Research Article

Cell-Free DNA Variables including Gene Mutations in CA15-3 Normal Breast Cancer Reflect Prognosis

Juan Xu,1,2 Wenxiu Chen,1 Ziwei Sun,1 Hailin Peng,2 Chenglin Zhou,2 and Shiyang Pan1

1Department of Laboratory Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
2Department of Laboratory Medicine, Taizhou People’s Hospital, Taizhou 225300, China

Correspondence should be addressed to Chenglin Zhou; jszhoucl@yeah.net and Shiyang Pan; sypan@njmu.edu.cn

Received 12 December 2021; Revised 16 January 2022; Accepted 31 January 2022; Published 24 February 2022

Academic Editor: Valentina Magri

Copyright © 2022 Juan Xu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background. Cell-free DNA (cfDNA) has attracted considerable attention in precision medicine. However, few data are available regarding to the prognostic value of cfDNA variables in CA15-3 normal breast cancer (BC) patients. Here, we aimed at investigating the prognostic value of cfDNA variables including gene mutations in CA15-3 normal BC patients.

Methods. A total of 68 BC patients with normal CA15-3 levels were enrolled. cfDNA concentration and integrity were assessed based on qPCR. cfDNA gene mutations were conducted by using next gene sequencing (NGS). The association between cfDNA variables and the prognosis of patients was analyzed.

Results. cfDNA concentration was related to tumor stage (P = 0.002), metastases (P = 0.001), and distant metastases (P < 0.001). The elevated copy number variants (CNV) were found in distant metastasis patients compared with patients without distant metastases (P = 0.008). Nineteen mutant genes were validated in enrolled CA15-3 normal BC patients. Thirty-two patients (47.0%) had single nucleotide variants (SNV), and 13 (19.1%) patients had TP53 mutations (TP53mut). SNV (P = 0.033) was related to tumor stage, and TP53mut was related to metastases (P = 0.016) and distant metastases (P = 0.006). In multivariate logistic analysis, cfDNA concentration was associated with metastases (OR = 3.404, 95% CI: 1.074-10.788, P = 0.037) and distant metastases (OR = 13.750, 95% CI: 1.473-128.358, P = 0.021). Cases with high cfDNA levels (>15.6 ng/ml), SNV, and TP53mut showed worse DFS compared with patients with low cfDNA levels (P < 0.001), without SNV (P = 0.002) and with TP53 wildtype (P < 0.001), respectively. In the multivariate Cox proportional hazard model, cfDNA concentration was an independent predictor of poor survival (HR = 5.786, 95% CI: 1.010-30.407, P = 0.038). Conclusions. Assessment of cfDNA concentration, CNV, SNV, and TP53mut could be useful in predicting prognosis for CA15-3 normal BC patients. The cfDNA concentration was an independent predictor prognostic factor in CA15-3 normal BC patients.

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer all around the world. In 2020, more than half a million people died from this disease worldwide [1]. In China, BC ranks first in terms of incidence and fifth in terms of mortality among all cancers in women, and the burden of BC incidence and mortality is rapidly growing [2]. BC patient prognosis is mainly determined by several clinical characteristics, such as stage, metastasis, and molecular subtypes [3]. To improve survival prediction for BC patients, researchers are ongoing on the search for new prognostic markers. Cancer antigen 15-3 (CA15-3) is overexpressed in multiple cancers and is known as a representative biomarker for malignant tumor [4]. In clinical routine, utilizing CA15-3 for predicting BC prognosis has been widely incorporated [5]. However, in a study of 1046 BC cases by Ebeling et al., CA15-3 was not identified as an independent prognostic biomarker [6]. Another study also reported no association between CA15-3 levels and survival outcome in BC patients younger than 40 years old [7]. Moreover, blood biomarkers were often measured for evaluating prognosis of BC patients,
Among these biomarkers, cfDNA has attracted considerable attention. cfDNA concentrations, which represent the quantity of cfDNA, are elevated in various cancers compared with healthy controls [13, 14]. cfDNA integrity (cfDI), which indicates the quality of cfDNA, is based on the ratio of longer DNA fragment to shorter ones of a specific genetic element, such as long interspersed nuclear element 1 (LINE1). cfDNA variables including concentration and integrity have been proposed as prognostic oncological biomarkers [15, 16]. However, usefulness of these cfDNA variables in CA15-3 normal BC patients has so far not been shown.

Approximately 10% of BC cases are related to genetic mutations. Mutations in the BRCA1 and BRCA2 genes are the most common high-risk mutations associated with BC [17]. Gene mutations in cfDNA have shown some prognostic significance, and these mutations can be detected in plasma cfDNA by next generation sequencing (NGS) [1]. There is a clear correlation between presence of TP53 mutations in cfDNA and adverse progression-free survival (PFS) outcome [18]. Several retrospective studies have also reported that ESR1 or PIK3CA mutations were related to shorter PFS and overall survival (OS) in hormone receptor-(HR-) positive metastatic BC [19].

In this study, we analyzed cfDNA variables including gene mutations in CA15-3 normal breast cancer. Gene mutations in cfDNA were confirmed by using NGS on a 50 genes panel. Our results showed that cfDNA concentration, copy number variation (CNV), single nucleotide variant (SNV), and TP53 mutations had the potential to predict the survival of CA15-3 normal BC patients. High concentration of cfDNA was an independent predictor of poor survival in CA15-3 normal BC patients.

### 2. Patients and Methods

#### 2.1. Patients

The study included 68 women identified from Taizhou People’s Hospital and diagnosed with BC with normal CA15-3 levels between August 2018 and August 2019. The diagnoses of all patients were confirmed by core biopsy. Blood samples were collected before surgery, and CA15-3 levels were detected by electrochemiluminescence. CA15-3 levels were determined by standard values for our institution: normal CA15-3 level was defined as serum CA15-3 above the cut-off value of 31.3 U/ml. Patients with elevated CA15-3 levels were defined as serum CA15-3 above the cut-off value of 31.3 U/ml. Patients with elevated CA15-3 levels were excluded. Follow-up information and clinical information were obtained using the institutional database. The last update of clinical information was completed on August 31, 2020. This study was conducted in accordance with the Declaration of Helsinki on 2013. Informed consent was obtained from all patients, and this study was approved by the ethics committee of the Taizhou People’s Hospital (KY 201804801, Taizhou, China).

#### 2.2. Sample Processing

Plasma samples were obtained from BC patients at the time of diagnosis, prior to surgery or therapy. Blood samples (2-3 ml) were collected in EDTA tubes and processed immediately. Plasma components were separated by centrifugation at 1600 g for 10 minutes at 4°C. Plasma was then transferred to new 1.5 ml EP tubes and stored at −80°C until analysis.

| Genes   | Genes | Genes | Genes | Genes |
|---------|-------|-------|-------|-------|
| ABL1    | AKT1  | ALK   | APC   | ATM   |
| BRAF    | CDH1  | CDKN2A| EGFR  | ERBB2 |
| ERBB4   | EZH2  | FBXW7 | FGFR1 | FGFR2 |
| FGFR3   | GNAS  | GNAQ  | HNF1A | HRAS  |
| IDH1    | JAK2  | JAK3  | IDH2  | KRAS  |
| MET     | MLH1  | MPL   | NOTCH1| NPM1  |
| NRAS    | PDGFR | PTPN11| RB1   | RET   |
| SMAD4   | SMARCB1|SMO   | SRC   | STK11 |
| CSF1R   | CTNNB1| FLT3  | GNA11 | KDR   |
| KIT     | PIK3CA| TP53  | PTEN  | VHL   |

#### Table 2: Clinical characteristics of the BC patients (n = 68).

| Characteristics | Total cases, N | % |
|-----------------|----------------|---|
| Age at diagnosis (years) |                |   |
| ≤50              | 32             | 47.06 |
| >50              | 36             | 52.94 |
| Molecular subtype |                |   |
| Triple-negative  | 6              | 8.82  |
| HER2+ (any ER/PR)| 14             | 20.59 |
| HER2+ (ER or/and PR) | 48           | 70.59 |
| Tumor size (cm)  |                |   |
| ≤2               | 31             | 45.59 |
| >2               | 37             | 54.41 |
| Tumor stage      |                |   |
| I/II             | 41             | 60.29 |
| III/IV           | 27             | 39.71 |
| Metastatic status|                |   |
| No               | 36             | 52.94 |
| Yes              | 32             | 47.06 |
| Distant metastatic status | |   |
| No               | 55             | 80.88 |
| Yes              | 13             | 19.12 |

HER2: human epidermal growth factor receptor 2; ER/PR: estrogen/progesterone receptor.
centrifuged a second time at 12000 g for 10 minutes at 4°C. The plasma was stored at -80°C until the time of DNA extraction.

2.3. Cell-Free DNA Extraction. Plasma was thawed on ice first, and then it was centrifuged at 12000 g for 5 minutes before DNA extraction. cfDNA was extracted from about 1 ml plasma using the CWhipro Circulating DNA Midi Kit (CWBIO, Beijing, China) in accordance with the manufacturer’s instructions. Extracted cfDNA was eluted in 40 μl elution buffer, and the final eluted cfDNA was immediately used for analysis or stored at -20°C.

2.4. Concentration and Integrity of cfDNA Measurement. The short and long DNA fragments of LINE1 (LINE1-97 bp and LINE1-259 bp) were measured by qPCR as previous study [20]. In brief, concentration of cfDNA was represented with LINE1-97 base pair (bp) fragment qPCR result (LightCycler LC480, USA). cfDI was based on the ratio of long and short fragment of the LINE1 element: LINE1-259/97. A reference standard curve was created by a serially diluted standardized solution of human genomic DNA (Thermo Fisher, USA). The LINE1-97 bp qPCR fragment was amplified using the forward (5'-AGGTGCGTGAG AGGATGT-3') and reverse (5'-GGAAATCGCCACACCTGACCT-3') primer; the LINE1-259 bp fragment was amplified using the forward (5'-TGCCGCAATAACATACGTG-3') and reverse (5'-AACAACAGGTGCTGGAGGAGG-3') primer.

2.5. Analysis of cfDNA by NGS. By using the Oncomine cfDNA assay system (Thermo Fisher Scientific, USA), we constructed the library for cfDNA sequencing. This assay system can detect 50 genes (see Table 1) with 207 amplicons including 2855 hotspots by using 10 ng of cfDNA. cfDNA was used to generate libraries using Fast cfDNA Library Prep Set for MGI (CWBIO, China) following the manufacturer’s instructions. Library circularization and MGI sequencing (MGISEQ-2000) were performed using the Circularization Kit for MGI (CWBIO, China) and MGISEQ-2000RS library Sequencing Kit (PE100, BGI), independently. Variant caller was configured to call high stringency somatic variants, and the hotspot_min Allele frequency was set to 0.01. ANNOVAR was used to annotate all variants. All data were manually reviewed to provide a precise interpretation by using the Integrated Genomics Viewer package (v2.3.25).

2.6. CNV Detection. Burrows-Wheeler Aligner software was used to perform alignment analysis for raw reads. Sequences that could be mapped to just one location in the hg19 reference human genome without mismatch were counted. GC bias correction was performed as described elsewhere before [21]. The read counts for 500 kb bin size and in each 0.5% of GC content bin were both got. \( M \) and \( M_i \) were the average counts across all GC content bins and in bin \( i \), respectively. Counts in each GC content bin were weighted by \( W_i = \frac{M_i}{M} \). Counts ratio was normalized by 500 kb bin counts/all counts, and a reference baseline was created. \( Z \) score was calculated by the following: (sample counts ratios – mean of

| Characteristics            | Concentration (ng/ml) | P     | Integrity index | P     | CNV index | P     |
|----------------------------|-----------------------|-------|-----------------|-------|-----------|-------|
| Age at diagnosis (years)   |                       |       |                 |       |           |       |
| ≤50                        | 21.74 ± 4.66          | 0.593 | 0.28 (0.12, 0.61) | 0.134 | 1.03 ± 0.09 | 0.310 |
| >50                        | 19.20 ± 1.66          | 0.21  | (0.08, 0.36)    |       | 1.14 ± 0.07 |       |
| ER/PR/HER2 status at diagnosis |                   |       |                 |       |           |       |
| TNBC                      | 34.28 ± 3.99          | 0.080 | 0.27 ± 0.09     | 0.695 | 1.14 ± 0.10 | 0.533 |
| HER2' (any ER/PR)         | 24.66 ± 9.94          | 0.30  | ± 0.09          |       | 1.10 ± 0.15 |       |
| HER2+ (ER or and PR+)     | 17.41 ± 1.47          | 0.32  | ± 0.46          |       | 1.08 ± 0.07 |       |
| Tumor size (cm) ≤2        | 18.42 ± 1.70          | 0.446 | 0.33 ± 0.05     | 0.658 | 1.01 ± 0.07 | 0.226 |
| >2                        | 22.05 ± 4.09          |       | 0.30 ± 0.06     | 1.16  | ± 0.09    |       |
| Tumor stage               |                       |       |                 |       |           |       |
| I/II                      | 13.76 (8.94, 18.85)   | 0.002 | **               | 0.36  | ± 0.05    | 0.141 | 1.03 (0.90,1.18) | 0.278 |
| III/IV                    | 25.02 (12.67, 35.30)  | 0.24  | ± 0.05          |       | 1.07 (0.92,1.55) |       |
| Metastatic status         |                       |       |                 |       |           |       |
| No                        | 12.72 (9.06, 18.18)   | 0.001 | **               | 0.37  | ± 0.06    | 0.123 | 1.00 ± 0.09 | 0.090 |
| Yes                       | 26.16 (15.25, 42.83)  |       | 0.25 ± 0.04     |       | 1.20 ± 0.07 |       |
| Distant metastatic status |                       |       |                 |       |           |       |
| No                        | 12.94 (9.41, 19.53)   | <0.001 | **               | 0.33  | ± 0.04    | 0.364 | 1.02 ± 0.06 | 0.008** |
| Yes                       | 33.62 (22.95, 41.09)  | 0.24  | ± 0.09          |       | 1.40 ± 0.12 |       |

*P < 0.05, **P < 0.01, ***P < 0.001.

Table 3: Association between concentration, integrity index, CNV index, and clinical characteristics of patients (n = 68) (x ± SD, M (P25, P75)).
reference counts ratios)/standard deviation of reference counts ratios. For data visualization, we further plotted Z score into genome-wide copy number by using ggplot2 [21]. The marker of CNVZ was scored by the formula avg. (abs (Z score)).

2.7. Statistics. Statistical analysis was performed using SPSS 22.0 software (IBM, USA). The independent-samples t-test, Mann–Whitney U tests, and one-way ANOVA analysis were used, where appropriate, to evaluate the significance of differences among different groups. Association between variables and clinical characteristics was evaluated by chi-square or Fisher exact test. Univariate and multivariate logistic regression analysis were performed to identify odds ratios (OR) for metastases and distant metastases. Disease-free survival (DFS) referred to the period between the date of surgery and the date of progression, last follow-up, or death. Kaplan-Meier analysis was used to plot survival curves, and the differences between survival curves were determined by log-rank test. A Cox regression model was utilized to evaluate hazard ratios (HR) for DFS. \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Patient Characteristics. Sixty-eight BC patients with normal CA15-3 were enrolled in this study. The median age of patients was 50 years which ranging from 29 to 85 years (see Table 2). The majority of tumor cases had negative HER2 status (70.6%), and 14 cases (20.59%) had positive HER2 status with ER or PR positive. Six cases (10.9%) had been diagnosed as triple-negative BC. Among the overall patients, 31 (45.6%) BC patients had a tumor size \( \leq 2 \) cm while 37 (54.41%) cases had a tumor > 2 cm. Furthermore, 41 cases (60.3%) were diagnosed with stage I/II BC, and 27 cases (39.7%) were diagnosed with stage III/IV BC. Thirty-six patients (52.9%) had metastases, and 13 patients (19.1%) had distant metastases. The median follow-up time was 12 months (range 3-22 months).

3.2. Association between cfDNA Concentration, Integrity, and CNV with Clinical Characteristics. The distributions of clinical characteristics factors according to concentration, integrity, and CNV of cfDNA are shown by in Table 3. The median cfDNA concentration, integrity, and CNV were 15.6 ng/ml (3.78-152.66), 0.23 ng/ml (0.01-1.61), and
3.3. Association between cfDNA Gene Mutations and Clinical Characteristics.

Gene mutations in cfDNA were observed in 32 (47.0%) of the 68 enrolled CA15-3 normal BC patients. Mutations were identified in TP53 (19.1%), PIK3CA (17.6%), KRAS (10.3%), EGFR (4.4%), NRAS (2.9%), CDKN2A (1.5%), CTNNB1 (2.9%), STK11 (1.5%), VHL (1.5%), HRAS (1.5%), and FBXW7 (1.5%). Overall, 9 patients harbored more than two mutated genes (see Figure 1). Patients with advanced stage (17/27) were more likely to have a high amount of SNV compared with stage I/II patients (15/41) (P = 0.033). TP53 mutations were more frequent in the metastatic (10/32) and distant metastatic (6/13) groups relative to the nonmetastatic (3/36) (P = 0.016) and nondistant metastatic group (7/55) (P = 0.006), respectively (see Table 4).

3.4. Association between cfDNA Variables and BC Prognosis.

A strong relationship between cfDNA concentration and metastatic status was observed both in univariable analysis and multivariable logistic regression analysis (OR = 3.404, 95% CI: 1.074-10.788, P = 0.037) (see Table 5). Logistic regression analysis also confirmed that high cfDNA concentration was associated with distant metastatic status in enrolled CA15-3 normal BC patients (OR = 13.750, 95% CI: 1.473-128.358, P = 0.021) (see Table 6).
analysis also showed that high cfDNA concentration was positively associated with metastatic and distant metastatic status. In the multivariate analysis, we identified that the concentration of cfDNA was an independent predictor correlated with adverse survival outcome.

BC is a distinctly heterogeneous tumor with various prognosis and is divided into four major molecular (triple-negative, HER2+, luminal-A, and luminal-B) subtypes. Nowadays, the selection of treatment is mainly based on the molecular subtype in clinical practice, and the prognosis of BC patients varies with molecular subtype [3]. Tumor biomarkers are widely used for monitoring cancer prognosis, of which CA15-3 is a conventional marker for BC. However, previous studies [22] have shown that less than 20% BC patients have elevated levels of serum CA15-3. Hence, in BC patients with normal CA15-3 levels, there was a lack of an accredited blood biomarker that assists the clinicians to monitor the outcome without recourse to expensive imaging.

Liquid biopsy is becoming more and more important in precision medicine. Many traditional biomarkers identified from liquid biopsy such as microRNA, circulating DNA, and circulating tumor cells have been investigated as prognostic markers in various kinds of tumors, including BC [8]. cfDNA variables are prominent biomarkers among them. Relationship between cfDNA variables and different molecular subtypes was reported extensively. However, as far as we know, this is the first study evaluating the prognostic value of cfDNA variables in BC patients with normal CA15-3 levels.

Studies have shown that concentration of cfDNA increases with disease progression, and monitoring dynamic cfDNA concentration is clinically important to evaluate prognosis in cancer patients. For example, Cheng et al. showed that cfDNA concentration could serve as an independent prognostic marker in metastatic BC patients [23]. Shibayama et al. found increased cfDNA concentration in BC patients with the increased number of organs with metastases [24]. These studies promised the prognosis value of cfDNA concentration in BC regardless of the CA15-3 levels. In agreement with previous studies, we focused on BC patients with normal CA15-3 levels, and our results

| Characteristics | Uniavariable analysis | Multivariate analysis |
|-----------------|-----------------------|----------------------|
| Age at diagnosis (year) | | |
| ≤50 | 1.000 | 1.000 |
| >50 | 0.387 | 0.145-1.030 | 0.057 |
| | | 0.316 | 0.098-1.018 | 0.054 |
| Tumor size (cm) | | |
| <2 | 1.000 | |
| ≥2 | 1.572 | 0.698-3.537 | 0.275 |
| Stage | | |
| I/II | 1.000 | 1.000 |
| III/IV | 0.840 | 0.317-2.228 | 0.726 |
| | | 0.813 | 0.247-2.678 | 0.733 |
| ER/PR/HER2 status at diagnosis | | |
| TNBC | 1.000 | |
| HER2+ (any ER/PR) | 0.500 | 0.068-3.675 | 0.497 |
| HER2- (ER or/and PR+) | 0.389 | 0.065-2.331 | 0.301 |
| cfDNA concentration | | |
| Low | 1.000 | 1.000 |
| High | 4.400 | 1.588-12.193 | 0.004** |
| | | 3.404 | 1.074-10.788 | 0.037* |
| cfDNA integrity | | |
| Low | 1.000 | |
| High | 0.489 | 0.186-1.286 | 0.147 |
| CNV | | |
| Low | 1.000 | |
| High | 2.046 | 0.777-5.386 | 0.147 |
| SNV | | |
| No | 1.000 | 1.000 |
| Yes | 3.818 | 1.396-10.443 | 0.009** |
| TP53 mutation | | |
| No | 1.000 | 1.000 |
| Yes | 8.905 | 1.795-44.186 | 0.007** |

*P < 0.05, **P < 0.01. Hosmer and Lemeshow Test: P = 0.936.
demonstrated that the concentration of cfDNA was related with stage, metastases, and distant metastases. Furthermore, cfDNA concentration was an independent predictor of DFS of BC with normal CA15-3 levels.

As to cfDNA integrity (cfDI), it remains controversial about the association of high cfDI with poor survival outcome in BC patients. Lam and his colleagues documented that high cfDI was correlated with poor RFS in newly diagnosed BC patients [25], whereas Cheng et al. and Madhavan et al. found opposite results in patients with metastatic BC [23, 26]. DNA fragments released by apoptotic cells ranged from approximately 180 to 200 bp. On the contrary, DNA fragments released by malignant cells in cancer patients vary in length size for their undergoing different pathophysiological processes [27]. Recent studies observed short fragments of cfDNA in cancer patients compared with healthy individuals [28]. Our study showed no association of cfDI with clinical characteristics in BC patients with normal CA15-3 levels. Notably, we used LINE1-based real-time PCR to determine cfDI instead of the automated gel electrophoresis used in previous studies. The differences in the methodology, enrolled patient cohorts, and timing of sample collection may partly explain the different results, but more researches and systematic reviews are required to identify the prognostic potential of cfDI in BC.

Previous studies have shown that mutations in genes including PIK3CA, TP53, ESR1, and ERBB2 can be used as prognostic biomarkers in BC, and these biomarkers can assist the implementation of personalized therapy for BC patients [29]. CNV and SNV are the most common variables of gene mutations related to BC [30]. A systematic review and meta-analysis revealed the potential application of cfDNA to identify the SNV and CNV in the most common genes associated with BC. They demonstrated that detecting SNV of cfDNA had a high sensitivity, specificity, and accuracy, but for CNV, there was a need for further exploration [31]. In our work, we observed significant increased levels of CNV and SNV in BC patients with distant metastases, and two gene mutations, TP53 and PIK3CA mutations, were more frequently. Approximately, 19.1% of patients carried TP53

| Characteristics               | OR   | 95% CI    | P   | OR   | 95% CI    | P   |
|------------------------------|------|-----------|-----|------|-----------|-----|
| Age at diagnosis (year)      |      |           |     |      |           |     |
| ≤50                          | 1.000|           |     | 1.000|           |     |
| >50                          | 1.543| 0.448-5.311| 0.492| 2.627| 0.467-14.782| 0.273|
| Tumor size (cm)              |      |           |     |      |           |     |
| <2                           | 1.000|           |     | 1.000|           |     |
| ≥2                           | 2.289| 0.824-6.355| 0.112|      |           |     |
| Stage                        |      |           |     |      |           |     |
| I/II                         | 1.000|           |     | 1.000|           |     |
| III/IV                       | 1.388| 0.410-4.694| 0.598| 0.862| 0.169-4.382| 0.858|
| ER/PR/HER2 status at diagnosis|      |           |     |      |           |     |
| TNBC                         | 1.000|           |     | 1.000|           |     |
| HER2+ (any ER/PR)            | 0.800| 0.102-6.249| 0.832|      |           |     |
| HER2- (ER or/and PR+)        | 0.341| 0.052-2.231| 0.262|      |           |     |
| cfDNA concentration          |      |           |     |      |           |     |
| Low                          | 1.000|           |     | 1.000|           |     |
| High                         | 18.000| 2.182-148.486| 0.007**| 13.750| 1.473-128.358| 0.021*|
| cfDNA integrity              |      |           |     |      |           |     |
| Low                          | 1.000|           |     | 1.000|           |     |
| High                         | 0.232| 0.058-0.938| 0.040*| 0.285| 0.055-1.462| 0.132|
| CNV                          |      |           |     |      |           |     |
| Low                          | 1.000|           |     | 1.000|           |     |
| High                         | 4.306| 1.066-17.389| 0.040*| 4.192| 0.718-24.494| 0.112|
| SNV                          |      |           |     |      |           |     |
| No                           | 1.000|           |     | 1.000|           |     |
| Yes                          | 4.638| 1.147-18.751| 0.031*| 1.363| 0.201-9.233| 0.751|
| TP53 mutation                |      |           |     |      |           |     |
| No                           | 1.000|           |     | 1.000|           |     |
| Yes                          | 5.878| 1.526-22.633| 0.010*| 4.154| 0.596-28.951| 0.151|

*P < 0.05, **P < 0.01. Hosmer and Lemeshow Test: P = 0.498.
Figure 2: Kaplan-Meier DFS curves based on cfDNA of BC patients \((n = 68)\). (a) cfDNA concentration. (b) SNV. (c) TP53 mutations. (d) Integrity. (e) CNV. (f) PIK3CA mutations. (g) KRAS mutations.
mutations are in accordance with the previous studies, which reported the frequency of somatic TP53 mutations ranging from 15% to 71%. Somatic TP53 mutation leads to disruption in the cell cycle, induced apoptosis, and affected DNA damage repair process, and the presence of TP53 mutations in cfDNA was associated with lower PFS independently of clinical treatment [18]. Approximately 17.6% of patients carried PIK3CA mutations. Even though the mutation frequency of PIK3CA was similar between invasive and ductal carcinoma in situ BC, PIK3CA gene mutation was reported to be driver mutation in both BC subtypes. BC patients with the PIK3CA mutations showed similar prognosis to patients without the PIK3CA mutations [32], and these findings are partly in accordance with our results in BC patients with normal CA15-3 levels.

There were several limitations in this study. First, this was a small and single institutional cohort without regarding the ethnic differences. Moreover, few recent studies focused on cfDNA variables including gene mutations in non-Asian BC patients with normal CA15-3 levels. Several studies in English suggested that analysis of cfDNA especially in combination with other biomarkers can serve as attractive prognostic for BC patients [23, 33, 34]. Other studies demonstrated that mutations detection in cfDNA may have important implications for prognosis in BC patients [35, 36]. A large-scale and multi-institutional study is required to confirm the results. In our study, 40% (27/68) BC patients with stage III-IV were enrolled. Many studies have shown that BC patients with III/IV do not have an acceptable blood marker that allows the clinician to monitor the outcome [33]. So, dedicated studies are warranted in this population. Second, the mutations were not detected in tumor because of the lack of matching tissue samples in the majority of patients. Combining mutation analysis in cfDNA and tissue samples would be a more powerful predictive marker for BC patients with normal CA15-3 levels. Third, as cfDNA

Table 7: Univariate/multivariate Cox proportional regression survival analysis.

| Characteristics                              | Univariable analysis | Multivariate analysis |
|----------------------------------------------|----------------------|-----------------------|
|                                              | HR 95% CI            | P                     |
|                                              | HR 95% CI            | P                     |
| Age at diagnosis (year)                      |                      |                       |
| ≤50                                          | 1.00                 |                       |
| >50                                          | 1.80 0.610-5.309     | 0.287                 |
| Tumor size (cm)                              |                      |                       |
| <2                                           | 1.00                 |                       |
| ≥2                                           | 0.799 0.342-1.862    | 0.603                 |
| Stage                                        |                      |                       |
| I/II                                         | 1.00                 |                       |
| III/IV                                       | 1.091 0.376-3.165    | 0.873                 |
|                                              | 0.346 0.088-1.355    | 0.127                 |
| ER/PR/HER2 status at diagnosis               |                      |                       |
| TNBC                                         | 1.00                 |                       |
| HER2+ (any ER/PR)                            | 0.367 0.071-1.900    | 0.232                 |
| HER2- (ER or/and PR+)                        | 0.348 0.090-1.340    | 0.125                 |
| cfDNA concentration                          |                      |                       |
| Low                                          | 1.00                 |                       |
| High                                         | 10.204 2.291-45.437  | 0.002**               |
|                                              | 5.786 1.101-30.407   | 0.038*                |
| cfDNA integrity                              |                      |                       |
| Low                                          | 1.00                 |                       |
| High                                         | 0.361 0.114-1.144    | 0.083                 |
| CNV                                          | 1.00                 |                       |
| Low                                          | 2.558 0.713-9.182    | 0.150                 |
| High                                         |                       |                       |
| SNV                                          | 1.00                 |                       |
| No                                           | 1.00                 |                       |
| Yes                                          | 6.042 1.666-21.904   | 0.006**               |
|                                              | 2.580 0.416-16.011   | 0.309                 |
| TP53 mutation                                |                      |                       |
| No                                           | 1.00                 |                       |
| Yes                                          | 7.414 2.528-21.741   | 0.001***              |
|                                              | 2.771 0.699-10.987   | 0.147                 |

*P < 0.05. Omnibus test: P < 0.001.
variables vary from person to person, it is valuable to evaluate the gene mutations at multiple time points before and after therapy.

5. Conclusions
We determined cfDNA variables including gene mutations in BC patients with normal CA15-3 levels. Nineteen mutant genes were validated in enrolled CA15-3 normal BC patients. cfDNA concentration, CNV, SNV, and TP53 mutations are shown to be prognostic predictors that associated with clinical characteristics and poor survival outcome. The concentration of cfDNA was an independent predictor prognostic factor in CA15-3 normal BC patients. These results were helpful for promoting the application of cfDNA detecting in the longitudinal monitoring of treatment management of BC patients with normal CA15-3 levels.

Data Availability
The datasets analyzed during this study are available from the corresponding author on reasonable request.

Conflicts of Interest
The authors have no conflicts of interest to declare.

Acknowledgments
This study was supported by the National Natural Science Foundation of China (81672100), the Taizhou Technology Support Program (SSF20170218), the Taizhou Social Development Project (TS201623), and the Project of Jiangsu Provincial Commission of Health (H2018072).

References
[1] S. Loibl, P. Poortmans, M. Morrow, C. Denkert, and G. Curigliano, "Breast cancer," Lancet, vol. 397, no. 10286, pp. 1750–1769, 2021.
[2] L. Fan, K. Strasser-Weippl, J. Li et al., "Breast cancer in China," The Lancet Oncology, vol. 15, no. 7, pp. e279–e289, 2014.
[3] F. Yu, F. Quan, J. Xu et al., "Breast cancer prognosis signature: linking risk stratification to disease subtypes," Briefings in Bioinformatics, vol. 20, no. 6, pp. 2130–2140, 2019.
[4] L. Perey, D. F. Hayes, P. Maimonis, M. Abe, C. O’Hara, and D. W. Kufe, "Tumor selective reactivity of a monoclonal antibody prepared against a recombinant peptide derived from the DF3 human breast carcinoma-associated antigen," Cancer Research, vol. 52, no. 9, pp. 2563–2568, 1992.
[5] J. Li, L. Liu, Z. Feng et al., "Tumor markers CA15-3, CA125, CEA and breast cancer survival by molecular subtype: a cohort study," Breast Cancer, vol. 27, no. 4, pp. 621–630, 2020.
[6] F. G. Ebeling, P. Steiber, M. Unch et al., "Serum CEA and CA 15-3 as prognostic factors in primary breast cancer," British Journal of Cancer, vol. 86, no. 8, pp. 1217–1222, 2002.
[7] X. Li, D. Dai, B. Chen, H. Tang, X. Xie, and W. Wei, "Determination of the prognostic value of preoperative CA15-3 and CEA in predicting the prognosis of young patients with breast cancer," Oncology Letters, vol. 16, no. 4, pp. 4679–4688, 2018.
[8] I. B. Hench, J. Hench, and M. Tolnay, "Liquid biopsy in clinical Management of Breast, lung, and colorectal cancer," Frontiers in Medicine, vol. 5, p. 9, 2018.
[9] S. Almirzaie, M. Bagherzadeh, and M. R. Akbari, "Liquid biopsy in breast cancer: a comprehensive review," Clinical Genetics, vol. 95, no. 6, pp. 643–660, 2019.
[10] D. Boral, M. Vishnoi, H. N. Liu et al., "Molecular characterization of breast cancer CTCs associated with brain metastasis," Nature Communications, vol. 8, no. 1, p. 196, 2017.
[11] I. Fridrichova and I. Zmetakova, "MicroRNAs contribute to breast cancer invasiveness," Cell, vol. 8, no. 11, p. 1361, 2019.
[12] J. Moss, A. Zick, A. Grinshpun et al., "Circulating breast-derived DNA allows universal detection and monitoring of localized breast cancer," Annals of Oncology, vol. 31, no. 3, pp. 395–403, 2020.
[13] J. Goodall, J. Mateo, W. Yuan et al., "Circulating cell-free DNA to guide prostate cancer treatment with PARP inhibition," Cancer Discovery, vol. 7, no. 9, pp. 1006–1017, 2017.
[14] G. M. Gressel, E. C. Maggi, B. E. Harmon et al., "Low molecular weight serum cell-free DNA concentration is associated with clinicopathologic indices of poor prognosis in women with uterine cancer," Journal of Translational Medicine, vol. 18, no. 1, p. 323, 2020.
[15] K. Waki, K. Yokomizo, K. Kavano, N. Tsuda, N. Komatsu, and A. Yamada, "Integrity of plasma DNA is inversely correlated with vaccine-induced antitumor immunity in ovarian cancer patients," Cancer Immunology, Immunotherapy, vol. 69, no. 10, pp. 2001–2007, 2020.
[16] F. Salvianti, C. Giuliani, L. Petrone et al., "Integrity and quantity of total cell-free DNA in the diagnosis of thyroid cancer: correlation with cytological classification," International Journal of Molecular Sciences, vol. 18, no. 7, p. 1350, 2017.
[17] K. B. Kuchenbäcker, J. L. Hopper, D. R. Barnes et al., "Risks of breast, ovarian, and contralateral breast cancer for BRCA1andBRCA2Mutation carriers," JAMA, vol. 317, no. 23, pp. 2402–2416, 2017.
[18] M. C. Garrido-Navas, A. Garcia-Diaz, and M. P. Molina-Valejo, "The polemic diagnostic role of TP53 mutations in liquid biopsies from Breast, Colon and Lung Cancers," Colon and Lung Cancers: Cancers, vol. 12, no. 11, pp. 3343, 2020.
[19] S. H. Sim, H. N. Yang, S. Y. Jeon, K. S. Lee, and I. H. Park, "Mutation analysis using cell-free DNA for endocrine therapy in patients with HR+/HER2+ metastatic breast cancer," Scientific Reports, vol. 11, no. 1, p. 5566, 2021.
[20] X. Wang, L. Wang, Y. Su et al., "Plasma cell-free DNA quantification is highly correlated to tumor burden in children with neuroblastoma," Cancer Medicine, vol. 7, no. 7, pp. 3022–3030, 2018.
[21] C. Ginestet, "ggplot2: elegant graphics for data analysis," Royal Statistical Society: Series A, vol. 174, no. 1, pp. 245-246, 2011.
[22] S. E. Nam, W. Lim, J. Jeong et al., "The prognostic significance of preoperative tumor marker (CEA, CA15-3) elevation in breast cancer patients: data from the Korean Breast Cancer Society Registry," Breast Cancer Research and Treatment, vol. 177, no. 3, pp. 669–678, 2019.
[23] J. Cheng, T. Holland-Letz, M. Wallwiener et al., "Circulating free DNA integrity and concentration as independent prognostic markers in metastatic breast cancer," Breast Cancer Research and Treatment, vol. 169, no. 1, pp. 69–82, 2018.
[24] T. Shibayama, S. K. Low, M. Ono et al., "Clinical significance of gene mutation in ctDNA analysis for hormone receptor-
positive metastatic breast cancer,” *Breast Cancer Research and Treatment*, vol. 180, no. 2, pp. 331–341, 2020.

[25] M. Lamminaho, J. Kujala, H. Peltonen, M. Tengström, V. M. Kosma, and A. Mannermaa, “High cell-free DNA integrity is associated with poor breast cancer survival,” *Cancers*, vol. 13, no. 18, p. 4679, 2021.

[26] D. Madhavan, M. Wallwiener, K. Bents et al., “Plasma DNA integrity as a biomarker for primary and metastatic breast cancer and potential marker for early diagnosis,” *Breast Cancer Research and Treatment*, vol. 146, no. 1, pp. 163–174, 2014.

[27] S. Jahr, H. Hentze, S. Englisch et al., “DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells,” *Cancer Research*, vol. 61, no. 4, pp. 1659–1665, 2001.

[28] H. R. Underhill, J. O. Kitzman, S. Hellwig et al., “Fragment length of circulating tumor DNA,” *PLoS Genetics*, vol. 12, no. 7, article e1006162, 2016.

[29] P. J. Stephens, P. S. Tarpey, and H. Davies, “The landscape of cancer genes and mutational processes in breast cancer,” *Nature*, vol. 486, no. 7403, pp. 400–404, 2012.

[30] A. A. Davis, S. Jacob, L. Gerratana et al., “Landscape of circulating tumour DNA in metastatic breast cancer,” *eBioMedicine*, vol. 58, article 102914, 2020.

[31] X. Yang, K. Zhang, C. Zhang, R. Peng, and C. Sun, “Accuracy of analysis of cfDNA for detection of single nucleotide variants and copy number variants in breast cancer,” *BMC Cancer*, vol. 19, no. 1, p. 465, 2019.

[32] A. Miron, M. Varadi, D. Carrasco et al., “PIK3CA mutations in in situ and invasive breast carcinomas,” *Cancer Research*, vol. 70, no. 14, pp. 5674–5678, 2010.

[33] D. Fernandez-Garcia, A. Hills, K. Page et al., “Plasma cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast cancer,” *Breast Cancer Research*, vol. 21, no. 1, p. 149, 2019.

[34] M. Panagopoulou, M. Karaglani, I. Balgkouranidou et al., “Circulating cell-free DNA in breast cancer: size profiling, levels, and methylation patterns lead to prognostic and predictive classifiers,” *Oncogene*, vol. 38, no. 18, pp. 3387–3401, 2019.

[35] Z. B. Liu, N. E. Ezzedine, A. K. Eterovic et al., “Detection of breast cancer stem cell gene mutations in circulating free DNA during the evolution of metastases,” *Breast Cancer Research and Treatment*, vol. 178, no. 2, pp. 251–261, 2019.

[36] F. Clatot, A. Perdrix, L. Beaussire et al., “Risk of early progression according to circulating ESR1 mutation, CA-15.3 and cfDNA increases under first-line anti-aromatase treatment in metastatic breast cancer,” *Breast Cancer Research*, vol. 22, no. 1, p. 56, 2020.