predicts a better response to HER2-specific therapies, such as anti-HER2 antibodies and HER2 tyrosine kinase inhibitors. The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommend HER2 testing at the time of initial breast cancer diagnosis and at disease progression [1–3]. In addition, HER2 status is frequently used by oncologists to identify a patient's eligibility for clinical trials. Thus, accurate and

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appropriate HER2 testing is imperative to identify patients who would likely benefit from standard or experimental HER2-specific treatment.

The U.S. Food and Drug Administration (FDA) has approved several assays for clinical HER2 testing. These include identification of *HER2* gene amplification by Fluorescence in situ hybridization (FISH) and HER2 protein overexpression by immunohistochemistry (IHC). To standardize clinical HER2 testing, an ASCO/CAP expert panel established recommendations for HER2 testing in 2007, with significant updates made in 2013 and 2018 [1–3]. The expert panel established criteria for patient eligibility, specimen handling, test validation and assessment, and reporting in order to reduce assay variation and inaccuracies arising from preanalytic, analytic, and postanalytic factors. Despite these standardizations, some degree of non-reproducibility still exists in practice between the interpretations of IHC and ISH results [3]. The ASCO/CAP 2007 guidelines used a 3-tiered system with positive, equivocal, and negative result categories using *HER2*/CEP17 ratio thresholds of < 1.8 and > 2.2 for negative and positive dual-probe assays, respectively, and *HER2* signal thresholds of < 4 and > 6 for negative and positive single-probe assays, respectively [2]. This led to conflicting HER2 single-probe and dual-probe results in a subset of cases, and in response the ASCO/CAP 2013 recommendations incorporated both a *HER2*/CEP17 ratio and *HER2* copy number, with *HER2*/CEP17 ratio ≥ 2 or *HER2* copy number ≥ 6 signifying a positive result [1]. However, the equivocal category continued to create confusion in determining the eligibility of patients for anti-HER2 therapy. Furthermore, some ISH cases with less common patterns resulted in discordant IHC and ISH results. These problematic cases included patients with *polysomy CEP17* with a *HER2*/CEP17 ratio < 2 but a high *HER2* copy number (i.e., *HER2* ≥ 6) or patients with *monosomy CEP17* with a *HER2*/CEP17 ratio ≥ 2 but a low *HER2* copy number (i.e., *HER2* < 4). Indeed, using criteria from the earlier guidelines, monosomy CEP17 cases were interpreted as *HER2* amplified by ISH but mostly as negative by IHC. Some of these patients went on to receive HER2-targeting therapy but showed no significant improvement in disease-free survival (DFS) and overall survival (OS) [4].

The ASCO/CAP 2018 guidelines attempt to more definitively classify these infrequent but challenging ISH results by incorporating IHC results into the interpretation of HER2 status [3, 4]. With the 2018 update, work-up of cases of monosomy CEP17 and polysomy CEP17 cases and equivocal cases with *HER2*/CEP17 ratio < 2 and *HER2* copy number ≥ 4 and < 6 would incorporate IHC results. Thus with the 2013 guidelines, monosomy CEP17 and polysomy CEP17 were interpreted as HER2-positive, but following the 2018 updates, which incorporated IHC results, only 0–8% of monosomy CEP17 and 6–25% of polysomy CEP17 cases were positive [3–5].

In addition to the standard HER2 testing modalities of IHC and FISH, *HER2* expression can also be measured using RNA analysis. Oncotype DX is an mRNA expression assay that uses quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to measure 21 genes (16 cancer-related genes and 5 reference genes) to predict tumor recurrence, typically in estrogen receptor (ER)-positive, HER2-negative, and node-negative breast cancers [6, 7]. Oncotype DX also provides quantitative data on the mRNA expression of *HER2* and reports them as positive, equivocal, or negative results. However, a previous study comparing Oncotype DX with IHC/FISH using the ASCO/CAP 2007 guidelines showed significant discordance between Oncotype DX and IHC/FISH for HER2 status in IHC/FISH-positive and -equivocal cases [8]. To date, there has not been an assessment of HER2 status concordance between Oncotype DX and IHC/FISH using the updated 2018 guidelines. The purpose of this study was to compare concordance of HER2 status between the testing modalities IHC, and FISH using the ASCO/CAP 2013 and 2018 guidelines with Oncotype DX in patients with equivocal and negative IHC/FISH results.

Materials and methods

Patient cohort

We retrospectively reviewed the medical records of 657 patients with ER-positive primary breast cancer who underwent Oncotype DX testing between May 2011 and May 2018 at The University of Texas MD Anderson Cancer Center. We included cases with HER2-negative or -equivocal results by IHC and/or FISH according to the ASCO/CAP 2007 and 2013 guidelines at the time of testing [9]. For this quality assurance study at MD Anderson, we collected data on patient age and gender; tumor histology, grade, and ER, progesterone receptor (PR), HER2, and Ki67 status; and Oncotype DX results. HER2 results by Oncotype DX assay from Genomic Health, Incorporated (GHI) were reported as positive (≥ 11.5 units), equivocal (10.7 to 11.4 units), or negative (< 10.7 units). Approval from the institutional review board was obtained for this study.

Immunohistochemistry

HER2 IHC performed at our institution ($n = 606$) used mouse monoclonal antibody AB8 (NeoMarkers) from 2011 until August 2016 ($n = 489$) and then rabbit monoclonal antibody 4B5 (Ventana) from August 2016 to 2018 ($n = 117$), following the manufacturers' instructions [10]. In 27 cases, HER2 IHC was performed at outside institutions, and the

slides were reviewed at our institution. In 24 cases, HER2 IHC was not performed or slides/results were not available for review. Interpretation of HER2 IHC followed ASCO/CAP 2013 guidelines [1].

Hormone receptor testing was performed on a Leica platform using the monoclonal antibodies ER clone 6F11 (Leica Biosystems, Buffalo Grove, IL) and PR clone PgR 1294 (Dako, Carpinteria, CA). Interpretation of ER and PR results followed modified ASCO/CAP 2010 guidelines [11]: positive if at least 10% positive tumor nuclei staining [12].

Fluorescence in situ hybridization

HER2 FISH testing at our institution was performed on IHC HER2-equivocal (score 2+) cases and a subset of IHC HER2-negative (score 0/1+) cases at the discretion of the pathologist/oncologist. Dual-color FISH was performed using a PathVysion HER2/neu DNA Probe kit (Abbott Molecular, Des Plaines, IL) following standard laboratory procedures according to the manufacturer's recommendations. The average number of *HER2* signals and the average number of chromosome probes (CEP17) per nucleus were assessed in 60 representative invasive tumor cells to generate a *HER2*-to-CEP17 ratio. For cases with equivocal results (*HER2*/CEP17 ratio < 2 and *HER2* copy number from 4 to 6), an additional 60 representative invasive tumor cells were counted for a total of 120 tumor cells. HER2 results following ASCO/CAP 2013 and 2018 guidelines were compared in Supplementary Table 1 [1, 3].

Statistical analysis

Statistical analyses of the association between HER2 subgroups and other categorical variables including gender, histology, grade, ER, PR, Ki67, and HER2 by Oncotype DX were assessed by the Fisher exact test. Correlation between age and HER2 subgroups was examined by the Student *t* test. The correlation of HER2 results between different assays and guidelines was examined using the Kruskal–Wallis test. A *p* value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

In total, we reviewed 657 primary invasive breast cancer cases with available Oncotype DX results. There were 654 (99.5%) women and 3 (0.5%) men (Table 1). Patients' age at initial diagnosis ranged from 30.6 to 84.0 years (median 58.0 years). Tumor histology was predominantly ductal (544, 82.8%), followed by lobular (75, 11.4%) and mixed ductal and lobular (38, 5.8%) carcinoma. Most cases were grade 2 (382, 58.1%), and the remaining were grade 1 (170, 25.9%) and grade 3 (105, 16.0%). All the cases were ER-positive by IHC and Oncotype DX, and most were PR-positive (536, 81.6%)

Table 1 Clinicopathologic features of primary invasive breast cancer tested by Oncotype DX

Characteristics	All patients ($n=657$)
Age, years	
Median	58.0
Range	30.6 to 84.0
Gender, *n* (%)	
Female	654 (99.5%)
Male	3 (0.5%)
Histology, *n* (%)	
Ductal	544 (82.8%)
Lobular	75 (11.4%)
Ductal and lobular	38 (5.8%)
Nottingham histologic grade, *n* (%)	
1	170 (25.9%)
2	382 (58.1%)
3	105 (16.0%)
Estrogen receptor, *n* (%)	
Positive (≥ 10%)	657 (100.0%)
Progesterone receptor, *n* (%)	
Negative (< 1%)	84 (12.8%)
Low Positive (1–9%)	37 (5.6%)
Positive (≥ 10%)	536 (81.6%)
Ki-67 staining, *n* (%)	
Low (< 17%)	340 (65.4%)
Moderate (17–35%)	125 (24.0%)
High (> 35%)	55 (10.6%)
N/A	137
HER2 by immunohistochemistry, *n* (%)	
0	305 (48.2%)
1+	260 (41.1%)
2+	68 (10.7%)
N/A	24
HER2 by FISH 2013 guidelines, *n* (%)	
Negative	281 (94.3%)
Equivocal	16 (5.4%)
Positive	1 (0.3%)
N/A	359
HER2 by IHC and FISH 2018 guidelines, *n* (%)	
Negative	280 (100%)
Positive	0
N/A	377
HER2 by Oncotype DX, *n* (%)	
Negative	654 (99.5%)
Equivocal	3 (0.5%)

and had low Ki67 staining (340/520, 65.4%). Of the 657 patients tested by Oncotype DX, 633 patients had HER2 IHC reviewed at our institution, 298 had HER2 testing by FISH, and 280 had both IHC and FISH reviewed at our institution. Most cases were HER2-negative, 89.3% (565/633) by IHC (score 0 and 1), 94.3% (281/298) by FISH 2013 guidelines, 100% (280/280) by combined FISH/IHC 2018 guidelines, and 99.5% (654/657) by Oncotype DX. There were HER2-equivocal cases, 10.7% (68/633) by IHC, 5.4% (16/298) by FISH 2013 guidelines, and 0.5% (3/657) by Oncotype DX, but none by combined FISH/IHC 2018 guidelines.

Concordance of IHC with FISH 2013 and 2018 guidelines and Oncotype

In 280 cases with HER2 results from the three testing modalities (IHC, FISH, and Oncotype DX), HER2 by IHC was equivocal in 67 and negative in 213 cases. There was no HER2 positive case since it is not indicated for Oncotype DX testing. In Table 2, the HER2 IHC results were compared to FISH results by 2013 guidelines, FISH results by 2018 guidelines, and HER2 results by Oncotype DX. Comparing the 213 IHC-negative cases to FISH results per the 2013 guidelines, 204 (95.8%) were FISH-negative, 8 (3.8%) were FISH-equivocal, and 1 (0.5%) was FISH-positive. Of 67 IHC-equivocal cases, 59 (88.1%) were FISH-negative and 8 (11.9%) were FISH-equivocal. The HER2 FISH-equivocal results according to 2013 guidelines was higher in IHC-equivocal group (11.9%) than in IHC-negative group (3.8%, $p=0.038$). According to the FISH 2018 guidelines, all (213/213, 100%) IHC-negative cases and all (67/67, 100%) IHC-equivocal cases were HER2-negative per the 2018 guidelines. Similarly, comparison of IHC to Oncotype DX demonstrated that majority of IHC-negative cases (212/213, 99.5%) and all IHC-equivocal cases (67/67,100%) are HER2-negative by Oncotype DX (Table 2).

The overall HER2-equivocal results were the highest in assay using IHC (67/280, 23.9%), followed by FISH according to 2013 guidelines (16/280, 5.7%), and Oncotype DX (1/280, 0.4%) (Table 2). No equivocal FISH results were obtained using the 2018 guidelines. The IHC-equivocal cases were all HER2-negative by FISH 2018 guidelines (67/67, 100%) and by Oncotype DX (67/67, 100%).

The HER2-negative agreement between IHC and FISH was lower according to the 2013 guidelines (95.8%, 204/213) than according to the 2018 guidelines (100%, 213/213, $p=0.004$). The negative agreement between IHC and Oncotype DX was 99.5% (212/213). The overall HER2 concordance between IHC and FISH was 75.7% (212/280) according to the 2013 guidelines and 76.1% (213/280) according to the 2018 guidelines. The overall HER2 concordance between IHC and Oncotype DX was 75.7% (212/280). The discordant results were predominantly between equivocal and negative categories.

Concordance between FISH and Oncotype DX

In Table 3, the HER2 FISH results classified using the 2013 guidelines were compared to HER2 results by FISH per 2018 guidelines and by Oncotype DX. All the 263 HER2-negative cases by FISH 2013 guidelines were consistently negative by FISH 2018 guidelines and by Oncotype DX. In comparison, the 16 HER2-equivocal cases by FISH 2013 guidelines were HER2-negative in all the 16 (100%) cases by FISH 2018 guidelines and HER2-negative in 15 (93.8%) cases by Oncotype DX. The 1 HER2-positive case by FISH 2013 guidelines was negative by both FISH 2018 guidelines and Oncotype DX.

In Table 4, the 16 equivocal and 1 positive cases by FISH 2013 guidelines were individually evaluated and compared to FISH 2018 guidelines, IHC and Oncotype DX. The 1 HER2-positive case by FISH 2013 guidelines was based on

Table 2 Concordance of HER2 IHC results with HER2 results by FISH and Oncotype DX

Assays	Total ($n=280$)	HER2 immunohistochemistry		*P* value
		Negative ($n=213$)	Equivocal ($n=67$)	
HER2 by FISH 2013 guidelines, *n* (%)				0.038
Negative	263 (93.9%)	204 (95.8%)	59 (88.1%)	
Equivocal	16 (5.7%)	8 (3.8%)	8 (11.9%)	
Positive	1 (0.4%)	1 (0.5%)	0	
HER2 by FISH 2018 guidelines, *n* (%)				1
Negative	280 (100%)	213 (100%)	67 (100%)	
Positive	0	0	0	
HER2 by Oncotype DX, *n* (%)				1
Negative	279 (99.6%)	212 (99.5%)	67 (100%)	
Equivocal	1 (0.4%)	1 (0.5%)	0	
Positive	0	0	0	

Comparison between negative and equivocal groups by Fisher exact test

Table 3 Concordance of HER2 FISH results by 2013 guidelines with HER2 results by FISH 2018 guidelines and Oncotype DX

Assays	Total ($n=280$)	HER2 FISH by 2013 guidelines			*P* value
		Negative ($n=263$)	Equivocal ($n=16$)	Positive ($n=1$)	
HER2 by FISH 2018 guidelines, *n* (%)					1
Negative	280 (100%)	263 (100%)	16 (100%)	1	
Positive	0	0	0	0	
HER2 by Oncotype DX, *n* (%)					0.057
Negative	279 (99.6%)	263 (100%)	15 (93.8%)	1	
Equivocal	1 (0.4%)	0	1 (6.3%)	0	
Positive	0	0	0	0	

Comparison between negative and equivocal groups by Fisher exact test

its *HER2*/CEP17 ratio = 2.0, but its *HER2* signals was at 3.5, less than 4 copies per cell. This case was reported as equivocal for *HER2* amplification using the 2007 guidelines and was HER2-negative by IHC (score 1 +), by FISH 2018 guidelines and by Oncotype DX (Table 2). The 16 equivocal cases per FISH 2013 guidelines were all interpreted as HER2-negative by FISH 2018 guidelines (16/16) and majority interpreted as HER2-negative by Oncotype DX (15/16). Of note, the 1 equivocal case by Oncotype DX was also interpreted as HER2-equivocal by FISH 2013 guidelines (*HER2*/CEP17 ratio of 1.5 and *HER2* signals of 4.1) but HER2-negative by IHC (score 1 +) and by FISH 2018 guidelines (Table 4; Fig. 1).

The HER2 negative agreement was 100% (263/263) between FISH 2013 guidelines and either FISH 2018 guidelines or Oncotype DX, and 99.6% (279/280) between FISH 2018 guidelines and Oncotype DX. The overall HER2 concordance was 94.3% (264/280) between FISH 2013 guidelines and Oncotype DX as compared to 99.6% (279/280) by the FISH 2018 guidelines and Oncotype DX (Table 5). The discordance was predominantly due to differences between the equivocal and negative categories using the 2013 guidelines.

Table 4 Cases with HER2-equivocal and monosomy CEP17 results by FISH 2013

Patient identifier	Fish					IHC	Oncotype DX	
	HER2 result, 2013	*HER2*/CEP17 ratio	HER2 copy #	CEP17 copy #	HER2 result, 2018	HER2 result (Score)	HER2 result	HER2 score
PT0630	Equivocal	1.2	4.5	3.6	Negative	Negative (0)	Negative	8.6
PT0222	Equivocal	1.9	5.9	3.1	Negative	Negative (0)	Negative	9.7
PT0056	Equivocal	1.1	4.1	3.6	Negative	Negative (1 +)	Negative	9.0
PT0647	Equivocal	1.1	5.0	4.4	Negative	Negative (1 +)	Negative	10.0
PT0262	Equivocal	1.1	5.0	4.7	Negative	Negative (1 +)	Negative	8.6
PT0162	Equivocal	1.3	4.3	3.2	Negative	Negative (1 +)	Negative	7.6
PT0024	Equivocal	1.5	4.1	2.8	Negative	Negative (1 +)	Equivocal	10.7
PT0670	Equivocal	1.9	4.3	2.2	Negative	Negative (1 +)	Negative	9.4
PT0450	Positive	2.0	3.5	1.7	Negative	Negative (1 +)	Negative	9.2
PT0610	Equivocal	1.0	4.2	4.1	Negative	Equivocal (2 +)	Negative	10.1
PT1280	Equivocal	1.5	4.1	2.7	Negative	Equivocal (2 +)	Negative	10.1
PT0658	Equivocal	1.5	5.0	3.3	Negative	Equivocal (2 +)	Negative	8.3
PT0854	Equivocal	1.6	4.9	3.0	Negative	Equivocal (2 +)	Negative	9.0
PT1088	Equivocal	1.6	4.1	2.5	Negative	Equivocal (2 +)	Negative	8.8
PT0091	Equivocal	1.7	5.0	3.0	Negative	Equivocal (2 +)	Negative	10.4
PT0812	Equivocal	1.8	4.1	2.3	Negative	Equivocal (2 +)	Negative	9.3
PT0708	Equivocal	1.9	5.0	2.7	Negative	Equivocal (2 +)	Negative	9.8

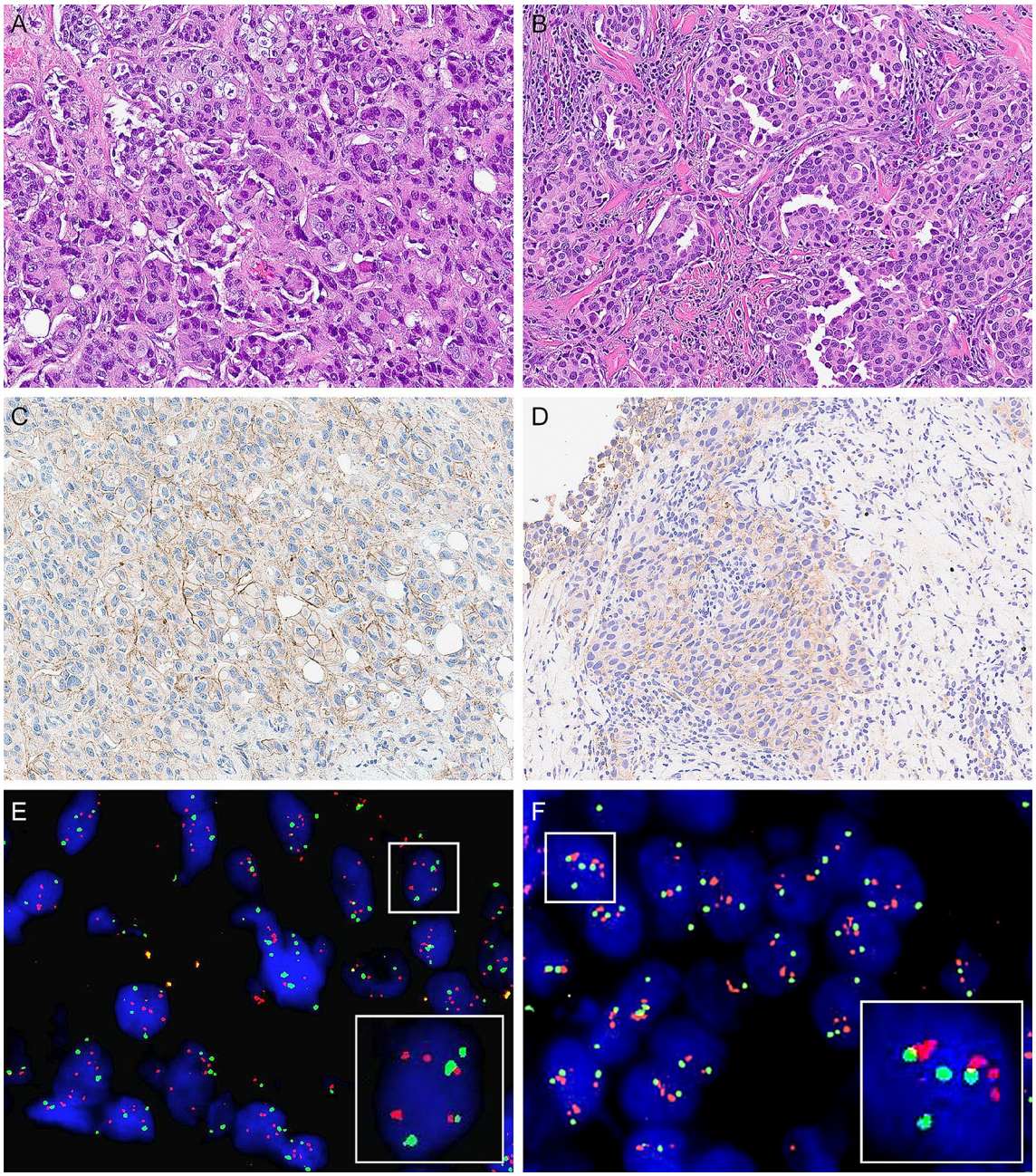

Fig. 1 Representative IHC and FISH images illustrate HER2-equivocal results. **a**,**c**,**e** Grade 2 invasive ductal carcinoma with positive ER, positive PR, and Ki67 proliferative index of 15%. HER2 IHC was equivocal (score 2+). HER2 FISH was equivocal (*HER2*/CEP17 ratio: 1.61, average *HER2* signals per cell: 4.08). HER2 by Oncotype DX was negative. **b**,**d**,**f** Grade 2 invasive ductal carcinoma with positive ER, positive PR, and Ki67 proliferative index of 25%. HER2 IHC was negative (1+). HER2 FISH was equivocal (*HER2*/CEP17 ratio: 1.5, average *HER2* signals per cell: 4.1). HER2 by Oncotype DX was equivocal

Correlation with clinical, pathological, and molecular features

The distribution of age, gender, histology, Nottingham histologic grade, and Ki67 score did not differ between the HER2-negative (score 0/1+) and HER2-equivocal (score 2+) groups by IHC (Supplementary Table 2) or between the HER2-negative (group 5) and HER2-equivocal (group 4) sets by FISH (Supplementary Table 3). PR-negative tumors were more frequent in the IHC HER2-equivocal group (16/67, 23.9%) than in the IHC HER2-negative group (20/213, 9.4%; $p=0.012$). No difference in PR-negative rate

Table 5 Concordance of HER2 IHC and FISH results by 2018 guidelines with HER2 results by Oncotype DX

Assays	Total ($n=280$)	HER2 by IHC/FISH 2018 guidelines		P value
		Negative ($n=280$)	Positive ($n=0$)	
HER2 by Oncotype DX, *n* (%)				1
Negative	279 (99.6%%)	279 (99.6%)	0	
Equivocal	1 (0.4%)	1 (0.4%)		
Positive	0	0	0	

was observed between the FISH HER2-equivocal group (3/16, 18.8%) and the FISH HER2-negative group (33/263, 12.5%; $p=0.542$).

Discussion

Despite efforts to standardize HER2 testing, discordant results have been reported with IHC and FISH. To improve the accuracy of HER2 testing, the ASCO/CAP guidelines were updated in 2018 to include both ISH and IHC results in the interpretation of HER2 status. In this study, we evaluated the concordance between HER2 results by IHC and FISH using the 2013 and 2018 ASCO/CAP guidelines and compared the results to mRNA expression by RT-PCR as reported by the Oncotype DX test. To our knowledge, this is the first study to evaluate the impact of the 2018 guidelines on the concordance between IHC/FISH and Oncotype DX assay results.

Comparison of HER2 testing by IHC and FISH

It has been suggested that some level of discordance is to be expected because IHC and FISH measure HER2 protein expression and *HER2* gene copy number, respectively. Indeed, discordance between IHC and FISH has been reported in 2.0% to 11.0% of cases [13–15]. HER2-overexpressed (IHC score 3+) and *HER2* non-amplified tumors are extremely rare (< 0.1%) but have been reported [4]. Most of these discrepancies involve cases with less common ISH patterns, such as a normal *HER2*/CEP17 ratio but high *HER2* copy number (polysomy CEP17).

False-negative IHC results have been reported in 1–3% of HER2-negative cases (scores 0/1+) using either the 2007 or 2013 guidelines [14, 16, 17]. These false-negative results were typically in cases with *HER2* amplification at low levels, monosomy CEP17, or polysomy CEP17 or in equivocal cases with an elevated reflex FISH ratio by alternative chromosome 17 probe. In a study by Press et al. based on the BCIRG-005 and BCIRG-006 trials, 7/9 (77.8%) polysomy CEP17 cases, 32/35 (91.4%) monosomy CEP17 cases, and 87/99 (87.9%) cases with low levels of *HER2* amplification (*HER2* ≥ 4.0–5.99 and ratio ≥ 2) were HER2-negative by IHC [4].

Similarly, discordance between single-probe and dual-probe HER2 ISH results categorized according to the ASCO/CAP 2007 and 2013 guidelines have been reported [13, 16]. Interestingly, patients with monosomy CEP17 were considered HER2-positive on the basis of an increased *HER2*/CEP17 ratio by the FDA and 2013 ASCO/CAP guidelines but did not show benefit from anti-HER2 therapy [4]. Of patients not receiving anti-HER2 treatment, the polysomy CEP17 group had worse DFS than the FISH HER2-negative group (*HER2*/CEP17 ratio < 2 and *HER2* < 4). The OS and DFS of patients with FISH HER2-equivocal results were not different from those of the FISH HER2-negative group [4].

Impact of 2018 ASCO/CAP guidelines on HER2 results

The previous 2013 ASCO/CAP guidelines acknowledged the important gaps in the literature specifically with respect to the equivocal category in the IHC and FISH tests, which produce continuous variables. However, patients are treated on the basis of a binary decision: patients with positive HER2 results receive anti-HER2 therapy and those with negative HER2 results do not receive anti-HER2 therapy. The "grey-zone" equivocal category creates a dilemma for oncologists: determining whether patients with equivocal results should or should not receive HER2-targeted therapy. Previous attempts to address this issue with alternative reference probes simply complicated the issue because loss of reference signal(s) does not entirely correlate with *HER2* amplification status [18]. In a study by Sneige et al. of the prognostic significance of HER2-equivocal cases utilizing alternative reference probes, the OS and DFS were similar between cases with unchanged equivocal status and cases upgraded to HER2-positive with SMS/RARA probes, suggesting that these alternative reference probes might erroneously upgrade HER2 status [19].

The 2018 ASCO/CAP guidelines integrate both IHC and dual-probe ISH results to further delineate ambiguous cases that fall in the monosomy CEP17, polysomy CEP17, and equivocal FISH categories (groups 2 through 4). Several recent studies have shown the impact of the 2018 ASCO/

CAP guidelines on increasing the HER2-negative rate and reducing the HER2-positive interpretation [5, 20–22]. These changes in HER2 interpretation mainly affect FISH groups 2 through 4, with no effect on FISH groups 1 and 5 (Supplementary Table 1). All the monosomy CEP17 FISH cases were re-classified from HER2-positive according to the 2013 guidelines to HER2-negative according to the 2018 guidelines [5, 20–22]. With the incorporation of IHC results in the 2018 guidelines, the majority of polysomy CEP17 cases remain HER2-positive, with a small proportion (10–25%) falling into the HER2-negative category [20–22]. The previous equivocal category, or group 4, is predominately considered HER2-negative (and rarely [0–6%] HER2-positive) according to the 2018 guidelines [21, 22]. Furthermore, as shown in a study from our institution (19), the survival analysis of equivocal cases was no different than HER2-negative cases. Our study showed that all IHC HER2-equivocal cases were HER2-negative by the 2018 guidelines and HER2-negative by Oncotype DX. Furthermore, there was an increase in the HER2-negative rate by FISH, from 263/280 (93.9%) per the 2013 guidelines compared to 280/280 (100%) using the combined IHC and FISH results of the 2018 guidelines. These changes reduced the equivocal FISH cases obtained with the 2013 guidelines which were converted to HER2 negative by 2018 guidelines. These results suggest that the 2018 guidelines can eliminate ambiguity and more accurately interpret the final HER2 results for equivocal cases.

Comparison of IHC, FISH, and RT-PCR by Oncotype DX

It is well established that Oncotype DX can predict the risk of recurrence in ER-positive HER2-negative tumors. However, the clinical implications of HER2 mRNA levels detected by Oncotype DX are still under investigation. Multiple previous studies have shown a high concordance (96% to 99%) between IHC, FISH, and Oncotype DX testing [8, 23–26]. Hanna et al. showed high concordance between HER2 IHC and Oncotype DX assay, with only a 2.7% discrepancy rate [25]. These discrepant cases included 9 IHC HER2-positive cases that were either HER2-negative (5) or -equivocal (4) by Oncotype DX and 4 IHC HER2-negative cases that were equivocal by Oncotype DX. The study suggested that HER2-positive cases could be undervalued by Oncotype DX due to preanalytical contamination or interference from benign cellular stroma and inflammatory cells.

Using the ASCO/CAP 2007 guidelines, Dabbs et al. showed high negative agreement (779/784, 99%) between IHC/FISH and Oncotype DX [8]. However, the positive agreement was low; only 10/36 (28%) HER2-positive cases by IHC/FISH were positive by Oncotype DX. The same study showed that all 23 HER2-equivocal cases by IHC/FISH were reported as HER2-negative by Oncotype DX. Similar findings have been reported in separate studies using either the 2007 or 2013 guidelines [24, 26, 27]. These discrepancies had been attributed to the suboptimal micro-dissection and heterogeneous amplification of *HER2* [28]. The authors concluded that Oncotype DX could not accurately identify HER2-positive cases and discouraged decisions preferentially made on the basis of Oncotype DX.

In our study, we also found high negative agreement between IHC/FISH and Oncotype DX. The negative agreement between IHC and Oncotype DX is 99.5% (212/213). The negative agreement between FISH per 2013 guidelines and Oncotype DX is 100% (263/263). Furthermore, HER2-equivocal cases by FISH per 2013 guidelines were predominantly HER2-negative by Oncotype DX (93.8%).

Our study included HER2-negative and HER2-equivocal cases at the time of reporting owing to the inclusion criteria for Oncotype DX testing. Therefore, the assessment of the utility of these testing modalities in HER2-positive cases is limited. There was only 1 HER2-positive case by FISH 2013 guidelines in this study. This "positive" case had FISH monosomy CEP17 and negative HER2 status by IHC, Oncotype DX, and the 2018 guidelines, suggesting that the latter negative result was a more accurate assessment of HER2 status in this case. In addition, clinical follow-up and survival analyses of our equivocal/ "discrepant" cases would be an area of future investigation. Further study of these cases with respect to response to HER2-targeted treatment and clinical outcome would advance our understanding of this challenging entity.

In summary, concordance between Oncotype DX and FISH assay increased from 94.3% using the 2013 guidelines to 99.6% using the 2018 guidelines. Similarly, high negative agreement was observed between the combined IHC/FISH and Oncotype DX results. Most importantly, we noted consistent classification of monosomy CEP17 and equivocal cases by FISH per the 2018 guidelines and Oncotype DX, indicating that the 2018 guidelines can accurately classify ambiguous categories to ensure the accuracy of HER2 results.

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Author contributions HC and CTA conceived the study. LM, CTA, NS, HC were involved in the design of the study. SD, IY, KL and HC were involved in the acquisition of data. CTA and HC were involved in study supervision. LM, CTA, SKG, RLB, YW and HC were involved in the analysis and interpretation of data. LM, CTA, HC and NS drafted the manuscript. All authors critically reviewed and approved the manuscript before submission.

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Data availability The datasets generated during the current study are not publicly available due to individual privacy but are available from the corresponding author on reasonable request.

Declarations

Conflict of interest All authors declare they have no conflicts of interest.

Ethical approval This project was approved by the Institutional Review Board of the M.D. Anderson Cancer (PA18-0796).

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