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ARTICLE

Clinical significance of quantitative categorization of HER2 fluorescent in situ hybridization results in invasive breast cancer patients treated with HER2-targeted agents

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Abstract

HER2 (*ERBB2*) gene status serves as a strong predictive marker of response to HER2-targeted agents in invasive breast cancers, albeit with heterogeneous response. Our aim was to determine the distribution and prognosis of HER2 groups by fluorescent in situ hybridization (FISH) using the updated 2018 American Society of Clinical Oncology–College of American Pathologist (ASCO–CAP) guidelines. We identified 226 cases of equivocal or positive HER2 FISH invasive breast cancer (interpreted by ASCO–CAP guidelines at the time of reporting) who received HER2-targeted agents from 2006 to 2017. We subcategorized Group 1 further into three subgroups: low amplified (HER2/CEP17 ratio ≥ 2.0–2.99, mean HER2/cell 4.0–5.9), amplified (HER2/CEP17 ratio ≥ 2.0–2.99, mean HER2/cell ≥ 6), and excessive amplification (HER2/CEP17 ratio ≥ 3.0, mean HER2/cell ≥ 4.0). Outcomes studied were recurrence, metastasis, second breast primary, disease-specific survival (DSS), and overall survival (OS). Univariate analysis showed that the five categories of HER2 FISH were significantly associated with OS ($p < 0.01$), specifically higher HER2 amplification was associated with fewer deaths. HER2 FISH status also statistically significantly relates to DFS ($p < 0.01$) and metastasis ($p = 0.01$) but not with recurrence or second breast primary in our study. Tumor type and HER2 ISH Groups are independent predictors for both OS and DFS in our cohort. The proposed Group 1 subcategories were significantly associated with OS ($p < 0.01$) and DFS ($p < 0.01$), excessive HER2 amplification was associated with longer median survival. The Cox regression models showed better survival outcomes for the excessive amplification subgroup than the low amplified subgroup, with OS (hazard ratio = 0.63, 95% CI 0.42–0.93) and DFS (HR = 0.55, 95% CI 0.37–0.83). We demonstrated that in HER2 FISH Group 1 patients, high HER2 amplification was significantly associated with longer OS and DFS; these patients seem to benefit more from HER2-targeted regimens. We recommend reporting these Group 1 subcategories when assessing HER2 FISH.

Introduction

Approximately 15–20% of invasive breast cancers show overexpression of human epidermal growth factor receptor type 2 (HER2) protein, a form of a transmembrane tyrosine kinase receptor, which is encoded by the *ERBB2* gene, located on chromosome 17 (17q12) [1, 2]. Prior to the use of HER2-targeted agents, these aggressive cancers were associated with adverse outcomes. The use of HER2-targeted therapy such as trastuzumab (Herceptin), lapatinib (Tykerb/Tyverb), pertuzumab (Perjeta), and ado-trastuzumab emtansine/T-DM1 (Kadcyla) has significantly improved survival and revolutionized the treatment of HER2-positive breast cancers [3–5]. However, these medications are costly and associated with devastating side effects like cardiotoxicity. Therefore, accurate assessment

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of HER2 status plays a pivotal role in the management of breast cancer in these patients. As a result, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) produced algorithm using immunohistochemistry (IHC) testing and in situ hybridization (ISH) pertaining to standardization and interpretation of HER2 diagnostic tests.

Since its introduction in 2007 [6], the ASCO–CAP guidelines have been updated twice, in 2013 [7] and 2018 [8]. The latest ASCO–CAP guidelines introduced five ISH categories, incorporated IHC testing with ISH in the analysis of a subset of cases, and eliminated the equivocal category. The equivocal category had existed in the preceding two guideline versions and had posed a challenge to many treating oncologists because they lacked clear guidelines on when and how to use HER2-targeted agents.

ASCO–CAP 2018 guidelines [8] introduced five groups based on HER2 testing using validated dual-probe ISH assay. The "classical groups" (Groups 1 and 5), i.e., when both the HER2/chromosome enumeration probe 17 (CEP17) ratio and the number of HER2 signals per cell are simultaneously amplified or not, had the same threshold as described in the 2013 updates. However, the "nonclassical groups" (Groups 2–4), when the ratio and the number of HER2 signals per cell results are discordant with only one being "amplified," had significant changes in this latest 2018 update. This yielded the following categories: Group 1 (HER2/chromosome enumeration probe 17 [CEP17] ratio ≥ 2 + HER2 signal ≥ 4 per cell), Group 2 (HER2/CEP17 ratio ≥ 2 + HER2 signal < 4 per cell), Group 3 (HER2/CEP17 ratio <2 + HER2 signal ≥ 6 per cell), Group 4 (HER2/CEP17 ratio < 2 + HER2 signal ≥ 4–<6 per cell), and Group 5 (HER2/CEP17 ratio < 2 + HER2 signal < 4 per cell). Additional work is required if a nonclassical group (Groups 2–4) is encountered; a final result is reached by combining the IHC with the ISH result, and if the IHC is 2+, then ISH should be reassessed by an additional observer, who has been blinded to previous ISH results, counting at least 20 cells that include the area of invasive cancer with IHC 2+ staining.

The aim of our study is to investigate the impact on invasive breast cancer patients of the categories defined by the 2018 ASCO–CAP guidelines on HER2 classification who received HER2-targeted agents. Also, to describe the histopathologic features and prognosis of HER2 ISH Groups.

Material and methods

Following institutional review board approval from the Henry Ford Health System, we retrospectively retrieved all cases of breast cancer from 2006 to 2017, either primary or metastatic, in which the fluorescent in situ hybridization (FISH) was either positive or equivocal at the time of reporting and the patient subsequently received HER2-targeted agents. Breast cancer patients who had not received HER2-targeted agents were excluded from the study regardless of the FISH results. In addition, cases in which FISH was not performed were excluded.

Histopathology features and other results were directly extracted from our institutional pathology reports, and the clinical information, including outcomes, was retrieved from the patients' electronic medical records. The recorded variables include the following: age, race, type of surgery, type of HER2-targeted therapy, and its complications and whether neoadjuvant, adjuvant, radiotherapy, or hormonal therapy was given. The following pathologic features were recorded: tumor type (ductal carcinoma of no special type [NST], lobular carcinoma, special ductal type carcinoma, or mixed ductal and lobular carcinoma), Nottingham grade, focality (unifocal or multifocal), presence of ductal carcinoma in situ (DCIS), presence of lobular carcinoma in situ, status of final margins for both invasive and DCIS, presence of lymphovascular invasion, treatment effect—if neoadjuvant therapy was given (pathological complete response [pCR], probable or no response) as described in the American Joint Committee on breast cancer staging at the time of reporting [9, 10], tumor stage (pT), lymph node stage (pN), number of positive sentinel lymph nodes, status, and percentage, of both estrogen receptors (ER) and progesterone receptors (PR) by following CAP cancer protocol guidelines and Allred scoring system [11], proliferation index by MIB1 IHC documented in the pathologic report as percentage of strongly positive tumor nuclei of invasive component [11], HER2 IHC (0–3+), and finally HER2 FISH ratio with the average HER2 signals per cell using the dual-probe FISH assay. The outcomes recorded in our study include development of metastasis, second primary breast cancer, recurrence, disease-free survival (DFS), and overall survival (OS).

The above variables were assessed by three of the authors (MA, BA, and HM). The final cohort included a total of 226 patients with breast cancer in which HER2 FISH was performed and HER2-targeted therapy was given.

HER2 immunohistochemistry (IHC)

ER (Clone EP1from Dako, Agilent Technologies, Inc., Santa Clara, CA, USA) and PR (Clone PgR 636 from Dako) stains were performed as standard of practice for all the invasive breast cancers and scored following Allred scoring system [11]. HER2 IHC (HercepTest from Dako) was performed at the time of routine specimen processing and CAP guidelines were followed especially those pertaining to specimen handling, fixation, cold ischemia time, and

interpretation of IHC tests [11]. Results were signed out by experienced breast pathologists.

HER2 fluorescence in situ hybridization (FISH)

Prior to routine sign out, ISH analysis for HER2 protein was performed using the dual-probe HER2/CEP17 FISH assay using Vysis dual FISH probe (FDA Approved PathVysion HER2 DNA Probe Kit from Abbott Molecular, Inc., Abbott Park, Illinois, USA).

HER2 FISH was performed on 226 specimens and HER2 FISH ratio with the average HER2 signals per cell were recorded for all cases. The test was interpreted using the ASCO–CAP guidelines at the time of reporting and then reclassified using the latest 2018 ASCO–CAP guidelines into five groups and by incorporating HER2 IHC, when applicable. FISH results were reviewed by three of the authors (MA, JS, and DC).

Statistical analysis

To assess significance of associations between different variables, we used Mann–Whitney U, chi-square, and Fisher's exact tests. For clinical outcomes, Kaplan–Meier curves were generated and compared using log rank tests. We performed multivariate analysis and additional univariate analysis using Cox proportional hazards models. A p value of <0.05 was considered statistically significant in our study. All tests were performed (by two authors) using R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria).

Results

Over this time period, our laboratory performed a total of 729 FISH tests, out of which 274 patients were either positive or equivocal at the time of reporting. Out of these 274 cases, 226 patients were treated with HER2-targeted agents. The median age of our cohort is 65 years (range 26–98); the racial distribution in our cohort was 58% Caucasians, 24% African Americans, 1.5% other races, and 16.5% unknown. The median HER2 FISH ratio was 2.47 (range 1.18–21) and HER2 signal/cell was 6.30 (range 2.09–21). Surgical intervention in the form of mastectomy, lumpectomy, or excision was recorded in 198 (88%) patients. A total of 70 (31%) patients received neoadjuvant chemotherapy, 153 (68%) hormonal therapy, 188 (83%) adjuvant chemotherapy, and 146 (65%) radiotherapy.

Almost all patients received trastuzumab (Herceptin), either alone in 145 (64%) patients or in combination with pertuzumab (Perjeta) in 72 (32%) patients or lapatinib (Tykerb/Tyverb) in 2 (1%) patients. The combination of lapatinib (Tykerb/Tyverb) with trastuzumab (Herceptin) was part of a clinical trial while the combination of pertuzumab (Perjeta) with trastuzumab (Herceptin) was part of standard of care for high-risk patients and noted more frequently in the neoadjuvant setting. The use of trastuzumab (Herceptin) alone was significantly associated with longer median OS (281.4 weeks, $p < 0.01$) and DFS (262.9 weeks, $p < 0.01$). Controlling for tumor type and 2018 ASCO–CAP ISH Groups (the two independent predicators for OS and DFS in our cohort) showed that the use of trastuzumab (Herceptin) with pertuzumab (Perjeta) or lapatinib (Tykerb/Tyverb) was independently associated with higher hazard ratio (HR) of death than trastuzumab (Herceptin) alone for OS (HR = 1.98, 95% CI 1.41–2.76; $p < 0.01$) and DFS (HR = 2.17, 95% CI 1.50–3.12; $p < 0.01$).

HER2-targeted medication side effects were reported in 27 (12%) patients, 23/226 (10% of the patients) suffered cardiac side effects. The median follow-up was 214.8 weeks (range 4.7–873.1).

Breast cancer cases were 88% ductal (NST), 5% special ductal, 4% lobular, 2% invasive mammary (not specified), and 1% mixed ductal and lobular. The Nottingham grade was 1/well-differentiated in 8 cases, 2/moderately differentiated in 90 cases, 3/poorly differentiated in 115 cases and it was not recorded in 13 cases. The tumor was unifocal in 47 cases while it was multifocal in 23 cases and was not recorded in 156 cases. DCIS was present in 111 cases while lobular carcinoma in situ was found in 7 cases. Only six cases had positive margins for invasive carcinoma and three cases had positive margins for DCIS. Lymphovascular invasion was seen in 56 cases. For patients who received neoadjuvant therapy, 9 patients had complete response with no residual cancer while 61 patients had either probable or no response. The tumors were staged as follows: ypT0 13 cases (6%), ypTis 1 case (0.5%), pT1 104 cases (46%) [1mic:1 case, 1a:6 cases, 1b:33 cases, and 1c:64 cases], pT2 56 cases (24%), pT3 8 cases (3.5%), pT4 6 cases (3%), and 38 (17%) unknown cases. Nodal staging was pN0 in 116 cases (52%), micrometastasis in 5 cases (2%), pN1 in 32 cases (14%), pN2 in 20 cases (9%), pN3 in 5 cases (2%), and in 48 (21%) the node status was unknown. The number of positive sentinel lymph nodes was 0 in 101 (45%) cases, 1 in 18 (8%) cases (including 1 with individual tumor cells and 1 micrometastasis), more than 1 in 11 (5%) cases, and the pN was unknown in 96 (42%) cases. ER was positive in 160 (71%) cases, while PR was positive in 132 (58%) cases. The median MIB1 was 27% (range 2–95). HER2 IHC was negative (0–1+) in 7 (3%) cases, equivocal (2+) in 192 (85%) cases, and positive (3+) in 25 (11%) cases.

Overall, 164 (73%) patients were alive without disease, 17 (7%) alive with disease, 34 (15%) died of breast cancer, and 11 (5%) died due to other causes. Forty-five (20%) patients developed metastasis, ten (4%) patients had local recurrence, and six (3%) patients had second breast primary.

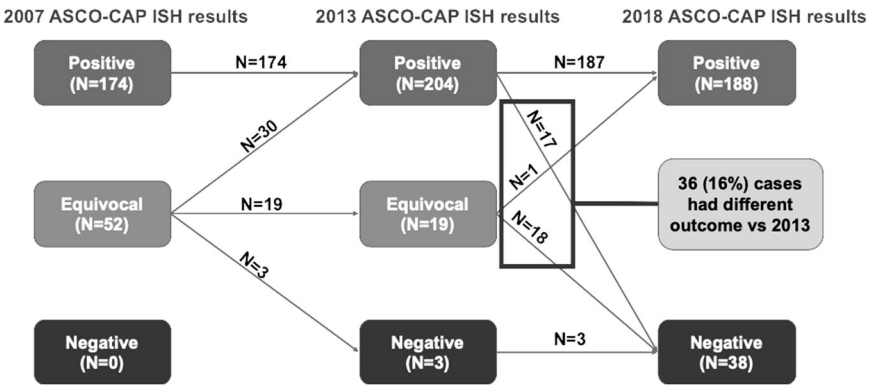


Fig. 1 Class migration of cases among different ASCO–CAP HER2 guidelines. ASCO–CAP American Society of Clinical Oncology–College of American Pathologist, ISH in situ hybridization.

Impact of ASCO–CAP ISH guidelines change

Figure 1 shows our cohort (patients who received HER2-targeted agents from 2006 to 2017) case distribution when 2007, 2013, and 2018 ASCO–CAP ISH guidelines are applied.

When applying 2007 ASCO–CAP ISH guidelines (amplified when ratio of HER2 to CEP17 > 2.2 or average HER2 gene copy-number > 6 signals/nucleus, equivocal when HER2/CEP17 ratio of 1.8–2.2 or average HER2 gene copy-number 4–6 HER2 signals/nucleus, and negative when HER2/CEP17 ratio of <1.8 or average HER2 gene copy-number of <4 signals/nucleus) to our cohort, 174 cases would have been categorized as ISH-positive and 52 cases would have been categorized as equivocal. As expected, no negative cases were encountered in our exclusively HER2-treated cohort.

When 2013 ASCO–CAP ISH guidelines were applied (amplified when dual-probe HER2/CEP17 ratio ≥ 2.0; with an average HER2 copy-number ≥ 4.0 signals/cell or <4.0 signals/cell, or dual-probe HER2/CEP17 ratio < 2.0; with an average HER2 copy-number ≥6.0 signals/cell, equivocal when dual-probe HER2/CEP17 ratio <2.0 with an average HER2 copy-number ≥4.0 and <6.0 signals/cell, and negative when dual-probe HER2/CEP17 ratio < 2.0 with an average HER2 copy-number <4.0 signals/cell), 204 cases would have been classified as ISH-positive, 19 equivocal cases, and 3 negative cases. The 2007–2013 change of guidelines resulted in 15% increase in positive cases (as most of the 2007 equivocal cases reclassified as positive in 2013) and 63% decrease in equivocal cases was observed.

The reclassification of cases according to 2018 ASCO–CAP ISH guidelines and after incorporating HER2 IHC results (in Groups 2–4) showed the following: 17 of 204 positive cases (2013 guidelines) were reclassified as negative according to the 2018 guidelines. Of the 19 equivocal cases from the 2013 guidelines, 1 case became positive (2018 criteria) while the rest of the cases were classified as negative. The final distribution of cases is as follows: 188 positive cases (8% reduction from 2013 guidelines) and 38 negative cases (92% increase in comparison to 2013 guidelines). The equivocal category was eliminated from the 2018 guidelines. Overall, 36 (15.9%) cases changed categories from the 2013 to the 2018 guidelines. Figure 1 summarizes the case migration according to different ASCO–CAP guidelines.

Using log rank analysis, the 2018 ASCO–CAP reclassification of cases was significantly associated with OS ($p = 0.003$) and DFS ($p = 0.001$). The 2013 ASCO–CAP classification was significantly associated with OS ($p = 0.02$) but not with DFS ($p = 0.07$). Significant statistical association was observed in both OS ($p < 0.01$) and DFS ($p < 0.01$) when 2007 ASCO–CAP guidelines were applied.

ASCO–CAP HER2 ISH Groups

Our cases were grouped using the 2018 ASCO–CAP newly introduced five ISH Groups: Group 1/classical amplified (HER2/CEP17 ratio ≥ 2 + HER2 signal ≥ 4 per cell), Group 2/"nonclassical" monosomy (HER2/CEP17 ratio ≥ 2 + HER2 signal < 4 per cell), Group 3/"nonclassical" co-amplified/polysomy (HER2/CEP17 ratio < 2 + HER2 signal ≥ 6 per cell), Group 4/"nonclassical" previously called equivocal (HER2/CEP17 ratio < 2 + HER2 signal ≥ 4–<6 per cell), and Group 5/classical non-amplified (HER2/CEP17 ratio < 2 + HER2 signal <4 per cell). HER2 IHC was referred to when needed (Groups 2–4) and as instructed in the latest guidelines to reach a final result as to whether the HER2 FISH is positive or negative. Figure 2 shows the distribution of our cohort by 2018 ASCO–CAP categories and shows different visual examples of HER2 FISH within each group. Table 1 shows the histopathologic features of breast cancer by HER2 FISH Group; no significant relationship between the groups and the variables was observed including HER2 IHC.

Univariate analysis using the log rank test showed that the HER2 FISH Groups were significantly associated with OS ($p < 0.01$; Fig. 3), higher HER2 amplification was associated with fewer deaths, possibly reflecting a better response to HER2-targeted therapy. HER2 FISH status also statistically significantly relates to DFS ($p < 0.01$; Fig. 4) and metastasis ($p = 0.01$) but not with recurrence ($p = 0.49$) or second primary ($p = 0.37$) in our cohort.

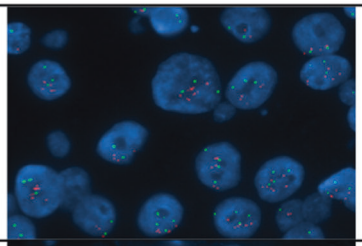
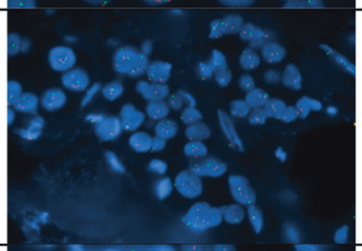
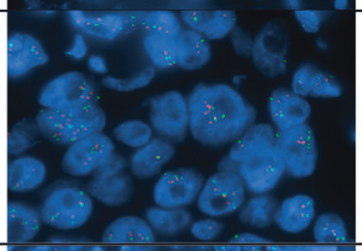
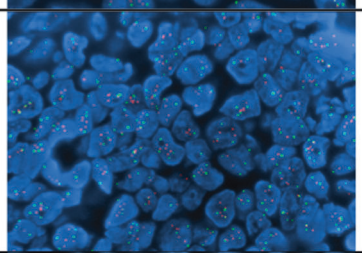
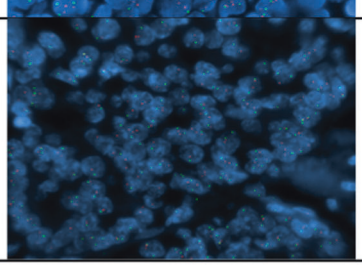
ASCO-CAP Group	Definition	Example of HER2 FISH	Number of cases
1	HER2/CEP17 ratio ≥2 + HER2 signal ≥4 per cell		176 (78%)
2	HER2/CEP17 ratio ≥2 + HER2 signal <4 per cell		17 (7.5%)
3	HER2/CEP17 ratio <2 + HER2 signal ≥6 per cell		11 (5%)
4	HER2/CEP17 ratio <2 + HER2 signal ≥4 -<6 per cell		19 (8.5%)
5	HER2/CEP17 ratio <2 + HER2 signal <4 per cell		3 (1%)
Total			226

Fig. 2 Case distribution among different ASCO–CAP Groups. ASCO–CAP American Society of Clinical Oncology–College of American Pathologist, CEP chromosome enumeration probe, FISH fluorescent in situ hybridization, HER2 human epidermal growth factor receptor 2.

The multivariate analysis showed that tumor type and HER2 ISH Groups are independent predictors for both OS and DFS in our HER2-treated cohort (Tables 2 and 3).

Group 1/classical amplified (HER2/CEP17 ratio ≥ 2 + HER2 signal ≥ 4 per cell)

The vast majority of our cohort belonged to this group, 176 (78%) out of 226 cases. The median age was 65 years (range 26–98), median HER2 FISH ratio was 2.795 (range 2–21), and median HER2 signal/cell was 6.99 (range 4.08–21). There were 159 (93%) cases of invasive ductal carcinoma NST, 6 (4%) cases were special ductal type carcinoma, 5 (3%) cases were lobular carcinoma, and 1 (1%) case was mixed carcinoma. Around 57% of Group 1 cases were poorly differentiated and 86% belonged to pathologic stage pT1 and pT2. Group 1 patients were given trastuzumab (Herceptin) alone in 114 (64%) patients, pertuzumab (Perjeta) and trastuzumab (Herceptin) in 57 (32%) patients, lapatinib (Tykerb/Tyverb) and trastuzumab

Table 1 Histopathologic characteristics of cases within each 2018 ASCO–CAP Groups.

	Group 1	Group 2	Group 3	Group 4	Group 5	*p* value	Total
Tumor type							
Ductal NST	159 (90%)	14 (82%)	9 (82%)	15 (79%)	3 (100%)	0.09	200 (89%)
Lobular	5 (3%)	1 (6%)	1 (9%)	1 (5%)	0		8 (4%)
Special type	6 (6%)	1 (6%)	1 (9%)	3 (16%)	0		11 (5%)
Mixed	1 (1%)	1 (6%)	0	0	0		2 (1%)
Nottingham grade							
Grade 1	6 (3%)	1 (6%)	0	1 (5%)	0	0.38	8 (4%)
Grade 2	65 (37%)	6 (35%)	7 (63%)	10 (53%)	2 (67%)		90 (40%)
Grade 3	93 (53%)	10 (59%)	3 (27%)	8 (42%)	1 (33%)		115 (51%)
Pathologic tumor stage (pT)							
ypT0 and ypTis	12 (7%)	0	0	1 (5%)	1 (33%)	0.69	14 (6%)
pT1	82 (47%)	10 (59%)	5 (45%)	7 (37%)	0		104 (46%)
pT2	42 (24%)	4 (24%)	4 (36%)	6 (32%)	0		56 (25%)
pT3	5 (3%)	1 (6%)	0	1 (5%)	1 (33%)		8 (4%)
pT4	4 (2%)	0	1 (9%)	1 (5%)	0		6 (3%)
Pathologic lymph node stage (pN)							
pN0	93 (53%)	12 (71%)	3 (27%)	8 (42%)	0	0.30	116 (51%)
pNmic	2 (1%)	0	2 (18%)	1 (5%)	0		5 (2%)
pN1	25 (14%)	1 (6%)	1 (9%)	3 (16%)	2 (67%)		32 (14%)
pN2	14 (8%)	2 (12%)	2 (18%)	2 (11%)	0		20 (9%)
pN3	4 (2%)	0	0	1 (5%)	0		5 (2%)
Neoadjuvant therapy							
Pathological complete response (pCR)	9/54 (17%)	0/3	0/4	0/8	0/1	0.11	9/70 (13%)
Estrogen receptor (ER)							
ER+	124 (70%)	12 (71%)	9 (81%)	12 (63%)	3 (100%)	0.51	160 (71%)
ER−	48 (27%)	5 (29%)	1 (9%)	7 (37%)	0		61 (27%)
Progesterone receptor (PR)							
PR+	97 (55%)	12 (71%)	9 (81%)	12 (63%)	2 (67%)	0.13	132 (58%)
PR−	75 (43%)	5 (29%)	1 (9%)	7 (37%)	1 (33%)		89 (39%)
HER2 IHC							
0–1+	5 (3%)	2 (12%)	0	0	0	0.29	7 (3%)
2+	146 (83%)	15 (88%)	10 (91%)	18 (95%)	3 (100%)		192 (85%)
3+	23 (13%)	0	1 (9%)	1 (5%)	0		25 (11%)

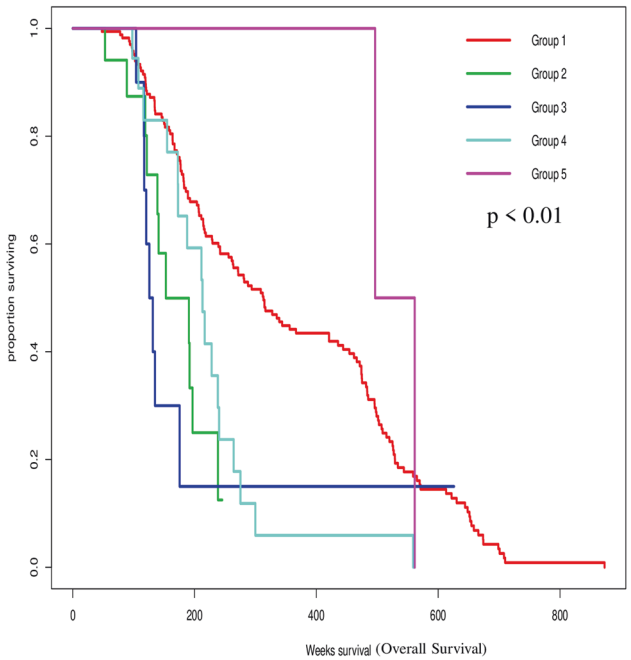
ASCO–CAP American Society of Clinical Oncology–College of American Pathologist, *ductal NST* ductal carcinoma of no special type, *HER2* human epidermal growth factor receptor 2, *IHC* immunohistochemistry.

(Herceptin) in 2 (1%) patients, and lapatinib (Tykerb/Tyverb) alone in 1 (0.5%) patient. Of note, 9 of 54 (17%) cases in Group 1 received neoadjuvant therapy and showed pCR. A higher pCR to neoadjuvant therapy was also found in Group 1 in comparison to the other Groups (Groups 2–5), which had no pCR ($p = 0.11$). Approximately 67% of cases were pN0 and the majority of the group cases were ER- and PR-positive, 72 and 56%, respectively. Most cases had HER2 IHC 2+ or 3+ staining (169 cases [97%], 2+: 146 cases [84%], and 3+: 23 cases [13%]).

With regards to outcomes, Group 1 patients had a better OS and DFS, suggesting that the higher HER2 amplification, the better the response to HER2-targeted medications. Due to the lack of enough negative control cases (i.e., Group 5 cases), Group 1 was used as the baseline comparison group for both OS and DFS.

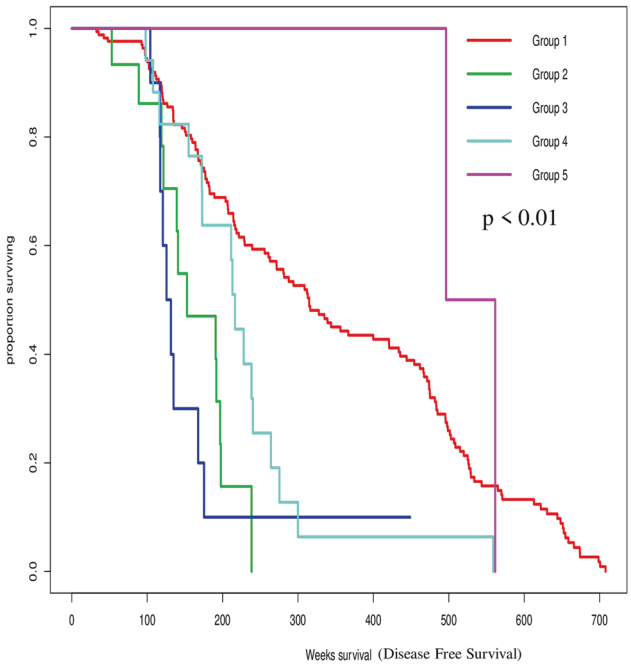
Group 1 subcategories

Since Group 1 (HER2/CEP17 ratio ≥ 2 + HER2 signal ≥ 4 per cell) is a broad group and the dominant category in terms of number of patients in our cohort (176 [78%] out of 226), and because it shows significant heterogeneity in response to HER2 agents, we proposed three subcategories



2018 ASCO-CAP HER2 FISH Group	Cox Model for Overall Survival
Group 1 (n=176)	Reference
Group 2 (n=17)	**HR =3.06, 95% CI 1.61-5.8; p<0.001**
Group 3 (n=11)	**HR =2.42, 95% CI 1.18-5.0; p=0.016**
Group 4 (n=19)	**HR =2.15, 95% CI 1.28-3.6; p=0.004**
Group 5 (n=3)	HR =0.52, 95% CI 0.13-2.1; p=0.358

Fig. 3 Kaplan–Meier plot (left) and Cox model (right) for overall survival by 2018 ASCO–CAP HER2 FISH Groups. CI confidence interval, FISH fluorescence in situ hybridization, HR hazard ratio. Bolded values indicate statistically significant results.



2018 ASCO-CAP HER2 FISH Group	Cox Model for Disease Free Survival
Group 1 (n=176)	Reference
Group 2 (n=17)	**HR =3.70, 95% CI 1.98-6.9; p<0.001**
Group 3 (n=11)	**HR =3.95, 95% CI 1.97-7.9; p<0.001**
Group 4 (n=19)	**HR =2.08, 95% CI 1.22-3.5; p=0.007**
Group 5 (n=3)	HR =0.63, 95% CI 0.15-2.5; p=0.512

Fig. 4 Kaplan–Meier plot (left) and Cox model (right) for disease-free survival by 2018 ASCO–CAP HER2 FISH Groups. CI confidence interval, FISH fluorescence in situ hybridization, HR hazard ratio. Bolded values indicate statistically significant results.

under "Group 1/classical amplification" category to look for any potential differences in outcomes in patients who received HER2-targeted agents. These three subcategories included low amplified (HER2/CEP17 ratio ≥ 2.0–2.99, mean HER2/cell 4.0–5.9), amplified (HER2/CEP17 ratio ≥ 2.0–2.99, mean HER2/cell ≥ 6), and excessive amplification (HER2/CEP17 ratio ≥ 3.0 and HER2/cell ≥ 4.0). The cutoffs for subcategories are based on the expert opinion of one experienced molecular and breast pathologist (DC) and published literature [5, 12].

Table 2 Cox regression for overall and disease-free survival using standard prognostic variables.

Prognostic variable	Hazard ratio	Lower .95	Upper .95	*p* value
Overall survival				
Tumor type	3.0388	1.7407	5.305	9.25×10^{-5}
Nottingham grade	0.9422	0.6846	1.297	0.7148
Pathologic tumor stage (pT)	1.1076	0.8480	1.447	0.4532
Pathologic lymph node stage (pN)	0.8504	0.6531	1.107	0.2290
ER	1.7590	0.9906	3.124	0.0539
PR	0.7111	0.4264	1.186	0.1915
HER2 IHC	0.7177	0.4879	1.056	0.0921
Disease-free survival				
Tumor type	2.6552	1.4963	4.712	0.0008
Nottingham grade	0.8722	0.6361	1.196	0.3960
Pathologic tumor stage (pT)	1.2423	0.9337	1.653	0.1364
Pathologic lymph node stage (pN)	0.8707	0.6706	1.131	0.2987
ER	1.6053	0.9130	2.823	0.1002
PR	0.6708	0.4031	1.116	0.1244
HER2 IHC	0.7595	0.5199	1.109	0.1547

ER estrogen receptor, *HER2* human epidermal growth factor receptor 2, *IHC* immunohistochemistry, *PR* progesterone receptor.

Table 3 Cox regression for overall and disease-free survival using only tumor type and ASCO–CAP 2018 ISH Groups.

Prognostic variable	Hazard ratio	Lower .95	Upper .95	*p* value
Overall survival				
Tumor type	2.180	1.345	3.532	0.0015
ASCO–CAP ISH Groups	1.296	1.111	1.513	0.0010
Disease-free survival				
Tumor type	1.837	1.111	3.040	0.0178
ASCO–CAP ISH Groups	1.325	1.136	1.546	0.0003

ASCO–CAP American Society of Clinical Oncology–College of American Pathologist, *ISH* in situ hybridization.

Figure 5 and Table 4 show case distribution and the histopathologic features of each entity. They demonstrate strikingly similar frequencies of almost all histopathologic features among the subgroups. Of note, no significant relationship between these subcategories and histopathologic features is noted except for HER2 IHC. The excessive amplification subcategory was significantly associated with HER2 IHC 3+ cases ($p < 0.01$).

Patients in the low-amplified subcategory received trastuzumab (Herceptin) alone in 30 (54%) patients while 26 (46%) patients received trastuzumab (Herceptin) with pertuzumab (Perjeta). Almost half of the amplified subcategory patients (20 patients) received trastuzumab (Herceptin) alone and the other half (22 patients) received trastuzumab (Herceptin) with pertuzumab (Perjeta). Excessive amplification patients were mainly treated with trastuzumab (Herceptin) alone (64 [85%] patients) and only 11 (15%) patients received trastuzumab (Herceptin) in combination with other drug [9 patients with pertuzumab (Perjeta) and 2 patients with lapatinib (Tykerb/Tyverb)]. Excessive amplification patients who received trastuzumab (Herceptin) alone had the longest median survival (483.4 weeks for OS and 481.6 weeks for DFS) as well as lowest HRs (Fig. 6) (OS HR = 0.60, 95% CI 0.37–0.96; $p = 0.035$ and DFS HR = 0.57, 95% CI 0.36–0.92; $p = 0.022$) even after controlling for tumor type in Cox regression model for OS (HR = 0.53, 95% CI 0.32–0.87; $p = 0.012$).

Complete response to neoadjuvant therapy was not statically significant between the excessive amplification subcategory and the other two categories ($p = 0.29$), despite 23% (6 out of 26 cases) rate of pCR after neoadjuvant therapy in excessive amplification group as opposed to 11% (3 out 28 cases) in the other two subcategories.

The proposed subcategories were significantly associated with OS ($p < 0.01$; Fig. 7) and DFS ($p < 0.01$) using log rank test, excessive HER2 amplification was associated with longest median survival (475 weeks for OS and 474 weeks for DFS), possibly reflecting a better response to HER2-targeted therapy. Group 1 subcategories were not statistically significantly associated with metastasis ($p = 0.15$), recurrence ($p = 0.07$), or second breast primary ($p = 0.81$).

The Cox regression models showed better survival outcomes for the excessive amplification subgroup than the low amplified subgroup, with OS (HR = 0.63, 95% CI 0.42–0.93; Fig. 7) and DFS (HR = 0.55, 95% CI 0.37–0.83). Similar findings were seen after including the rest of the ASCO–CAP HER2 ISH groups in the regression model (Fig. 8).

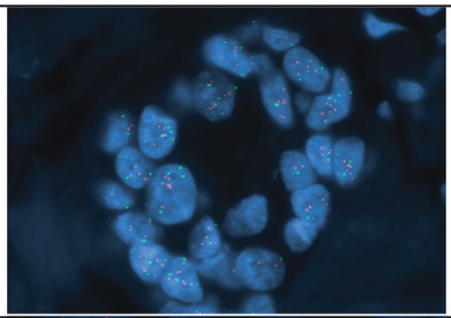
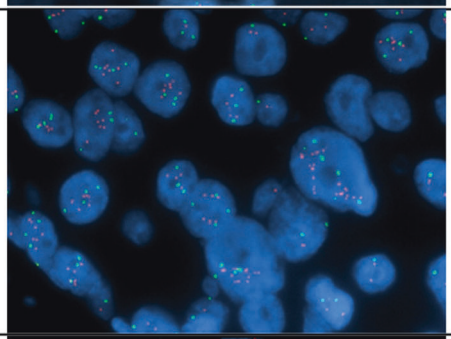
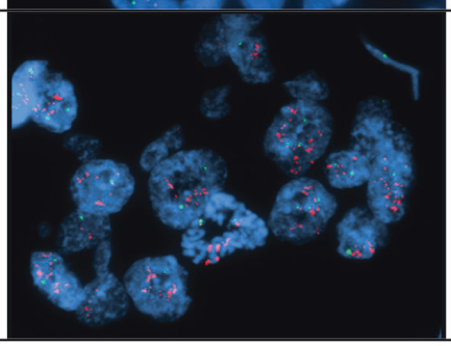
Group 1 subcategories	Definition	Example of HER2 FISH	Number of cases
Low amplified	HER2/CEP17 ratio ≥2.0-2.99, mean HER2/cell 4.0-5.9		57 (32%)
Amplified	HER2/CEP17 ratio ≥2.0-2.99, mean HER2/cell ≥6		44 (25%)
Excessive amplification	HER2/CEP17 ratio ≥3.0, mean HER2/cell ≥ 4.0		75 (43%)
Total			176

Fig. 5 Case distribution of Group 1 subcategories along with their ISH definitions. CEP chromosome enumeration probe, HER2 human epidermal growth factor receptor 2, ISH in situ hybridization.

Group 2/"nonclassical" monosomy (HER2/CEP17 ratio ≥ 2 + HER2 signal < 4 per cell)

Our cohort had 17 (7.5%) cases that showed HER2/CEP17 ratio ≥ 2 + HER2 signal < 4 per cell. The median age was 64 years (range 43–89), median HER2 FISH ratio was 2.15 (range 2–3.28), and median HER2 signal/cell was 3.50 (range 2.08–3.98). Fifteen (88%) cases had 2+ staining for HER2 IHC and 2 (22%) cases had 0–1+ staining. Since there were no 3+ cases, all cases in this cohort are categorized as negative for HER2 FISH according to the latest guidelines (2+ cases require a count from an additional observer and if the count remains the same then negative result is rendered).

When compared to Group 1 category, Group 2 showed inferior survival outcomes for both OS (HR = 3.06, 95% CI 1.61–5.8; Fig. 3) and DFS (HR = 3.70, 95% CI 1.98–6.9; Fig. 4), possibly suggesting inferior response to HER2-targeted therapy.

Group 3/"nonclassical" co-amplified/polysomy (HER2/CEP17 ratio < 2 + HER2 signal ≥ 6 per cell)

Only 11 out of 226 (5%) cases were recorded in this group. The median age was 66 years (range 43–83), median HER2 FISH ratio was 1.56 (range 1.38–1.92), and median HER2 signal/cell was 6.37 (range 6.20–8.22). The tumors were HER2 IHC 2+ (10 cases) and HER2 IHC 3+ (1 case). All of our cases in this cohort are categorized as ISH-positive according to the latest guidelines (2+ cases require a count from an additional observer and if the count remains the same then positive result is rendered).

When compared to Group 1 category, Group 3 showed inferior clinical outcomes for both OS (HR = 2.42, 95% CI

Table 4 Histopathologic characteristics of cases within Group 1 subcategories.

	Low amplified	Amplified	Excessive amplification	*p* value
Tumor type				
Ductal NST	51 (89%)	39 (89%)	69 (92%)	1.00
Lobular	1 (2%)	0	4 (5%)	
Special type	2 (4%)	3 (7%)	1 (1%)	
Mixed	1 (2%)	0	0	
Nottingham grade				
Grade 1	1 (2%)	2 (5%)	3 (4%)	0.67
Grade 2	19 (33%)	18 (41%)	28 (37%)	
Grade 3	34 (60%)	19 (43%)	40 (53%)	
Pathologic tumor stage (pT)				
ypT0 and ypTis	2 (4%)	2 (5%)	8 (11%)	0.08
pT1	25 (44%)	19 (43%)	38 (52%)	
pT2	16 (28%)	10 (23%)	16 (21%)	
pT3	5 (9%)	0	0	
pT4	3 (5%)	0	1 (1%)	
Pathologic lymph node stage (pN)				
pN0	29 (51%)	20 (45%)	44 (59%)	0.66
pNmic	0	0	2 (3%)	
pN1	8 (14%)	8 (18%)	9 (12%)	
pN2	6 (11%)	4 (9%)	4 (5%)	
pN3	2 (4%)	0	2 (3%)	
Neoadjuvant Therapy				
Pathological complete response (pCR)	1/18 (6%)	2/10 (20%)	6/26 (23%)	0.29
Estrogen receptor (ER)				
ER+	39 (68%)	33 (75%)	52 (69%)	0.59
ER−	17 (30%)	9 (20%)	22 (29%)	
Progesterone receptor (PR)				
PR+	28 (49%)	29 (66%)	40 (53%)	0.15
PR−	28 (49%)	13 (30%)	34 (45%)	
HER2 IHC				
0–1+	1 (2%)	3 (7%)	1 (1%)	<0.01
2+	54 (95%)	41 (93%)	51 (68%)	
3+	1 (2%)	0	22 (29%)	

Ductal NST ductal carcinoma of no special type, *HER2* human epidermal growth factor receptor 2, *IHC* immunohistochemistry.

1.18–5.0; Fig. 3) and DFS (HR = 3.95, 95% CI 1.97–7.9; Fig. 4), possibly suggesting inferior response to HER2-targeted therapy.

Group 4/"nonclassical" equivocal (HER2/CEP17 ratio < 2 + HER2 signal ≥ 4–< 6 per cell)

There were 19 (8.5%) cases recorded. The median age was 66 years (range 42–82), median HER2 FISH ratio was 1.64 (range 1.18–1.97), and median HER2 signal/cell was 5.10 (range 4.28–5.98). According to the 2018 ASCO–CAP guidelines, cases under this category with HER2 2+ are ISH-negative, if the count remains the same from an additional observer, while HER2 3+ are classified ISH-positive. From our cohort, 18 cases had 2+ staining for HER2 IHC, i.e., negative, while only 1 case had 3+ staining for HER IHC and was classified HER2-positive.

The case that had 3+ IHC was a poorly differentiated invasive ductal carcinoma in a 69-year-old patient, had lymphovascular invasion with pT2 pN2a disease, ER was 100% and PR was 65%. The patient received adjuvant chemo- and radiotherapy along with hormonal treatment and is still alive with no evidence of metastasis, second primary, or recurrence.

When compared to Group 1 category, Group 4 showed inferior clinical outcomes for both OS (HR = 2.15, 95% CI 1.28–3.6; Fig. 3) and DFS (HR = 2.08, 95% CI 1.22–3.5; Fig. 4), possibly suggesting inferior response to HER2-targeted therapy.

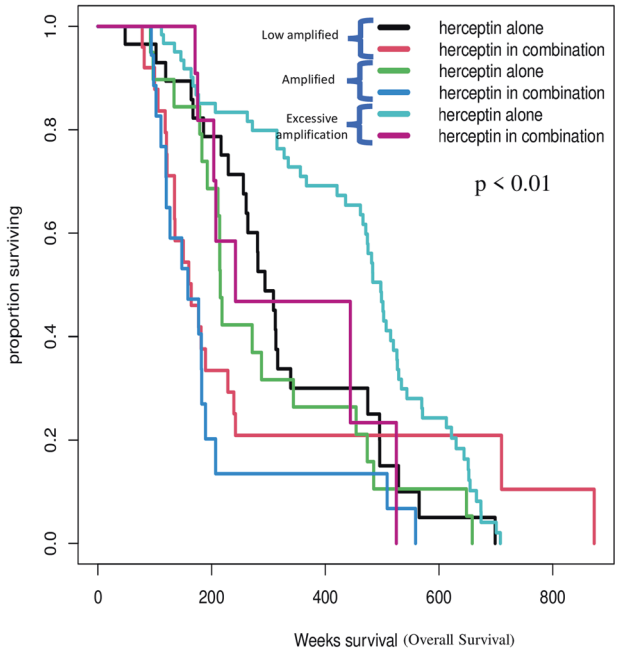
Group 5/classical non-amplified (HER2/CEP17 ratio <2 + HER2 signal <4 per cell)

Three patients (1%) belonged to this group, all of whom received HER2-targted therapy based on equivocal results from 2007 guidelines as these cases were diagnosed in 2007, 2008, and 2010. The median age was 59 years (range 42–86), median HER2 FISH ratio was 1.84 (range 1.82–1.96), and median HER2 signal/cell was 3.82 (range 3.40–3.90). HER IHC 2+ was recorded for all cases.

Due to low case volume in this cohort, survival outcomes cannot be ascertained. However, out of three patients only two are alive while the third patient passed away due to breast cancer and metastatic disease. One of the living patients developed metastasis, suggesting poor response to HER2-targeted therapy.

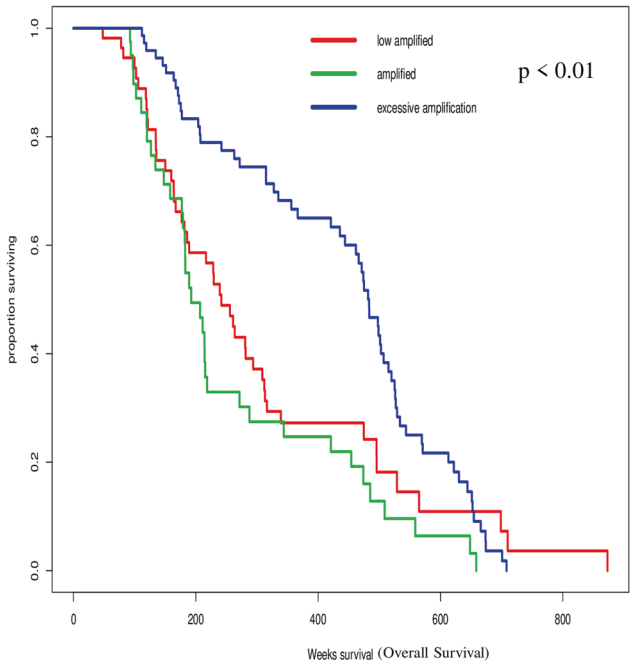
HER2 signals per cell

Adopted from published literature, cases were also grouped into three categories based on average HER2 copy-number per cell alone, using a cutoff system of <6 copies ($n = 99$ cases), 6–10 copies ($n = 76$ cases), and >10 copies ($n = 51$ cases) to investigate whether this classification predicts outcome and is the increasing success of anti-HER2 therapy in this setting driven more by the "numerator" than the ratio. Cases with >10 copies of HER2 per cell showed superior survival outcomes for both OS (HR = 0.45, 95% CI 0.30–0.66; Fig. 9) and DFS (HR = 0.42, 95% CI 0.28–0.63; Fig. 9) in comparison to cases with <6 copies of HER2-signals per cell.



Group 1 subcategories and HER2 targeted agents received	Cox Model for Overall Survival	Cox Model for Disease Free Survival
Low amplified- Herceptin alone (n=30)	Reference	Reference
Low amplified- Herceptin in combination (n=26)	HR =1.10, 95% CI 0.59-2.03; p=0.765	HR =1.46, 95% CI 0.76-2.83; p=0.256
Amplified- Herceptin alone (n=20)	HR =1.21, 95% CI 0.66-2.19; p=0.543	HR =1.01, 95% CI 0.55-1.89; p=0.957
Amplified- Herceptin in combination (n=22)	**HR =2.32, 95% CI 1.23-4.39; p=0.010**	**HR =2.09, 95% CI 1.08-4.06; p=0.029**
Excessive amplification- Herceptin alone (n=64)	**HR =0.60, 95% CI 0.37-0.96; p=0.035**	**HR =0.57, 95% CI 0.36-0.92; p=0.022**
Excessive amplification- Herceptin in combination (n=11)	HR =0.98, 95% CI 0.42-2.26; p=0.952	HR =1.01, 95% CI 0.41-2.47; p=0.981

Fig. 6 Kaplan–Meier plot of overall survival (left) and Cox models for both overall survival and disease-free survival (right) by Group 1 subcategories and HER2-targeted agents received. CI confidence interval, HR hazard ratio. Bolded values indicate statistically significant results.



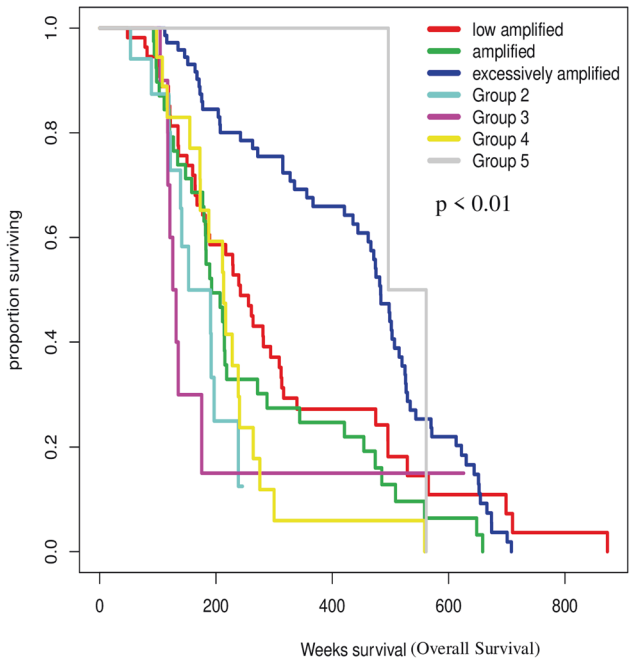
Group 1 subcategories	Cox Model for Overall Survival
Low amplified (n=57)	Reference
Amplified (n=44)	HR =1.42, 95% CI 0.91-2.22; p=0.123
Excessive amplification (n=75)	**HR =0.63, 95% CI 0.42-0.93; p=0.019**

Group 1 subcategories	Cox Model for Disease Free Survival
Low amplified (n=57)	Reference
Amplified (n=44)	HR =1.15, 95% CI 0.73-1.83; p=0.547
Excessive amplification (n=75)	**HR =0.55, 95% CI 0.37-0.83; p=0.004**

Fig. 7 Kaplan–Meier plot of overall survival (left) and Cox models for both overall survival (top right) and disease-free survival (bottom right) by Group 1 subcategories. CI confidence interval, HR hazard ratio. Bolded values indicate statistically significant results.

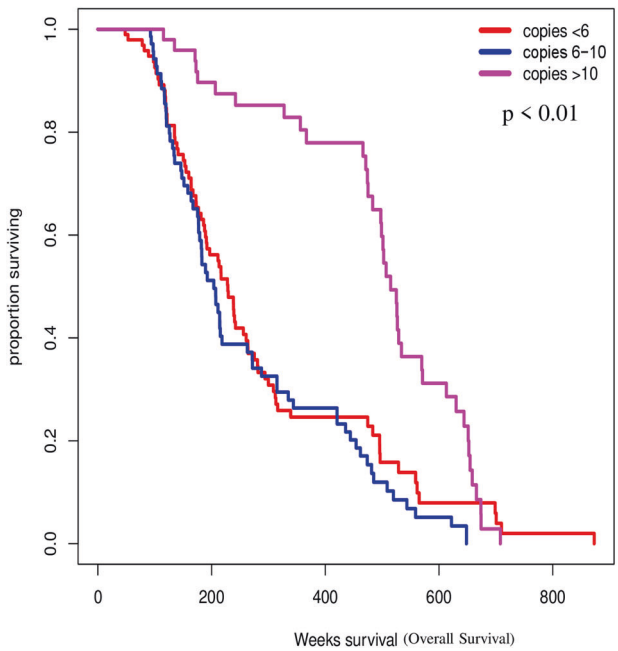
Discussion

We retrospectively applied different classification systems of HER2 FISH based on the latest ASCO–CAP guidelines to a unique cohort of patients that was exclusively treated with HER2-targeted agents in a non-trial setting in our institution from 2006 to 2017 and studied their prognostic significance. Limited data exists in the literature assessing the association of level of HER2 amplification and response to HER2-targeted agents. There are some reports showing a



Group 1 subcategories and 2018 ASCO-CAP HER2 ISH Group	Cox Model for Overall Survival	Cox Model for Disease Free Survival
Group 1- Low amplified (n=57)	Reference	Reference
Group 1- Amplified (n=44)	HR =1.44, 95% CI 0.92-2.26; p=0.107	HR =1.17, 95% CI 0.73-1.85; p=0.518
Group 1- Excessive amplification (n=75)	**HR =0.62, 95% CI 0.42-0.92; p=0.018**	**HR =0.54, 95% CI 0.36-0.81; p=0.003**
Group 2 (n=17)	**HR =2.73, 95% CI 1.38-5.41; p=0.004**	**HR =2.96, 95% CI 1.51-5.78; p=0.001**
Group 3 (n=11)	HR =2.06, 95% CI 0.96-4.43; p=0.064	**HR =3.11, 95% CI 1.49-6.50; p=0.003**
Group 4 (n=19)	**HR =1.88, 95% CI 1.06-3.32; p=0.031**	HR =1.61, 95% CI 0.90-2.90; p=0.109
Group 5 (n=3)	HR =0.44, 95% CI 0.11-1.81; p=0.252	HR =0.46, 95% CI 0.11-1.91; p=0.285

Fig. 8 Kaplan–Meier plot of overall survival (left) and Cox models for both overall survival and disease-free survival (right) by Group 1 subcategories and 2018 ASCO–CAP HER2 ISH Groups 2–5. CI confidence interval, HR hazard ratio. Bolded values indicate statistically significant results.



Average HER2 signals per cell categories	Cox Model for Overall Survival
<6 copies (n=99)	Reference
6 to 10 copies (n=76)	HR =1.29, 95% CI 0.92-1.81; p=0.144
> 10 copies (n=51)	**HR =0.45, 95% CI 0.30-0.66; p <0.01**

Average HER2 signals per cell categories	Cox Model for Disease Free Survival
<6 copies (n=99)	Reference
6 to 10 copies (n=76)	HR =1.18, 95% CI 0.84-1.67; p=0.347
> 10 copies (n=51)	**HR =0.42, 95% CI 0.28-0.63; p <0.01**

Fig. 9 Kaplan–Meier plot of overall survival (left) and Cox models for both overall survival (top right) and disease-free survival (bottom right) by average HER2 signals per cell. CI confidence interval, HR hazard ratio. Bolded values indicate statistically significant results.

positive relation between level of HER2 amplification by ISH and rate of pathological complete response to trastuzumab-based neoadjuvant treatment [13, 14]. In our cohort, 17% of cases in Group 1 that received neoadjuvant therapy showed pCR. This was not statistically significant when compared to other groups. Twenty-three percent of patients in the excessive amplification subcategory showed pCR to neoadjuvant therapy, but again this was not

statically significant when compared to the other two subcategories (11% in the other two subcategories of Group 1).

About 95% of all performed HER2 FISH results belonged to the classical category either amplified (Group 1) or non-amplified (Group 5) [15]. The threshold of both groups remained unchanged by the new guidelines and it is well-documented that cases with HER2 overexpression demonstrate clinical benefit when HER2-targeted agents like trastuzumab (Herceptin) [16, 17], lapatinib (Tykerb/Tyverb) [18], pertuzumab (Perjeta) [19], and ado-trastuzumab emtansine/T-DM1 (Kadcyla) [20, 21] are used.

The vast majority of our cohort received trastuzumab (Herceptin) alone (145 (64%) patients) while the remaining patients received trastuzumab (Herceptin) in combination with pertuzumab (Perjeta) or lapatinib (Tykerb/Tyverb) in 74 (33%) patients. The use of trastuzumab (Herceptin) alone was significantly associated with longer median OS and DFS. Similarly, excessive amplification subcategory patients who received trastuzumab (Herceptin) alone had the longest median survival as well as lowest HRs for OS and DFS. We acknowledge that our results might have been confounded by the fact that this is a retrospective review and the fact that we used pertuzumab (Perjeta) and lapatinib (Tykerb/Tyverb) in higher risk patients than those who received trastuzumab (Herceptin) alone.

Data on the histopathologic features and prognosis are limited in the literature on the nonclassical category (Groups 2–4) due to their rare inclusion in clinical trials and partly due to their low incidence (5% of cases) [5, 8]. In our study and from our unique cohort, the incidence of nonclassical cases was 21%. Table 1 demonstrates the histopathologic characteristics of each nonclassical group.

Group 1, defined as ISH-positive and HER2 amplified, compromised the vast majority of cases (176 [78%] out 226) and was superior to the other groups in having fewer deaths and better survival outcomes. This finding suggests that the higher HER2 expression the better response to HER2-targeted medications. We also have demonstrated that some heterogeneity exists in relation to response to HER2 treatments because our three proposed subcategories (low-amplified, amplified, and excessive amplification) showed significant association with OS ($p < 0.01$; Fig. 7) and DFS ($p < 0.01$). In addition, the highest HER2/CEP17 ratio (excessive amplification subgroup) was associated with the longest median survival for both OS and DFS (Fig. 8) when compared to the rest of the ASCO–CAP HER2 ISH Groups, supporting our theory that these cases would have a better response to HER2-targeted therapy. This novel finding warrants additional investigation as our findings suggest different response to HER2 therapy within the same category. Although one would argue that these cases represent HER2 IHC 3+ cases, and thus, scant ISH data in the literature since most laboratories do not perform ISH when the HER2 IHC is 3+. From our cohort, excessive amplification subcategory was indeed enriched with HER 3 + cases (22 [30%] cases, $p < 0.01$), but the vast majority of cases (51 [~70%] cases) were HER IHC 2+. The current HER2 ISH interpretation does not stratify classic amplification as high or low in Group 1 cases. We recommend reporting these subcategories or include a comment that specifies high or low amplification, which is similar in concept to the "low positive" category, which was recently introduced in the ASCO–CAP update on ER testing when the staining is between 1 and 10% [22]. However, larger and prospective longitudinal studies are needed to validate our findings.

Group 2 is seen in cases with loss of a chromosome (true monosomy) or part of the chromosome (CEP17) [5]. When such a case is encountered in dual-probe ISH, additional work is required to render a final result. In our study, only 17 (7.5%) cases were recorded in this group. It also contained the highest number of 0–1+ HER2 IHC cases in the nonclassical category, 12%, with no HER 3+ cases. After applying the new ASCO–CAP guidelines, all of our Group 2 cases would have been classified as ISH-negative. Despite the low number of cases, our cases showed inferior survival outcomes compared to Group 1, possibly suggesting inferior response to HER2-targeted therapy, a finding that supports the current reporting scheme and the literature as described by Press et al. [15] and Risio et al. [23].

Group 3 cases are seen when the ratio is negative, but HER2 signals are more than 6 per cell, and are due to gain of the pericentromeric region of chromosome 17 rather than the rare true polysomy, i.e., gain of an entire chromosome [24–26]. In our study, Group 3 contained the lowest number of patients in the nonclassical category (11 patients [5% of cohort]) with 91% of the cases being HER IHC 2+. Group 3 has limited data on response to HER2-targeted therapies with one study showing that patients in this group who did not receive HER2-targeted therapy had a worse outcome (OS and DFS) than Group 5 or ISH non-amplified cases [15]. In addition, it is well-described in the literature that Group 3 comprises two heterogeneous groups: one amplified and the other is not amplified. Press et al. [27] found that the amplified group had an average of 12.3 HER2 signals per cell and was predominately HER2 IHC 2 + and 3+, while the non-amplified group had an average of 6.8 HER2 signals per cell and was predominately HER2 IHC 0–1+. From our study, the average HER2 signals per cell in this category was 6.79 and all of our cases were HER2 IHC 2+ (ten cases) and 3+ (one case), thus considered ISH-positive based on the current guidelines. In terms of survival, Group 3 cohort showed inferior survival outcomes in contrast to Group 1, suggesting that these cases belong to the non-amplified group despite having HER2 IHC 2+ and 3+ and contradict the designation of ISH-

positive according to the latest reporting scheme. However, due the low number of cases in this group, it is hard to draw a definite conclusion, and larger prospective studies are needed.

Group 4, formerly referred to as ISH equivocal, has frequent cells with slight increase in HER2 signals (≥4–<6) per cell with simultaneous mild increase in mean CEP17 signals per cell or non-clustered scattered distribution of cells with ≥6 signals of HER2 per cell [5]. In our study, 19 (8.5%) patients were recorded with 5% (1 case) being HER2 IHC 3+, 95% (18 cases) being HER2 IHC 2+ and no HER2 IHC 0–1+ cases. In order to adjudicate these cases, ASCO–CAP guidelines require incorporating IHC results and recommend against the use of alternative chromosome 17 probes [8]. From our cohort, the one HER2 3+ case is the only case classified as amplified while the rest were non-amplified. In one study, Group 4 cases were included in a clinical trial that received chemotherapy but not HER2-targeted agents, and the outcomes were similar to the negative control (non-amplified cases/Group 5) [15]. Given that 18 out of 19 cases are ISH-negative, these cases, despite the low number, showed inferior clinical outcomes than Group 1 and possibly suggests an inferior response to HER2-targeted therapy. This finding supports the current reporting scheme to consider these cases (especially the HER2 2+) as ISH-negative. However, larger studies are needed to validate this finding.

Group 5, classical non-amplified, had three cases (1% of the entire cohort). These were old equivocal cases based on older guidelines in which the treating oncologist decided to treat with HER2-targeted agents. We acknowledged that the small number of cases was insufficient for definitive evaluation for outcome or response to HER2-targeted therapy.

In contrary to a systematic review and meta-analysis by Xu et al. [12], which showed that different HER2 gene copy-number level had no prognostic value on survival for those who received trastuzumab-based adjuvant treatment, categorizing the cases solely based on the average number of HER2 signals per cell (<6 copies, 6–10 copies, and >10 copies) enabled us to predict clinical endpoints in our cohort. Cases with >10 copies of HER2 showed longer median OS and DFS when compared to the other categories, suggesting superior response to HER2-targeted agents and suggesting that the increasing success to HER2-targeted agents could also be driven by the absolute number of HER2 signals rather than the ratio. Larger and prospective longitudinal studies are needed for validation.

To summarize the impact of implementing the new 2018 ASCO–CAP guidelines in our cohort, 36 (15.9%) cases changed their category compared to the 2013 guidelines, a rate which is slightly higher than what Zare et al. reported (10.7%) [28]. The 2018 ASCO–CAP reclassification of cases was significantly associated with OS ($p = 0.003$) and DFS ($p = 0.001$) using the log rank test.

Low number of cases in some of the groups is one of the limitations of our study. Despite that, our HER2-treated cohort provides a solid start for future studies. Another drawback of this cohort pertains to the inherent limitation of FISH slides that cannot be rescored using current ASCO–CAP guidelines for an additional blinded observer scoring, a step that is now required for HER2 2+ in Groups 2–4 cases. In addition, HER2 IHC interpretation was updated in the 2018 ASCO–CAP update [8], it resulted in an increase in HER2 0–1+ cases and a decrease in 2+ cases, all of the HER2 IHC results provided in our study were recorded based on prior guidelines. Finally, our study lacked data on HER2 heterogeneity. The literature data on the latter are limited with conflicting definitions and a reported rate of <1% of total cases as described in a study by Ballard et al. [5].

In conclusion, our retrospective, single-institution, HER2-treated cohort study supports the new ASCO–CAP 2018 guidelines binary reporting scheme and we recommend reporting each specific group due to the heterogeneity in response to HER2-targeted agents. We also recommend reporting ASCO–CAP Group 1 ISH subcategories for every patient since the current HER2 ISH interpretation does not stratify classic amplification as high or low and our data showed that excessive amplification is associated with the longest median survival for both OS and DFS, subject to larger and prospective longitudinal studies to validate our findings.

Compliance with ethical standards

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

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