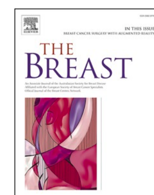
The Breast 62 (2022) 114–122

Contents lists available at ScienceDirect

The Breast

journal homepage: www.journals.elsevier.com/the-breast



HER2 copy number quantification in primary tumor and cell-free DNA provides additional prognostic information in HER2 positive early breast cancer

Xiaobin Chen [a,b,c,1], Yuxiang Lin [a,b,c,1], Zhengwen Jiang [d,1], Yan Li [a,b,c], Yihua Zhang [e], Ying Wang [d], Feng Yu [d], Wenhui Guo [a,b,c], Lili Chen [a,b,c], Minyan Chen [a,b,c], Wenzhe Zhang [a,b,c], Chuan Wang [a,b,c,*], Fangmeng Fu [a,b,c,**]

[a] *Department of Breast Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, 350001, China*
[b] *Department of General Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, 350001, China*
[c] *Breast Cancer Institute, Fujian Medical University, Fuzhou, Fujian Province, China*
[d] *Genesky Biotechnologies Inc., Shanghai, 201315, China*
[e] *Department of Dermatology, Fujian Medical University First Affiliated Hospital, Fuzhou, Fujian Province, 350000, China*

A R T I C L E I N F O

Keywords:
HER2
Copy number variation
Cell-free DNA
CNVplex
Prognosis

A B S T R A C T

Background: The quantitative relationship between *HER2* copy number and prognosis in *HER2* positive adjuvant setting remain controversial, and few studies have focused on adjuvant setting to illustrate the potential clinical relevance of *HER2* in cfDNA. Our study aim to develop a novel method in *HER2* quantification and explore the relationship between *HER2* copy number in primary tumors or cfDNA and prognosis in HER2 positive early breast cancer.
Methods: Two hundred and two patients with early breast cancer were prospectively included in a study where primary tumors, matching non-cancer breast tissue, corresponding plasma, and the plasma from 20 healthy volunteers were collected. Cox proportional hazard analysis was employed to determine the prognostic value of *HER2* gene copy number in tissue and cfDNA. Tissue based nomograms and time-dependent decision curve analysis were used to evaluate the practicality of *HER2* copy number stratification.
Results: HER2 amplification by CNVplex demonstrated a robust concordance with FISH (concordance 89.2%). A three-tiered system of tissue and a two-tiered system of cfDNA classification were shown to be independent prognostic factors. A tissue copy number-based nomogram was fitted and further evaluation revealed a good performance in discrimination (c statistic 0.801) and calibration.
Conclusions: We first report CNVplex as a viable alternative for *HER2* detection. Quantitative evaluation of *HER2* presents tremendous potential for use in risk stratification. We also uncover the potential for using *HER2* copy number in cfDNA as a biomarker for prognosis in a HER2 positive adjuvant setting.

1. Introduction

Breast cancer is one of the most commonly diagnosed cancers, accounting for 24.2% in newly diagnosed cancer cases [1]. The amplification/overexpression of *C-erbB2* has been reported in approximately 15%–20% of breast cancers. It plays a pivotal role in oncogenesis, cancer angiogenesis, and progression, indicating a more aggressive phenotype and a poor prognosis for breast cancer patients [2–4]. The addition of anti-HER2 therapy (trastuzumab; trastuzumab plus pertuzumab; trastuzumab plus TKI) to adjuvant chemotherapy have significantly improved the prognosis of HER2 positive breast cancer [5–7]. Nonetheless, about 20–30% of patients experience recurrence despite trastuzumab administration with curative intent, and there remain unmet clinical needs in risk stratification [5,8].

* Corresponding author. Department of Breast Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, 350001, China.
** Corresponding author. Department of Breast Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, 350001, China.
E-mail addresses: dr_chuanwang@fjmu.edu.cn (C. Wang), ffm@fjmu.edu.cn (F. Fu).
[1] These authors contribute equally to this work.

https://doi.org/10.1016/j.breast.2022.02.002
Received 23 April 2021; Received in revised form 7 November 2021; Accepted 3 February 2022
Available online 8 February 2022
0960-9776/© 2022 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Despite being widely accepted as a poor prognostic factor for breast cancer, the quantitative relationship between the *HER2* copy number and clinical outcome remains elusive [9,10]. Accurate and quantitative evaluation of *HER2* copy number is the precondition to assess the dose-response or prognostic effect of *HER2* copy number in HER2 positive breast cancer treated with trastuzumab. Although multiple approaches have been reported in *HER2* copy number detection, such as chromogenic in site hybridization (CISH) [11], silver in site hybridization (SISH) [12], multiplex ligation dependent probe amplification (MLPA) [13], droplet digital PCR (ddPCR) and next generation sequencing (NGS) [14], FISH has been recommended as the gold standard of *HER2* copy number detection. However, it is also expensive and time consuming [15–17]. An easier and more cost-effective approach may have the potential to improve clinical practice.

Having established that there is high intratumoral heterogeneity in breast cancer, conventional tissue-based approaches of *HER2* detection may lead to an inaccurate assessment [18,19]. Tumor specific genetic alterations in cfDNA may mirror a comprehensive genetic landscape of cancer [20,21]. Previous studies have demonstrated the potential for using the *HER2* copy number in cfDNA as a biomarker to predict the trastuzumab response and/or the prognosis [22]. However, most studies monitoring *HER2* copy number via cfDNA focused on advanced cancer or the neoadjuvant setting harboring a high tumor burden. Circulating tumor DNA can represent only a very small proportion of cfDNA in the adjuvant setting with a relatively low tumor burden, indicating that a highly sensitive method is needed in this population. Limitations in methodology have limited the application of cfDNA in an adjuvant setting.

Herein, we develop an assay based on the high throughout ligation dependent probe amplification (CNVplex) technology to determine the *HER2* copy number of the primary tumor and cfDNA in the HER2 positive adjuvant setting. Our aim is to assess the feasibility of using CNVplex in *HER2* copy number quantification, assess the potential utility of cfDNA in the adjuvant setting as a non-invasive approach to determine *HER2* copy number. Explore the feasibility of *HER2* copy number in tumor and cfDNA function as a molecular prognostic biomarker in the HER2 positive adjuvant setting.

2. Materials and methods

2.1. Patient inclusion and study design

As shown in Fig. 1, a total of 202 patients who had been pathologically diagnosed with early breast cancer and underwent surgery followed by adjuvant therapy between January 2015 to June 2017 at Fujian Medical University Union hospital were included in our study. Primary cancer tissue and corresponding non-cancerous breast tissue were collected from the 202 patients. Matched plasma prior to surgery

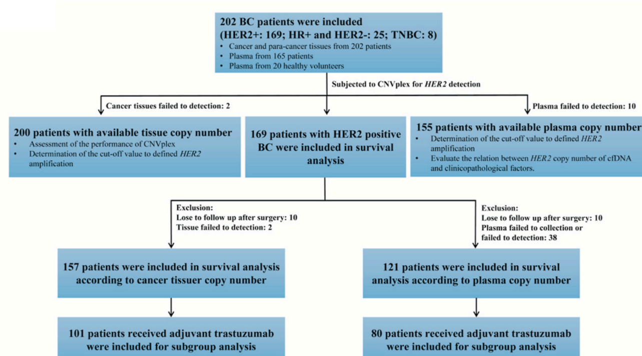


Fig. 1. Schematic representation of the study design. BC: Breast cancer; HR: Hormone receptor status; TNBC: Triple negative breast cancer.

was collected from 165 patients. All 202 patients, as indicated, received adjuvant chemotherapy, radiotherapy, and endocrine therapy according to local guideline. Among 202 participants, 148 patients were diagnosed as *HER2* amplification, while 37 patients were classified as non-amplification by FISH and 17 patient tumors did not get FISH analysis. Patients categorized as HER2 positive breast cancer were included in survival analysis to investigate the association between *HER2* copy number in tumor tissue or cfDNA and prognosis.

The study protocol was approved by ethics committee of Fujian Medical University Union Hospital (No. 2014021) in December 26, 2014. Written informed consent was obtained from all subjects before their participation.

2.2. Sample collection

Tumor, para-cancerous samples, and corresponding plasma samples were prospectively collected from participants. Tumor tissues and non-cancerous breast tissues were derived from surgical specimen and were immediately snap-frozen in liquid nitrogen and then stored at −80 °C. Blood samples were collected in EDTA tubes from patients and healthy volunteers and centrifuged at 1600 g for 10 min to separate plasma. The supernatant was then centrifuged at 16000 g for 10 min to further remove cell debris. Plasma samples were isolated and stored at −80 °C within 2 h of collection.

2.3. Genomic and circulating free DNA extraction

Genomic DNA of the participants was extracted from tissues using the TIANamp Genomic DNA Kit (TIANGEN, LOT# U8701), cfDNA was extracted from the plasma using MagMAXTM Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, LOT#2009058) according to the manufacturer's instructions. The concentration and quality of cfDNA was assessed by Bioanalyzer 2100 (Agilent Technologies), CfDNA samples with high molecular weight DNA would be excluded from the study.

2.4. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) assay

IHC was performed on 4-μm-thick formalin fixed paraffin embedded specimens. The sections were deparaffinized in xylene, then dehydrated through three alcohol changes and transferred to Ventana wash solution. Epitope retrieval was conducted using cell conditioning solution at 100 °C for 35 min, and Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. The slides were then incubated with Ventana anti-HER2/neu (4B5) rabbit monoclonal primary antibody at 37 °C for 30 min and developed in DAB for 10 min. Finally, sections were counterstained with hematoxylin and mounted. The expression of HER2 was evaluated according to ASCO/CAP guideline [23].

FISH for detection of *HER2* gene amplification was performed by using a PathVysion HER2 DNA probe kit (20 Assays) according to the manufacturer's instructions. Briefly, the kit contains a HER2/neu probe and a chromosome 17 centromere (*CEP17*) probe, which were labeled with spectrum Orange and spectrum Green respectively. Both the absolute *HER2* signal and the ratio of *HER2/CEP17* signals were recorded. And the status of *HER2* amplification was assessed according to the updated ASCO/CAP guideline [23].

2.5. HER2 copy number detection

CNVplex was employed to determine the *HER2* copy number of tumor tissue and plasma, which was modified from a multiplex ligation-dependent probe amplification (MLPA). A total of 193 pairs of probes were designed to evaluate HER2 and reference genes. To ensure two copies/cell for a reference gene and exclude the influence of chromosome 17 polysomy or co-amplification of *CEP17* and *HER2*, 64 pairs of probes that cover 21 chromosomes (2n = 42) were designed to

investigate reference genes, while 129 pairs of probes were designed for *HER2* detection. The workflow of CNVplex has been described previously [24,25]. In general, *HER2* copy number in normal breast tissue and cfDNA from healthy volunteers was defined as two copies, which were used for the reference sample in the tumor and cfDNA from patients *HER2* detection assays, respectively. Peak height (H value) of each detected genomic locus was calculated, R (region 1 of cancer or cfDNA from patients) = H (targeted region 1 of cancer or cfDNA from patients)/H (reference region of cancer or cfDNA from patients), R (region 1 of control group) = H (region 1 of control group)/H (reference of control group), RR = R (region 1 of cancer or cfDNA from patients)/R (region 1 of control group). The copy number of the control group and reference gene were defined as 2 copies, so the copy number of region 1 of cancer or cfDNA from patients = RR (region 1 of cancer or cfDNA from patients)*2 copies. After this procedure, the copy number of 129 loci of *HER2* were generated, the highest and lowest of three values were removed. Then, the mean copy number of the remaining 123 values was calculated to defined the *HER2* copy number of the sample. All detected *HER2* or reference genomic loci and the sequence of specific probe combining areas are listed in Supplementary Table 1.

2.6. Statistical analysis

A non-parametric test (Kruskal-Wallis test or Mann-Whitney test) was performed for subgroup comparison, and Bonferroni correction for multiple tests. Receiver operating characteristic (ROC) curves were generated to optimize the cut-off value of tissue/cfDNA copy number for detecting *HER2* amplification. Kappa tests were employed to evaluate the consistency between *HER2* amplification detected by CNVplex and FISH. A chi-square test was used to assess the association between *HER2* amplification in tumor/cfDNA detected by CNVplex and clinicopathological factors. Spearman correlation coefficients were calculated to determine the relationship between the copy number in tissue and cfDNA. Univariate and multivariate Cox proportional hazard models was used to confirm independent prognostic values of *HER2* copy number, and Cox regression coefficients were employed to construct a nomogram to predict the 3-year disease free survival probability. Harrell's concordance index (c statistic) and calibration curve were used for discrimination and calibration evaluation. Time dependent decision curve analysis (DCA) was employed to determine the clinical value (net benefit) of a nomogram. Statistical analysis was done with R software version 3.6.3, and P values were considered to be significant at $P < 0.05$.

3. Results

Tumor and corresponding non-tumor breast tissues were collected from 202 patients, and matched plasma samples were collected from 165/202 patients. In 10 plasma samples and two tumor samples we failed to detect *HER2* due to insufficient specimen volume. Patient demographic data are listed in Table 1.

3.1. Assessment of CNVplex performance in HER2 copy number detection

We first compared FISH with corresponding tissue copy number as determined by CNVplex and found that the copy number was strongly related to the *HER2* amplification signal (Fig. 2a). The copy number of FISH-positive tumor is significantly higher than that of FISH non-amplification tumor and non-cancer tissues (Fig. 1b). We also found a positive correlation between the staining intensity of the HER2 protein (by IHC) and *HER2* copy number in the tissue (Fig. 2c). The cutoff value of *HER2* gene copy number by CNVplex to evaluate *HER2* amplification was determined by receiver operating characteristic (ROC) curve, with an area under curves (AUC) of 0.963 (95% CI, 0.937–0.988) (Fig. 2d). The cutoff value, sensitivity, and specificity were 2.38, 0.915, and 0.906, respectively. With this cutoff value, we divided the copy number of tissues into non-amplification (<2.38) and amplification (≥2.38). A high concordance (Kappa coefficient, 0.699; P < 0.001) of *HER2* gene amplification detected by FISH and CNVplex was observed (Table 2). Together, these data suggest CNVplex is a quantitative, precise approach of *HER2* copy number detection, and suggests a role for CNVplex as a surrogate to detect *HER2* amplification.

Table 1
Baseline clinicopathological characteristics of patients included in study.

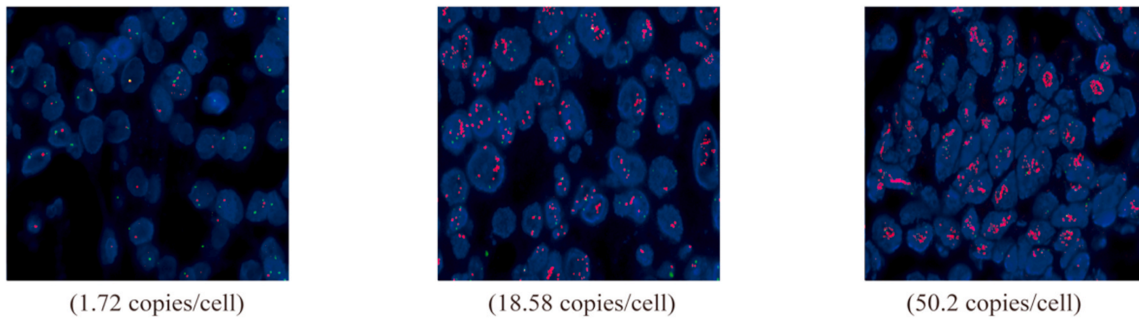
Variables	Patients (n)	Percentage
Total	202	100%
Age		
<48	75	36.9%
≥48	127	63.1%
T stage [a]		
T1	52	25.6%
T2	123	60.6%
T3	27	13.8%
N stage [a]		
Negative	97	48.0%
Positive	105	52.0%
Stage		
I	32	15.9%
II	152	75.2%
III	18	8.9%
HR [b]		
Negative	93	46.0%
Positive	109	54.0%
***HER2* status** [c]		
Non-amplification	37	18.3%
Amplification	148	73.3%
Unknown	17	8.4%
HER2 expression [c]		
0-1	18	8.9%
2	33	16.3%
3	151	74.8%
Ki67		
<35%	66	32.5%
≥35%	136	67.5%
Histologic grade		
I-II	86	42.9%
III	116	57.1%
Subtype		
ERBB2+	169	83.6%
HR+ and ERBB2-	25	12.4%
TNBC	8	4.0%

Abbreviation: TNBC = Triple negative breast cancer. HR = Hormone receptor status.
[a] Tumor size and axillary lymph node status was evaluated by ultrasound and was defined in accordance with AJCC breast cancer staging manual 8th.
[b] HR: Hormone Receptor: hormone receptor positive was defined as estrogen or progesterone receptor staining intensity >0% by IHC.
[c] HER2 status was evaluated by FISH, and HER2 expression by IHC according to American Society of Clinical Oncology/College of American Pathologists human epidermal growth factor receptor 2 (HER2) testing in breast cancer guideline.

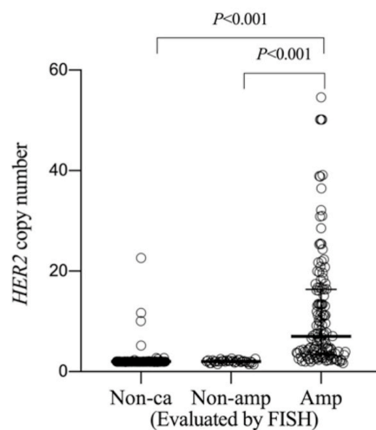
3.2. HER2 copy number of cfDNA and its association with clinical characteristics

To demonstrate the practicability of cfDNA as a biomarker to predict *HER2* amplification in tumor tissue, ROC curves were generated (AUC, 0.703; 95%CI, 0.602–0.804; P = 0.001) (Fig. 3a), the cutoff value, sensitivity, and specificity were 1.98, 0.602, and 0.704 respectively. The *HER2* amplification by cfDNA presents a relatively limited concordance with FISH (Kappa coefficient, 0.196; P = 0.004) (Table 2). We next explored the factors which were attributed to the variants of plasma *HER2* copy number among individuals. The copy number of cfDNA in the FISH positive cohort was significantly higher than in the FISH negative cohort (Fig. 3b), and it was proportional to the copy number in cancer tissue detected by CNVplex (Fig. 3c). A large tumor size (T2/3) and axillary lymph nodes metastasis tended to be associated with a higher copy number with borderline statistical significance (Fig. 3d and

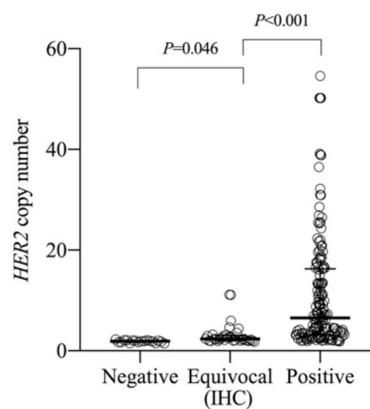
A



B



C



D

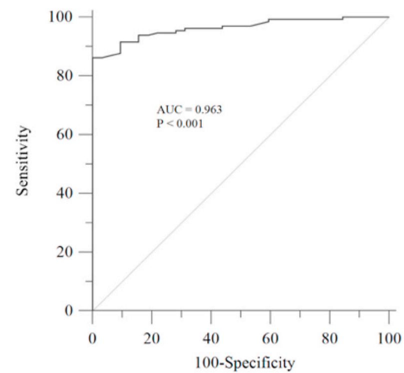


Fig. 2. Assessment of CNVplex in *HER2* copy number detection. (A) Assessment of consistency between *HER2* amplification signal and copy number detected by CNVplex. **(**B) The distribution of *HER2* copy number in different tissues. Each circle indicates a patient. Non-ca: Non-cancer tissue; Non-amp: Non-amplification; Amp: Amplification. The median copy number of *HER2* amplified breast cancer tissues was 7.02 (interquartile range, 3.36–16.34), which is significantly higher than that for FISH non-amplification cancer tissues (median, 2.03; interquartile range, 1.87–2.23; $P < 0.001$), and also higher than the non-cancer tissues (median, 1.99; interquartile range 1.97–2.01; $P < 0.001$). (C) The distribution of *HER2* copy number according to HER2 protein staining intensity by Immunohistochemistry. Negative: IHC scoring 0,1; Equivocal: IHC scoring 2; Positive: IHC scoring 3. (D) Tissue *HER2* copy number (by CNVplex) was obtained from 200 patients. A cutoff value of 2.38 was determined using receiver operating characteristic (ROC) analysis to defined *HER2* amplification.

Table 2
Concordance of *HER2* amplification (tissue/plasma) detected by CNVplex and FISH.

		FISH Non-amplification	Amplification	Total
Tissue	Non-amplification	33	16	49
	Amplification	4	132	136
	Total	37	148	185
Kappa coefficient = 0.699, $P < 0.001$				
Concordance = 89.2%				
		FISH Non-amplification	Amplification	Total
Plasma	Non-amplification	19	47	66
	Amplification	8	71	79
	Total	27	118	145
Kappa coefficient = 0.196, $P = 0.004$				
Concordance = 62.1%				

e). We also found that high Ki67 (≥35%) and negative hormone receptor was significantly correlated with a higher *HER2* copy number (Fig. 3f and g).

3.3. Quantitative relationship of HER2 copy number and prognosis

To explore the prognostic impact of the *HER2* copy number in HER2 positive breast cancer patients, 169 patients diagnosed as HER2 positive breast cancer were included in survival analysis (101 of them received standard adjuvant trastuzumab therapy). Ten of 169 patients were excluded from survival analysis because of loss follow up after the completion of surgery. Twenty-eight recurrences were observed at a median follow-up of 37 months (Interquartile range 33–41). Finally, there were 157 patients with available tissue *HER2* copy number data defined by CNVplex (2/159 patients were excluded because of failure of detection) that were used to investigate the association of copy number and prognosis, and 121/159 had their cfDNA copy number analyzed. X-tile was employed to optimize a cutoff value and both a three-tiered scoring system of tissue (Fig. 4a) and a two-tiered scoring system of cfDNA copy number (Fig. 4b) were generated. Both high/intermediate copy number in tumor tissue and high copy number in cfDNA had significant prognostic value for univariate survival analysis.

3.4. Development of multivariate cox proportional hazard model

Multivariate survival analysis was fitted to confirm the prognostic value of the *HER2* copy number scoring system. Both the three-tiered system of tissue (Table 3) and the two-tiered system of plasma (Table 4) remained highly prognostic in a multivariate survival model adjusted for age, tumor size, axillary nodal status, hormone receptor status, histological grade, and Ki67, which are widely accepted as prognostic factors for breast cancer. Additionally, multivariate Cox regression coefficients were employed to generate a tissue-based nomogram to predict the probability of 3-year disease free survival

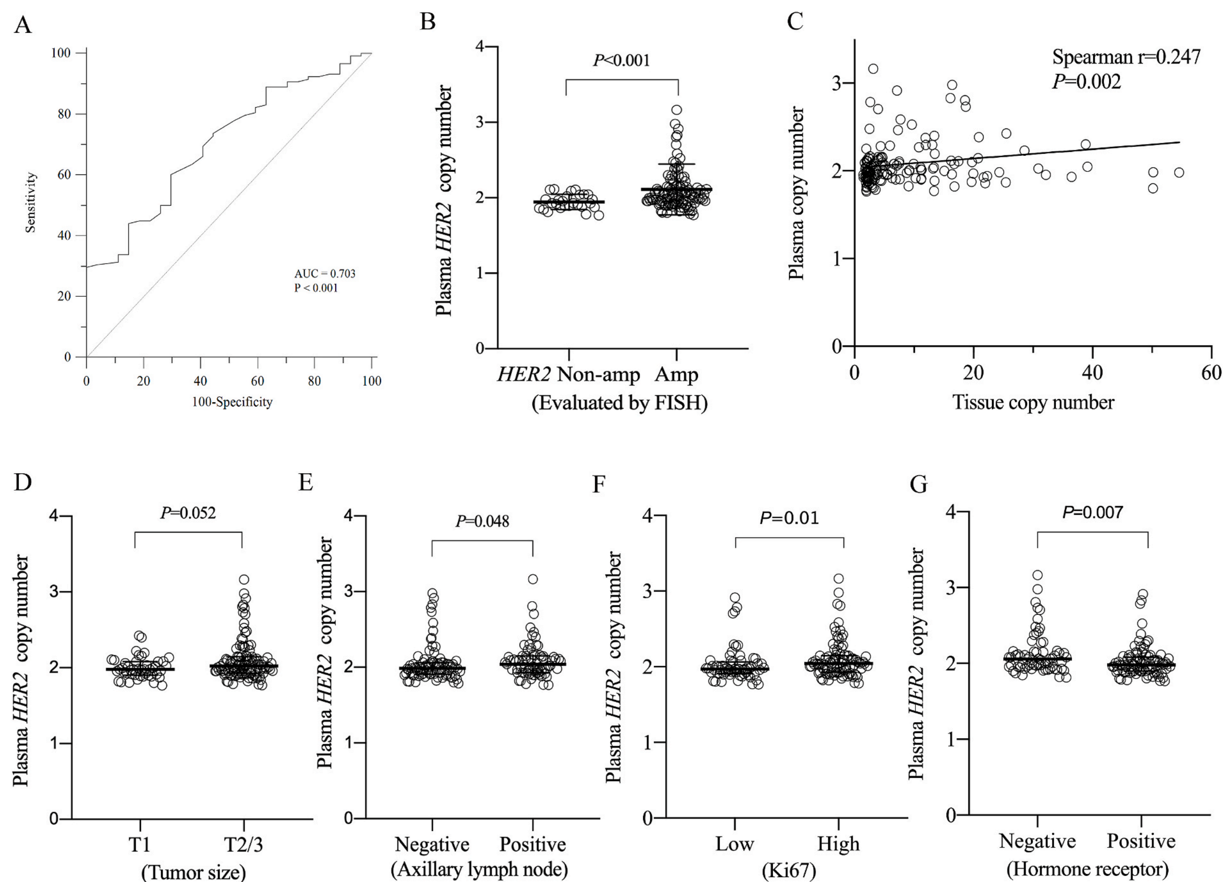


Fig. 3. Assessment of CNVplex in plasma (cfDNA) *HER2* copy number detection. (A) *HER2* copy number of cfDNA was acquired from 155 patients, 118 of them were diagnosed as *HER2* amplification by FISH. A cut-off value of 1.98 was determined by ROC analysis with an AUC of 0.703. (B) cfDNA copy number in *HER2* amplification (defined by FISH) setting was significantly higher than the non-amplification setting (Mann-Whitney test). (C) cfDNA copy number was positively correlated with tissue copy number, evaluated by Spearman correlation coefficient. (D–G) Large tumor size (T2/3), axillary lymph node metastasis, high Ki67, and hormone receptor negativity tend to present higher cfDNA copy number (Mann-Whitney test).

(Fig. 5a). The performance of the nomogram was assessed by Harrell's concordance index (c statistic, 0.801; 95%CI, 0.752–0.928) and by calibration curve (Fig. 5b). The performance of the nomogram for discrimination and calibration both show a good fit. We could conclude that the *HER2* copy number stratification of tumor tissue has a tremendous potential in prognosis prediction, and we also unraveled the independent prognostic value of cfDNA copy number.

3.5. Evaluation of clinical benefit with time-dependent decision curve analysis

Time-dependent decision curve analysis (DCA) was conducted to further confirm the incremental clinical benefit of *HER2* copy number stratification in tumor tissue [26]. Clinicopathological characteristics which have independent prognostic value in multivariate survival analysis (excluding tissue copy number) (Table 3) were included to construct clinical based prediction models. The *HER2* gene copy number evaluated by FISH (amplification/non-amplification) was added to the clinical based model to generate a FISH based model, but it did not provide additional net benefit as expected. While the addition of the tissue copy number stratification system to the clinical based model provides a significantly higher benefit across the range of risk threshold (Fig. 5c).

3.6. HER2 copy number stratification remain prognostic in trastuzumab treated patients

We have revealed that a high copy number of *HER2* in either tumor tissue or cfDNA indicated a poor prognosis in HER2 positive early breast cancer. To further determine whether the addition of trastuzumab affects the prognostic value of *HER2* copy number, patients diagnosed as HER2 positive breast cancer who received trastuzumab therapy were included in a subgroup analysis. Among these patients, high copy number in tumor tissue (Fig. 3c) and plasma (Fig. 3d) were found to be an independent prognostic indicator in multivariate survival analysis (Supplementary Tables 2 and 3).

4. Discussion

Accurate *HER2* gene evaluation in HER2 equivocal breast cancer is the foundation of trastuzumab administration in clinical practice due to the prognostic and therapeutic impact [27]. FISH has been recommended as the gold standard in *HER2* copy number detection which uses *CEP17* as the single reference control. However, chromosome 17 polysomy may result in a low *HER2/CEP17* ratio, leading to underestimating *HER2* amplification. Recent studies revealed that an additional *CEP17* signal (>2 copies/cell) might also be induced by co-amplification of *CEP17* and *HER2* [28–30]. Regardless of the mechanism of *CEP17* amplification, it may result in misclassification of *HER2* status, and a multi-gene reference is needed for accurate *HER2* detection [31,32].

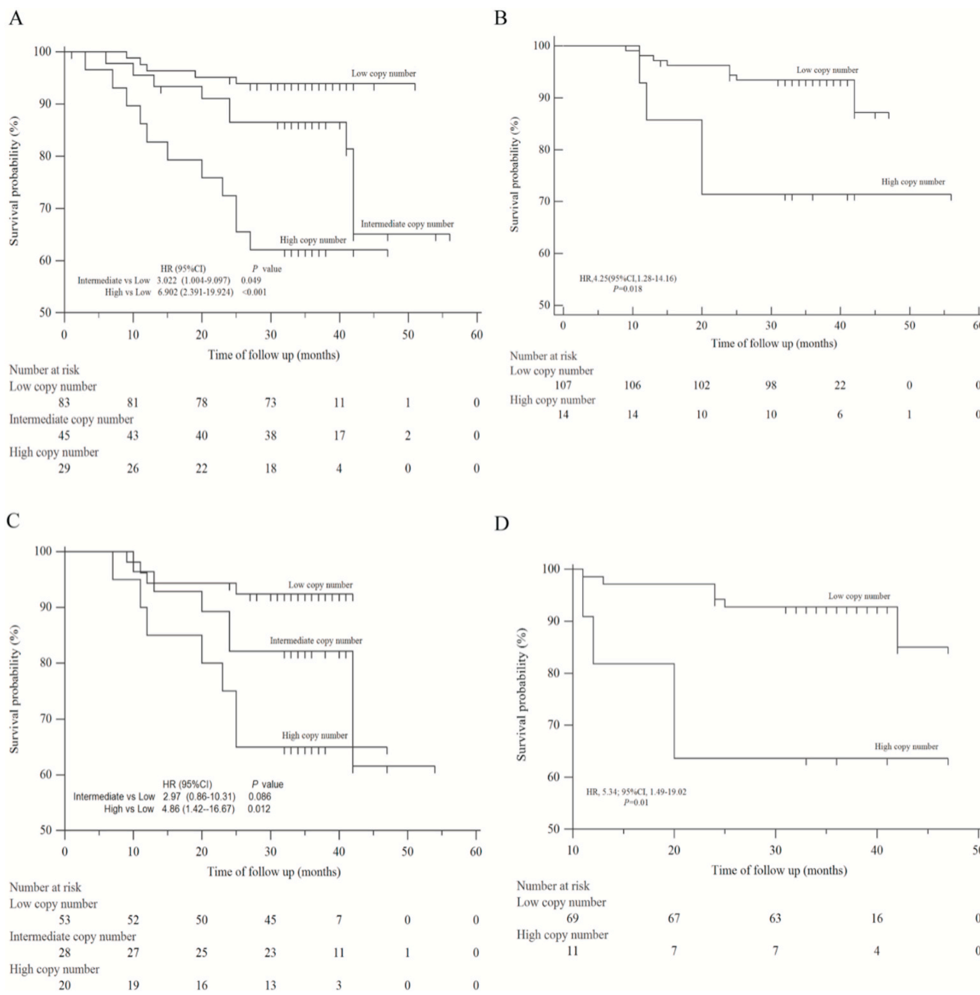


Fig. 4. Disease free survival among patients with different *HER2* copy number. (A) Disease free survival by tissue *HER2* copy number stratification, x-tile was employed to optimized cutoff value. Patients with a high (>17.5) or intermediate (6.1–17.5) copy number present poorer prognosis than the low group (<6.1). (B) Disease free survival by cfDNA *HER2* copy number, the cutoff value was also generated by X-tile. (C) Subgroup analysis of patients receiving trastuzumab administration, high copy number of tissue present significant prognostic value, intermediate copy number of tissues also present inferior survival, despite it did not reach statistical significance. (D) Subgroup analysis of patients receiving trastuzumab therapy, high copy number of cfDNA present significant prognostic value in univariate survival analysis.

Table 3
Tissue based multivariate survival model.

Variables	Unadjusted univariate model			Adjusted multivariate model		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Tissue copy number						
Low (<6.1)	1			1		
Intermediate (6.1–17.5)	3.02	1.01–9.10	0.049	4.51	1.39–14.59	0.012
High (>17.5)	6.91	2.39–19.92	<0.001	10.51	3.36–32.83	<0.001
Age						
<48	1			1		
≥48	1.54	0.64–3.71	0.33	3.31	1.13–9.66	0.029
cT						
T1	1			1		
T2	0.88	0.31–2.48	0.813	1.47	0.50–4.32	0.484
T3	2.88	0.91–9.08	0.071	5.59	1.50–20.85	0.01
cN						
Negative	1			1		
Positive	2.42	1.01–5.79	0.048	3.53	1.39–8.94	0.008
HR						
Negative	1			1		
Positive	0.79	0.35–1.76	0.564	2.22	0.83–5.87	0.109
Ki67						
<35%	1			1		
≥35%	0.49	0.23–1.10	0.085	0.38	0.15–0.94	0.036
Histological grade						
I-II	1			1		
III	0.76	0.34–1.67	0.493	0.76	0.33–1.77	0.519

Table 4
Plasma based multivariate survival model.

Variables	Unadjusted univariate model			Adjusted multivariate model		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Plasma copy number						
Low (<2.4)	1			1		
High (≥2.4)	5.04	1.36–18.68	0.016	5.51	1.43–21.22	0.013
Age						
<48	1			1		
≥48	1.72	0.46–7.11	0.418	1.72	0.43–6.84	0.439
cT						
T1	1			1		
T2	1.14	0.31–4.30	0.846	0.75	0.18–3.11	0.694
T3	1.41	0.15–13.56	0.766	0.49	0.04–5.96	0.577
cN						
Negative	1			1		
Positive	3.55	0.89–14.21	0.082	3.66	0.88–15.16	0.073
HR						
Negative	1			1		
Positive	0.3	0.08–1.11	0.071	0.52	0.12–2.18	0.372
Ki67						
<35%	1			1		
≥35%	1.97	0.53–7.32	0.31	1.64	0.43–6.35	0.472
Histological grade						
I-II	1			1		
III	0.86	0.27–2.71	0.793	0.59	0.16–2.18	0.436

For the present study we developed a quantitative method, CNVplex, which was modified from MLPA. CNVplex has been employed to identify low frequency germline amplification at chromosome 15q13.3 that is associated with an increased risk of HBV-related hepatocellular cancer, and has also been reported in prenatal screening of fetal aneuploidy [24,33]. To our knowledge, this is the first report of CNVplex for *HER2* gene copy number detection. High concordance (165/185 89.2%) among CNVplex and FISH was observed, suggesting the potential of using CNVplex as a viable alternative for *HER2* amplification detection. Among the 20 patients discordant for FISH and CNVplex results, 16 of them were defined as *HER2* amplification by FISH but did not show amplification by CNVplex, while four patients have amplification by CNVplex but not by FISH. We speculate that it may result from high intratumoral heterogeneity in the distribution of *HER2* amplification tumor cells across the tumor.

We also found that the cfDNA-based copy number is a promising biomarker for predicting the FISH results, but it is less accurate than the tissue-based copy number detected by CNVplex. Poor prediction accuracy was also observed by Shoda et al. in an advanced setting of gastric cancer using real-time quantitative chain reaction for detecting the cfDNA *HER2* copy number [34]. The accuracy was improved by Siravegna et al. where the maximum mutant allele fraction that implicates the proportion of tumor DNA in cfDNA was employed for cfDNA *HER2* copy number correction, and the adjusted cfDNA copy number had a stronger correlation with tissue than the unadjusted number [22]. In our study, further analysis revealed that the plasma *HER2* copy number was significantly associated with the tissue copy number although it was a weak correlation. Patients with large tumors (T2/3), axillary lymph node metastasis, high Ki67 (≥35%), and hormone receptor negativity tend to present with a higher cfDNA copy number. We can infer that the

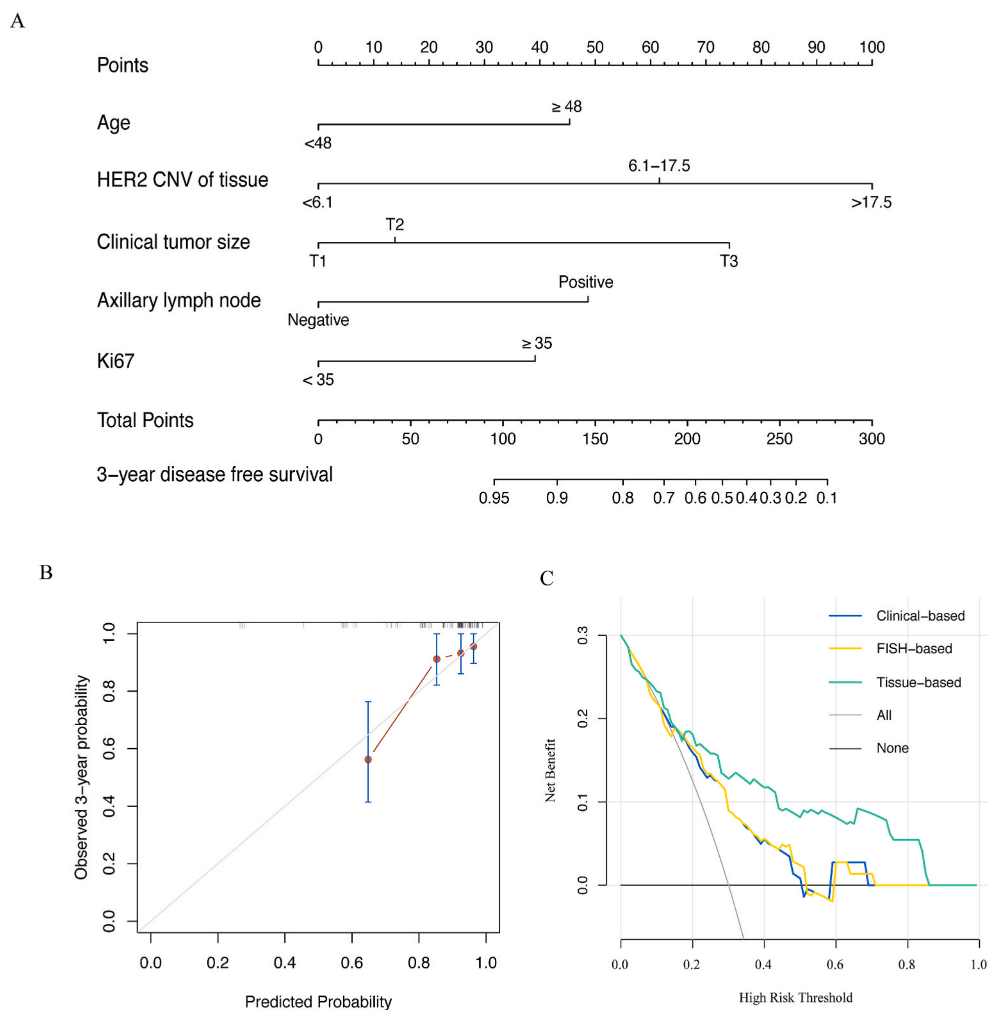


Fig. 5. Development of tissue copy number-based nomogram and nomogram evaluation. (A) Tissue copy number based nomogram (Tissue-based model) for predicting 3-year disease free survival in adjuvant setting of HER2 positive breast cancer was generated, variables present independent prognostic value in multivariate survival analysis (Table 3) were included in nomogram. (B) Calibration plot of observed 3-year disease free survival probability (y-axis) over predicted probability (x-axis). (C) Time-dependent decision curve analysis was generated to evaluate clinical benefit of model. The high-risk threshold represents the risk of 3-year recurrence predicted by each model, patients were recommended for intervention if they exceed this threshold. Net benefit balanced the clinical benefit and harm from model. The addition of FISH to clinical model did not improve net benefit of clinical-based model, while tissue copy number stratification bring substantial benefit.

HER2 copy number of cfDNA was not only affected by the copy number in tissue, but also by the tumor burden and hormone receptor status [35]. High Ki67 was also associated with a higher *HER2* copy number and it might result from a propensity of hyperproliferative tumors to shed DNA into the blood [36]. These factors may explain why cfDNA copy number is inferior to tissue-based copy number in predicting the *HER2* status.

According to guideline from American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) human epidermal growth factor receptor 2 (HER2) testing [23], HER2 can be defined as positive when IHC result is 3+, and further examination for *HER2* gene amplification can be omitted. But previous studies have demonstrated that patients with different degrees of *HER2* amplification may experience different courses of disease, thus *HER2* copy number evaluation has the potential to provide additional therapeutic or prognostic information. High *HER2* gene copy number has been reported to be associated with higher rates of a pathological complete response in patients treated with trastuzumab in a neoadjuvant setting [37]. Similar result was also presented by Guiu et al. where they draw the conclusion that, although presented with higher rate of pCR, high *HER2* copy number may still indicated a poor prognosis (HR, 2.819; $P = 0.057$) [38]. High copy number has indicated an aggressive phenotype and presents a poor prognosis in an adjuvant setting when trastuzumab was not routinely administered [39,40]. Borley et al. reported that high amplification of *HER2* was connected with a superior prognosis than intermediate copy number under trastuzumab administration, which implies that a high copy number seems predict better trastuzumab response than an intermediate copy number [10]. But, interestingly, neither the expanded analysis of the N9831 or the HERA trial failed to demonstrate a linear dose-effect between *HER2* copy number evaluated by FISH and the trastuzumab response, patients with different *HER2* copy numbers derived similar benefits from trastuzumab [41,42].

In our study, a three-tiered scoring system of tissue and a two-tiered system of cfDNA were generated and the prognostic value of the *HER2* copy number stratification remained significant for patients received adjuvant trastuzumab. Indicating that a high copy is still a marker of poor prognosis even when trastuzumab was given. A similar conclusion was presented by Xuan et al. [9]. The HER2 protein is a common coreceptor that can mediate a signaling pathway by homodimerization or heterodimerization. The anti-tumor effect of trastuzumab is mainly due to downregulation of the HER2 signaling and more importantly, antibody dependent cell-mediated cytotoxicity (ADCC). While it exerts a limited effect on dimerization inhibition, this incomplete inhibition of the HER2 enabling sustained signaling from uninhibited HER2 protein [43–46]. The overexpression of HER2 is mainly driven by *HER2* amplification, and they show a positive correlation [47]. Hence, we can speculate that *HER2* amplification leads to HER2 overexpression and subsequent overactivation of HER2 signaling, thus resulting in a poor prognosis. Incomplete inhibition of HER2 signaling mediated by trastuzumab cannot reverse the poor prognosis associated with highly amplified *HER2* in breast cancer patients.

Our study has several limitations. First of all, there were only 165 plasma samples that matched tissue samples, while 37 plasma samples were not obtained. Second, although there was good performance in discrimination and calibration, our tissue-based nomogram is based on a relatively small cohort, and an external validation cohort is needed to confirm the model. Third, the *HER2* copy number detection was only performed in frozen tissues instead of formalin fixed paraffin-embedded (FFPE) tissues and that might be inconvenient in routine clinical practice. More importantly, the differences between frozen and FFPE tissues might result in a diverse interpretation between *HER2* copy number and prognosis. Large scale studies are needed to further confirm the quantitative association between *HER2* copy number and individual prognosis.

In conclusion, we are the first to report the application of CNVplex for *HER2* detection and it was proven to be an accurate method for frozen tissue and cfDNA evaluation. Quantitative stratification of the *HER2* gene copy number in tumor tissue for HER2 positive breast cancer can provide an accurate prediction of individual prognosis and discriminate high-risk patients from low-risk patients. We also introduce the feasibility of using cfDNA in an adjuvant setting to predict *HER2* amplification of tumor tissue and act as a prognostic biomarker.

Declarations

Funding: This study was supported by grants from Joint Funds for the Innovation of Science and Technology, Fujian Province (2018Y9055, 2019Y9103) and Joint Key Funds for the Health and Education of Fujian Province (2019-WJ-23).

Availability of data and materials

Data were available from corresponding authors upon reasonable request.

Code available

Not applicable.

Authors' contributions

FF and WC conceived and designed the study. LY, ZW and ZY collected the data. GW, CL and CM analyzed the data. W and Y offer technical assistance. CX, LY and JZ wrote the manuscript. All authors contributed toward data analysis, drafting, and critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Ethic approval and consent to participate

The study protocol was approved by ethics committee of Fujian Medical University Union Hospital, written informed consent was obtained from all subjects before their participation.

Consent for publication

We have obtained consent to publish from the participants.

Declaration of competing interest

The authors declare no conflict of interests.

Acknowledgement

We thank the patients who participated in this study and Genesky Biotechnologies for technical assistance. We acknowledge the assistance of Jinxing L, Qian Mei, Liuwen Yu, Jing Li, Peng Zhou.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2022.02.002.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J Clin 2018;68:394–424.
[2] Borley A, Mercer T, Morgan M, Dutton P, Barrett-Lee P, Brunelli M, et al. Impact of HER2 copy number in IHC2+/FISH-amplified breast cancer on outcome of adjuvant trastuzumab treatment in a large UK cancer network. Br J Cancer 2014;110:2139–43.
[3] Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. Nat Rev Dis Prim 2019;5:66.

[4] Slomski A. Adjuvant therapy for HER2-positive breast cancer. JAMA 2019;322:1134.

[5] Cameron D, Piccart-Gebhart MJ, Gelber RD, Procter M, Goldhirsch A, de Azambuja E, et al. 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. Lancet 2017;389:1195–205.

[6] Piccart M, Procter M, Fumagalli D, de Azambuja E, Clark E, Ewer MS, et al. Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer in the APHINITY trial: 6 Years' follow-up. J Clin Oncol 2021;39:1448–57.

[7] Martin M, Holmes FA, Ejlertsen B, Delaloge S, Moy B, Iwata H, et al. Neratinib after trastuzumab-based adjuvant therapy in HER2-positive breast cancer (ExteNET): 5-year analysis of a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2017;18:1688–700.

[8] Chumsri S, Li Z, Serie DJ, Mashadi-Hossein A, Colon-Otero G, Song N, et al. Incidence of late relapses in patients with HER2-positive breast cancer receiving adjuvant trastuzumab: combined analysis of NCCTG N9831 (alliance) and NRG Oncology/NSABP B-31. J Clin Oncol 2019;37:3425–35.

[9] Xuan Q, Ji H, Tao X, Xu Y, Zhang Q. Quantitative assessment of HER2 amplification in HER2-positive breast cancer: its association with clinical outcomes. Breast Cancer Res Treat 2015;150:581–8.

[10] Borley A, Mercer T, Morgan M, Dutton P, Barrett-Lee P, Brunelli M, et al. Impact of HER2 copy number in IHC2+/FISH-amplified breast cancer on outcome of adjuvant trastuzumab treatment in a large UK cancer network. Br J Cancer 2014;110:2139–43.

[11] Miligy IM, Toss MS, Gorringe KL, Lee AHS, Ellis IO, Green AR, et al. The clinical and biological significance of HER2 over-expression in breast ductal carcinoma in situ: a large study from a single institution. Br J Cancer 2019;120:1075–82.

[12] Koh J, Nam SK, Lee YW, Kim JW, Lee KW, Ock CY, et al. Trastuzumab specific epitope evaluation as a predictive and prognostic biomarker in gastric cancer patients. Biomolecules 2019;9.

[13] Silva ANS, Coffa J, Menon V, Hewitt LC, Das K, Miyagi Y, et al. Frequent coamplification of receptor tyrosine kinase and downstream signaling genes in Japanese primary gastric cancer and conversion in matched lymph node metastasis. Ann Surg 2018;267:114–21.

[14] Yang SR, Bouhlal Y, De La Vega FM, Ballard M, Kuo CJ, Vilborg A, et al. Integrated genomic characterization of ERBB2/HER2 alterations in invasive breast carcinoma: a focus on unusual FISH groups. Mod Pathol 2020;33:1546–56.

[15] Koudelakova V, Berkovcova J, Trojanec R, Vrbkova J, Radova L, Ehrmann J, et al. Evaluation of HER2 gene status in breast cancer samples with indeterminate fluorescence in situ hybridization by quantitative real-time PCR. J Mol Diagn 2015;17:446–55.

[16] Khoury T, Sait S, Hwang H, Chandrasekhar R, Wilding G, Tan D, et al. Delay to formalin fixation effect on breast biomarkers. Mod Pathol 2009;22:1457–67.

[17] Shah MV, Wiktor AE, Meyer RG, Tenner KS, Ballman KV, Green SJ, et al. Change in pattern of HER2 fluorescent in situ hybridization (FISH) results in breast cancers submitted for FISH testing: experience of a reference laboratory using US food and drug administration criteria and American society of clinical Oncology and college of American Pathologists guidelines. J Clin Oncol 2016;34:3502–10.

[18] Parikh AR, Leshchiner I, Elagina L, Goyal L, Levovitz C, Siravegna G, et al. Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. Nat Med 2019;25:1415–21.

[19] Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, Bruhm DC, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. Nature 2019;570:385–9.

[20] Chabon JJ, Hamilton EG, Kurtz DM, Esfahani MS, Moding EJ, Stehr H, et al. Integrating genomic features for non-invasive early lung cancer detection. Nature 2020;580:245–51.

[21] Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating tumor DNA analysis in patients with cancer: American society of clinical Oncology and college of American Pathologists Joint review. J Clin Oncol 2018;36:1631–41.

[22] Siravegna G, Sartore-Bianchi A, Nagy RJ, Raghav K, Odegaard JI, Lanman RB, et al. Plasma HER2 (ERBB2) copy number predicts response to HER2-targeted therapy in metastatic colorectal cancer. Clin Cancer Res 2019;25:3046–53.

[23] Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of American Pathologists clinical practice guideline focused update. Arch Pathol Lab Med 2018;142:1364–82.

[24] Xu C, Wang T, Liu C, Li H, Chen X, Zhu H, et al. Noninvasive prenatal screening of fetal aneuploidy without massively parallel sequencing. Clin Chem 2017;63:861–9.

[25] Zhang X, Xu Y, Liu D, Geng J, Chen S, Jiang Z, et al. A modified multiplex ligation-dependent probe amplification method for the detection of 22q11.2 copy number variations in patients with congenital heart disease. BMC Genom 2015;16.

[26] Chen SY, Yang Y, Qi SN, Wang Y, Hu C, He X, et al. Validation of nomogram-revised risk index and comparison with other models for extranodal nasal-type NK/T-cell lymphoma in the modern chemotherapy era: indication for prognostication and clinical decision-making. Leukemia 2020;35:130–42.

[27] Oh DY, Bang YJ. HER2-targeted therapies - a role beyond breast cancer. Nat Rev Clin Oncol 2020;17:33–48.

[28] Tse CH, Hwang HC, Goldstein LC, Kandalaft PL, Wiley JC, Kussick SJ, et al. Determining true HER2 gene status in breast cancers with polysomy by using alternative chromosome 17 reference genes: implications for anti-HER2 targeted therapy. J Clin Oncol 2011;29:4168–74.

[29] Yeh IT, Martin MA, Robetorye RS, Bolla AR, McCaskill C, Shah RK, et al. Clinical validation of an array CGH test for HER2 status in breast cancer reveals that polysomy 17 is a rare event. Mod Pathol 2009;22:1169–75.

[30] Vranic S, Teruya B, Repertinger S, Ulmer P, Hagenkord J, Gatalica Z. Assessment of HER2 gene status in breast carcinomas with polysomy of chromosome 17. Cancer 2011;117:48–53.

[31] Donaldson AR, Shetty S, Wang Z, Rivera CL, Portier BP, Budd GT, et al. Impact of an alternative chromosome 17 probe and the 2013 American Society of Clinical Oncology and College of American Pathologists guidelines on fluorescence in situ hybridization for the determination of HER2 gene amplification in breast cancer. Cancer 2017;123:2230–9.

[32] Hanna WM, Ruschoff J, Bilous M, Coudry RA, Dowsett M, Osamura RY, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol 2014;27:4–18.

[33] Cao P, Yang A, Wang R, Xia X, Zhai Y, Li Y, et al. Germline duplication of SNORA18L5 increases risk for HBV-related hepatocellular carcinoma by altering localization of ribosomal proteins and decreasing levels of p53. Gastroenterology 2018;155:542–56.

[34] Shoda K, Masuda K, Ichikawa D, Arita T, Miyakami Y, Watanabe M, et al. HER2 amplification detected in the circulating DNA of patients with gastric cancer: a retrospective pilot study. Gastric Cancer 2015;18:698–710.

[35] Zhang X, Zhao W, Wei W, You Z, Ou X, Sun M, et al. Parallel analyses of somatic mutations in plasma circulating tumor DNA (ctDNA) and matched tumor tissues in early-stage breast cancer. Clin Cancer Res 2019;25:6546–53.

[36] Volik S, Alcaide M, Morin RD, Collins C. Cell-free DNA (cfDNA): clinical significance and utility in cancer shaped by emerging Technologies. Mol Cancer Res 2016;14:898–908.

[37] Arnould L, Arveux P, Couturier J, Gelly-Marty M, Loustalot C, Ettore F, et al. Pathologic complete response to trastuzumab-based neoadjuvant therapy is related to the level of HER-2 amplification. Clin Cancer Res 2007;13:6404–9.

[38] Guiu S, Gauthier M, Coudert B, Bonnetain F, Favier L, Ladoire S, et al. Pathological complete response and survival according to the level of HER-2 amplification after trastuzumab-based neoadjuvant therapy for breast cancer. Br J Cancer 2010;103:1335–42.

[39] Fuchs EM, Kostler WJ, Horvat R, Hudelist G, Kubista E, Attems J, et al. High-level ERBB2 gene amplification is associated with a particularly short time-to-metastasis, but results in a high rate of complete response once trastuzumab-based therapy is offered in the metastatic setting. Int J Cancer 2014;135:224–31.

[40] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.

[41] Ross JS. Disease-free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 Year of trastuzumab: the HERA trial. Breast Dis Year Bk Q: Year Book Quarter 2010;21:378–80.

[42] Perez EA, Reinholz MM, Hillman DW, Tenner KS, Schroeder MJ, Davidson NE, et al. HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. J Clin Oncol 2010;28:4307–15.

[43] Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell 2002;2:127–37.

[44] Goutsouliak K, Veeraraghavan J, Sethunath V, De Angelis C, Osborne CK, Rimawi MF, et al. Towards personalized treatment for early stage HER2-positive breast cancer. Nat Rev Clin Oncol 2020;17:233–50.

[45] Gennari R, Menard S, Fagnoni F, Ponchio L, Scelsi M, Tagliabue E, et al. Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. Clin Cancer Res 2004;10:5650–5.

[46] Cooley S, Burns LJ, Repka T, Miller JS. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. Exp Hematol 1999;27:1533–41.

[47] Li AC, Zhao J, Zhao C, Ma Z, Hartage R, Zhang Y, et al. Quantitative digital imaging analysis of HER2 immunohistochemistry predicts the response to anti-HER2 neoadjuvant chemotherapy in HER2-positive breast carcinoma. Breast Cancer Res Treat 2020;180:321–9.