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

ABSTRACT

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].
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 [5,10]. 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 situ hybridization (CISH) [11], silver in situ 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 throughput 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 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), CFNA 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 (CPE17) probe, which were labeled with spectrum Orange and spectrum Green respectively. Both the absolute HER2 signal and the ratio of HER2/CPE17 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

Fig. 1. Schematic representation of the study design. BC: Breast cancer; HR: Hormone receptor status; TNBC: Triple negative breast cancer.
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 were used to confirm independent prognostic values of HER2 copy number for predicting survival. The workflow of CNVplex has been described previously [24,25].

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.

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

| 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 | | |
| T1 | 52 | 25.6% |
| T2 | 123 | 60.6% |
| T3 | 27 | 13.8% |
| N stage | | |
| Negative | 97 | 48.0% |
| Positive | 105 | 52.0% |
| Stage | | |
| I | 32 | 15.9% |
| II | 152 | 75.2% |
| III | 18 | 8.9% |
| HR | | |
| Negative | 93 | 46.0% |
| Positive | 109 | 54.0% |
| HER2 status | | |
| Non-amplification | 37 | 18.3% |
| Amplification | 148 | 73.3% |
| Unknown | 17 | 8.4% |
| HER2 expression | | |
| 0-1 | 18 | 8.9% |
| 2 | 32 | 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. 
<sup>a</sup> Tumor size and axillary lymph node status was evaluated by ultrasound and was defined in accordance with AJCC breast cancer staging manual 8th. 
<sup>b</sup> HR: Hormone Receptor: hormone receptor positive was defined as estrogen or progesterone receptor staining intensity >0% by IHC. 
<sup>c</sup> 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.
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.

### 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%

### 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; Equivocal: IHC scoring 1; 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.

![Image](image_url)
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 under-estimating 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          | 1         | 1   | 1.39–14.59 | 0.012     |
| 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          | 1         | 1   | 1.13–9.66  | 0.029     |
| ≥48                | 1.54 | 0.64–3.71 | 0.33     | 3.31 | 1.13–9.66  | 0.029     |
| cT                 |     |            |           |     |            |           |
| T1                 | 1   | 1          | 1         | 1   | 1.05–4.32  | 0.484     |
| 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          | 1         | 1   | 1.39–8.94  | 0.008     |
| Positive           | 2.42 | 1.01–5.79 | 0.048    | 3.53 | 1.39–8.94  | 0.008     |
| HR                 |     |            |           |     |            |           |
| Negative           | 1   | 1          | 1         | 1   | 0.83–5.87  | 0.109     |
| Positive           | 0.79 | 0.35–1.76 | 0.564    | 2.22 | 0.83–5.87  | 0.109     |
| Ki67 <35%          | 1   | 1          | 1         | 1   | 0.15–0.94  | 0.036     |
| ≥35%               | 0.49 | 0.23–1.10 | 0.085    | 0.38 | 0.15–0.94  | 0.036     |
| Histological grade |     |            |           |     |            |           |
| I-II               | 1   | 1          | 1         | 1   | 0.33–1.77  | 0.519     |
| III                | 0.76 | 0.34–1.67 | 0.493    | 0.76 | 0.33–1.77  | 0.519     |
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

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                         | 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                         | 1 1                        |
| ≥48                | 1.72 0.46–7.11 0.418        | 1.72 0.43–6.84 0.439       |
| cfT                |                             |                            |
| T1                 | 1 1                         | 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       |
| eN                 |                             |                            |
| Negative           | 1 1                         | 1 1                        |
| Positive           | 3.55 0.89–14.21 0.082       | 3.66 0.88–15.16 0.073      |
| HR                 |                             |                            |
| Negative           | 1 1                         | 1 1                        |
| Positive           | 0.3 0.08–1.11 0.071         | 0.52 0.12–2.18 0.372       |
| Ki67 <35%          | 1 1                         | 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                         | 1 1                        |
| III                | 0.86 0.27–2.71 0.793        | 0.59 0.16–2.18 0.436       |

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 Guiz 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. [8]. The HER2 protein is a common co-receptor 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

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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.

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Appendix A. Supplementary data

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

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