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Original article

# Genome-wide chromosomal instability by cell-free DNA sequencing predicts survival in patients with metastatic breast cancer

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ABSTRACT

*Background:* Genome-wide chromosomal instability, instead of specific somatic mutations or copy-number alterations in selected genes, is a significant property of cancer and may suggest a new strategy for treatment. Here we utilized cell-free DNA (cfDNA) sequencing to display the whole picture of chromosomal instability in patients with metastatic breast cancer (MBC), and evaluate its predictive value for patient survival.

*Methods:* The clinical data of 65 patients who had frozen plasma and planned to change the therapeutic regimen were retrospectively enrolled. Low-coverage whole-genome sequencing of cfDNA was performed to generate the chromosomal instability represented by chromosomal instability (CIN) score.

*Results:* Tumors with diverse status of hormone receptor and HER2 represented diverse chromosomal instability across the whole genome. According to the receiver operating characteristic curve and the statistical distribution, CIN score exceed 3881 was defined as "High". 32 (53.3%) patients with high CIN score had similar clinicopathologic characteristics compared with low CIN score patients. The median overall survival of patients with high CIN score was 21.2 months (95% CI 14.1–28.3), which was significantly inferior to those with low CIN score (not reached, $P = 0.006$). Regardless of various treatment regimens, the median progression free survival in patients with high CIN score was 7.3 months, which was significantly worse than those in the low CIN score population (11.0 months, $P = 0.034$). Multivariate analysis revealed that CIN score was an independent prognostic factor, with hazard ratio of 3.563 ($P = 0.005$).

*Conclusions:* To our knowledge, this is the first study illustrating the prognostic value of chromosomal instability derived from cfDNA in MBC.



## 1. Background

Breast cancer is the most common cancer in women in China and worldwide [1,2]. This heterogeneous disease is clinically categorized into three basic therapeutic groups with diverse genetic alterations [3]: hormone receptor positive (HR+) group, HER2 amplified (HER2+HR-) group, and triple-negative breast cancer (TNBC). Despite tremendous advances in the treatment of breast cancer, metastatic breast cancer (MBC) virtually remains an incurable disease, with a median overall survival (OS) of approximately three years and a 5-year survival of only 25% [4]. Precise characterization of genomic profiling may provide indications for novel treatment strategies for these patients.

Extensive efforts have focused on the genomic features of primary breast cancer, instead of the metastatic disease [3,5–8].

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Meanwhile, only a few studies in small cohorts of patients have launched to interrogate the genomic features of MBC [9–12]. Intratumor heterogeneity [13,14], as well as infeasibility of repeated tissue biopsy may be the major causes, especially in the setting of metastatic disease.

Tumor derived cell-free DNA (cfDNA), an approach which is minimally influenced by clonal heterogeneity, has emerged as a potential way to resolve this problem. The advantage of cfDNA analysis with next generation sequencing lies in minimally invasive but more comprehensive genomic profiling when compared with tissue aspiration biopsy. Prior applications of cfDNA have mainly focused on tracking specific somatic mutations or copy-number alterations (CNAs) in targeted panels of genes [15–19], which may not provide the full picture of the genome.

Recently, analysis of genome-wide chromosomal instability emerges as a novel application of cfDNA. Multiregional tissue biopsy in lung cancer reveals that, patients with a high proportion of CNAs (instead of mutations) were at significantly higher risk for disease recurrence [14]. The genome-wide chromosomal instability from cfDNA was also concordant with of treatment resistance in patients with metastatic TNBC or multiple myeloma [5,13]. Except for TNBC [5], there is no relevant analysis in other molecular subtypes of MBC revealing the association between chromosomal instability and patient survival.

Here we developed an Ultrasensitive Chromosomal Aneuploidy Detector (UCAD) exclusively using cfDNA. Through low-coverage whole-genome sequencing of cfDNA, this technology could profile genome-wide chromosomal instability without the need for prior knowledge of tumor mutations in tissue. We aimed to 1) evaluate the association of chromosomal instability with patient survival, and 2) identify key CNAs that are enriched in different subtypes of MBC.

## 2. Methods

### 2.1. Patients

Medical records of patients with MBC treated at National Cancer Center, Cancer Hospital Chinese Academy of Medical Sciences, from March 2015 to October 2015 were retrospectively reviewed. Patients with stage IV MBC who were about to change line of therapy and had at least one blood draw for cfDNA were eligible. Exclusion criteria included early-stage breast cancer and patients who did not have a cfDNA analysis due to insufficient blood samples. 65 patients were selected by applying these criteria. Clinicopathologic data were abstracted from the medical record. Patients with HER2 score 3+ by immunohistochemistry (IHC) or HER2 amplification by fluorescence in situ hybridization (FISH) were defined as HER2 positive. Use of patients' clinicopathologic data and cfDNA draws were approved by the institutional review board of Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CH-BC-018), and written informed consent was obtained from all patients.

### 2.2. Plasma collection and DNA extraction

Plasma samples were collected once per person on the first day of new lines of metastatic therapy in EDTA Blood Collection Tubes. Samples were processed within 2 h of collection by centrifugation at 3200g for 10 min at room temperature. Plasma was separated and stored at −80 °C until DNA extraction. Total genomic DNA and cfDNA were isolated from plasma using the QIAseq cfDNA Extraction kit (Qiagen).

### 2.3. Low-pass whole-genome sequencing

The detailed procedure of next generation sequencing has been previously described [20,21]. DNA was fragmented into an average size of 300bp (cfDNA without fragmentation), and then 100 ng of fragmented genomic DNA (cfDNA 10 ng) was used for preparation of sequencing libraries (NEBnext Ultra II). 8bp barcoded sequencing adaptors were then ligated with DNA fragments and amplified by polymerase chain reaction. Purified sequencing libraries were massively parallel sequenced by Illumina HiSeq Xten platform. About 4G sequencing raw data per sample were filtered and aligned to the human reference genome to average coverage 2.1x.

### 2.4. Gene-level copy number analyses

CNAs were derived by the UCAD pipeline (Supplementary Table 1). An online version of the pipeline is available on website http://www.istopcancer.net/pgweb/cn/istopcancer.jsp. Sequencing coverage for each 200 K bin was calculated followed by GC normalization. The sequencing coverage were further normalized by a set of controls plasma samples from 9 post-surgery early stage breast cancer patients and 7 health individuals (Supplementary Table 2). The Z-score for each bin was calculated by formula $Z = \frac{C_{test} - average(C_{control})}{sd(C_{control})}$, where $C_{test}$ and $C_{control}$ are the coverage of the bin. The normalized bin values were sent to segmentation calls by algorithm circular segmentation algorithm as provided by R package DNA copy. If the standard deviation of copy ratios between adjacent bins was>30, samples would be excluded because of poor-quality sequence data. The chromosomal overall copy number changes were then summarized by chromosomal instability (CIN) score $CIN\_Score = \sum_{all\ segments} V_{segment} \times L_{segment}$, where V is the Z-score value of a segment, and L is the length of a segment in basepair. An elevated chromosomal instability was defined by CIN score greater than average(controls)+6*stdev(controls). Gene CNA from cfDNA was defined as copy number ≥5, similar to the fluorescence in situ hybridization (FISH) method for detecting HER2 gene amplification [22].

### 2.5. Statistical analyses

All statistical analyses were performed in IBM SPSS Statistics 24. Receiver Operating Curve (ROC) was used to identify the cutoff value of CIN score for overall survival. Contrasts in clinicopathologic characteristics between different CIN score group were evaluated using Pearson's $\chi^2$ tests. After checking the assumptions of normality distribution, Kruskal-Wallis H test was used to assess the correlation between CIN scores and clinicopathologic subtypes. The association of CIN to categorical clinicopathologic factors was further evaluated using Multivariate Logistic Regression analyses. Survival was measured from the date of new treatment initiation after cfDNA collection. Progression-free survival and overall survival were estimated through the Kaplan–Meier method. Multivariable analysis was performed by the Cox proportional hazard model.

## 3. Results

### 3.1. Patients characteristics

We identified 65 patients with MBC at a single tertiary care institution, with plasma samples collected between March 2015 and October 2015 under institutional review board–approved protocols (Fig. 1). The clinical and pathological characteristics of

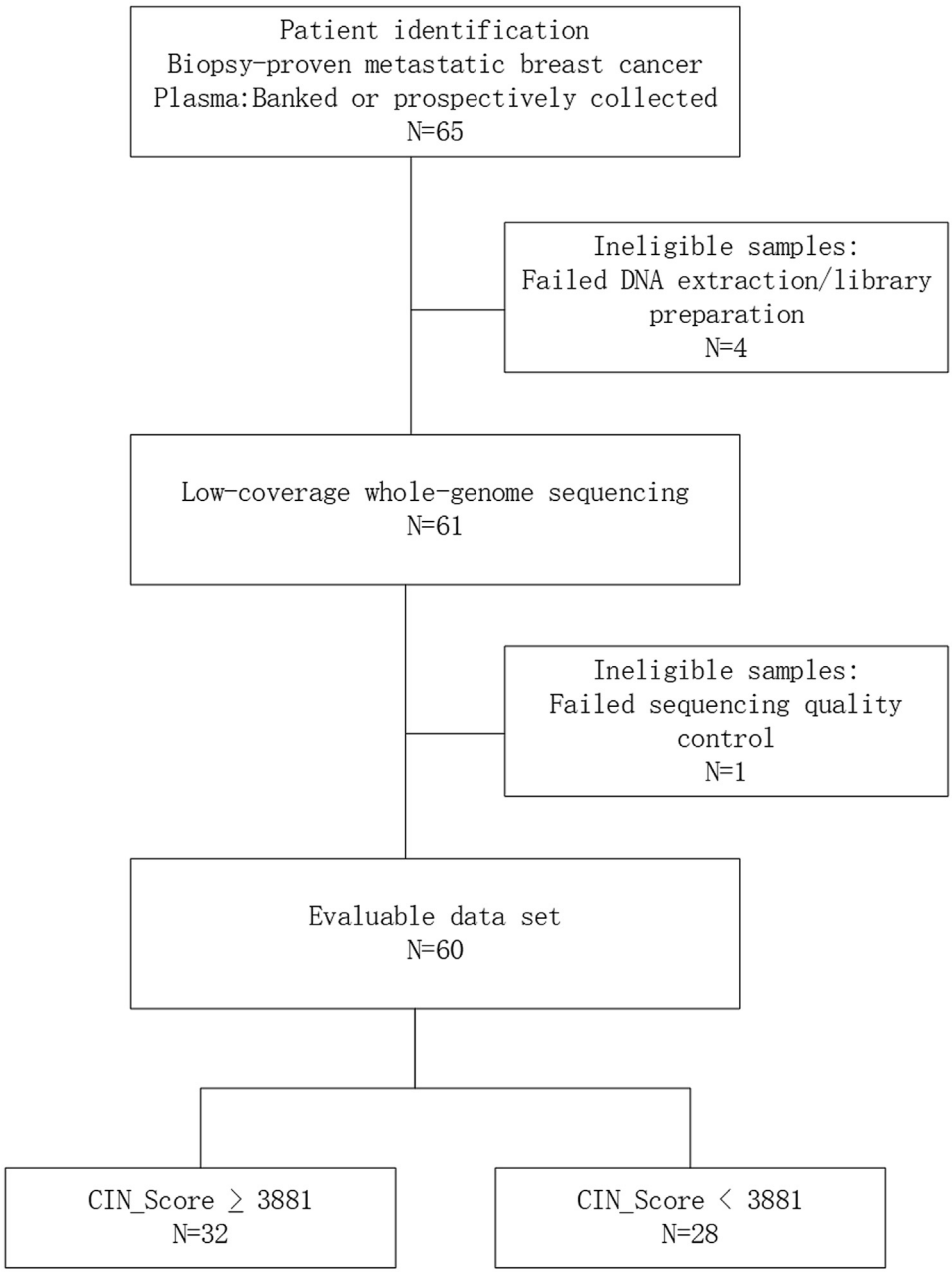


**Fig. 1.** REporting recommendations for tumor MARKer prognostic studies (REMARK) diagram.

the evaluable patients are detailed in Table 1. Thirty-two (53.3%) patients had visceral disease when the blood samples were collected, while 7 (11.7%) patients had bone-only metastasis. Twenty-eight (43.1%) out of 65 patients had no prior systemic therapies for MBC. 23.3% were stage IV at primary diagnosis, 81.7% had been treated with chemotherapy previously and 55.0% had received prior endocrine therapy in the adjuvant and/or metastatic setting. The median follow-up after blood collection was 24.3 months (range, 0.2 to 37.2), with a median PFS of 7.7 months (95%CI 6.4 to 9.0) and median OS of 28.4 months (95%CI 19.1 to 37.6).

### *3.2. Chromosomal instability and its correlation with patients' characteristics*

Evaluable sequencing data were acquired from 60 patients, with 32 (53.3%) samples resulted to have high CIN score. The choice of CIN's cutoff value is mainly based on the statistical distribution of CIN scores (Supplementary Fig. 1). It can be found that around CIN = 4000, patients can be divided into two groups. Then, we use the ROC curve to find the most appropriate cutoff value in the range of about 4000 (Supplementary Table 3). Instead of using the Yoden Index (CIN = 6885, sensitivity 73.9% and specificity 77.4%), we chose 3881 in consideration of maximizing sensitivity on the premise of relatively high sensitivity (sensitivity 78.3% and specificity 64.5%).

Compared to patients with low CIN score, patients with high CIN score had similar clinicopathologic characteristics (Table 1). The median CIN scores were 12084 in patients younger than 40 years, 5124 in patients 40–60 years old, and 1479 in patients older than 60 (P = 0.082). The median CIN scores of patients with different

**Table 1**
Cohort clinicopathologic characteristics.

| Characteristics | All Patients (n = 60) | CIN low (n = 28) | CIN high (n = 32) | P |
|---|---|---|---|---|
| Age at blood collection | | | | 0.305 |
| <40 years | 11(18.3) | 3(10.7) | 8(25.0) | |
| 40–60 years | 37(61.7) | 18(64.3) | 19(59.4) | |
| >60 years | 12(20.0) | 7(25.0) | 5(15.6) | |
| Primary receptor status | | | | 0.202 |
| HR-positive | 41(68.3) | 16(57.1) | 25(78.1) | |
| HR-negative, HER2- positive | 12(20.0) | 8(28.6) | 4(12.5) | |
| HR-negative, HER2-negative | 7(11.7) | 4(14.3) | 3(9.4) | |
| AJCC stage at primary diagnosis | | | | 0.644 |
| I-III | 42(70.0) | 21(75.0) | 21(65.6) | |
| IV | 14(23.3) | 5(17.9) | 9(28.1) | |
| Unknow | 4(6.7) | 2(7.1) | 2(6.3) | |
| Visceral disease, n (%) | | | | 0.972 |
| Yes | 32 (53.3) | 15 (53.6) | 17 (53.1) | |
| No | 28 (46.7) | 13 (46.4) | 15 (46.9) | |
| Bone-only metastasis | | | | 0.068 |
| Yes | 7 (11.7) | 1 (3.6) | 6 (18.7) | |
| No | 53 (88.3) | 27 (96.4) | 26 (81.3) | |
| Lines of metastatic therapy | | | | 0.221 |
| 0 | 28(46.7) | 16(57.1) | 12(37.5) | |
| 1–2 | 21(35.0) | 9(32.1) | 12(37.5) | |
| ≥3 | 11(18.3) | 3(10.7) | 8(25.0) | |
| Prior endocrine therapy | | | | 0.212 |
| ≥1 | 33(55.0) | 13(46.4) | 20(62.5) | |
| None | 27(45.0) | 15(53.6) | 12(37.5) | |
| Prior chemotherapy | | | | 0.307 |
| Neo/adjuvant only | 17(28.3) | 10(35.7) | 7(21.9) | |
| Metastatic +/− adj | 32(53.3) | 12(42.9) | 20(62.5) | |
| None | 11(18.3) | 6(21.4) | 5(15.8) | |

Data presented as No. (%) unless otherwise noted.
Abbreviations: AJCC, American Joint Committee on Cancer Staging; HER2, human epidermal growth factor receptor 2; HR, hormone receptor.

receptor status were: 6660 in HR + subtype, 2152 in HER2+HR- subtype, and 2723 in the TNBC subtype(P = 0.168). Multivariate logistic regression analyses revealed that none of age (P = 0.073), disease stage at primary diagnosis (P = 0.353), molecular subtype (P = 0.136), or previous lines of systemic therapy (P = 0.067) was correlated with genome-wide chromosomal instability represented by CIN score.

While elevated chromosomal instability were readily detected in cfDNA from 48 out of 60 MBC patients (80.0%), no elevation was detected in cfDNA from 9 healthy blood donors or 9 postoperative patients with breast cancer (0%, $X^2 = 20.029$, P < 0.001) (all CIN scores are listed in Supplement Table 4). Overall, altered chromosome regions were remarkably discordant among different subtypes of MBC (Fig. 2). For patients with HR + MBC, frequent focal gains were identified, including 8p11.23 (10/41, 24.4%), 8p11.21 (12/41, 29.3%), 20q13.11 (6/41, 14.6%) where potential oncogene FGFR1, IKBKB and SGK2 was located. Meanwhile, frequent HER2 (17q21.1) CNAs were found in patients with HER2+HR- MBC (9/12, 75%), but none of the other regions as mentioned above. For patients with TNBC, the most frequently CNA were 11q13.2–13.4(3/7, 42.8%), 1q23.1(3/7, 42.8%), 9p24.1(2/7, 25.6%) and 8p11.21(2/7, 25.6%), where potential oncogene CCND1, NTRK1, CD274 and IKBKB are located.

There were 38 patients with confirmed HER2 status and evaluable for CNA of ERBB2 gene in cfDNA. The ERBB2 copy numbers in cfDNA estimated by UCAD pipeline were shown in theSupplementary Table 5. Through the UCAD pipeline, 14 out of 16 HER2 positive patients had HER2 gene CNA in cfDNA, while 21 out of 22 HER2 negative patients had no CNA of HER2 gene in cfDNA. HER2 CNA from cfDNA had a sensitivity and specificity of 87.5% (14/16) and 95.5% (21/22), respectively. Positive predictive values and negative predictive values were 93.3% (14/15) and 91.3% (21/23), respectively. Thus, both methods (IHC/FISH versus UCAD pipeline) of HER2 status test showed almost identical results (McNemer test, P = 1.000). The diagnostic results of these two methods are in good agreement (Kappa = 0.837, P < 0.001).

### 3.3. Chromosomal instability associated with drug resistance and poor survival in MBC

As shown in Fig. 3, frequent chr08 and chr17 gains were found in treatment resistant patients, where oncogenes MYC (8q24.21), IKBKB(8p11.21) were located (P = 0.010 and 0.051 respectively). Chr3q, chr11 were also tends to correlates with treatment resistances, where oncogene PIK3CA (3q26.32) and CCND1(11q13.3) are located (P = 0.065 and 0.083 respectively). Chr09 loss and chr07 long arm loss are also found to correlate with treatment resistance (P = 0.039 and 0.021 respectively).

Patients with high CIN score had significantly worse OS compared with other patients, median 21.2 months (95% CI 14.1–28.3) versus not reached (P = 0.006; Fig. 4A). For patients with high CIN score, the 1-, 2-, 3-year survival rate were 77.3%, 39.3%, 20.4%, respectively. For those with low CIN score, the 1-, 2-, 3-year survival rate were 92.3%, 80.3%, 64.2%, respectively. Besides, patients with high CIN had worse PFS from blood draw compared with those in the low CIN group (median 7.3 vs 11.0 months, P = 0.034; Fig. 4B). Univariate analysis revealed that patients with high CIN or extensively pretreated had significantly worse prognosis (Supplementary Fig. 2). Notably, genome-wide chromosomal instability remained to be an independent prognostic factor in the multivariate Cox proportional hazards model (hazard ratio, 3.563; 95%CI, 1.481 to 8.572; P = 0.005; Table 2).

## 4. Discussion

Using an innovative cfDNA-exclusive UCAD approach, we

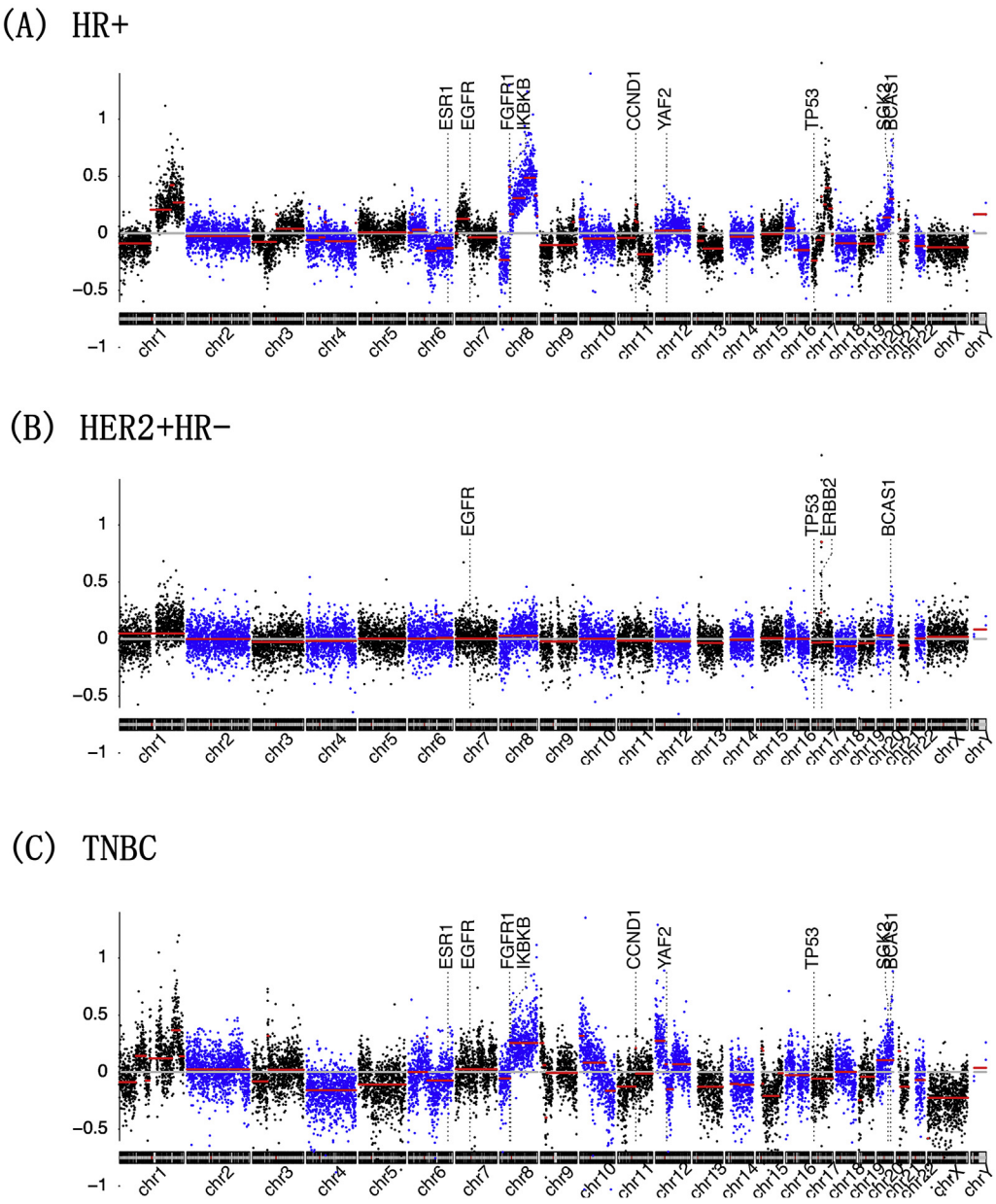


**Fig. 2.** Copy number plots of four representative examples of cfDNA with copy number (normalized log2 ratio) indicated on the y-axis and chromosome on the x-axis. (A) hormone receptor positive breast cancer (HR+) (B) hormone receptor negative HER2 positive breast cancer (HER2+HR-) (C) triple-negative breast cancer (TNBC).

demonstrate that genome-wide chromosomal instability represented by CIN score is a significant independent prognostic biomarker in patients with MBC. We illustrated the prognostic value of chromosomal instability derived from cfDNA in various subtypes of MBC.

Genomic analysis of MBC could be well recapitulated by tumor cfDNA with minimal invasion [15,23]. The correlation between CNAs in specific genes or chromosome regions and patient's survival prognosis have been illustrated in several studies with diverse results [8,24–26]. From a more macro standpoint, our study focused on genome-wide chromosome instability, and demonstrated its independent prognostic value in patients with MBC for the first time. We demonstrated that CIN score as a genomic biomarker is uncorrelated with most clinicopathologic characteristics, including patient's age or receptor status. A prospective cohort study will be launched to dynamically monitor genome-wide chromosomal instability during treatment, and investigate its association with response to specific systemic therapies.

Besides the analysis of whole genome on the chromosome level, our results also went deep into CNA profiles of specific chromosome regions and gene locus. In this complex MBC cohort with different subtypes, the hotspot regions of CNA are mainly consistent with the literature on the CNA derived from tumor tissue in breast cancer [27,28]. By enlarging the sample size and dynamic monitoring during treatment, we will aim to identify novel cancer drivers among CNAs enriched in patients with drug resistance.

HER2 gene CNA was successfully identified from cfDNA in our cohort, showing excellent concordance with that in tumor tissue by FISH or IHC. Based on these results, it's reasonable to use HER2 CNA in cfDNA as a predictive surrogate for trastuzumab response. We've planned to verify the correlation of HER2 CNA in cfDNA to trastuzumab response and patient prognosis in large cohorts.

| Chr | CNV segment (hg19) Start | End | Key genes | Statistics Category | P |
|---|---|---|---|---|---|
| chr08 | 136,400,000 | 144,000,000 | MYC | R | 0.010 |
| chr08 | 96,400,000 | 136,200,000 | | R | 0.004 |
| chr08 | 47,000,000 | 96,200,000 | | R | 0.013 |
| chr08 | 41,600,000 | 42,400,000 | IKBKB | R | 0.051 |
| chr08 | 36,400,000 | 39,600,000 | FGFR1 | R | 0.100 |
| chr12 | 0 | 133,600,000 | MDM2, KRAS | R | 0.061 |
| chr17 | 47,000,000 | 48,600,000 | | R | 0.028 |
| chr17 | 73,000,000 | 77,400,000 | | R | 0.031 |
| chr17 | 77,600,000 | 80,800,000 | | R | 0.077 |
| chr03 | 105,000,000 | 197,800,000 | PIK3CA | R | 0.065 |
| chr11 | 66,200,000 | 80,200,000 | CCND1,FGF3,FGF4 | R | 0.083 |
| chr20 | 33,200,000 | 49,200,000 | | R | 0.193 |
| chr02 | 92,000,000 | 242,800,000 | | S | 0.462 |
| chr15 | 23,600,000 | 102,200,000 | | S | 0.273 |
| chr18 | 0 | 77,800,000 | | S | 0.398 |
| chr14 | 19,000,000 | 107,000,000 | | S | 0.114 |
| chr11 | 0 | 66,000,000 | | S | 0.197 |
| chr04 | 49,600,000 | 190,200,000 | | S | 0.124 |
| chr11 | 80,400,000 | 134,800,000 | | S | 0.128 |
| chr20 | 0 | 25,000,000 | | S | 0.248 |
| chr01 | 0 | 120,200,000 | CDKN2C | S | 0.355 |
| chr10 | 42,800,000 | 135,200,000 | PTEN | S | 0.231 |
| chr09 | 0 | 123,800,000 | CDKN2A | S | 0.115 |
| chr21 | 15,000,000 | 47,800,000 | | S | 0.071 |
| chr04 | 0 | 48,800,000 | | S | 0.063 |
| chr09 | 130,200,000 | 141,000,000 | | S | 0.039 |
| chr07 | 62,800,000 | 158,600,000 | | S | 0.021 |


**Fig. 3.** Heatmap view of chromosomal copy number changes associated with treatment responses. Red color indicates copy number gains. Green color indicates copy number losses. Patient samples with treatment response PD (disease progression), SD (stable disease) and PR (partial response) were listed from left to right.

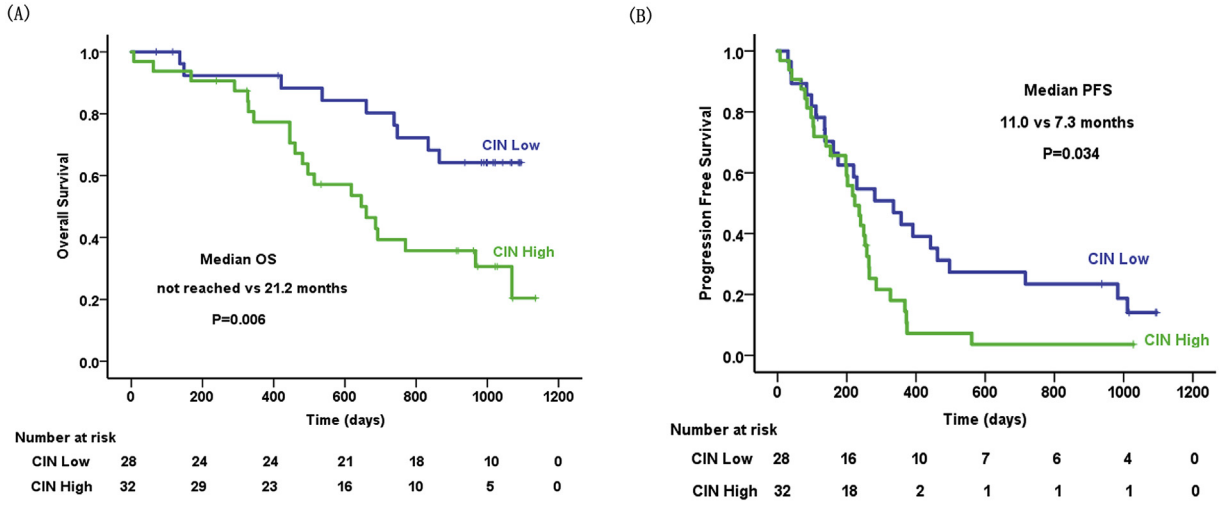


**Fig. 4.** Kaplan-Meier curve of (A) overall survival and (B) progression free survival from blood draw for patients with metastatic breast cancer stratified by chromosomal instability. High CIN score was associated with significantly worse overall survival and progression free survival.

One of the limits of this study is that, this is a single-institution retrospective study with a small number of participants. All of the blood samples are archived specimens. Despite the relatively small sample size of our study, genome-wide chromosomal instability remains to be an independent prognostic factor in multivariate analysis. Larger studies focused on specific subtypes of MBC, such as HR + group and HER2+ group, are planned to verify the prognostic value of CIN score as well as the reliability of the UCAD system.

## 5. Conclusions

Here we characterized the genome-wide chromosomal instability of MBC with different molecular subtypes, utilizing cfDNA as a minimally invasive method. Genome-wide chromosomal instability by UCAD is reliable and predicts patient survival. The UCAD technique has the potential to identify unique genomic features of MBC and may advance our understanding of intratumor heterogeneity and novel therapeutic targets.

## Availability of data and material

The clinical and pathological datasets are available in the Mendeley Data repository, https://data.mendeley.com/datasets/nsxd9gwg8f/draft?a=4624c0c3-a076-45f0-88f0-1fcd09f9ae6f.

The precompiled software was available online http://istopcancer.net/pgweb/cn/download.

For any of the processed data, users can also query the database http://www.istopcancer.net/pgweb/cn/istopcancer.jsp to see all the details.

**Table 2**
Multivariable Cox proportional hazards model of overall survival from blood draw.

| Variables | Hazard Ratio | 95%CI | | P |
|---|---|---|---|---|
| | | Lower | Upper | |
| CIN High | 3.563 | 1.481 | 8.572 | 0.005 |
| Age at blood draw | | | | 0.344 |
| <40 | Ref | Ref | Ref | Ref |
| 40–60 | 1.856 | 0.653 | 5.279 | 0.246 |
| >60 | 1.020 | 0.228 | 4.566 | 0.980 |
| Primary stage IV at diagnosis | 1.318 | 0.493 | 3.523 | 0.582 |
| Primary receptor status | | | | 0.004 |
| HR+ | Ref | Ref | Ref | Ref |
| HR-HER2+ | 2.105 | 0.683 | 6.485 | 0.195 |
| TNBC | 7.368 | 2.272 | 23.886 | 0.001 |
| Line of metastatic therapy at blood draw | | | | 0.035 |
| 0 | Ref | Ref | Ref | Ref |
| 1–2 | 1.378 | 0.542 | 3.501 | 0.500 |
| ≥3 | 4.285 | 1.379 | 13.311 | 0.012 |

## Declaration of competing interest

Ziliang Qian is a salaried employee of Prophet Genomics Inc., a provider of cancer genome-based diagnostic testing. The remaining authors declare no conflict of interest.

## Acknowledgement

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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.2020.07.004.

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