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ARTICLE

Intense basolateral membrane staining indicates *HER2* positivity in invasive micropapillary breast carcinoma

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Abstract

Invasive micropapillary carcinoma is characterized by the inside-out growth of tumor clusters and displays incomplete membrane immunostaining of *HER2*. According to the 2018 American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) *HER2*-testing recommendation, moderate to intense but incomplete staining could be scored as immunohistochemical 2+. Furthermore, the criteria of immunohistochemical 3+ for this staining pattern are not mentioned. One hundred and forty-seven cases of invasive micropapillary carcinoma with moderate-to-intense *HER2* immunostaining were enrolled. Invasive micropapillary carcinoma components of all cases were scored as immunohistochemical 2+ based on the 2018 ASCO/CAP recommendation. The invasive micropapillary carcinoma component varied from 10% to 100% (mean, 80%). Invasive micropapillary carcinoma components of all 147 tumors exhibited reversed polarity and incomplete basolateral *HER2* membrane staining. One hundred and seventeen of the tumors (80%, 117/147) had moderate staining, and 38 (32%, 38/117) showed *HER2* gene amplification by fluorescence in-situ hybridization. *HER2* gene was amplified in all the remaining 30 tumors (20%, 30/147) that exhibited intense basolateral membrane staining. Besides, average *HER2* signals per cell and ratio of *HER2*/CEP17 were significantly higher in the intense-staining tumors compared with the moderate-staining tumors ($p < 0.0001$). Follow-up data were available for 140 patients. None of the patients were died. The follow-up time ranged from 1 month to 99 months (median, 57 months). Thirteen (9%, 13/140) patients exhibited disease progression (recurrence or metastasis). *HER2* gene amplification was correlated inversely with estrogen receptor ($p = 0.000$) and progesterone receptor ($p = 0.000$) expression, and positively with histological grade ($p = 0.003$) and disease progression ($p = 0.000$). Invasive micropapillary carcinoma with intense clear linear basolateral membrane immunostaining indicates *HER2* positivity, even if the staining is incomplete. They should be classified as immunohistochemical 3+ rather than immunohistochemical 2+, which would avoid further fluorescence in-situ hybridization-testing procedure and greatly save the related time, labor, and financial costs. Ultimately, ensure all patients with *HER2* gene amplification obtain effective targeted therapy in time.

Introduction

HER2 is known as a key driver for tumorigenesis and its overexpression caused by *HER2* gene amplification has been observed in many solid tumors [1, 2]. Notably, overexpression of *HER2* occurs rather highly in invasive breast cancers, ranging from 13 to 20% [3, 4], as compared with other tumors. It has been reported that *HER2* overexpression is associated with poor clinical outcomes of prostate cancer, lung cancer, gastric cancer, as well as breast cancer. As a well-established therapeutic target, mounting evidence has indicated that targeting *HER2* could improve the outcomes of *HER2*-positive breast cancer patients [5, 6].

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Therefore, it is critical to accurately assess *HER2* expression of tumors of breast cancer patients, and to correctly identify patients with *HER2*-positive tumors who may benefit from the target therapy, while to spare patients with *HER2*-negative tumors from anti-*HER2* therapies and the toxic effects of these costly drugs.

To improve the reproducibility and standardization of the assessment of *HER2* expression in invasive breast cancer, ASCO/CAP established updated comprehensive guideline recommendations for *HER2* testing [7] in 2018, following the earlier guideline in 2013 [8]. In the newly updated guideline, *HER2* immunohistochemical 3+ requires that >10% of invasive tumor cells exhibit intense and complete membrane staining. Invasive micropapillary carcinoma (invasive micropapillary carcinoma) is characterized by the inside-out growth of tumor clusters in a pseudopapillary arrangement. *HER2* staining in invasive micropapillary carcinoma tumors was incomplete, with basolateral membrane staining (or U-shaped) [9]. Based on 2018 ASCO/CAP guideline, invasive micropapillary carcinoma with moderate to intense but incomplete staining should be reported as immunohistochemical 2+, requiring an additional fluorescence in-situ hybridization. However the criteria of *HER2* immunohistochemical 3+ for invasive micropapillary carcinoma are not mentioned in the guideline.

We sought to provide additional information for further improvement of the *HER2* immunohistochemical evaluation criteria in invasive micropapillary carcinoma tumors. One hundred and forty-seven cases with varied proportion of invasive micropapillary carcinoma and moderate-to-intense *HER2* immunostaining in more than 10% of tumor cells of invasive micropapillary carcinoma were enrolled. Both immunohistochemical and fluorescence in-situ hybridization for *HER2* were reevaluated. They were considered as immunohistochemical 2+ by the guideline in 2018 [7].

Materials and methods

Sample collection

This study was approved by the research ethics committees from the authors' institutions. Hematoxylin and eosin and immunohistochemical staining of *HER2* of invasive breast cancers previously diagnosed as invasive micropapillary carcinoma or invasive carcinoma of no special type with invasive micropapillary carcinoma components in the Department of Pathology in Fudan University Shanghai Cancer Center between 2010 and 2017 were retrieved and reviewed. Cases that exhibited moderate-to-intense incomplete *HER2* membrane staining and with fluorescence in-situ hybridization results would be enrolled. And they would be scored as immunohistochemical 2+ according to the 2018 ASCO/CAP recommendation [7]. A total of 147 cases were included finally, including 100 in-house cases and 47 consultation cases. Clinical features, including clinical presentations, treatments and follow-up information were obtained from patient clinical histories. Tumor stages of primary tumor without preoperative treatment were evaluated according to the eighth edition of the AJCC staging system [10]. Seventeen patients who had received neoadjuvant chemotherapy prior to surgery were excluded before tumor staging.

Diagnostic criteria for invasive micropapillary carcinoma

The surgical specimens were routinely processed into 4-μm sections and stained with hematoxylin–eosin. To identify invasive micropapillary carcinoma cases, we used the criteria described in the WHO histologic classification of breast tumors (2019 version) [11]. The tumors examined in this study were reviewed independently by two breast pathologists to confirm the diagnosis. All 147 tumors were grouped basing on the proportion of invasive micropapillary carcinoma component. Group A was pure invasive micropapillary carcinoma with more than 90% of invasive micropapillary carcinoma component. Group B was mixed invasive breast carcinoma of no special type and invasive micropapillary carcinoma with proportion of invasive micropapillary carcinoma between 10 and 90%. Group C was invasive breast carcinoma of no special type with focal invasive micropapillary carcinoma (<10%). The invasive micropapillary carcinoma component of each tumor was graded histologically according to a modified Bloom and Richardson scoring system [12]. Relevant histologic features such as lymphatic vessel invasion and lymph node status were also noted.

Immunohistochemistry and fluorescence in-situ hybridization testing and the evaluation criteria

All 147 tumors were immunohistochemically assessed for estrogen receptor, progesterone receptor, *HER2*, Ki-67, and epithelial membrane antigen. All of the antibodies used in this study were from Roche Ventana. The staining was performed with the Ventana BenchMark ultra autostainer (Ventana Medical System Inc., Roche, Tuscon, AZ, USA). The scoring recommendation from the ASCO/CAP [7] was used to evaluate *HER2* status. Staining was considered positive for estrogen receptor and progesterone receptor when nuclear staining was observed in 1% or more of the

tumor cells [13]. The Ki-67 labeling index was determined by counting the number of Ki-67-positive cancer nuclei from a total of 1000 cancer cells. We reevaluated both immunostaining and fluorescence in-situ hybridization results for *HER2* in the micropapillary component of all 147 tumors.

Immunochemical staining of *HER2* was reevaluated by two certified breast pathologist according to the criterion described in previous reports [7, 14]. Intense staining was visible to the naked eye with clear liner membrane staining at low magnification (×4) as shown in Fig. 2. Moderate staining was visible at medium magnification (×10–20) [7, 14]. All 147 patients were finally divided into two groups based on *HER2* immunostaining intensity (moderate or intense).

All 147 tumors were further examined using the Abbott–Vysis *HER2* fluorescence in-situ hybridization assay. *HER2* and CEP17 signals were manually counted by two certified molecular pathologists independently. Each individually scored 20 tumor cells from nonoverlapping areas and calculated the corresponding *HER2*/CEP17 ratios. If their counts are comparable, an average was used to determine the final copy numbers. If results were significantly discrepant, the counts were repeated. Results were interpreted and reported according to the 2018 ASCO/CAP guideline [7].

Based on the definitions adopted by the 2013 St. Gallen Consensus Panel [15], all included tumors were further classified into the following immunohistochemical surrogate subtypes: luminal A-like subtype (estrogen receptor+, progesterone receptor(+, ≥20%), *HER2*- and Ki-67 <20%), Luminal B-like (*HER2*-negative subtype: estrogen receptor+, *HER2*-, Ki-67 >20% or progesterone receptor −/+; *HER2*-positive subtype: estrogen receptor+, *HER2* overexpression with any progesterone receptor or Ki-67 index), *HER2* overexpression (non-luminal) (*HER2* gene amplification or *HER2* immunohistochemical 3+), triple negative (negative for both hormonal receptors and *HER2*).

Statistical analysis

Descriptive statistics were calculated. The Pearson chi-square test was used to evaluate the relationship between *HER2* immunostaining intensity and *HER2* gene overexpression in the micropapillary tumor component, and the relationship of *HER2* protein and gene expression with clinicopathological characteristics and disease progression. The Fisher exact test was performed when necessary. All statistical tests were two-sided, and p values less than 0.05 were considered significant. All analyses were performed in SPSS (version 17.0, SPSS Company, Chicago, IL).

Results

Clinical features

All the patients were female. The median age at diagnosis was 47 years (range 24–80 years). The initial symptom in the majority of patients was a palpable firm breast mass. One of the patients also presented with swelling of the right breast. The tumor was present on the right side of the breast in 63 patients and left side in 84 patients. Ten patients exhibited a family history of cancers, including breast cancer, thyroid cancer, malignant lymphoma, hepatic carcinoma (all, $n = 1$), lung cancer ($n = 2$), and colorectal cancer ($n = 3$). One patient's father suffered from both lung cancer and colorectal cancer.

Pathological features

The tumor size was ranged from 0.1 to 10 cm in diameter, with mean and median diameters of 2.3 cm and 1.9 cm, respectively. The tumors were firm solitary masses with ill-defined margins. The cut surface was usually described as solid and white or grayish-white. The clinicopathological characteristics of the 147 cases are summarized in Table 1.

Microscopically, morula-like clusters of cancer cells in pseudopapillary structures and surrounded by clear stromal spaces (Fig. 1a, b). This pattern was similar to extensive lymphovascular invasion; however, these spaces were devoid of endothelial lining cells. Nuclear atypia was generally severe, with pleomorphism, hyperchromasia, and macronucleoli. Invasive micropapillary carcinoma exhibited reversed polarity (Fig. 1c). Using the Nottingham modification of the Bloom–Richardson grading system [12], 106 tumors (72%, 106/147) were classified as grade 2, and 41 tumors (28%, 41/147) as grade 3. Lymphatic vessel invasion was observed in 83 tumors (56%, 83/147).

The ratio of invasive micropapillary carcinoma component ranged from 10 to 100%, with mean and median of 80%, 40%, respectively. According to the grouping criteria above, more than half of the tumors (54%, 79/147) were categorized into group A, and the rest 68 (46%, 68/147) tumors were into group B (Table 1). Fifty six (38%, 56/147) of pure invasive micropapillary carcinoma (Group A) showed invasive micropapillary carcinoma ratio of 100%. A component of invasive breast carcinoma of no special type was found in all of the remaining 91 tumors.

Surrogate molecular subtype

The positive rates of estrogen receptor and progesterone receptor were 84% (124/147) and 80% (118/147), respectively (Table 1). Most cases were of a luminal subtype (luminal A, 22%, 33/147; luminal B, 61%, 90/147),

Table 1 General characteristic of 147 patients.

Characteristics	*n*	proportion
Age (24–80 years, median 47 years)		
≤50	64	44%
>50	83	56%
Tumor site		
Left	84	57%
Right	63	43%
Tumor size (0.1–10 cm, median 1.9 cm)		
≤2	49	33%
2–5	67	46%
>5	8	5%
Nonavailable[a]	23	16%
Histological grade		
G2	106	72%
G3	41	28%
Ratio group of invasive micropapillary carcinoma (10–100%, median 40%)		
Group A (90–100%)	79	54%
Group B (10–90%)	68	46%
Lymph-vascular invasion		
Absent	64	44%
Present	83	56%
Lymph node status		
Negative	42	29%
Positive	90	61%
pN1	32	24%
pN2	29	22%
pN3	29	22%
Nonavailable[b]	15	10%
AJCC staging		
I	18	12%
II	45	31%
III	47	32%
Others[c]	37	25%
Estrogen receptor		
Positive	124	84%
Negative	23	16%
Progesterone receptor		
Positive	118	80%
Negative	29	20%
HER2 fluorescence in-situ hybridization groups		
Group 1	67	46%
Group 3	1	1%
Group 4	4	3%
Group 5	75	51%
Surrogate molecular subtype		
Luminal A	33	22%
Luminal B	90	61%
HER2-positive	45	31%
HER2-negative	45	31%
HER2 overexpression	23	16%
Triple negative	1	1%

[a,b]Clinicopathological information of some consultation cases were nonavailable, including surgical operations, tumor size, and lymph node status

[c]Seventeen patients who received neoadjuvant chemotherapy prior to surgery were ruled out. Besides, some consultation cases cannot be staged for incomplete clinicopathological information

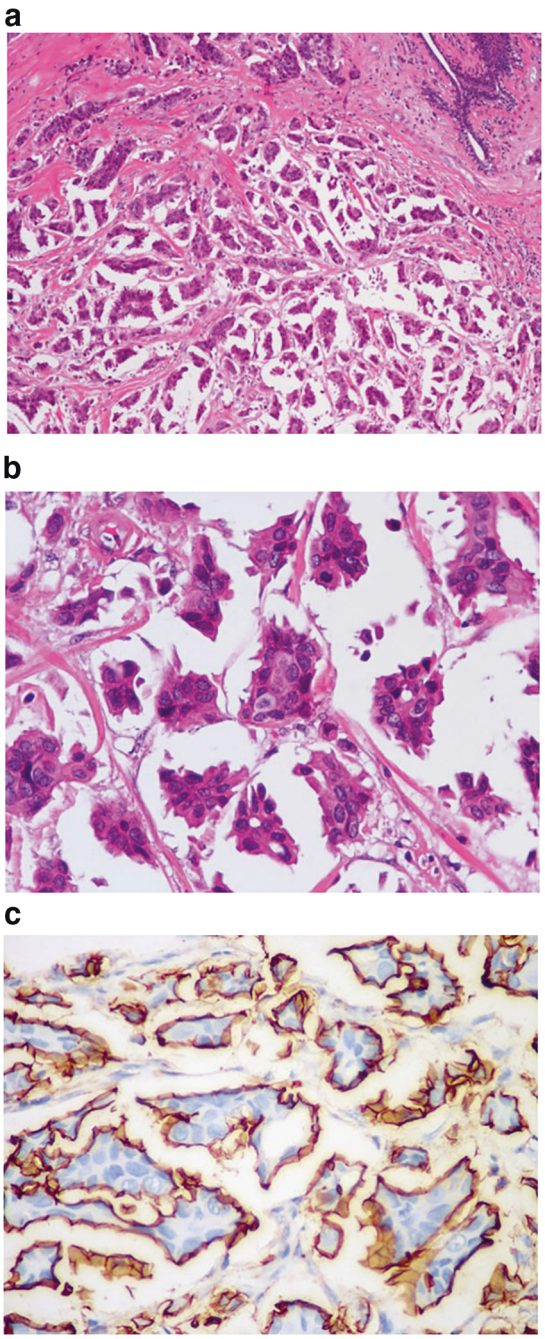

Fig. 1 Invasive micropapillary carcinoma of the breast. a The tumor is composed of pseudopapillary and tubuloalveolar structures floating in empty, clear spaces lined by delicate strands of stroma (H&E, ×100). **b** High-power magnification shows hyperchromatic, high-grade nuclei in the tufts of neoplastic cells. A mitotic figure is present (upper right) (H&E, ×400). **c** Immunohistochemical staining for epithelial membrane antigen shows cell membrane positivity only toward the stromal pole (immunoperoxidase, ×400).

whereas the triple-negative subtype and *HER2* subtype accounted for 1% (1/147) and 16% (23/147), respectively. Half (50%, 45/90) of the luminal B subtype displayed *HER2* gene amplification.

Table 2 Correlation of *HER2* immunostaining intensity with *HER2* gene amplification.

HER2 gene status	*HER2* immunostaining		χ^2	p
	Moderate incomplete staining $N = 117$	Intense incomplete staining $N = 30$		
Amplification ($n = 68$)	38 (32%)	30 (100%)		
Nonamplification ($n = 79$)	79 (68%)	0 (0)	43.790	0.000
Total	117 (100%)	30 (100%)		

HER2 gene amplification and protein expression

Both immunohistochemistry and fluorescence in-situ hybridization results of *HER2* were reevaluated in 147 tumors (Table 2). *HER2* signals per cell varied from 1.4 to 35, with mean and median value of 8.1 and 3.65, respectively. According to the 2018 ASCO/CAP guideline [7], there were 67 patients (46%, 67/147) of group 1, 1 patient (1%, 1/147) of group 3, 4 patients (3%, 4/147) of group 4, and 75 patients (51%, 75/147) of group 5 (Table 1). Group 1 was the classical *HER2*-positive group and group 5 was the classical *HER2*-negative group. Both group 3 and group 4 tumors displayed *HER2* moderate immunostaining (immunohistochemical 2+). After recounting another 30 cells, the grouping results of group 3 and group 4 remain unchanged. Therefore, the only patient of group 3 was determined as *HER2* positive while four patients of group 4 were reported as *HER2* negative. Finally, 68 patients (46%, 68/147) were determined as *HER2* gene amplification (Table 2).

All invasive micropapillary carcinoma components exhibited moderate or intense membrane staining of *HER2* in more than 10% of tumor cells. Consistent with previous reports, all 147 tumors showed incomplete basolateral membrane staining, while no immunoreactivity staining was observed on the basal side of the stromal-facing membrane. Intense basolateral membrane staining (Fig. 2a, c) of *HER2* was observed in thirty tumors (20%, 30/147) of invasive micropapillary carcinoma. All of them displayed *HER2* gene amplification by fluorescence in-situ hybridization (Fig. 2b, d). Whereas, one hundred and seventeen tumors (80%, 117/147) had moderate staining (Fig. 3a, c), and only thirty eight of the 117 tumors (32%, 38/117) exhibited *HER2* gene amplification. Overall, *HER2* gene amplification was identified in 68 (46%, 68/147) tumors, including all thirty tumors with intense *HER2* membrane staining and thirty eight tumors with moderate *HER2* staining (Table 2). The positive ratio of fluorescence in-situ hybridization testing for former cases was 100% (30/30). Intense *HER2* membrane-staining tumors were more likely to be *HER2* positive than moderately stained ones ($p = 0.000$). Besides, the mean and median *HER2* signals per cell were significantly higher ($p < 0.0001$) in the intense-staining tumors (14.5, 13), in contrast with the moderate-staining tumors (5.65, 3.65) (Fig. 4a). The mean and the median ratio of *HER2*/CEP17 in intense-staining tumors was 7.5 and 5.65, respectively. While the mean and the median ratio of *HER2*/CEP17 (2.4, 1.45) in moderate-staining tumors were much lower ($p < 0.0001$) than those in intense-staining tumors (Fig. 4b).

Correlation of *HER2* expression with clinicopathological characteristics

Analysis of correlation of *HER2* expression with clinicopathological characteristics were summarized in Table 3. Estrogen receptor and progesterone receptor status has a significant negative correlation with *HER2*-positive rates both on protein expression ($p = 0.000$) and gene amplification ($p = 0.000$). Higher the positive rates of estrogen receptor lower the *HER2*-positive rates and lower the *HER2* intense membrane-staining rates. Similarly, there is also a negative correlation between progesterone receptor status and *HER2*-positive rates ($p = 0.000$). Further stratification analysis (Table S1) revealed that patients with moderate staining of *HER2* gene amplification showed significantly higher frequencies of estrogen receptor-positive than those with intense staining of *HER2* gene amplification ($p = 0.025$, Fig. 5a). Among patients with moderate *HER2* staining, the positive rates of estrogen receptor ($p = 0.000$, Fig. 5b) and progesterone receptor ($p = 0.002$, Fig. 5d) were significantly higher in patients without *HER2* gene amplification than in patients with *HER2* gene amplification. When only patients with *HER2* gene amplification were included, the correlation between *HER2* staining intensity and PR-positive rate was not statistically significant ($p = 0.081$, Fig. 5c).

Correlation between *HER2* staining intensity and histological grade was not statistically significant ($p = 0.257$, Table 3). While *HER2* gene amplification was positive correlated with histological grade ($p = 0.003$, Table 3). This correlation could also be seen in further stratification analysis ($p = 0.005$, Table S1). The correlation between *HER2*-positive rates and age, tumor size, lymph node metastasis, lymphatic vessel invasion, and AJCC staging were not statistically significant ($p > 0.05$, Table 3).

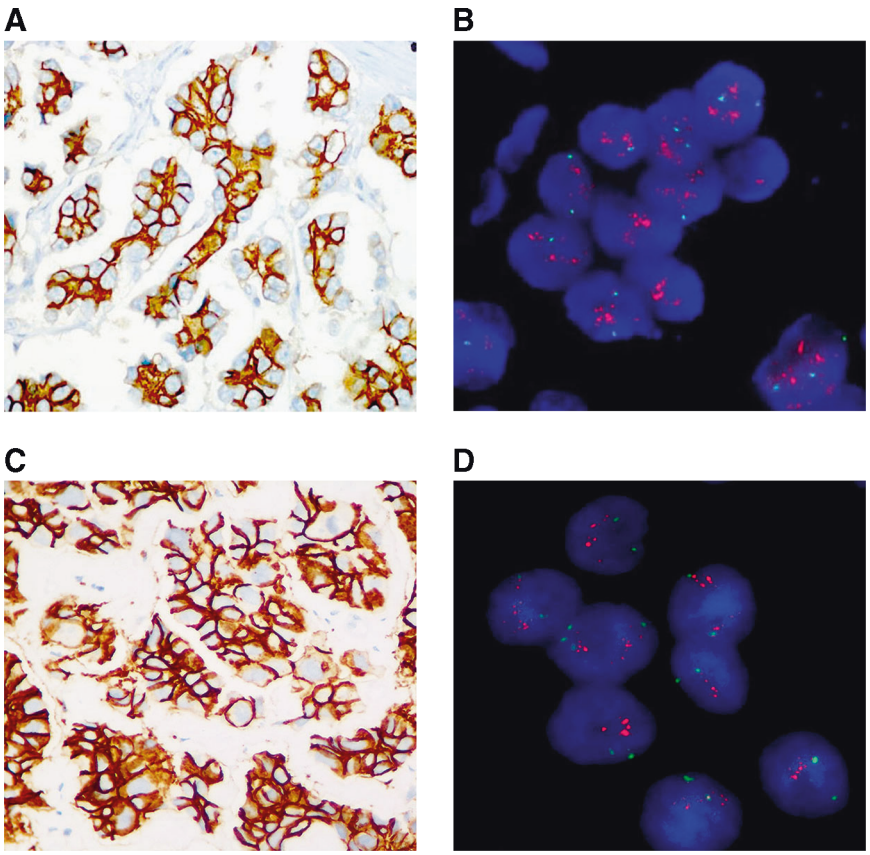

Fig. 2 Example of invasive micropapillary carcinoma tumors with intense basolateral *HER2* membrane staining and corresponding fluorescence in-situ hybridization images. **a**, **c** Invasive micropapillary carcinoma shows intense and incomplete basolateral membrane staining. **b**, **d** Fluorescence in-situ hybridization image corresponding to **a** and **c** presented *HER2* amplification with clustered *HER2* signals, respectively.

Treatment and clinical follow-up

All patients received surgery. Seventeen patients received neoadjuvant chemotherapy prior to surgery. All of the remaining 130 patients were primary breast cancer without preoperative treatment. Modified radical mastectomy was the initial therapy for 110 (75%, 110/147) of the 147 patients. Conservative surgery with sentinel lymph node biopsy was performed on ten (7%, 10/147) patients. Simple mastectomy with sentinel lymph node biopsy was performed on 12 (8%, 12/147) patients. Surgical operations of remaining 15 consultation patients (10%, 15/147) were unknown. Chemotherapy was given to 100 (77%, 100/130) patients, 29 (22%, 29/130) patients also received *HER2* target therapy, 56 (43%, 56/130) received radiation therapy, and 64 (49%, 64/130) received hormone therapy. Of the 147 patients, 90 (61%, 90/147) had metastases to axillary lymph nodes (range, 1–35 positive lymph node). Sixty-five of these patients (62%, 65/90) have more than three lymph nodes involvement, with a mean positive lymph node number of 11. Nine patients exhibited metastasis in all lymph nodes that were examined. In addition to 17 patients who had received neoadjuvant chemotherapy prior to surgery, 20 of the 47 consultation patients were also excluded during AJCC staging, due to the lack of complete clinical pathological information. The other 110 patients were staged, with 18 patients (12%, 18/147) of stage I, 45 patients (31%, 45/147) of stage II, and 47 patients (32%, 47/147) of stage III.

Follow-up data were available for 140 patients. The follow-up time ranged from 1 month to 99 months, with mean and median postsurgical intervals of 38 and 57 months, respectively. Thirteen (9%, 13/140) patients exhibited disease progression (recurrence or distant metastasis). Seven patients (5%, 7/140) exhibited chest wall recurrence 3 months to 72 months after the initial surgery, and three patients (2%, 3/140) displayed lung metastases with postsurgical intervals of 7, 8, and 42 months, respectively. One patient (1%, 1/140) exhibited both liver and multiple lymph node metastases. Brain (1%, 1/140) and eyelid (1%, 1/140) metastases occurred in one patient, respectively. None of the patients died.

In our study, half (50%, 31/62) of the 62 available patients with *HER2* gene amplification received trastuzumab-based therapy. Twenty four (77%, 24/31) of them are alive without disease progression. The follow-up time ranged from 7 months to 77 months, with mean and median postsurgical intervals of 31 months and 60 months. Six of them (19%, 6/31) occurred disease progression 3 months to 38 months after the initial trastuzumab-based therapy. The follow-up information of the last one patient was unavailable.

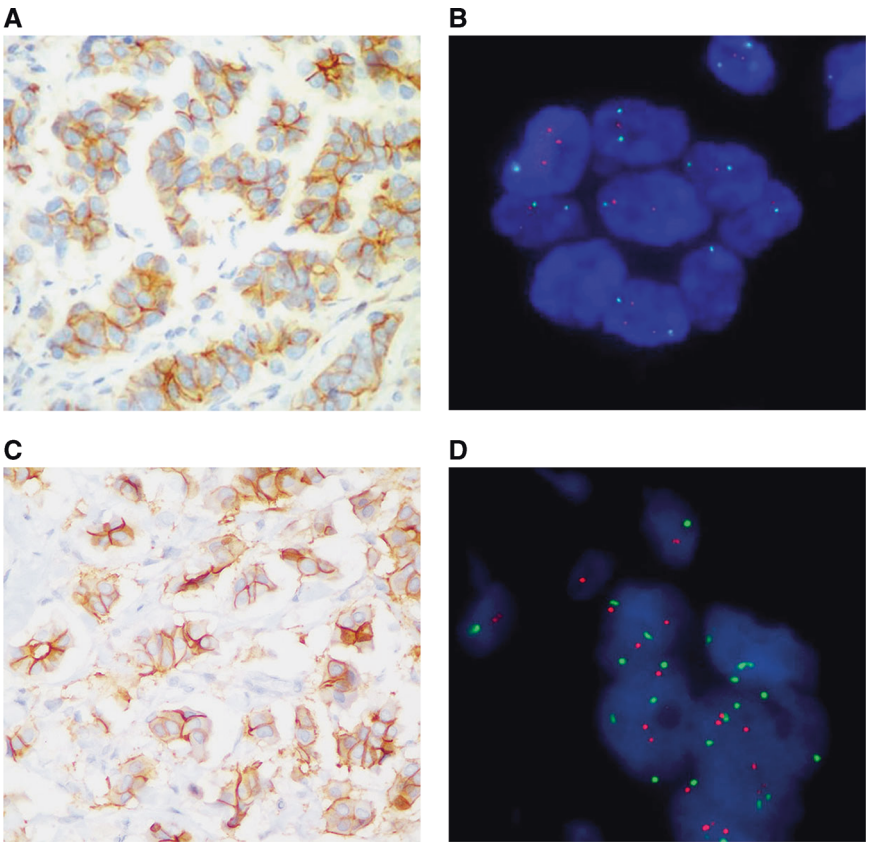

Fig. 3 Example of invasive micropapillary carcinoma tumors with moderate basolateral *HER2* membrane staining and corresponding fluorescence in-situ hybridization images. **a**, **c** Invasive micropapillary carcinoma displays moderate and incomplete basolateral membrane staining. **b** Fluorescence in-situ hybridization image corresponding to **a** exhibited normal *HER2* signals with *HER2* (red)/CEP17 (green) ratio of 1.1 and average *HER2* signal of 1.7. **d** Fluorescence in-situ hybridization image corresponding to **c** displayed *HER2* (red)/CEP17 (green) ratio of 0.8 and average *HER2* signal of 2.3.

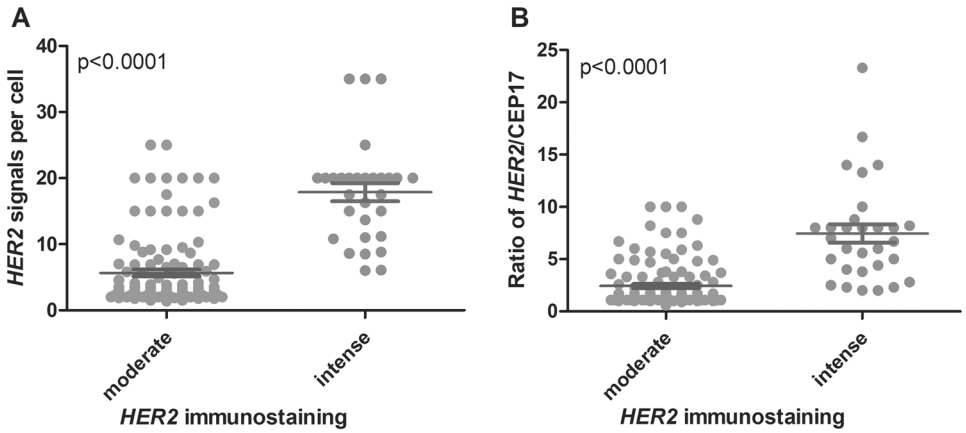

Fig. 4 *HER2* signals per cell and ratio of *HER2*/CEP17 in the moderate- and intense-staining tumors. Both the average *HER2* signals per cell (**a**, $p < 0.0001$) and ratio of *HER2*/CEP17 (**b**, $p < 0.0001$) were obviously higher in intense *HER2*-staining tumors than in moderate-staining ones.

Correlation of *HER2* expression with disease progression

According to the statistical analysis, patients with intense membrane *HER2* staining (25%, 7/28) and *HER2* gene amplification (19%, 12/64) were more prone to disease progression ($p = 0.003$, $p = 0.000$, Fig. 6a, Table S2) than those with moderate membrane staining (5%, 6/112) and *HER2* gene non-amplification (1%, 1/76). Further analysis of patients with *HER2* moderate staining revealed that disease progression rates were much higher in patients with *HER2* gene amplification than those without *HER2* gene amplification ($p = 0.006$, Fig. 6b, Table S2). While in patients with *HER2* gene amplification, the correlation between *HER2* staining intensity and disease progression was not statistically significant ($p = 0.259$, Fig. 6c, Table S2).

Of all AJCC-staged 110 patients, 87 patients obtained follow-up information. Ten of the 87 patients (11%, 10/87) occurred disease progression, including 8 patients of stage III and 2 patients of stage II. *HER2*-positive rates showed positive correlation with disease progression rates in stage

Table 3 Correlation of *HER2* expression with clinicopathological characteristics.

Parameters	*HER2* immunostaining		χ^2	p	*HER2* gene status		χ^2	p
	Moderate incomplete staining $N = 117$	Intense incomplete staining $N = 30$			Nonamplification $N = 79$	Amplification $N = 68$		
Age			0.640	0.424			0.638	0.424
≤50 years	49 (33%)	15 (10%)			32 (22%)	32 (22%)		
>50 years	68 (46%)	15 (10%)			47 (32%)	36 (24%)		
Tumor size (cm)[a]			2.093	0.351			3.324	0.190
≤2	39 (31%)	10 (8%)			29 (23%)	20 (16%)		
2–5	56 (45%)	11 (9%)			38 (31%)	29 (23%)		
>5	5 (4%)	3 (2%)			2 (2%)	6 (5%)		
Histologic grade			1.443	0.257			8.782	**0.003**
G2	87 (59%)	19 (13%)			65 (44%)	41 (28%)		
G3	30 (20%)	11 (7%)			14 (10%)	27 (18%)		
Estrogen receptor			27.481	**0.000**			26.759	**0.000**
Positive	108 (73%)	16 (11%)			78 (53%)	46 (31%)		
Negative	9 (6%)	14 (10%)			1 (1%)	22 (15%)		
Progesterone receptor			17.273	**0.000**			19.361	**0.000**
Positive	102 (69%)	16 (11%)			74 (50%)	44 (30%)		
Negative	15 (10%)	14 (10%)			5 (3%)	24 (16%)		
Lymph-vascular invasion			2.810	0.094			0.287	0.592
Absent	55 (37%)	9 (6%)			36 (24%)	28 (19%)		
Present	62 (42%)	21 (14%)			43 (29%)	40 (27%)		
Lymph node status[b]			5.872	0.118			0.943	0.331
Positive	72 (55%)	18 (14%)			51 (39%)	39 (30%)		
Negative	32 (24%)	10 (8%)			20 (15%)	22 (17%)		
AJCC staging[c]			1.141	0.565			0.671	0.715
I	16 (15%)	2 (2%)			12 (11%)	6 (5%)		
II	35 (32%)	10 (9%)			25 (23%)	20 (18%)		
III	39 (35%)	8 (7%)			27 (25%)	20 (18%)		

[a,b,c]Nonavailable cases were ruled out before statistical analysis

Bold values indicate statistical significance $p < 0.05$

III breast cancer ($p = 0.004$, Fig. 6d), but not in stage I or II patients (Table S3).

Discussion

Breast cancer remains a significant public health concern. More than a million new cases are diagnosed each year, resulting in 400,000 deaths worldwide [16, 17]. *HER2* is overexpressed in 15–20% of all breast cancers (*HER2*-positive breast cancer) and is associated with a worse clinical outcome without therapy [18, 19]. Multiple randomized controlled trials have shown that therapy with trastuzumab, a monoclonal antibody against *HER2*, is the standard *HER2*-positive breast cancer treatment in both neoadjuvant and metastatic settings [20, 21]. Invasive micropapillary carcinoma is a specific histological type of breast cancer. Mixed forms account for 2.6–7.4% of all invasive breast carcinomas, with pure invasive micropapillary carcinoma reported to be much less than mixed cases [22–26]. The most common histological type that mixed with invasive micropapillary carcinoma has been reported to be invasive carcinoma of no special type [25]. It is also known for its distinctive clinical features and a high incidence of lymphatic vessel invasion and axillary lymph node metastasis.

In our study, hormone receptors exhibited a significant inverse association with *HER2* gene amplification ($p = 0.000$). A previous large cohort study showed that expression of estrogen receptor and progesterone

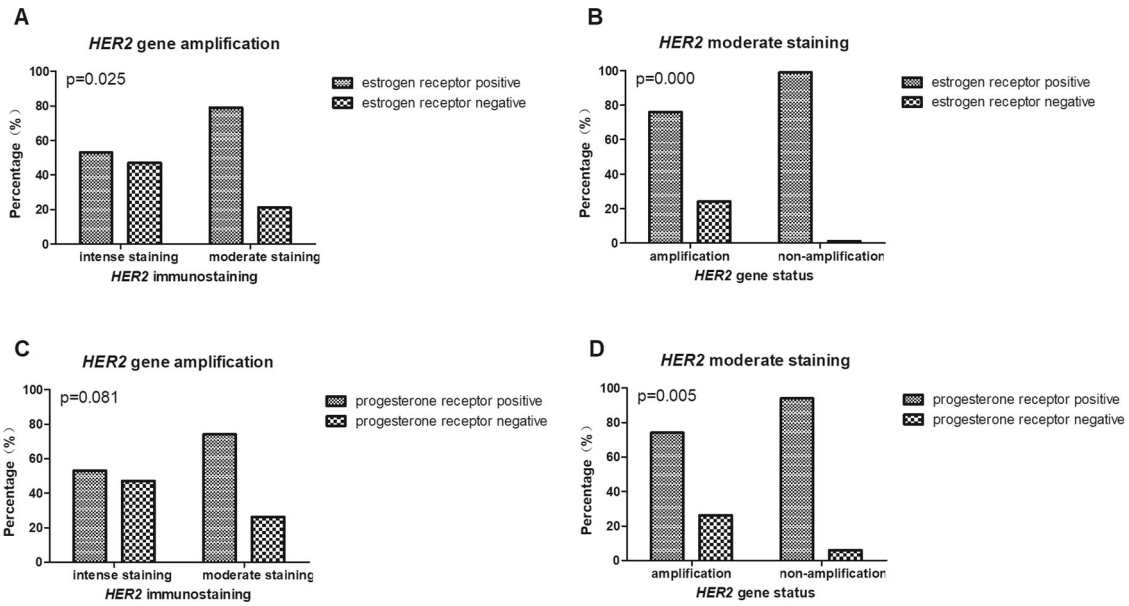

Fig. 5 Correlation between estrogen receptor and progesterone receptor with *HER2* expression. *HER2* expression was correlated inversely with estrogen receptor and progesterone receptor expression. Patients with moderate staining of HER2 gene amplification shows significantly higher frequencies of estrogen receptor-positive than those with intense staining of HER2 gene amplification (**a**, $p = 0.025$). Among patients with moderate HER2 staining, the estrogen receptor (**b**, $p = 0.000$) and progesterone receptor (**d**, $p = 0.002$) positive rate was significantly higher in patients without HER2 gene amplification than in patients with HER2 gene amplification. When only patients with HER2 gene amplification were included, the correlation between *HER2* staining intensity and PR-positive rate was not statistically significant (**c**, $p = 0.081$).

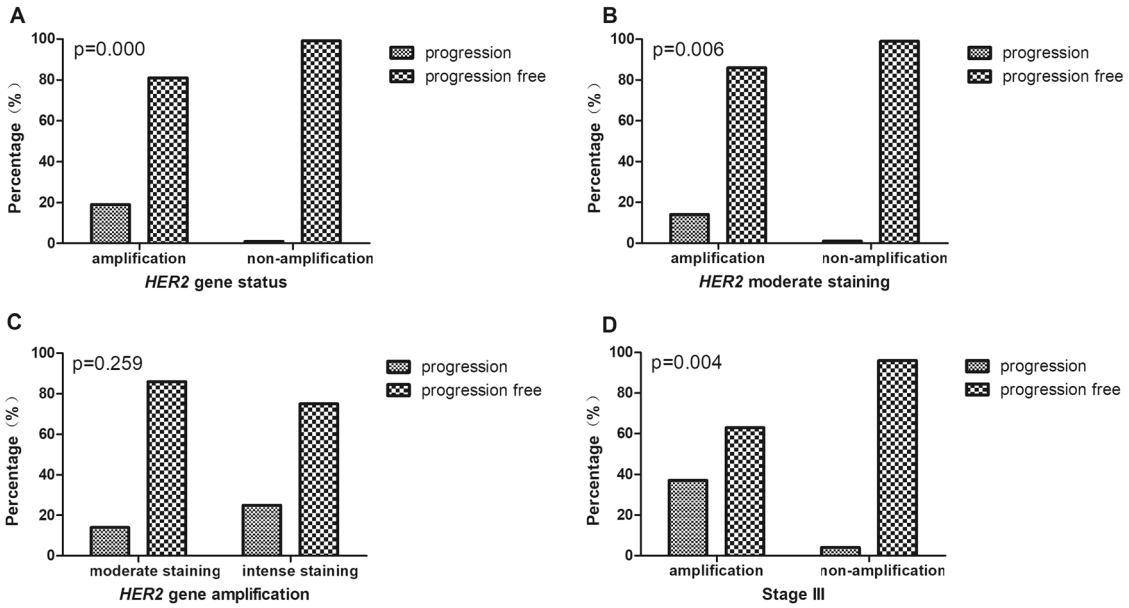

Fig. 6 Correlation between *HER2* status with disease progression and AJCC staging. Tumors with *HER2* gene amplification were more prone to occur disease progression than those without *HER2* gene amplification (**a**, $p = 0.000$). This trend could still be observed when cases were limited to the moderately stained tumors (**b**, $p = 0.006$). While in patients with *HER2* amplification, the correlation between *HER2* staining intensity and disease progression was not statistically significant (**c**, $p = 0.259$). *HER2* gene amplification showed positive correlation with disease progression in stage III breast cancer (**d**, $p = 0.004$).

receptor were significantly reduced in *HER2*-positive tumors compared with *HER2*-negative tumors [27]. Similar results were found in other studies where the amplification of *HER2* gene was correlated with a decreased positivity of estrogen receptor and progesterone receptor [27–30].

HER2 gene status is an important factor affecting prognosis and treatment choice for both primary and metastatic breast carcinoma [31]. An accurate diagnostic *HER2* assessment is essential to appropriately treat patients with trastuzumab regiments. It is clear that accurate patient identification of *HER2* receptor status, and thus clinical benefit, is dependent on the quality of *HER2* testing. The first-line method most commonly used to determine *HER2* status is immunohistochemistry, which is relatively inexpensive and routinely used in pathology laboratories, making its implementation simple. Immunohistochemistry results in a *HER2* score ranging from 0 (no expression) to immunohistochemical 3+ (strong complete tumor cell membrane expression).

Invasive micropapillary carcinoma exhibit a unique growth pattern with clusters of tumor cells displaying inverted polarity immersed in a spongy stroma, which is clearly visualized by epithelial membrane antigen or MUC1 staining [11]. Positive staining for E-cadherin showed sharp cell membrane staining but no immunoreactivity along the retracted stromal–membrane interface [32, 33]. The immunohistochemical staining features of *HER2* in invasive micropapillary carcinoma are quite similar to that of E-cadherin. Polarity reversal might account for the unique *HER2* immunostaining pattern observed in invasive micropapillary carcinoma.

According to the 2018 ASCO/CAP recommendation [7], incomplete membrane staining that is faint/barely perceptible and in >10% of tumor cells should be judged as *HER2* 1+. While *HER2* immunohistochemical 2+ needs complete weak-to-moderate membrane staining. However, tumors showing incomplete moderate-to-intense staining cannot be classified into any of these groups. The ASCO/CAP already noticed this situation and annotates specially that the moderate to intense but incomplete staining of *HER2* by immunohistochemistry are not covered by those conventional definitions. If encountered, they can be considered immunohistochemical 2+ equivocal, requiring an additional fluorescence in-situ hybridization test. The criteria of *HER2* immunohistochemical 3+ for invasive micropapillary carcinoma are not mentioned in the guideline. The criteria still need to be better characterized. All cases enrolled in our study showed moderate-to-intense incomplete (basolateral or U-shaped) *HER2* staining in more than 10% of tumor cells. Therefore, they were at least *HER2* immunohistochemical 2+ cases. Sixty eight of them (46%, 68/147) displayed *HER2* gene amplification, including all thirty tumors with intense *HER2* membrane staining and thirty eight tumors with moderate *HER2* staining. Similar results were found in other study where the amplification rate of *HER2* gene is 100% for all invasive micropapillary carcinoma tumors with intense staining [34]. Another study had shown that 17% of invasive micropapillary carcinoma tumors with *HER2* immunohistochemical 2+ displayed gene amplification [35]. *HER2* immunostaining only at the basolateral membrane had also been seen in previous study [34, 36], which described a relatively high-*HER2* amplification rate (95%) in invasive micropapillary carcinoma. However, our study observed that intense basolateral membrane reactivity in ≥10% of tumor cells indicate *HER2* positivity in invasive micropapillary carcinoma. These tumors should be scored as immunohistochemical 3+ directly. Therefore, the further expensive, time-consuming, and labor-intensive fluorescence in-situ hybridization testing of those tumors could be omitted and the corresponding cost could also be saved simultaneously.

In addition to invasive micropapillary carcinoma, gastric cancer shows basolateral *HER2* membrane staining, and focal staining is commonly reported in gastric cancer [13, 37, 38]. Gastric tumor cells often show incomplete *HER2* membrane reactivity, which can be basolateral or lateral in distribution [37]. One key feature of gastric cancer-specific scoring criteria is the characterization of strong incomplete membrane staining as *HER2*-positive if >10% cells or >5 clustered cells are stained in surgical or biopsy samples, respectively [13, 39]. Based on current evidence, lateral- or U-shaped *HER2* membrane staining in gastric cancer is regarded as 3+ staining [13, 40]. Hence, *HER2* assessment in invasive micropapillary carcinoma tumors with basolateral membrane staining in >10% tumor cells should be determined with staining intensity; tumors with intense staining should be classified as *HER2* immunohistochemical 3+ directly. Besides, considering that the interpretation of *HER2* immunohistochemical staining is somewhat subjective even with clear interpretation criteria [7, 14], subsequent FISH tests must be performed in case of uncertainty.

The prognosis of patients with *HER2*-positive breast cancer can be improved significantly by *HER2* targeted therapy. In our study, most (77%, 24/31) of the 31 patients received trastuzumab-based therapy are alive without disease progression. The follow-up time ranged from 7 to 77 months, with mean and median postsurgical intervals of 30 and 60 months. Other six (19%, 6/31) patients exhibited disease progression 3 months to 38 months after the initial trastuzumab-based therapy. Our study shows that *HER2* gene amplification was positively correlated with disease progression rates in stage III breast cancer, but not in stage I or II. Similar results were found in a previous study where *HER2* gene was found to be confined to advanced breast cancer [41].

Conclusion

Patients with *HER2*-positive breast cancer have a poor outcome in the absence of *HER2* target therapy. Therefore,

the importance of including patients with the potential to respond to targeted therapy is paramount. Accurate patient identification, and thus clinical benefit, requires quality *HER2* testing. *HER2* diagnostics are now mandatory, with immunohistochemistry as the first-line test, followed by fluorescence in-situ hybridization in immunohistochemical 2+ cases. Given that intense clear linear U-shaped membrane *HER2* immunostaining means gene amplification, we propose that invasive micropapillary carcinoma tumors with intense clear linear basolateral membrane *HER2* membrane staining, although incomplete, should be regarded as immunohistochemical 3+ rather than immunohistochemical 2+, which would avoid further fluorescence in-situ hybridization-testing procedure and greatly save the related time labor and financial costs. Ultimately, ensure all patients with *HER2* gene amplification obtain effective targeted therapy in time.

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Compliance with ethical standards

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

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