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Research Article

Clinical and genomic characteristics of HER2-ultralow breast cancer and implications for T-DXd therapy

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Abstract

Background: The clinical benefit of T-DXd in advanced breast cancer with hormone receptor-positive (HR+), human epidermal growth factor receptor 2 (HER2)-ultralow tumors in the DESTINY-Breast06 trial has drawn attention to this subtype.

Methods: We re-evaluated 473 pathological specimens from 302 HER2-negative breast cancer patients in our next generation sequencing database, classifying HER2-negative status into HER2-ultralow, IHC 0 without membrane staining (MS−) and HER2-low. Clinicopathologic characteristics and genomic profiles were analyzed by HER2 status.

Results: Overall, 35.5% of primary and 49.0% of metastatic HER2-IHC 0 tumors were reclassified as ultralow. Subtype analysis based on HR status showed no distinct clinicopathological characteristics in the HER2-ultralow subgroup. Upon metastasis, 40% of HER2-ultralow primary tumors converted to IHC 0 (MS−) and 46.7% to HER2-low. In the metastatic tumors, 60% of HER2-IHC 0 (MS−) and 50% of HER2-ultralow translated to other HER2 statuses in re-obtained samples. HER2-ultralow status was associated with worse disease-free survival than HER2-IHC 0 (MS−) and HER2-low status in HR-negative breast cancer, but no differences of overall survival were observed. The median progression-free survival for first-line chemotherapy was 7.2 months in HR+ HER2-low, 6.8 months in ultralow, and 8.8 months in IHC 0 (MS−) patients ($P = 0.06$). PIK3CA mutations were more common in the HER2-low subtype than in HER2-ultralow tumors in the HR− subtype.

Conclusion: In conclusion, HER2-ultralow status is not associated with distinct clinicopathologic or genomic characteristics. HER2-IHC 0 (MS−) and ultralow statuses often coexist within the same patient.

Keywords: HER2 ultralow breast cancer; T-DXd; HER2-IHC 0 without membrane staining (MS−); DESTINY-Breast06; genetic alterations

Introduction

In the era of trastuzumab, a binary algorithm has been employed to classify breast cancer into either human epidermal growth factor receptor 2 (HER2)-positive or HER2-negative to distinguish whether the patient will benefit from trastuzumab [1]. HER2-negative breast cancer, defined as immunohistochemistry (IHC) 0, IHC 1+, or IHC 2+/*in situ* hybridization (ISH)-negative, does not benefit from anti-HER2 treatment. Actually, HER2-negative breast cancer is within a continuum of HER2 expression, comprising nearly 85% of all cases [2]. The DESTINY-Breast04 phase III clinical trial indicated that T-DXd, a novel antibody–drug conjugate, significantly improved progression-free survival (PFS) and overall survival (OS) compared with standard chemotherapy in HER2-low breast cancer, which is defined as breast cancer with an IHC score of 1+/2+ and negative ISH results. The results of the DESTINY-Breast04 clinical trial have led to the identification of a new therapeutic subtype, HER2-low breast cancer, and the approval of T-DXd for the treatment of unresectable or metastatic HER2-low breast cancer [3]. According to a real world study, 41.5% of whole breast cancer patients are HER2-low phenotype [4]. Numerous studies have explored the clinicopathologic and molecular characteristics of this subtype and HER2-low breast cancer should not be considered a distinct molecular entity based on the current evidence [5].

T-DXd is designed to target HER2-expressing cancer cells and release chemotherapy agents inside cancer cells; these released chemotherapy agents can also be taken up by neighboring cancer cells without HER2 expression, causing cell death, a phenomenon known as the "bystander effect" [6]. Furthermore, as other novel HER2-targeting drug conjugates are being developed [7], defining the HER2 expression threshold at which anti-HER2 drug conjugates can provide benefit has long been a significant research question. The phase II DAISY study was the first clinical trial to observe the efficacy of T-DXd in HER2 IHC 0 breast cancer with heavy previous treatment, with an objective response rate of 29.7% [6]. Importantly, the HER2 IHC 0 category can be further subdivided based on membrane staining that is faint in ≤10% of tumor cells (HER2 ultralow) or not observable at all

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(IHC 0 without membrane staining, IHC 0 MS−) [8]. The released results of the DESTINY-Breast06 phase III clinical trial evaluating the efficacy of T-DXd compared with standard chemotherapy in patients with hormone receptor-positive (HR+), HER2-low or HER2-ultralow metastatic breast cancer demonstrated that T-DXd showed a clinically meaningful PFS benefit in both HER2-low and HER2-ultralow breast cancer patients. This potential efficacy of T-DXd in HER2-ultralow breast cancer has heightened interest in this phenotype and raised the question of whether HER2-ultralow breast cancer should be treated as a distinct subtype.

Currently, the research on HER2-ultralow breast cancer is very limited. We will review the HER2-negative breast cancer patients in our next generation sequence (NGS) database, reassess the HER2 expression status, and distinguish those with HER2-IHC 0 MS− expression and HER2-ultralow expression to explore the clinicopathologic and molecular characteristics of HER2-ultralow breast cancer. We will also investigate the efficacy of standard chemotherapy among the different HER2 subtypes in the real world as supplementary data for the DESTINY-Breast06 study. Our data and findings will contribute to understanding the similarities and differences among HER2-IHC 0 MS−, HER2-ultralow, and HER2-low breast cancer, providing an accurate depiction of HER2 expression in HER2-negative breast cancer. This understanding may help in the interpretation of the results of the DESTINY-Breast06 trial and benefit future clinical practice.

Methods

Study population and data extraction

The study was based on our own NGS database, which was derived from the initial screening phase (the first stage) of the China-Breast-Umbrella study, a two-stage, multi-cohort clinical trial initiated in September 2017. The study protocol was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (approval number: 1705172–9). The first stage of the study only required histologically confirmed, locally advanced or metastatic breast cancer patients with an accessible lesion for biopsy. During the screening of the first stage, patients underwent NGS testing based on their tissue or circulating tumor DNA (ctDNA) samples, and if they were identified as having actionable therapeutic targets, they proceeded to the second stage. The main inclusion criteria for the first stage were as follows. (i) Histologically confirmed locally advanced or metastatic breast cancer, with willingness to provide a tumor tissue or blood sample for genetic testing. (ii) Considered by the investigator to be suitable for systemic therapy, irrespective of the number of prior treatment lines. (iii) Documented disease progression on the last line of therapy or within 3 months after its discontinuation. (iv) Eastern Cooperative Oncology Group (ECOG) performance status of 0–2. In the second stage, the patients were assigned to specific treatment cohorts based on their specific targetable alterations and each cohort had its own specific inclusion criteria. All patients provided written informed consent for the use of their blood and tissue samples for research purposes.

For this study, patients from the NGS database were included if they had at least one pathological result indicating HER2-negative status and the corresponding IHC slides were available for re-evaluation. During November 2017 to September 2021, our NGS database included 445 patients with 520 cancer-related gene panels and 134 patients with other cancer-related gene panels based on ctDNA or tissue samples. Only genomic data of patients with 520 cancer-related gene panels based on tissues were extracted for mutational and copy number variation (CNV) analysis.

The clinicopathological parameters of all patients from our NGS database, including age, pathological type, estrogen receptor (ER) status, progesterone receptor (PR) status, HER2 IHC score, HER2 fluorescent ISH (FISH) result, Ki67 score, details of metastatic sites, the received treatments, time of recurrence or metastasis, and survival were gathered by the electronic medical records.

Tumor genomic analysis

DNA isolation and targeted sequencing were performed at Burning Rock Biotech (Guangzhou, China) according to optimized protocols as described in detail previously [9, 10]. Formalin-fixed, paraffin-embedded (FFPE) tumor samples were used to extract tissue DNA using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). Target capture was performed using a commercial panel consisting of 520 cancer-related genes (OncoScreen Plus, Burning Rock Biotech). The genes included in the panel are listed in supplementary Table 1 (see online supplementary material). Samples were compared with paired white blood cells to eliminate the majority of clonal hematopoiesis-related variants. CNV was calculated based on the ratio between the depth of coverage in tumor samples and the average coverage of an adequate number ($n > 50$) of samples without CNV as references per capture interval. Tumor mutational burden (TMB) per sample was computed as the ratio between nonsynonymous variants detected and the total coding region size of the gene panel.

Clustering analysis was performed using non-negative matrix factorization (NMF) based on Euclidean distance. The R package NMF was implemented to estimate the best rank using Lee's algorithm with the following initial parameters: 2 : 8 for rank, and numeric random seed (seed = 123 456).

Evaluation of HER2 expression

All IHC staining was performed by a Ventana Benchmark automated immunostainer (Ventana Medical System Inc, Roche) with the Bench-Mark ULTRA advanced staining system operator guide. ULTRA Cell Conditioning Solution (ULTRA CC1, pH = 8.5) was used to perform antigen retrieval at 90°C to 100°C. HER2 expression was tested by prediluted Ventana 4B5 antibody. Positive and negative controls of HER2 were included in each slide run.

The HER2 IHC scores were reassessed by two breast cancer specialist pathologists as per the latest 2018 American Society of Clinical Oncology/College of American Pathologists guideline and the exact percentage of cells with faint HER2 staining in the tumors with HER2 IHC 0 was also recorded [18]. In cases of discordant readings, the two pathologists discussed the results. For HER2 IHC results where a consensus still could not be reached, a conclusion was made after discussion with the chief pathologist.

HR and HER2 classification

HER2 statuses included in this study were as follows: HER2-positive (IHC 3+ or IHC 2+/FISH+), HER2-negative (IHC 0, IHC 1+ or IHC 2+/FISH−), HER2-low (IHC 1+ or IHC 2+/FISH−), HER2-ultralow (IHC 0 with membrane staining) and HER2-IHC 0 MS− (IHC 0 without membrane staining). For an exploratory analysis, we further classify the HER2-ultralow category into two distinct, mutually exclusive subgroups using 5% (the median value of the 1–10% range) as a cutoff: (i) >0% to <5% membrane staining, or (ii) ≥5% to ≤10% membrane staining. Based on the IHC data, a positive ER or PR status of tumors was considered as positive HR

status; and ≥1% of cancer cells with nuclear staining of ER or PR was considered to indicate ER or PR positivity.

In our NGS database, the HER2 status of primary tumor was used to investigate the difference of disease-free survival (DFS) and clinicopathologic features including age of diagnose, T stage, N stage, and pathology grade. The HER2 statuses utilized to analyze metastatic patterns and OS in our NGS database were defined according to the most recent pathological results from metastatic biopsies. The HER2 status used to analyze PFS of first-line chemotherapy was defined based on the most recent pathological results at the time chemotherapy was administered. The pathological results of the tissues themselves were used to investigate the association between mutation profiles and HER2 statuses. When analyzing the evolution of HER2 status from primary to metastatic breast cancer, the HER2 status was retrieved from the pathological results from the last re-evaluated status in primary tumors or the last re-evaluated status in metastatic tumors, and HR status was defined according to primary tumors.

Statistical analysis

Correlations between HER2 status and clinicopathological features were evaluated using the Chi-square test (for categorical parameters with more than two categories) and Fisher's exact test (for binary parameters). Fisher's exact test was used to analyze the association between gene mutation status and the subgroup. Mean ± the standard error of the mean (SEM) and median ± 95% confidence intervals (CIs) were computed for continuous variables. Ordinary one-way Analysis of Variance (ANOVA) and Kruskal–Wallis nonparametric test were used to study the distribution of continuous variables across different groups. DFS was defined as the time from surgery to locoregional or distant metastasis. OS was defined as the time from diagnosis of locoregional relapse or distant metastasis to death or last follow-up. PFS of first-line chemotherapy was defined as time from initiation of first-line chemotherapy to progression or death. The last follow-up time was 1 April 2022, with a median follow-up time of 32 months. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate Cox proportional hazard models were adopted to calculate hazard ratios and 95% CIs. Analyses were carried out using Graph Prism and R statistics package (R version 4.1.2).

Results

Patient cohorts and HER2 ultralow status

A total of 414 breast cancer patients with a previous HER2-negative tumor from our NGS database were screened. Finally, 302 patients were included, who had 473 available archived IHC slides for re-evaluation. This cohort included 1 patient with locally advanced breast cancer, 260 patients with distant metastasis followed radical operation, and 41 patients with *de novo* stage IV breast cancer. Among these patients, 164 patients had only one re-evaluated HER2 IHC result, while 138 patients had two or more re-evaluated HER2 IHC results. In all, 182 patients had the NGS results from a 520 cancer-related gene panel based on tissue samples (supplementary Fig. 1, see online supplementary material). The concordance rate of HER2 IHC between the re-evaluated results and the recorded results was 91.3% across the entire population. Of the HER2 IHC 0 breast cancers, 11.1% were identified as HER2 IHC 1+, while the remaining 88.9% were still classified as HER2 IHC 0 (supplementary Fig. 2A, see online supplementary material).

To identify HER2 ultralow or IHC 0 (MS−) tumors, we re-evaluated tumors with HER2 IHC 0 status and recorded the percentage of cells with faint HER2 staining in each sample. Of the 107 primary tumors with HER2 IHC 0 expression, 69 (64.5%) showed no faint HER2 staining, 31 (29.0%) had 0–5% cells faintly stained, and 7 (6.5%) exhibited ≥5% of cells faintly stained. In 100 tumors with HER2 IHC 0 in a metastatic setting, the percentage of tumors with 0%, 0–5%, and ≥5% of cells with faint HER2 staining were 51.0%, 29.0%, and 20.0%, respectively. Notably, the increase of tumors with ≥5% of cells with faint HER2 staining was mainly contributed by HR+ breast cancer. Among HR+ metastatic tumors, 31.0% had ≥5% of cells faintly stained, which was significantly higher than the 8.6% observed in primary breast cancer. Conversely, in HR− breast cancer, the composition of HER2 IHC 0 tumors between primary and metastatic tumors did not change significantly (Fig. 1A).

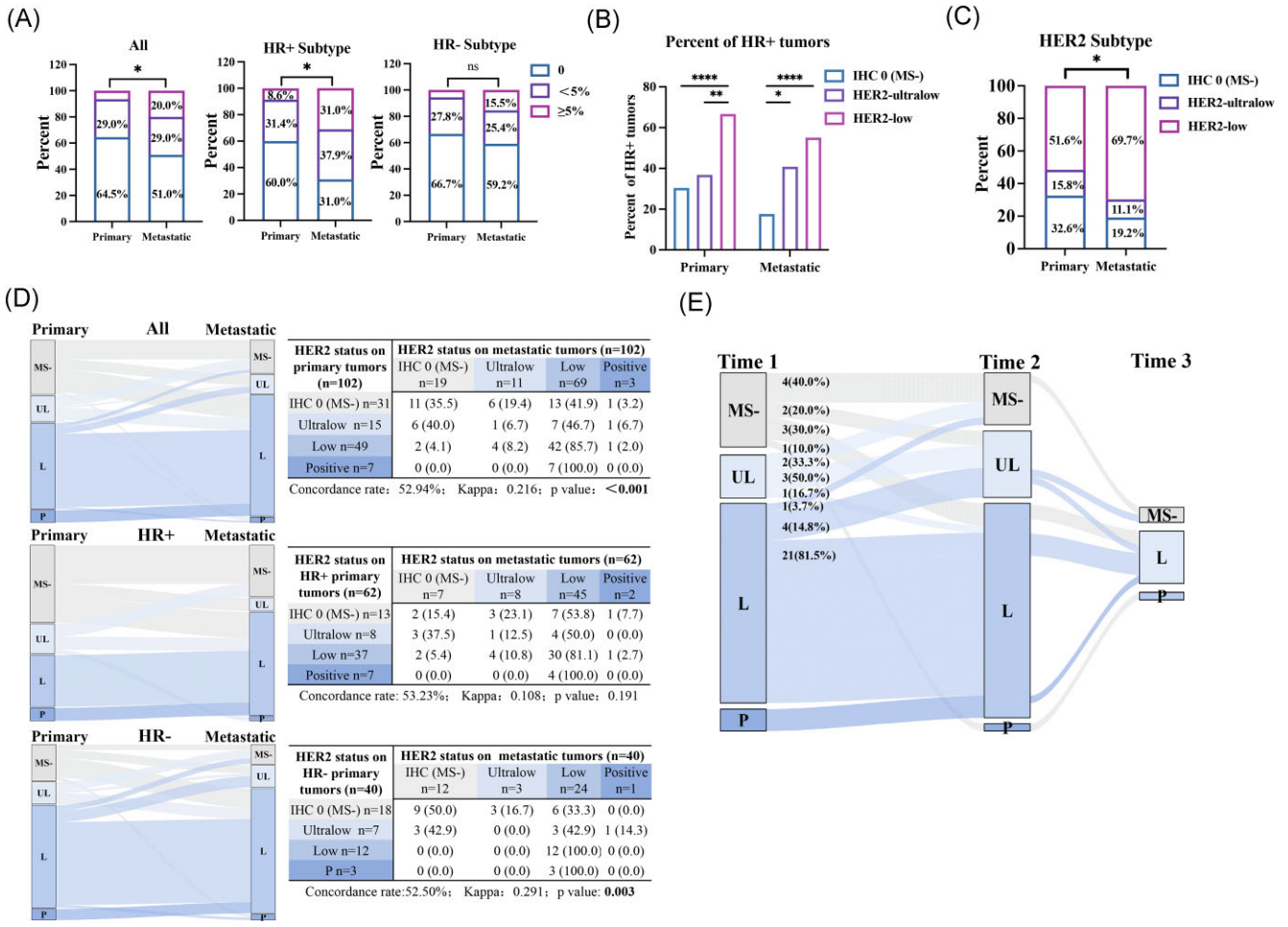
Clinicopathological characteristics by HER2 subtypes

The distribution of clinicopathologic features divided by HER2 status on primary breast cancer is summarized in Table 1. HER2-low tumors exhibited a higher proportion of HR-positive statuses, which was significantly greater than in HER2 ultralow or IHC 0 (MS−) tumors (67.35%, 38.64% and 28.38%, respectively, $P < 0.0001$ and $P = 0.0013$). In primary tumors, there were no differences in the proportion of HR+ between HER2 ultralow and IHC 0 (MS−) breast cancer (Table 1 and Fig. 1B). However, in metastatic tumors, both HER2-low and ultralow breast cancer showed a higher prevalence of HR+ phenotypes compared to IHC 0 (MS−) tumors (55.1%, 40.8%, and 17.6%, respectively, $P < 0.0001$ and $P = 0.01$) (Fig. 1B). Further analysis of the percentage of ER positive cells in each tumor also showed the trend (supplementary Fig. 2B). Regarding pathological grade, subgroup analysis based on HR status revealed that HR-HER2-IHC 0 (MS−) tumors had a higher grade than HR-HER2-low tumors, suggesting that HR-HER2-IHC 0 (MS−) could be a more aggressive subtype (supplementary Table 2, see online supplementary material). Compared to HR-HER2-IHC 0 (MS−) and HR-HER2-ultralow breast cancers, HR-HER2-low breast cancer had the lowest T stage (supplementary Table 2). There were no significant differences in Ki67 scores among different HER2 subtypes when stratified by HR status in both primary and metastatic settings (supplementary Table 2 and supplementary Fig. 2C).

Differences in the metastatic sites among the different HER2 statuses, as defined by metastatic tissues, were analyzed based on our records of initial and most recent metastatic sites (supplementary Table 3, see online supplementary material). There was no significant association between the number of initial metastatic sites and HER2 status. Notably, all HR+ HER2-ultralow patients eventually developed bone metastasis, while HR-HER2-low breast cancer was associated with a higher incidence of liver metastasis (supplementary Table 3).

Intra-patient heterogeneity of HER2 status

The prevalence of the HER2-low population is significantly higher in the advanced setting compared to in the early setting, which is consistent with previous studies (Fig. 1C) [11, 12]. In our NGS database, 102 patients had paired primary and metastatic breast cancer samples, allowing us to analyze the evolution of HER2 statuses between primary and metastatic tumors. In the entire population, 19.4% and 41.9% of HER2-IHC 0 (MS−) primary tumors transitioned to HER2-ultralow and HER2-low in the metastatic



HER2 status on primary tumors (n=102)	HER2 status on metastatic tumors (n=102)			
	IHC 0 (MS-) n=19	Ultralow n=11	Low n=69	Positive n=3
IHC 0 (MS-) n=31	11 (35.5)	6 (19.4)	13 (41.9)	1 (3.2)
Ultralow n=15	6 (40.0)	1 (6.7)	7 (46.7)	1 (6.7)
Low n=49	2 (4.1)	4 (8.2)	42 (85.7)	1 (2.0)
Positive n=7	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)

Concordance rate: 52.94%; Kappa: 0.216; p value: **<0.001**

HER2 status on HR+ primary tumors (n=62)	HER2 status on metastatic tumors (n=62)			
	IHC 0 (MS-) n=7	Ultralow n=8	Low n=45	Positive n=2
IHC 0 (MS-) n=13	2 (15.4)	3 (23.1)	7 (53.8)	1 (7.7)
Ultralow n=8	3 (37.5)	1 (12.5)	4 (50.0)	0 (0.0)
Low n=37	2 (5.4)	4 (10.8)	30 (81.1)	1 (2.7)
Positive n=7	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)

Concordance rate: 53.23%; Kappa: 0.108; p value: 0.191

HER2 status on HR- primary tumors (n=40)	HER2 status on metastatic tumors (n=40)			
	IHC (MS-) n=12	Ultralow n=3	Low n=24	Positive n=1
IHC 0 (MS-) n=18	9 (50.0)	3 (16.7)	6 (33.3)	0 (0.0)
Ultralow n=7	3 (42.9)	0 (0.0)	3 (42.9)	1 (14.3)
Low n=12	0 (0.0)	0 (0.0)	12 (100.0)	0 (0.0)
P n=3	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)

Concordance rate:52.50%; Kappa: 0.291; p value: **0.003**

Figure 1. Clinicopathologic characteristics according to HER2 status. (**A**) Percentage of HER2 IHC 0 primary and metastatic tumors with no, 0–5% and ≥5% of cells with faint HER2 staining in all populations, HR-positive, and HR-negative breast cancer. (**B**) Frequency of HR+ disease according to HER2 phenotypes in primary and metastatic tumors. (**C**) Percentage of different HER2 subtypes in primary and metastatic breast cancer. (**D**) Evolution of HER2 status from primary to metastatic tumors in the same patient, shown for the overall population and stratified by HR status. The tables within the figure provide the absolute percentages and statistical details. In the entire population, 19.4% and 41.9% of primary HER2-IHC 0 (MS−) tumors transitioned to HER2-ultralow and HER2-low in the metastatic setting, respectively. Concurrently, 40.0% of primary HER2-ultralow tumors converted to IHC 0 (MS−) and 46.7% converted to HER2-low. Notably, in the HR+ subgroup, 23.1% and 53.8% of primary HER2-IHC 0 (MS−) tumors converted to HER2-ultralow and HER2-low, respectively. HR status was assessed according to the pathology of the primary tumor. (**E**) HER2 status conversion among metastatic tumors at different timepoints. MS−: HER2-IHC 0 (MS−); UL: HER2-ultralow; L: HER2-low; P: HER2-positive. ∗$0.01 \le P < 0.05$, ∗∗$0.001 \le P < 0.01$, ∗∗∗$0.0001 \le P < 0.001$, ∗∗∗∗$P < 0.0001$

setting, respectively. Meanwhile, among primary tumors classified as HER2-ultralow, 40.0% converted to IHC 0 (MS−) and 46.7% converted to HER2-low upon metastasis (Fig. 1D). Notably, among HR+ primary tumors that were initially HER2-IHC 0 (MS−), 23.1% converted to HER2-ultralow and 53.8% converted to HER2-low. These conversion rates are higher than those observed in the HR− subgroup. Interestingly, only 1 (6.7%) patient with the HER2-ultralow primary tumor maintained the ultralow expression in metastatic tumor, indicating the high instability of the HER2-ultralow phenotype. It is notable that there was one case each of HER2-IHC 0 (MS−), HER2-ultralow, and HER2-low subtypes that converted to HER2-positive status in metastatic lesions (Fig. 1D). In HR− breast cancer, HER2 expression subtypes were more consistent between primary and metastatic tumors than in HR+ breast cancer (kappa value: 0.291 vs 0.108 and *P* value: 0.003 vs 0.191) (Fig. 1D). Univariate logistic regression analysis did not identify any factors associated with the increased HER2 expression (supplementary Table 4, see online supplementary material).

In 46 patients with more than one metastatic tumor sample, collected from the same or different metastatic organs at the different timepoints, the transition of HER2 status was frequently observed (Fig. 1E). At the second timepoint, 60% and 50% of HER2-IHC 0 (MS−) and ultralow metastatic tumors transitioned to another HER2 statuses, respectively. At the third timepoint, the transition remained evident.

Survival analysis across HER2 subgroups

Kaplan–Meier analysis showed that median DFS for HER2-low, HER2-ultralow and HER2-IHC 0 (MS−) patients were 26.1, 18.4 and 18.3 months, respectively. Subgroup analysis based on HR status demonstrated that HR-HER2-ultralow breast cancer had the shortest median DFS compared to HR-HER2-IHC 0 (MS−) and HER2-low breast cancer patients ($P = 0.048$ and $P = 0.035$) (Fig. 2A). Univariate Cox analysis also demonstrated that HER2-ultralow patients had a shorter DFS than HER2-low patients within the HR− subgroup (hazard ratio = 0.51, 95% CI = 0.28–0.91, $P = 0.023$)

Table 1. Clinicopathological characteristics of patients stratified by HER2 status on primary breast cancer[a].

	IHC 0 (MS−) (*n* = 74)		IHC 0 (UL) (*n* = 44)		Low (*n* = 98)		*P* value
Age, years							
≤ 45	39	52.70%	27	61.36%	46	46.94%	MS- vs UL: 0.36 MS- vs L: 0.45 UL vs L: 0.11
>45	35	47.30%	17	38.64%	52	53.06%	
HR status							
Negative	53	71.62%	27	61.36%	32	31.65%	MS- vs UL: 0.25 MS- vs L: **<0.0001** UL vs L: **0.001**
Positive	21	28.38%	17	38.64%	66	67.35%	
T stage							
pT1	18	31.58%	8	25.81%	31	44.29%	MS- vs UL: 0.57 MS- vs L: 0.14 UL vs L: 0.07
pT2-4	39	68.42%	23	74.19%	39	55.71%	
NA	17		13		28		
N stage							
pN0	19	30.65%	9	25.00%	29	38.67%	MS- vs UL: 0.55 MS- vs L: 0.32 UL vs L: 0.15
pN+	43	69.35%	27	75.00%	46	61.33%	
NA	12		8		23		
Ki67 percent (%)							
≤ 40	39	53.42%	23	53.49%	53	60.92%	MS- vs UL: 0.99 MS- vs L: 0.34 UL vs L: 0.42
> 40	34	46.58%	20	46.51%	34	39.08%	
NA	1		1		11		
Grade							
I-II	12	18.46%	10	32.36%	26	41.94%	MS- vs UL: 0.13 MS- vs L: **0.004** UL vs L: 0.37
III	53	81.54%	21	67.74%	36	58.06%	
NA	9		13		36		
Initial stage of breast cancer							
Early	66	89.19%	36	81.82%	77	78.57%	MS- vs UL: 0.26 MS- vs L: 0.06 UL vs L: 0.66
Advanced	8	10.81%	8	18.18%	21	21.43%	

[a]pN+: with lymph node metastasis; L: HER2-low; MS−: HER2-IHC 0 (MS−); UL: HER2-ultralow; Bolded text indicates statistical significance. *P* value was calculated by Chi-square test. Not Available (NA) values for categorical variables are shown but were excluded from the statistical analysis.

(supplementary Table 5, see online supplementary material). In multivariable analyses that included Ki67 score, N stage, HR status, and HER2 status, HER2-ultralow status remained associated with a worse DFS compared HER2-IHC 0 (MS−) and HER2-low breast cancer (Table 2). Besides, higher Ki67 score, lymph node invasion, and HR-negative status predicted a shorter DFS in all patients in multivariate Cox analysis (Table 2). No significant differences in OS were observed among the various HER2 statuses in the entire population, or within the HR-positive and the HR-negative subgroups (Fig. 2B and supplementary Table 5). Higher Ki67 score and HR-negative status were associated with a shorter OS in the overall population (supplementary Table 5).

The DESTINY-Breast06 study investigated PFS of T-DXd versus first-line chemotherapy in patients with HR+ HER2-low or -ultralow metastatic breast cancer. We also explored the median PFS (mPFS) of the first-line chemotherapy in HR+ HER2-negative metastatic breast cancer in a real-world setting based on our NGS database. The mPFS for HER2-low, -ultralow and -IHC 0 (MS−) subtypes were 8.8, 6.8 and 7.2 months, respectively (Fig. 2C). There were no significant differences in primary endocrine resistance, the number of prior lines of endocrine therapy, prior CDK4/6 inhibitor or fulvestrant use, and choice of chemotherapy among the three HER2 statuses (supplementary Table 6, see online supplementary material). These results were consistent with the reported mPFS from the DESTINY-Breast06 study [13].

Mutation landscape among different HER2 statuses

To further explore the molecular characteristics across different HER2 subtypes, we analyzed the NGS results of tissue samples from 181 patients in our database. The waterfall plot illustrated the distribution of genomic alterations, including mutation and CNV profiles, according to the HER2 statuses determined from the tumor samples subjected to NGS testing (Fig. 3A). In HER2-low, ultralow, and IHC 0 (MS−) breast cancer, the most altered genes were *TP53* (55% in the HER2-low cohort, 77% in ultralow, 83% in IHC 0 (MS−), *PIK3CA* (42%, 26%, 29%), *MYC* (27%, 28%, 21%), and *CCND1* (25%, 17%, 19%) (Fig. 3A). The *ERBB2* amplification rate was low across the HER2-low, ultralow and IHC 0 (MS−) subtypes (6.5%, 2.1%, and 2.4%, respectively) (Fig. 3A). No significant differences

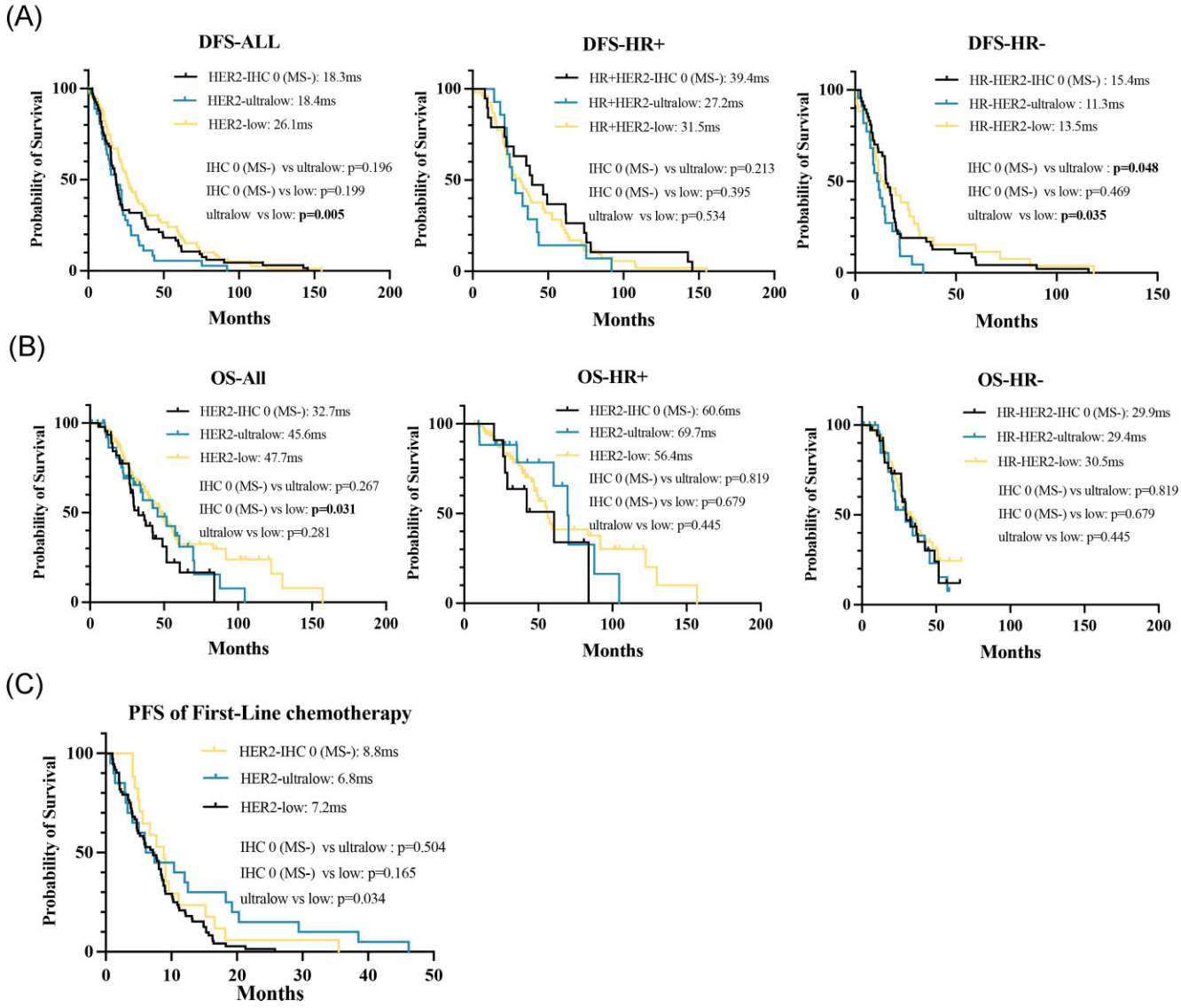


Figure 2. Kaplan–Meier curves of DFS and OS. DFS (**A**) and OS (**B**) were compared among different HER2 statuses in the overall population and HR-positive and HR-negative subgroups. Median DFS and OS are also represented. For DFS analysis, HR and HER2 status were assessed according to the last recorded pathological results of primary tumors; for OS analysis, HR and HER2 status were assessed according to the last recorded pathological results of metastatic tumors. (**C**) PFS of first-line chemotherapy among different HER2 statuses in HR+ breast cancer patients. HR and HER2 status were assessed according to the last recorded pathology results before first-line chemotherapy.

Table 2. Results from multivariate Cox proportional hazard models for DFS[a].

Parameter		Hazard ratio (95%CI)	*P* value
Ki67%		1.02 (1.01–1.03)	<0.001
N Stage	N+ vs N−	1.73 (1.22–2.47)	0.002
HR Status	HR+ vs HR−	0.59 (0.41–0.84)	0.004
HER2 Status			
	MS− vs ultralow	0.59 (0.38–0.92)	0.022
	Low vs ultralow	0.62 (0.40–0.97)	0.038

[a]HR and HER2 status according to results on primary breast cancer; HR−: HR-negative; HR+: HR-positive; L: HER2-low; MS−: HER2-IHC 0 (MS−).

in TMB were observed among HER2 subtypes in the overall population, HR+ breast cancer or HR− breast cancer (Fig. 3B). The *TP53* mutation rate was lower in HER2-low breast cancer than in ultralow and IHC 0 (MS−) breast cancer ($P = 0.001$). HER2-IHC 0 (MS−) breast cancer exhibited a higher frequency of *KRAS* mutations compared to HER2-low and ultralow breast cancer (14.29%, 4.3%, and 2.13%, respectively, $P = 0.034$) (Fig. 3C). However, when stratified by HR status, no significant differences of *TP53* and *KRAS* mutations among the HER2 statuses were observed (Fig. 3D). The most commonly altered genes showed no differences among the HER2 statuses in HR+ or HR− breast cancer. Only *PIK3CA* mutations were more common in the HER2-low subtype than in HER2-

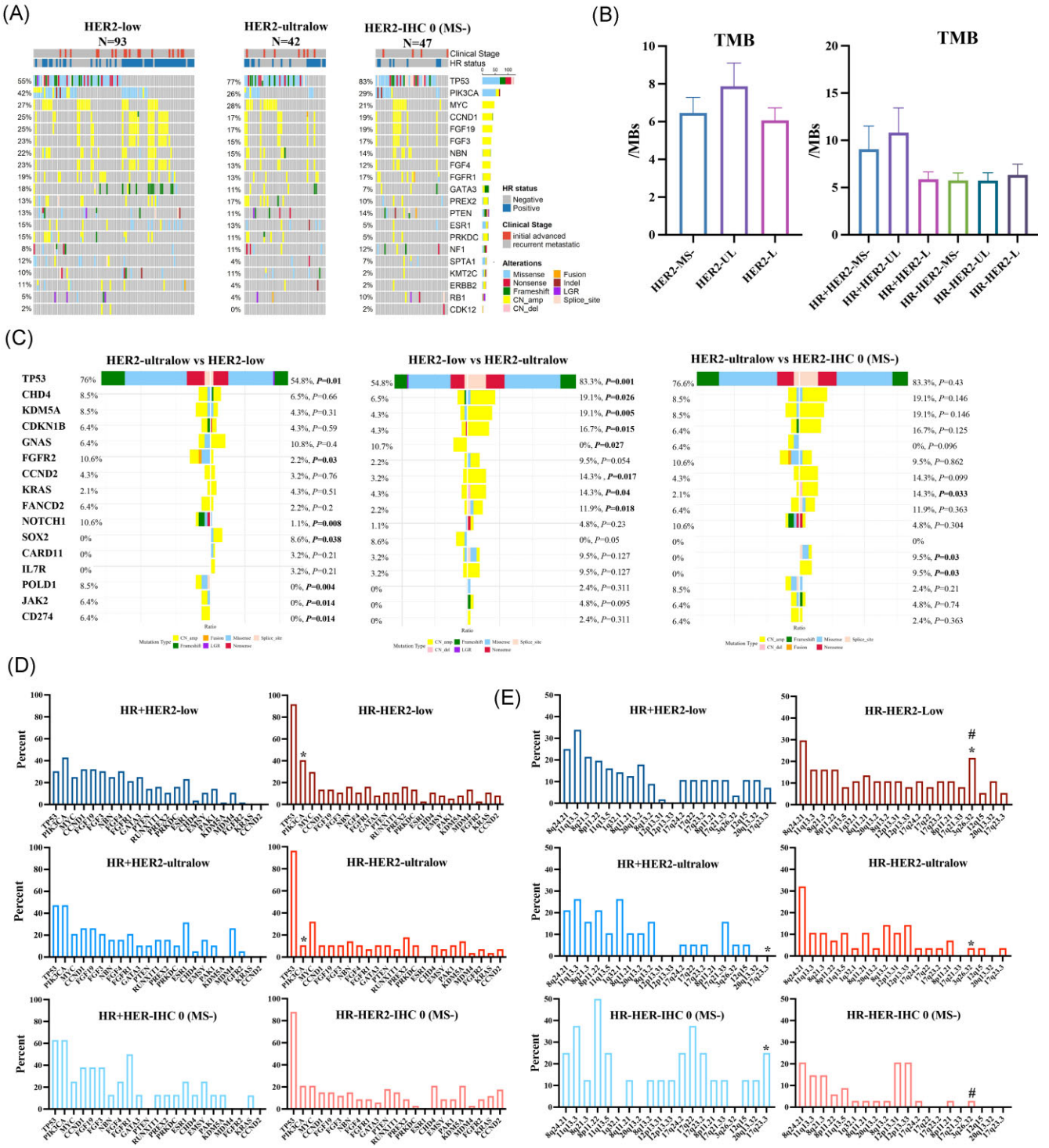


Figure 3. Mutation profiles across different HER2 statuses. (**A**) Genomic alterations of the tissue samples stratified by HER2 status determined from the tumor samples subjected to NGS testing in our NGS cohort. (**B**) TMB stratified by HR and HER2 status from our NGS database. (**C**) Frequency of the most common genomic alterations by HER2 status. (**D**) Mutation rates of common genes according to HR and HER2 status in our NGS database. (**E**) Amplification rates of common amplified segments according to HR and HER2 status in our NGS database. ∗ and # represent a significant difference among the annotated subtypes.

ultralow tumors in the HR− subtype (40.5% and 10.7%, respectively, $P = 0.0078$) (Fig. 3D).

In HR+ breast cancer, HER2-IHC 0 (MS−) breast cancer was found to have more frequent gains in 17q23.3 (*PPM1D* and *CD79B*). In HR− breast cancer, HER2-low tumors showed more common gains of 3q26.32 (*PIK3CA*) than HER2-ultralow and IHC 0 (MS−). Aside from these two segments, no other segments were observed to be differently amplified among the different HER2 statuses based on HR status subtype analysis (Fig. 3E).

HER2-negative breast cancer is a complex clinical entity with a wide spectrum of HER2 expression. To explore whether the en-

richment of molecular characteristics is associated with HER2 expression levels, NMF analysis based on the mutational profiles of HER2-negative breast cancer was performed. The most ideal value for cophenetic consensus was 2; therefore, the cohort was divided into two molecularly distinct clusters (supplementary Fig. 3A, 3B, see online supplementary material). HR-positive breast cancer had a higher prevalence of cluster 2 phenotypes compared to HR-negative breast cancer (59.1% vs 40.8%, $P = 0.03$). However, no differences of cluster phenotypes were observed among different HER2 statuses (supplementary Fig. 3C). This suggests that the molecular cluster is associated with HR status, but not with HER2 status.

Discussion

Given the therapeutic efficacy of T-DXd in HER2-ultralow breast cancer, it is essential to comprehensively understand this subset to help with appropriate clinical management. Based on our NGS database, our study was the first to investigate the similarities and differences in clinicopathological and genomic characteristics among HER2-IHC 0 (MS−), ultralow and HER2-low breast cancer.

Of the tumors previously scored as IHC 0, 11.1% were re-evaluated as HER2-IHC 1. This highlights the importance of re-evaluation when T-DXd is considered as a potential therapy for breast cancer patients. Our findings firstly revealed that almost half of metastatic lesion biopsies with HER2 IHC 0 status were reclassified as HER2-ultralow status. We also found that HER2-ultralow and IHC 0 (MS−) primary tumors had a similar proportion of HR+ phenotypes, both of which were lower than those in HER2-low tumors. However, in metastatic tumors, IHC 0 (MS−) tumors exhibited a lower proportion of HR+ status compared to HER2-low and ultralow tumors. A recent study also showed that higher percentages of ER-positive cells were observed in HER2-low or HER2-ultralow metastases compared to HER2-IHC 0 (MS−) metastases on a lesion-level [14]. For HER2 IHC 0 breast cancer, tumors with null HER2 expression had a lower proportion of HR positive phenotype only in the metastatic setting. In the HR+ HER2-IHC 0 lesions, the proportion of cases with ultralow HER2 expression was significantly higher in metastatic lesions compared to primary lesions, a difference that was not observed in the HR- HER2-IHC 0 lesions. These findings suggest that HR expression promotes ultralow HER2 expression during disease progression. The interplay between HER2 and ER pathways in breast cancer has been discussed for a long time [15–17]. Overall, these results lead to the conclusion that the interaction between low and ultralow HER2 expression and HR expression becomes more pronounced during disease progression.

Due to the significant proportion and impact of HR+ phenotype, HR status was included in the analysis. Subtype analysis based on HR status revealed no distinct clinicopathological characteristics among HER2-IHC 0 (MS−), HER2-ultralow, and HER2-low breast cancer patients. Although HER2-ultralow status was associated with a worse DFS compared to HER2-IHC 0 (MS−) and HER2-low breast cancer, no specific poor prognostic factors were identified to be associated with HER2 ultralow status. Additionally, no significant differences in OS were observed among the different HER2 statuses. The prognostic impact of HER2-ultralow expression needs to be validated in larger populations. A substantial number of previous studies have demonstrated that no significant molecular differences were observed between HER2-low and HER2-IHC 0 breast cancer [5, 9, 18]. In our study, we further refined the HER2 IHC 0 subtype into ultralow and IHC 0 (MS−) and, for the first time, dissected the molecular features among there subtypes using our NGS data. Once again, no distinct genomic alterations were observed in HER2 ultralow or IHC 0 (MS−) breast cancer after stratification based on HR status. Therefore, the minor differences in HER2 expression did not lead to differences at the clinicopathological or molecular levels.

Our study provides a comprehensive analysis of the spatial and temporal heterogeneity of HER2 status within individual patients. Frequent conversions were observed among HER2-IHC 0 (MS−), HER2-ultralow, and HER2-low categories, both between paired primary and metastatic lesions and among different metastatic sites. Notably, the magnitude of this discordance was substantial: over half of HER2-IHC 0 (MS−) primary tumors and nearly all HER2-ultralow primary tumors evolved to a different HER2 status in the metastatic tumors. Our previous study demonstrated that HER2 low expression was more common in the metastatic tumor than primary tumor, underscoring the dynamic nature of HER2 expression throughout disease progression [9]. In the metastatic setting, almost half of the tumors shifted to a different HER2 status when another biopsy was performed. These findings indicate that HER2-ultralow and IHC 0 (MS−) statuses are highly unstable over time and across different lesions. A recent study also found that most patients harbored HER2-IHC 0, ultralow, and low lesions simultaneously [14]. Taken together, this body of evidence strongly suggests that relying on a single biopsy to define a patient's HER2 status is insufficient and may not be representative of the overall disease burden.

As we know, the essential mechanism of T-DXd against tumors is the bystander effect [19]. According to the results released by the Destiny-Breast06 trial, it was shown that the cutoff value of HER2 expression for T-DXd efficacy is HER2-ultralow expression. Based on our research, we can speculate that even patients with HER2-IHC 0 (MS−) status may have a response to T-DXd, because the simultaneous presence of HER2-IHC 0 (MS−), HER2-ultralow, or HER2-low status across all lesions is commonly observed, particularly in the metastatic disease. Indeed, the DASIY study demonstrated a modest anti-tumor activity in 40 HER2-IHC 0 patients having heavy treatment, including HER2-IHC 0 (MS−) patients. From another perspective, this finding also explains the similar response to T-DXd in HER2-ultralow breast cancer to that in HER2-low breast cancer observed in the Destiny-Breast06 trial. It is difficult to define a breast cancer patient strictly as HER2-ultralow or HER2-low subtype; we can only define individual lesions as HER2-ultralow or HER2-low subtype. To accurately assess HER2 status of all lesions in one patient, HER2-PET would be a potential tool in the future [20].

In our NGS database, we also investigated the PFS of first-line chemotherapy in HR+ HER2-negative advanced breast cancer and our findings indicated that the effectiveness of first-line chemotherapy in the real world is limited. The mPFS of first-line chemotherapy whether in HR+ HER2-low, HR+ HER2-ultralow, or HR+ HER2-IHC 0 (MS−) breast cancer is considerably lower than that in the T-DXd group reported in the Destiny-Breast06 trail. This further highlighted the efficacy of T-DXd and suggests that treatment strategies for HR+ HER2-IHC 0 (MS−) breast cancer may also need to be adjusted. An ongoing clinical trial, Destiny-Breast15 (NCT05950945), is expected to evaluate T-DXd in breast cancer patients, including those with HER2-IHC 0 (MS−) expression and will help answer the question of whether HER2-IHC 0 (MS−) breast cancer can benefit from T-DXd [21].

Our study had several limitations. Firstly, the number of patients included was relatively small. Particularly when patients are stratified into subgroups based on HR and HER2 status, the

number of patients in the HR− subgroup becomes very limited. Therefore, the results of prognosis analysis among different HER2 subgroups in HR− breast cancer must be interpreted with caution. However, a comprehensive investigation, including clinicopathologic and molecular aspects, was conducted for these patients. Secondly, the number of samples available from each patient was limited. In clinical practice, obtaining multiple tissue specimens from a single patient for re-evaluation is indeed challenging. Although the number of included samples was limited, the high degree of heterogeneity in HER2 status observed in this study was certain. Thirdly, due to the limited number of patients undergoing genomic analysis, especially within the HR− subgroup, the results should be interpreted with caution. To avoid bias, we only conducted genomic analysis on tumors tested with the 520 cancer-related gene panel. Additionally, we did not perform analysis based on ctDNA because it is difficult to define HER2 status associated with ctDNA. Fourthly, it is difficult to determine whether fading affects the interpretation of HER2 ultralow status. Considering that re-staining archival tissues preserved for a long time also carries the risk of antigen loss, re-verification is not feasible. However, only 5.3% of the HER2 IHC 0 slides were prepared before 2017.

Currently, re-evaluation of tumors with HER2-IHC 0 status can identify HER2-low and HER2-ultralow statuses, which provides an additional opportunity for T-DXd treatment for these patients. Furthermore, compared to HER2-IHC 0 (MS−) tumors, HER2-ultralow and HER2-low tumors exhibited more HR+ statuses in the metastatic disease. A positive HR status appears to promote ultralow HER2 expression during disease progress. No distinct clinicopathologic and genomic characteristics were observed in HER2-ultralow breast cancer when analyzed by subtype based on HR status. It is also common for HER2-IHC 0 (MS−) and HER2-ultralow statuses to co-exist in one patient, highlighting the need for caution when using HER2-ultralow expression as a response threshold for T-DXd. We anticipate that targeted imaging, or other novel techniques, will provide better assessment of HER2 status beyond IHC, and the upcoming Destiny-Breast15 trial will offer further insights into the response threshold of T-DXd.

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Author contributions

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Supplementary data

Supplementary data is available at *PCMEDI Journal* online.

Conflict of interests

None declared.

Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethics Committee of Fudan University Shanghai Cancer Center (approval number: 1705172-9). The patients/participants provided their written informed consent to participate in this study.

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