Diagnostic Pathology

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Exploring the heterogeneity of HER2 gene status and expression in non-positive breast cancer patients: insights from immunohistochemistry and fluorescence in situ hybridization

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Abstract

Breast cancer became the most prevalent malignancy among women, and HER2 expression status is critical for treatment decisions. With the emergence of ADC drugs, HER2 low-expressing patients who previously did not respond well to traditional anti-HER2 therapies may now benefit. In this study, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) were applied to assess HER2 expression in 349 patients with HER2-non-positive breast cancer. Our analysis revealed that HER2-low tumors exhibited fewer grade III tumors (39.74% and 55.65%, respectively, $P=0.005$) and higher positivity for estrogen receptor (ER, 88.89% vs. 61.74%, $P<0.001$) and progesterone receptor (PR, 84.62% vs. 57.39%, $P<0.001$) compared to HER2-ZERO tumors. Of the 349 cases, IHC was ultimately evaluated in 327, the antibodies demonstrated only 64.22% (95% CI: 58.76–69.42%) agreement between clone 4B5 and clone EP3. Pathologist 1, who had more extensive working experience, demonstrated higher consistency (94.19%) with the gold standard when using clone EP3, compared to Pathologist 2 (74.31%). FISH analysis revealed significant differences in HER2/CEP17 ratio and average HER2 copy numbers between HER2-ZERO and HER2-low tumors, but no clear cut-off value could be identified. Notably, HER2/CEP17 ratio mostly between 1 and 2, with HER2-ZERO tumors primarily ≤ 1.4, and average HER2 copy numbers were mostly ≥ 2 and < 4, with HER2-ZERO tumors primarily ≤ 2.5. Despite distinct clinicopathological features, FISH remains inadequate for distinguishing HER2-low from HER2-ZERO expression. Further studies are needed to improve HER2 assessment in this challenging subset of patients.

Keywords Breast cancer, HER2-low, FISH, Immunohistochemistry staining

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Introduction

In recent years, breast cancer has become the most commonly diagnosed cancer in women [1]. The hormone receptor (HR) and epidermal growth factor receptor 2 (HER2, or ERBB2) are well-recognized biomarkers for the treatment of breast cancer [2], with HER2 determined by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Approximately 80-85% of breast cancers are defined as HER2 negative, while the majority of these tumors still express detectable levels of HER2 protein on cell membranes. This includes two thirds of HR positive and one third of triple-negative tumors with low HER2 expression (HER2 IHC score of 1 + or 2+/ISH not amplified) [3]. Unfortunately, anti-HER2 monoclonal antibodies that target HER2-low expression have failed to demonstrate any clinical benefit [4]. However, several clinical trials have shown promising results with anti-HER2 antibody-drug conjugates (ADCs) in the treatment of HER2-low breast cancer [5–10]. In particular, trastuzumab-deruxtecan (T-Dxd), has recently been approved by the US Food and Drug Administration as the first targeted therapy to treat HER2-low breast cancer [11].

Given the efficacy of ADCs in treating HER2-low breast cancer, more challenges have been identified in the interpretation of HER2 protein expression by immunohistochemistry (IHC) in clinical practice. A study conducted among 18 pathologists at Yale University examined the interpretation of HER2 IHC scores for 170 breast cancer biopsies. The findings suggested that the current standard HER2 IHC assay exhibits poor scoring accuracy in the low range (0 and 1+) [12]. This poor agreement in HER2 IHC scoring can lead to misassignment of ADCs therapy. To address this discordance, several studies have been designed to identify an effective quantitative method for distinguishing HER2-low from HER2-ZERO [13–15]. Additionally, the DESTINY-Breast06 trial is evaluating T-DXd in patients with HER2-ultra low breast tumors (IHC > 0 to < 1+), providing insights that may improve the identification of patient populations expected to benefit from HER2-targeted antibody-drug conjugates [16]. This trial also suggests that we should investigate the true statuses of HER2 gene in tumors with different HER2 protein expression levels. In this study, we applied FISH to perform a comprehensive stratification of HER2 gene among HER2 negative breast cancer tumors, including HER2-ZERO and HER2-low. Our aim was to reveal the actual status of HER2 in the large population of HER2 negative breast cancer patients, who account for 80-85% of all cases.

Materials and methods

Study subjects and clinical data

In this study, we retrospectively reviewed patients with invasive ductal breast carcinoma who underwent surgeries without pre-operative chemotherapy or radiotherapy at Union Hospital, Tongji Medical College, Huazhong University of Science and Technology between 2018 and 2019. The original medical records were thoroughly reviewed, wherein the HER2 status was initially assessed via immunohistochemistry (IHC) using clone 4B5. From this cohort, we selected cases categorized as IHC-0, IHC-1+, and IHC-2+, with the latter group already undergoing fluorescence in situ hybridization (FISH, Wuhan HealthCare Biotechnology Co., Ltd) testing to exclude any IHC-2+/FISH + scenarios. Ultimately, a total of 349 cases of HER2-negative were enrolled and the results of IHC using clone 4B5 were reviewed. All of cases were then divided into three groups: HER2-ZERO ($n = 115$), HER2-1+ ($n = 104$) and HER2-2+/FISH- ($n = 130$), according to the World Health Organization Classification of Tumors of the Breast, 5th edition. The HER2 status was re-examined using both IHC and FISH in all cases to further investigate HER2 expression in HER2-ZERO and HER2-low breast cancer. The interpretations were independently performed by two experienced pathologists (Fig. 1).

The study was approved by the Medical Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology and informed consent was obtained from all patients. The experiments performed in this study adhere to the current laws of China regarding the use of human subjects.

Fluorescence in situ hybridization

The patients in Group HER2-2+/FISH- were formerly confirmed to be HER2 negative using the human HER2 gene amplification test kit (Wuhan HealthCare Biotechnology Co., Ltd) via FISH. To assess HER2 gene expression, the LBP DNA probe Kit (Guangzhou LBP Medicine Science & Technology Co., Ltd.) was utilized to determine the status of all enrolled patients. For each sample, HER2 and CEP17 copy numbers were evaluated in approximately 30 invasive tumor cells, enabling the calculation of both the average HER2 copy numbers and HER2/CEP17 ratio.

Immunohistochemistry

All patients enrolled in this study were initially tested using clone 4B5 and then retested with clone EP3. Here is the detailed information about the antibodies used: clone 4B5 (Ventana, Roche) and clone EP3 (Guangzhou LBP Medicine Science & Technology Co., Ltd.), both of which are rabbit monoclonal antibodies. Finally, 327/349 cases of IHC were evaluated. The IHC staining results for clone EP3 were independently interpreted by two pathologists. In cases of disagreement, a senior pathologist conducted further evaluation to establish the gold standard. The results were interpreted according to the 2023 ASCO

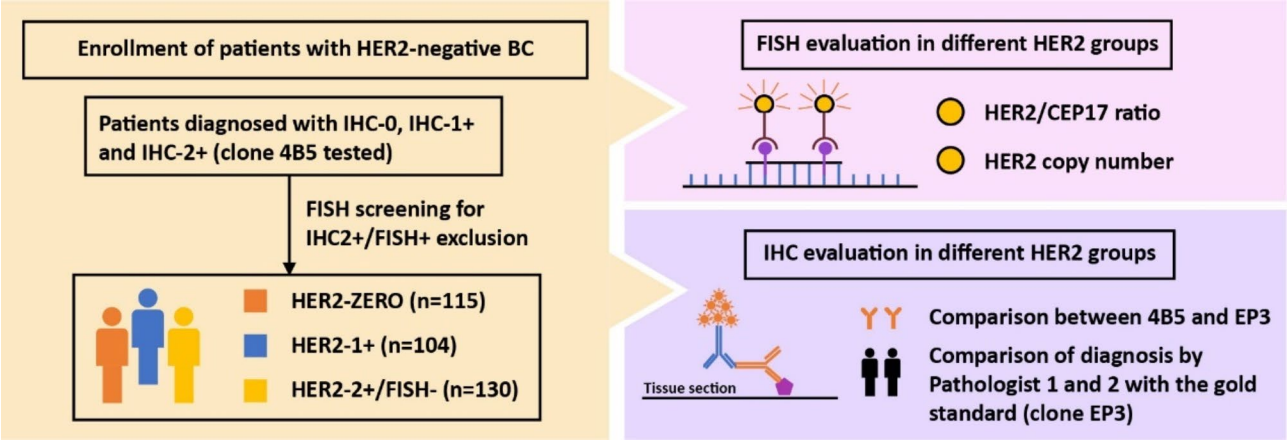

Fig. 1 Flow chart of study design. Step 1, a total of 349 cases of patients with HER2-negative breast cancer were enrolled, based on the archive IHC results using clone 4B5 for all patients and FISH testing (Wuhan HealthCare Biotechnology Co., Ltd) for those with IHC-2 + status. Step 2, IHC testing using clone EP3 and FISH testing (Guangzhou LBP Medicine Science & Technology Co., Ltd.) were re-administered to ascertain the HER2 status of all enrolled patients with HER2-negative breast cancer. A comparative analysis was conducted between the results obtained using clone 4B5 and clone EP3, as well as between the interpretations made by pathologists and the gold standard established by clone EP3. BC indicates breast cancer

guidelines [17] as follows: ① HER2-ZERO: no staining or incomplete membrane staining that is faint/ barely perceptible in ≤ 10% of tumor cells; ② HER2-1+: incomplete membrane staining that is faint/ barely perceptible in > 10% of tumor cells; ③ HER2-2+: weak to moderate complete membrane staining observed in > 10% of tumor cells.

Statistical analysis

To assess the clinicopathologic feature differences among HER2-ZERO, HER2-1+, and HER2-2+/FISH- groups, as well as between HER2-ZERO and HER2-low groups, the χ2 test or two-sided Fisher's exact test were utilized. To evaluate the consistency of HER2 expression interpretation by IHC using two distinct HER2 clone antibodies, as well as to assess the agreement between the interpretations of two pathologists compared to the gold standard, the paired t-test was utilized. The Fisher's exact test was used to estimate the differences in HER2 gene distribution between HER2-ZERO, HER2-1+, and HER2-2+/FISH- groups. Finally, the normal distribution of FISH results across various groups was examined using the Shapiro-Wilk test and one-way analysis of variance was utilized to measure the differences in FISH results among the HER2-ZERO, HER2-1+, and HER2-2+/FISH- groups.

Results

Clinicopathologic characteristics of the HER2-low and HER-ZERO breast cancer cohort

In this study, we enrolled 349 HER2-negative breast cancer patients, including 115 patients in the HER2-ZERO group, 104 patients in the HER2-1 + group, and 130 patients in the HER2-2+/FISH- group. These patients were generally reclassified into two groups: HER2-ZERO (HER2-ZERO, $n = 115$) and HER2-low (HER2-1 + and HER2-2+/FISH-, $n = 234$). The classification used in this study is based on the HER2 IHC score obtained through clone 4B5. In the entire breast cancer cohort, we found that HER2-low had fewer grade III tumors than HER2-ZERO (39.74% and 55.65%, respectively, $P = 0.005$, χ2 test). ER (88.89% and 61.74%, respectively, $P < 0.001$, χ2 test) and PR (84.62% and 57.39%, respectively, $P < 0.001$, χ2 test) positivity was more common in HER2-low tumors than HER2-ZERO. When stratified by HER2 expression by IHC, a similar situation was found between HER2-ZERO, HER2-1 + and IHC-2 in histologic grade, ER and PR expression ($P < 0.05$, χ2 test) (Table 1).

Dai et al. delved into the distinctive nature of HER2-low breast cancer across various hormone receptor statuses [18]. We, in turn, examined the nuances between HER2-ZERO and HER2-low in different hormone receptor contexts. According to the baseline clinicopathologic features observed in HR negative breast cancer, larger tumor size was more common in HER2-low than HER2-ZERO ($P = 0.004$, two-sided Fisher's exact test), and in HER2-1 + than HER2-ZERO (85.71% and 47.62%, respectively, $P = 0.005$, two-sided Fisher's exact test) (Table 2). In HR positive breast cancer, Ki67 labeling index was higher in HER2-low than HER2-ZERO ($P = 0.004$, χ2 test) and in HER2-2+/FISH- and HER2-ZERO ($P = 0.016$, χ2 test) (Table 3).

Comparison of HER2 IHC interpretation using two antibodies and pathologist concordance with the "EP3 gold standard"

We conducted a thorough review of the 327/349 cases of archived slides stained with clone 4B5, alongside the newly stained slides utilizing clone EP3. The overall

Table 1 Baseline clinicopathologic characteristics of HER2 HER2-ZERO, HER2-1 + and HER2-2+/FISH- breast cancer cohort

	HER2-ZERO (*n* = 115)	HER2-low (*n* = 234)	*P**	HER2-ZERO (*n* = 115)	HER2-1+ (*n* = 104)	HER2-2+/ FISH-(*n* = 130)	*P**
Age (years)							
< 50	54	114	0.757	54	60	54	0.047
≥ 50	61	120		61	44	76	
Grade							
I-II	51	141	0.005	51	57	84	0.006
III	64	93		64	47	46	
pT							
pT1	65	123	0.889	65	56	67	0.440
pT2	48	103		48	41	62	
pT3	2	5		2	4	1	
NA	0	3		0	3	0	
pN							
pN0	56	124	0.389	56	56	68	0.656
pN1	32	66		32	28	38	
pN2	19	24		19	10	14	
pN3	7	18		7	10	8	
NA	1	2		1	0	2	
Ki67 labeling index							
< 20%	40	73	0.501	40	32	41	0.791
≥ 20%	75	161		75	72	89	
ER							
Negative	44	26	< 0.001	44	14	12	< 0.001
Positive	71	208		71	90	118	
PR							
Negative	49	36	< 0.001	49	17	19	< 0.001
Positive	66	198		66	87	111	

* χ2 test or two-sided Fisher's exact test

Values that are not available (NA) were excluded from *P*-value calculation

concordance rate between 4B5 and EP3 was 64.22% (95% CI: 58.76-69.42%), indicating a relatively general consistency between 4B5 and EP3 staining in retrospectively selected tumor formalin fixed paraffin embedded (FFPE) samples (Fig. 2A). Among the 327 samples, the staining results of the two antibodies were completely consistent in 210 cases (64.22%), while discrepancies were observed in 117 cases. The specific discrepancies were as follows: 43 samples were scored as 0 with 4B5 but as either 1+ (41 cases) or 2+ (2 cases) with EP3; 31 samples were scored as 1 + with 4B5 but as either 0 (9 cases) or 2+ (22 cases) with EP3; 43 samples were scored as 2 + with 4B5 but as either 0 (3 cases) or 1+ (40 cases) with EP3. Among the 327 samples, no case was interpretated as score 3+.

We further compared the interpretations of EP3 stained slides by two pathologists with the EP3 gold standard. The results revealed that Pathologist 1 demonstrated higher concordance with the gold standard (Fig. 2B), achieving an overall concordance rate of 94.19% (95% CI: 91.07-96.47%). In contrast, Pathologist 2 showed lower concordance with the gold standard (Fig. 2C), with overall concordance rate of 74.31% (95% CI: 69.22-78.96%). Pathologist 1 has 7 years of experience in IHC slide interpretation, with most errors in this study occurring in slides scored as 1 + and 2+. Pathologist 2, with 5 years of IHC slide interpretation experience, predominantly made errors in slides scored as 0 and 2+. Furthermore, Pathologist 2 exhibited a strong tendency to interpret results as 1+, categorizing 67.9% of the slides in this study as 1+.

Distinct profiles of HER2/CEP17 ratio in breast cancer with different HER2 protein expression patterns

To determine the HER2 amplification status within each group, we employed FISH analysis. According to the 2023 ASCO guidelines[17], HER2 amplification was observed in 0.9% (1/105) of patients in the HER2-ZERO group, 2.88% (3/104) in the HER2-1 + group, and 1.54% (2/130) in the HER2-2+/FISH- group. However, only the three patients within the HER2-1 + group who tested positive for HER2 exhibited clear HER2 amplification, with HER2/CEP17 ratio of 9.68, 10.34, and 15.77, respectively. Conversely, the other HER2-positive cases in the HER2-ZERO and HER2-2+/FISH- groups were found to be very close to the threshold for HER2 amplification. When the IHC values of these three HER2-positive patients were reassessed using clone EP3, it was found that one patient was

Table 2 Baseline clinicopathologic characteristics of HER2 HER2-ZERO, HER2-1 + and HER2-2+/FISH- in HR negative breast cancer cohort

	HER2-ZERO (*n* = 42)	HER2-low (*n* = 25)	*P**	HER2-ZERO (*n* = 42)	HER2-1+ (*n* = 14)	HER2-2+/FISH-(*n* = 11)	*P**
Age (years)							
< 50	19	11	0.921	19	7	4	0.831
≥ 50	23	14		23	7	7	
Grade							
I-II	3	3	0.664	3	1	2	0.397
III	39	22		39	13	9	
pT							
pT1	22	4	0.004	22	1	3	0.005
pT2	20	20		20	12	8	
pT3	0	1		0	1	0	
pN							
pN0	22	18	0.342	22	12	6	0.393
pN1	10	2		10	0	2	
pN2	5	2		5	1	1	
pN3	4	2		4	1	1	
NA	1	1		1	0	1	
Ki67 labeling index							
< 20%	1	1	1.000	1	0	1	0.345
≥ 20%	41	24		41	14	10	

* χ2 test or two-sided Fisher's exact test

Values that are not available (NA) were excluded from *P*-value calculation

Table 3 Baseline clinicopathologic characteristics of HER2 HER2-ZERO, HER2-1 + and HER2-2+/FISH- in HR positive breast cancer cohort

	HER2-ZERO (*n* = 73)	HER2-low (*n* = 209)	*P**	HER2-ZERO (*n* = 73)	HER2-1+ (*n* = 90)	HER2-2+/FISH-(*n* = 119)	*P**
Age (years)							
< 50	35	103	0.892	35	53	50	0.053
≥ 50	38	106		38	37	69	
Grade							
I-II	48	138	0.966	48	56	82	0.600
III	25	71		25	34	37	
pT							
pT1	43	114	0.664	43	51	63	0.497
pT2	28	88		28	33	55	
pT3	2	4		2	3	1	
NA	0	3		0	3	0	
pN							
pN0	34	106	0.250	34	44	62	0.498
pN1	22	64		22	28	36	
pN2	14	22		14	9	13	
pN3	3	16		3	9	7	
NA	0	1		0	0	1	
Ki67 labeling index							
< 20%	39	72	0.004	39	32	40	0.016
≥ 20%	34	137		34	58	79	

* χ2 test or two-sided Fisher's exact test

Values that are not available (NA) were excluded from *P*-value calculation

reinterpreted as 2+, while the other two remained classified as 1+.

The majority of patients in each group exhibited a HER2/CEP17 ratio between 1 and 2. Notably, more patients in the HER2-2+/FISH- group had a HER2/CEP17 ratio ranging from 1 to 2 compared to the HER2-1 + group among all patients (95.38% vs. 85.58%, $P = 0.036$, two-sided Fisher's exact test) (Fig. 3A) and

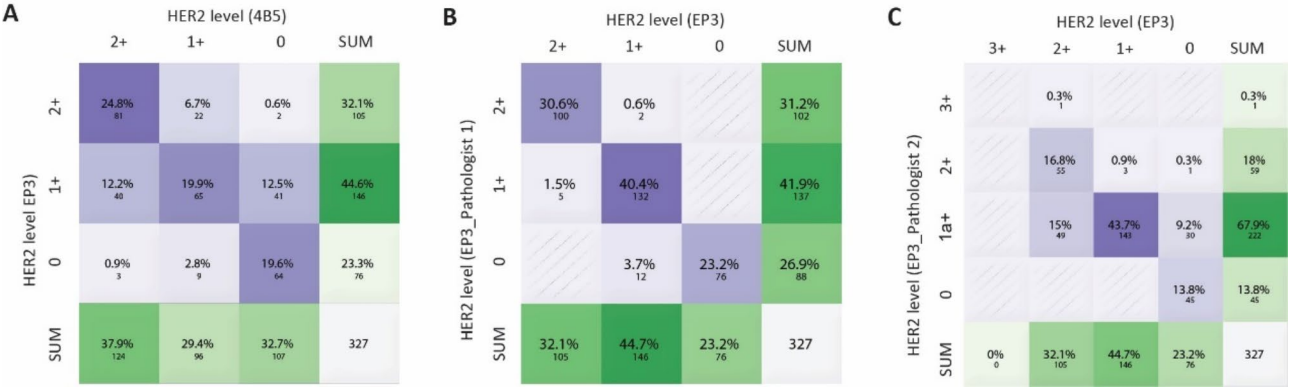

Fig. 2 Consistency comparison of HER2 IHC interpretation using two antibodies and pathologist concordance with the "EP3 gold standard". (**A**) Consistency comparison of HER2 IHC interpretation using clone 4B5 and clone EP3. (**B**) Consistency comparison between Pathologist 1's IHC results and the gold standard using clone EP3. (**C**) Consistency comparison between Pathologist 2's IHC results and the gold standard using clone EP3

hormone receptor (HR)-positive patients (95.80% vs. 86.67%, $P=0.045$, two-sided Fisher's exact test) (Fig. 3C). No statistically significant differences were observed among HR-negative patients across the HER2-ZERO, HER2-1+, and HER2-2+/FISH- groups ($P>0.05$, two-sided Fisher's exact test) (Fig. 3B). More precisely, the HER2/CEP17 ratio was primarily limited to 1.4 or less, with frequencies of 94.34%, 85.39%, and 83.87% observed in the HER2-ZERO, HER2-1+, and HER2-2+/FISH- groups, respectively. Significant differences in HER2/CEP17 ratio were observed between the HER2-ZERO and HER2-1 + groups ($P=0.036$) as well as between the HER2-ZERO and HER2-2+/FISH- groups ($P=0.012$). Furthermore, a higher proportion of patients in the HER2-ZERO group demonstrated HER2/CEP17 ratio not exceeding 1.4 compared to the HER2-low group (94.34% vs. 84.51%, $P=0.012$).

Regarding the distribution of HER2/CEP17 ratio, log2 transformation was performed due to the non-normal distribution of the values within each group, as confirmed by Shapiro-Wilks test. Based on the classification of HER2 expression by clone 4B5, HER2/CEP17 ratio was found to be significantly higher in the HER2-low group compared to the HER2-ZERO group after log2 transformation ($P=0.026$) (Fig. 3D and E). Among the comparison of three groups, the HER2/CEP17 ratio was found to be higher in the HER2-2+/FISH- compared to the HER2-ZERO before ($P=0.039$) (Fig. 3F) and after log2 transformation ($P=0.021$) (Fig. 3G).

Although significant difference was observed in HER2/CEP17 ratio between the HER2-ZERO and HER2-low groups, no effective cut off value could be determined. The square of the ROC for HER2/CEP17 ratio was 0.528, indicating that HER2/CEP17 ratio is not an effective value for distinguishing between HER2-ZERO and HER2-low.

Distinct profiles of average HER2 copy numbers in breast cancer with different HER2 protein expression patterns

The majority of patients in each group exhibited average HER2 copy numbers between 1 and 4. No statistically significant differences were found among the entire breast cancer cohort, HR-negative or -positive cohort across HER2-ZERO, HER2-1 + and HER2-2+/FISH- groups ($P>0.05$, two-sided Fisher's exact test) (Fig. 4A-C).

Specifically, the average HER2 copy numbers did not exceed 2.5 in the majority of cases, with frequencies of 80.87%, 69.00%, and 69.84% in the HER2-ZERO, HER2-1+, and HER2-2+/FISH- groups, respectively. It showed significant difference between the HER2-ZERO and HER2-1 + groups ($P=0.044$), as well as between the HER2-ZERO and HER2-2+/FISH- groups ($P=0.048$). Furthermore, a higher proportion of patients in the HER2-ZERO group exhibited average HER2 copy numbers not exceeding 2.5 compared to the HER2-low group (80.87% vs. 69.47%, $P=0.024$).

Regarding the distribution of average HER2 copy numbers, log2 transformation was performed due to the non-normal distribution of the values within each group, as confirmed by Shapiro-Wilks test. Based on the classification of HER2 expression by clone 4B5, the average HER2 copy numbers showed a significant decrease in the HER2-low group compared to the HER2-ZERO group after log2 transformation ($P=0.001$) (Fig. 4D and E). Among comparison of three groups, the average HER2 copy numbers were higher in the HER2-2+/FISH- group compared to the HER2-ZERO group before ($P=0.005$) (Fig. 4F) and after log2 transformation ($P=0.003$) (Fig. 4G). After applying log2 transformation, a similar trend was also observed between the HER2-1 + and HER2-ZERO groups ($P=0.012$) (Fig. 4G).

Although significant difference was observed in average HER2 copy numbers between the HER2-ZERO and HER2-low groups, no effective cut off value could be determined. The square of the ROC for average HER2

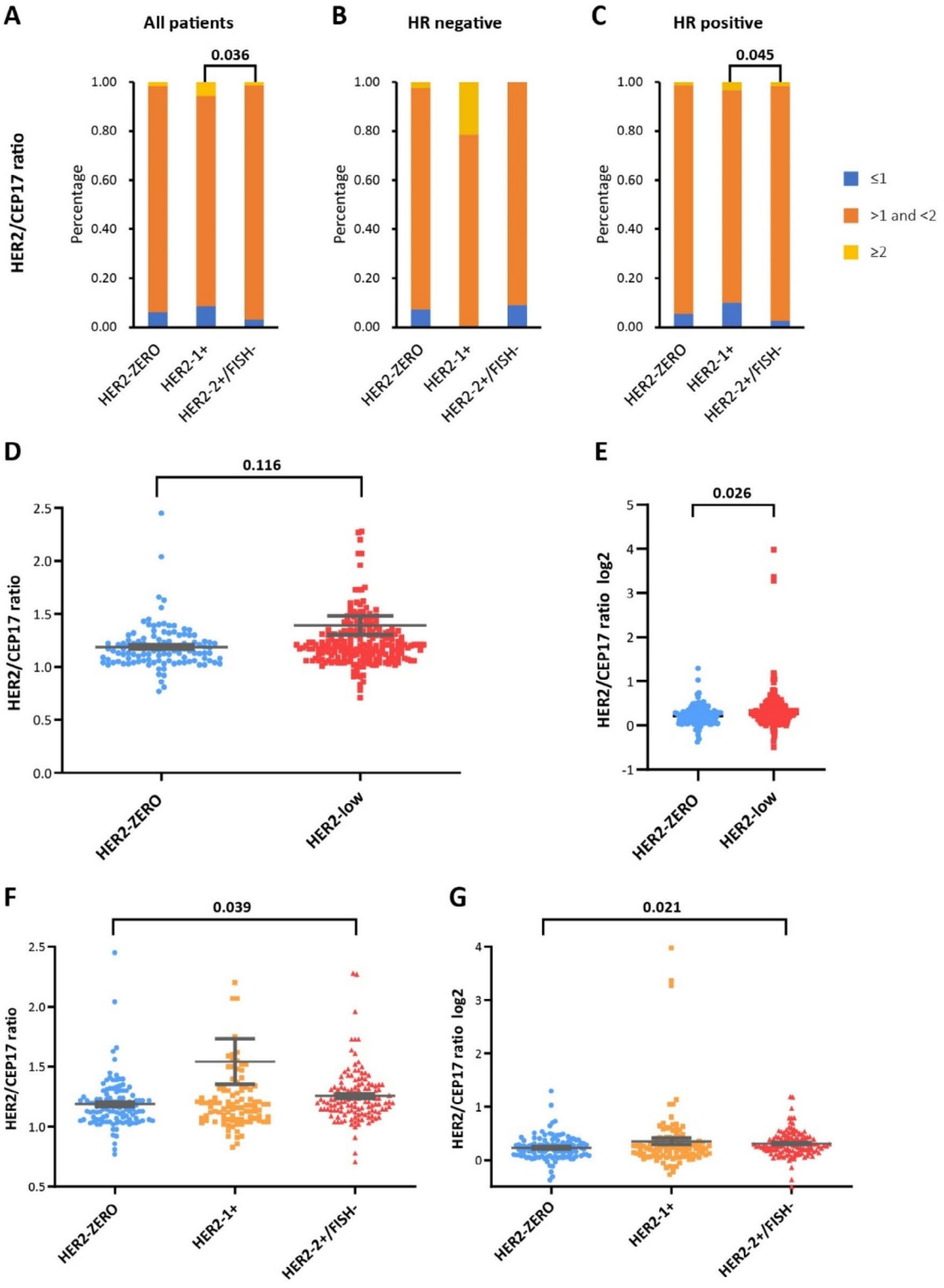

Fig. 3 The distribution and comparison of HER2/CEP17 ratio in breast cancer cohort. (**A**) Distribution of HER2/CEP17 ratio in the entire breast cancer cohort. (**B**) Distribution of HER2/CEP17 ratio in HR negative breast cancer cohort. (**C**) Distribution of HER2/CEP17 ratio in HR positive breast cancer cohort. No significant difference of HER2/CEP17 ratio was found between HER2-ZERO and HER2-low ($P=0.116$) (**D**), while it turned to be higher in HER2-low compared to HER2-ZERO after log2 transformation ($P=0.026$) (**E**). HER2/CEP17 ratio was higher in HER2-2+/FISH- compared to HER2-ZERO before ($P=0.039$) (**F**) and after ($P=0.021$) (**G**) log2 transformation

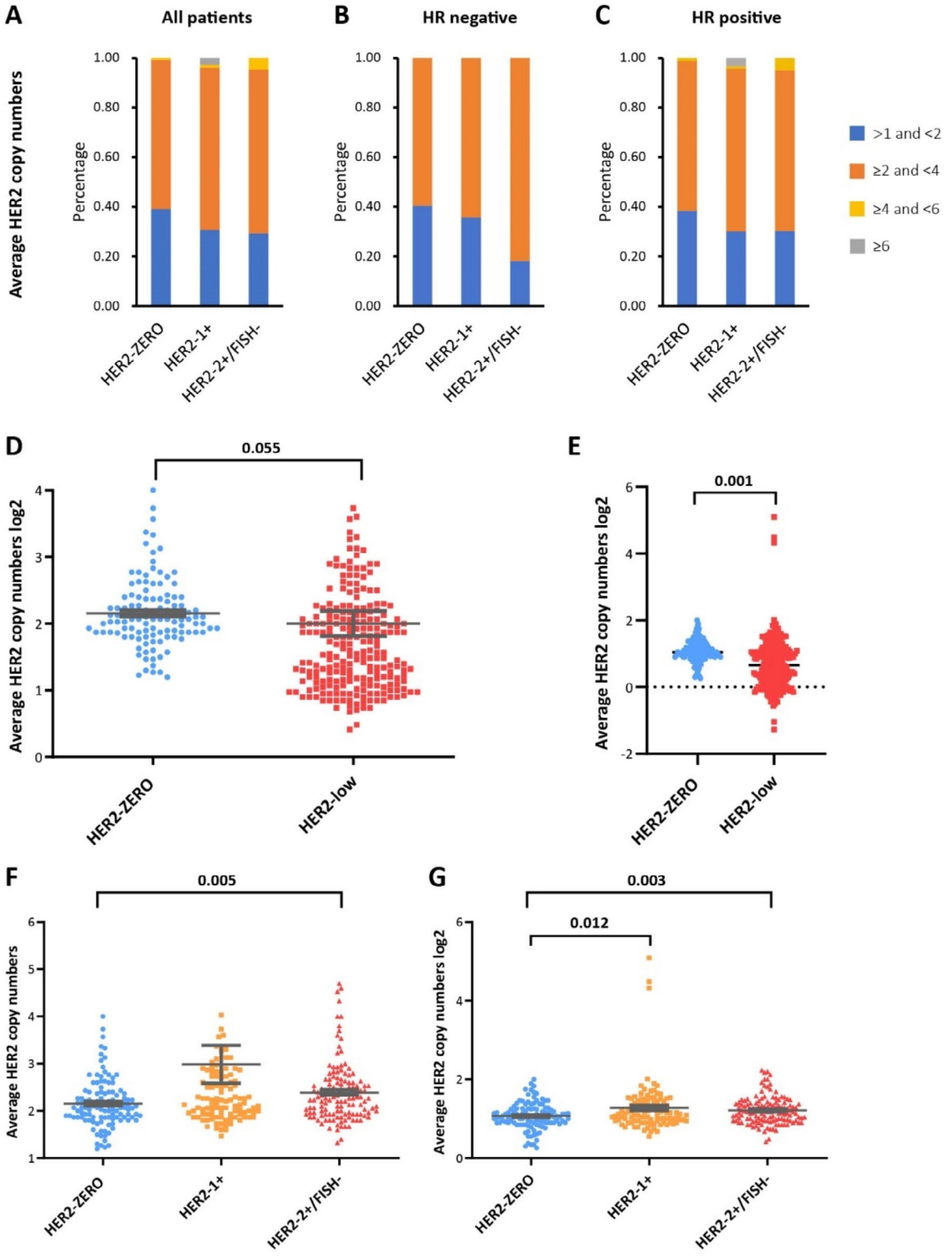

Fig. 4 The distribution and comparison of average HER2 copy numbers in breast cancer cohort. (**A**) Average HER2 copy numbers in the entire breast cancer cohort. (**B**) Average HER2 copy numbers in HR negative breast cancer cohort. (**C**) Average HER2 copy numbers in HR positive breast cancer cohort. No significant difference of average HER2 copy numbers was found between HER2-ZERO and HER2-low ($P=0.055$) (**D**), while it turned to be higher in HER2-ZERO compared to HER2-low after log2 transformation ($P=0.001$) (**E**). Average HER2 copy numbers was higher in HER2-2+/FISH- than HER2-ZERO before ($P=0.005$) (**F**) and after ($P=0.003$) (**d**) log2 transformation, and it also higher in HER2-1 + than HER2-ZERO after log2 transformation ($P=0.012$) (**G**)

copy number was 0.595, suggesting that average HER2 copy numbers is not an effective value for distinguishing between HER2-ZERO and HER2-low.

Then, the groups were reclassified based on HER2 IHC score using clone EP3. Only a significant difference was found between the HER2-ZERO and HER2-2+/FISH- groups in average HER2 copy numbers after log2 transformation ($P = 0.013$).

Discussion

Human epidermal growth factor receptor 2 (HER2) is a well recognized prognostic and therapeutic biomarker in breast cancer and it was defined by immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH). These patients of breast cancer with HER2 positive can be benefit from anti-HER2 therapy, which is only account for 15-20% of breast cancer. In standard care for breast cancer patients, HER2-ZERO, HER2-1 + and HER2-2+/FISH- are all classified as HER2 negative and no clinical significance in distinguishing between these categories because of no clinical benefits in patients of breast cancer in the first generation of HER2-targeted therapies.

However, recent data on the efficacy of new ADC therapies in patients with breast cancer with low HER2 protein expression opens the door to a new population of HER2-low breast cancer, indicating a new era of HER2 classification. In the current study, we found a significance between HER2-ZERO and HER2-low in histological grade and expression of estrogen receptor (ER), progesterone receptor (PR) and Ki67. In the cohort of Lei-Jie Dai et al., no significant difference between HER2-ZERO and HER2-low in clinicopathological characteristics was identified, while there is significant difference in RNA and protein level and the prognosis between HER2-ZERO and HER2-low [18]. In consideration of the efficacy of ADC therapies in patients with HER2-low breast cancer and the significant difference between it from HER2-ZERO and HER2-positive, how to distinguish HER2-low from HER2-ZERO seems of great importance. In this study, EP3 was used to compare with the archive original medical records of HER2 which was tested by 4B5. The consistency comparison between 4B5 and EP3 revealed a general consistency with 64.22%. Regarding the inter-observer consistency, we found that Pathologist 1, who has more extensive working experience, shows higher consistency with the gold standard. In clinical practice, the status of HER2 is defined by IHC and FISH, while the clone of HER2 antibody, different pathologists and different laboratory are all can have an impact on the interpretation of the result of IHC of HER2 [12, 19]. In general, the accordance is not good. However, based on the comparison of pathologists' results with the gold standard in this study, it is evident that enhancing training in HER2 interpretation can effectively improve pathologists' diagnostic acumen, thereby fostering greater consistency. On the other hand, some researches designed an AI to improve this situation and it seems to give us a good result [20]. However, it is worth noting that a potential limitation exists in the study. The definitions of the HER2-ZERO and HER2-low groups were predetermined, potentially introducing a degree of bias in their designation. Consequently, these findings highlight the limitations of IHC in accurately distinguishing between HER2-low and HER2-ZERO cases.

To further confirm the expression of HER2 at the gene level in these "HER2-non-positive" cases, FISH analysis was employed. In the HER2-1 + group, three cases were observed with clear HER2 amplification, while the HER2-ZERO and HER2-2+/FISH- groups each exhibited one HER2-positive case that was near the cut-off value. This phenomenon has also been noted in other studies, with respective percentages of 23.2%, 7.4%, and 4.1% for IHC-diagnosed cases of 2+, 1+, and 0 [21]. In this study, cases of HER2-2+/FISH + were excluded based on previously archived FISH results, resulting in a relatively low positive proportion within the HER2-2 + group. Additionally, in a comparative study of the DaKo Hercep Test and Ventana PATHWAY Anti-HER2 (4B5), three HER2-amplified cases were missed by 4B5 [22], potentially due to its IHC protocol [23]. Considering cost-effectiveness, criteria for HER2 positivity by IHC and FISH have been established to identify the most likely HER2-amplified cases. However, these criteria inevitably miss cases diagnosed as IHC 0 or 1 + that are later confirmed as HER2-amplified by FISH.

FISH reveals the status of the HER2 gene, whereas IHC detects the expression of the HER2 protein. The processes of gene transcription and translation are intricate, and the expression of a particular protein can be influenced by numerous factors, not solely by gene amplification. In fact, overexpression of the HER2 protein may not necessarily be linked to HER2 gene amplification, as evidenced in ER-positive ductal carcinoma in situ [24].

To reveal the expression profiles of HER2 gene in different HER2 protein expression patterns, we compared the HER2/CEP17 ratio and the average HER2 copy numbers between HER2-ZERO and HER2-low groups, indicating significant differences. Furthermore, we established cut-off values for both HER2/CEP17 ratio and average HER2 copy numbers, but neither exhibited satisfactory sensitivity and specificity. A critical stratification analysis of HER2/CEP17 ratio revealed that the majority of cases had HER2/CEP17 ratio concentrated between 1 and 2 (92.17%) and not exceeding 1.4 (94.34%). Notably, both of these ranges were more prevalent in HER2-ZERO cases compared to HER2-low cases. Similarly, the analysis of HER2 average copy numbers yielded a similar

observation, with a higher frequency of cases with signals not exceeding 2.5 in HER2-ZERO compared to HER2-low.

These findings suggest that while FISH can reveal population-level differences between HER2-ZERO and HER2-low cases, it remains challenging to make such distinctions in individual patients. Therefore, the accurate definition of HER2-ZERO and HER2-low remains a critical issue in practical pathology.

Multiple potential methods have been developed to assess the status of HER2, including the reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) assay. A study conducted by Shu L and colleagues compared HER2 mRNA levels between HER2 0 and 1 + tumors, revealing that both exhibited significantly lower levels compared to IHC 2+/FISH – tumors [25]. However, it is worth noting that some bias may still exist due to the interpretation of IHC results used to categorize the tumors. In "HER2-non-positive" cases, the expression of the HER2 gene tended to be closer to normal in a larger proportion of the HER2-ZERO group. Nevertheless, FISH remains a viable tool for identifying the relative and specific gene expression levels, which raises the question of what threshold of HER2 gene expression would render a patient eligible for ADC therapy. Prior to the advent of ADC therapy, anti-HER2 therapy was regarded as efficacious in cases of HER2 positivity. However, the response to anti-HER2 therapy has been observed to be less effective in IHC-2+/FISH + tumors compared to IHC-3 + tumors [26]. It appears that both the expression of HER2 protein and the amplification of the HER2 gene contribute to the efficacy of anti-HER2 therapy. Among the vast population of patients who are considered "HER2-non-positive," ADC therapy has emerged as a promising therapeutic option. The DESTINY-Breast 04 study revealed that regardless of the HR status, T-Dxd therapy in HER2-low cases significantly enhances progression-free survival (PFS) and overall survival (OS) when compared to chemotherapy. Specifically, the risk of disease progression and death decreased by 50% and 36%, respectively [5]. Furthermore, certain studies have hinted at the promising potential of novel ADCs in improving PFS and OS in patients with HR+/HER2- metastatic breast cancer [27–29]. The DESTINY-Breast 06 study encompassed patients with both HR-positive breast cancer featuring HER2-low and HER2-ultralow expression (IHC-0 with membrane staining). The results indicated that treatment with T-Dxd resulted in longer progression-free survival compared to chemotherapy [30].

HER2 is a membrane protein, acting as the anchor for ADCs. ADCs are comprised of four crucial components: the target antigen, the antibody construct, a payload (most frequently a cytotoxic agent), and a linker moiety that bridges the payload and the antibody [31]. Notably, the pharmacological mechanism of ADCs does not solely rely on high HER2 expression. Instead, its primary mode of action is attributed to its bystander benefit and high loading ratio [27]. Further study should be focus on the real expression profile of HER2 gene and protein in patients with HER2-low and HER2-ultralow, who were treated with ADC drugs, to defined the relation to the effectiveness of ADC therapy in those patients.

Conclusion

In conclusion, the IHC assessment of HER2 can be influenced by factors such as the clone of antibodies used and the observers' interpretation. However, we can enhance the consistency of these assessments by providing additional training in HER2 interpretation. FISH may not be reliable methods for distinguishing HER2-low from HER2-ZERO status, while it can be employed to assess the HER2 gene expression status, providing valuable insights for ADC therapy decisions. In the future, it is imperative to delve deeper into the HER2 gene expression patterns in breast cancer patients with low HER2 expression who are likely to respond favorably to ADC drug therapy. This will enable us to identify these patients with greater precision and efficacy, thereby guiding the treatment strategies for other similar patients.

Author contributions

Jingmin Zhong: Data curation, funding acquisition, writing-original draft and editing. Beibei Gao: Data curation, formal analysis, writing-review. Qingjie Wang: Data curation. Jun He: Methodology. Danjv Luo: Methodology. Cheng Zhang: Methodology. Jun Fan: Writing-review. Xiu Nie: Supervision, writing-review. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Conflict of interest

The authors have no conflicts of interest to declare.

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