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Plasma cell-free DNA integrity plus circulating tumor cells: a potential biomarker of no distant metastasis breast cancer

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Cell-free DNA integrity (cfDI) is a promising diagnostic and prognostic biomarker in breast cancer. However, no specific study has evaluated the diagnostic ability of cfDI in patients with no distant metastasis breast cancer (no-MBC) and benign breast tumor (BBT) to date. We assessed the plasma cfDI of 84 patients with no-MBC and 30 patients with BBT using quantitative PCR and compared it with circulating tumor cells (CTCs) and carbohydrate antigen 153 (CA153). The no-MBC group had significantly lower mean cfDI (0.58) than the BBT group (0.74, $p = 0.004$). Subgroup analysis showed that decreased cfDI seem to be associated with risk factors such as age < 45 years (mean cfDI = 0.52), triple-negative breast cancer (mean cfDI = 0.56), Ki67 > 14% (mean cfDI = 0.57), tumor size > 2 cm (mean cfDI = 0.58), and positive lymph node status (mean cfDI = 0.56), but had no statistical significance. McNemar's test suggested that cfDI had stronger diagnostic power than CTCs, cfDNA concentration, or CA153 ($p < 0.001$). Spearman's rho showed that the correlation coefficient between cfDI and CTCs was 0.278 ($p = 0.04$) in the no-MBC group. Receiver operating characteristic curve analysis also suggested that cfDI was superior to CTCs or CA153. Combined with CTCs, cfDI reduced the false positive rate from 50% to 10.71% and increased the area under the curve value from 0.66 to 0.68. Our results suggest that cfDI is a potential diagnostic biomarker of no-MBC. Using cfDI and CTCs as a combined diagnostic tool for no-MBC could improve diagnostic sensitivity and specificity but more samples will be needed.

Key words: cell-free DNA, cell-free DNA integrity, circulating tumor cell, no distant metastasis breast cancer

In the clinic, breast cancer (BC) is diagnosed by pathological examination. However, as information acquired from a single biopsy provides a spatially and temporally limited snapshot of a tumor, serial sampling of the tumor to monitor disease status or treatment response represents a prerequisite for personalized therapy [1-3]. Blood-based biomarkers have advantages over tissue biopsy because they can be accessed via minimally invasive procedures, and multiple samples can be obtained over a specific period [4]. Therefore, circulating biomarkers, which can provide more information on the tumor status, may be an excellent alternative. Traditional circulating biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 153 (CA153), have low sensitivity and specificity [5]. Recently, cell-free DNA (cfDNA) in blood has attracted increasing attention, and different cancer-associated cfDNA molecular characteristics, including copy number aberrations, methylation changes, single-nucleotide mutations, cancer-derived viral sequences, and chromosomal rearrangements, have been extensively studied [6]. Furthermore, cfDNA integrity (cfDI), which measures the extent of cfDNA fragmentation, has also been exploited as a diagnostic and prognostic biomarker in cancer [7]. As a biomarker, cfDI has practical advantages, including high sensitivity, non-invasiveness, and repeatability. For example, serum can be obtained easily, and the quick and well-established quantitative PCR (qPCR) method, requiring small amounts of blood, is a relatively cost-efficient technology [8]; in the circulation, DNA has a shorter half-life, ranging from 15 min to several hours, which can represent the real-time status of a tumor [9, 10]. Some studies have proven that cfDNA is detected sooner and more frequently than circulating tumor cells (CTCs) [11, 12]. However, previous studies have formed inconsistent conclusions on cfDI in patients with cancer. Using qPCR with different amplicon sizes, some studies have demonstrated a higher proportion of longer DNA and increased cfDI in patients with cancer as compared with subjects without a ma-

lignant condition [13-15]. On the other hand, contradictory evidence has shown that the shorter DNA molecules preferentially carry tumor-associated copy number aberrations and that cfDNA from tumor tissues might be shorter than that of nonmalignant cells [16, 17]. Reduced cfDI and increased cfDNA concentration could serve as diagnostic markers of primary BC and metastatic BC [18]. Although studies have assessed the clinical significance of cfDI in patients with BC and in healthy volunteers, few studies have focused on patients with benign BC (BBT) and no distant metastasis BC (no-MBC). Moreover, some studies have compared the diagnostic sensitivity of cfDI with CTCs but reached inconsistent conclusions [19-22]. In these studies, CTCs and cfDNA were often detected using different research platforms with differing detection sensitivity. Therefore, more studies using the same detection platform are needed for different breast diseases.

The absolute plasma DNA level is influenced by DNase I [14]. Some reports have suggested that DNase I activity in the circulation of patients with malignancies, such as prostate tumor, is decreased significantly compared with that of healthy controls [23, 24]. However, DNase I levels and whether cfDI is influenced by DNase I in breast tumor have not been studied to date.

In this study, we discuss the clinical significance of cfDI between patients with BBT and patients with no-MBC and investigated the diagnostic sensitivity of cfDI and CTCs using the same platform. Furthermore, we also tested plasma DNase I levels to explore whether they are related with cfDI in breast tumor.

Materials and methods

Subjects and plasma sample preparation. Between 2013 and 2015, 114 patients were recruited at the Department of Breast Surgery of Jiangsu Province Hospital. The subjects included patients with no-MBC (n = 84) and patients with BBT (n = 30). The no-MBC cohort consisted of patients with early BC (n = 57) and locally advanced BC (n = 27). All subjects were females. Blood was collected from the patients at the time of diagnosis before they underwent any therapy. Patients with BBT comprised individuals with no clinically diagnosed malignancies, autoimmune diseases, or infection. This study was approved by the ethical and scientific committee of our institution.

To reduce blood contamination by epithelial cells from the skin, the first 2 ml blood was discarded and the collection tube was disconnected before the needle was withdrawn at the end of the procedure. Peripheral blood (10 ml in EDTA) was collected and then shipped at room temperature within 2 h to the molecular diagnostic laboratory for immediate processing by Ficoll density gradient centrifugation (STEMCELL Technologies, Canada) according to the protocol. The supernatant plasma was centrifuged again at 2,000 ×*g* for 10 min at 4°C to minimize any contamination from blood cells or cell debris. Mononuclear cells were collected and dissolved in 1 ml RNAiso Plus (TaKaRa, Japan). All samples were stored at −80°C until further use.

DNA extraction from plasma. DNA was extracted from 200 µl plasma using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the kit protocol, and the final eluate was collected and stored at −20°C. Samples from different groups were always extracted together to avoid batch effects.

RNA extraction and complementary DNA (cDNA) synthesis. Total RNA was extracted according to the RNAiso Plus protocol. RNA quantification and purity assessment were performed by optical density measurement at 260 nm and 280 nm. RNA reverse transcription was carried out using the PrimeScript RT Master Mix system (TaKaRa). The cDNA was synthesized from 1 µg total RNA isolated from the peripheral blood mononuclear cells of the patients in a total volume of 20 µl according to the manufacturer's instructions.

Estimation of cfDI and cfDNA concentration with Alu repetitive elements. The cfDI and cfDNA concentration were derived by analyzing Alu repetitive elements. For this, a short (111 bp) and long (260 bp) fragment were measured in triplicate by qPCR using an Absolute SYBR Green assay with the Step One Plus Real-Time PCR System (Applied Biosystems, USA). Primers were designed according to a previous report (Supplementary Table 1) [18]. PCR was performed with FastStart Universal SYBR Green Master (Rox, Germany) according to the manufacturer's instructions in a final volume of 20 µl containing 10 µl 2× SYBR Green, 0.2 µl 10 µM PCR forward primer, 0.2 µl 10 µM PCR reverse primer, 1 µl DNA template, and 8.6 µl distilled water (dH_2O). The thermal cycling conditions were 10 min at 95°C and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 15 s.

A known-concentration DNA standard was divided into five copies and diluted to 1 ng/ml, 10 ng/ml, 100 ng/ml, 1000 ng/ml, and 10000 ng/ml. DNA concentration–Ct value standard curves were constructed. The respective absolute concentration of the long and short fragment was calculated and the cfDI was subsequently calculated as the ratio of long fragment concentration to short fragment concentration. The total cfDNA concentration of a sample was derived from the short fragment concentration.

CTC detection. CTCs were detected using a previously reported qPCR method [25]. The marker genes included cytokeratin 19 (*CK19*), human mammaglobin (h*MAM*), and small breast epithelial mucin (*SBEM*). The housekeeping gene was beta-cytoplasmic actin 2 (β-actin, *ACTB*). All PCR were performed using the fluorescent SYBR Green I methodology. PCR was performed with FastStart Universal SYBR Green Master (Rox) in a final volume of 20 µl containing 10 µl 2× SYBR Green, 0.4 µl 10 µM PCR forward primer, 0.4 µl 10 µM PCR reverse primer, 2 µl cDNA template, and 7.2 µl dH_2O. The thermal cycling conditions were 10 min at 95°C and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 15 s. The *CK19*, h*MAM*, and *SBEM* mRNA quantities were analyzed in triplicate, and normalized against the *ACTIN* control gene. Results were expressed as relative gene expression using the comparative threshold cycle

($2^{-\Delta\Delta Ct}$) method. A relative gene expression value of 1 of the mRNA markers was derived from the mRNA expression in the peripheral blood from a healthy female volunteer. Gene positivity was defined as expression above the cut-off threshold, which was set for each gene marker at three sd from the mean expression in BBT samples. CTC positivity was defined as positivity for at least one marker.

Detection of CA153 and plasma DNase I levels. CA153 was detected in the central laboratory of Jiangsu Province Hospital. DNase I levels were tested with an enzyme-linked immunosorbent assay kit (Cloud-Clone, USA) according to the manufacturer's protocol.

Data analysis. Statistical analysis was performed using SPSS 20 (IBM, USA). The normal distribution of the parameters was assessed and most followed normal distribution, except DNase I, which was calculated using the nonparametric test. Comparisons between two groups were made using Student's *t*-test; comparisons between three groups were made using one-way analysis of variance (ANOVA), and homogeneity of variance was evaluated using Bartlett's test. Paired data in 2 × 2 contingency tables were tested using McNemar's test. Receiver operating characteristic (ROC) analysis was performed to assess the discriminatory power of cfDI, cfDNA concentration, CTCs, and CA153 between two groups, and the corresponding area under the curve (AUC) was calculated. For binary variables such as CTCs, binary logistic analysis first was performed to produce predicted probabilities. Correlation coefficients were calculated with Pearson analysis for continuous variables and with Spearman's rho for binary variables.

Results

Study inclusion and patient characteristics. Table 1 lists the clinical and histological data of the patients, including age, menopausal status, pathological type, molecular type, and Ki67 index.

Standard curve setting. We constructed standard curves (Supplementary Figure 1). The R^2 of each curve was >0.99. The PCR efficiencies of all primer pairs were >70% [ALU (111 bp): 88.92%, ALU (260 bp): 71.55%]. Primer specificity was confirmed by melt curve analysis, and no multiple peaks were found. The concentration–Ct value formula was: $Y_{(111bp)} = -3.62X + 25.41$, $Y_{(260\ bp)} = -4.266X + 29.993$.

Comparison of cfDI between patients with no-MBC and patients with BBT. The mean cfDI (0.58) of the no-MBC group was significantly lower than that of the BBT group (0.74, p = 0.004). The mean ALU concentration of the no-MBC group (135.74 ng/ml) was greater than that of the BBT group (121.62 ng/ml), but had no statistical significance (p = 0.89). Subgroup analysis showed no significant difference. But we found that decreased cfDI appeared to be associated with high risk factors of prognosis, such as age < 45 years (mean cfDI = 0.52), triple-negative BC [TNBC (mean cfDI = 0.56)], Ki67 > 14% (mean cfDI = 0.57), tumor size > 2 cm (mean cfDI = 0.58), and positive lymph node status (mean cfDI = 0.56, Table 2).

Table 1. Patient characteristics

	Benign breast tumor		Breast cancer	
Age (years)				
Median (range)	42.5(20-67)		49 (28-82)	
Menopausal status (n, %)				
Premenopausal	25	21.9	49	43.0
Postmenopausal	5	4.4	35	30.7
Histological type (n, %)				
Fibroadenoma	8	7.0		
Intraductal papilloma	12	10.5		
Nodal mastopathy	10	8.8		
DCIS			2	1.8
IDC			70	61.4
Other			12	10.5
Molecular type (n, %)				
Luminal A			7	6.1
Luminal B			48	42.2
HER2			21	18.4
TNBC			8	7.0
Ki67* (n, %)				
≤14%			13	11.4
>14%			67	58.8

*Part of the information was lost. TNBC = triple-negative breast cancer.

Table 2. Differences in cfDI values between groups

Group	Number (%)	cfDI	p
Benign breast tumor	30 (26.3)	0.74	0.004
Breast cancer	84 (73.7)	0.58	
Age (years)			0.090
<45	28 (24.6)	0.52	
45-55	31 (27.2)	0.66	
>55	25 (21.9)	0.56	
Menopausal status*			0.690
Pre-menopausal	48 (42.1)	0.59	
Post-menopausal	34 (29.8)	0.57	
Molecular subtype			0.940
Luminal	55 (48.3)	0.59	
HER2	21 (18.4)	0.59	
TNBC	8 (7.0)	0.56	
Ki67*			0.090
≤14	13 (11.4)	0.69	
>14	67 (58.8)	0.57	
Tumor diameter (cm)*			0.390
≤2	35 (30.7)	0.65	
>2cm	21 (18.4)	0.58	
Lymph node status*			0.180
N0	37 (32.5)	0.66	
N1-N3	17 (14.9)	0.56	

*Part of the information was lost. TNBC = triple-negative breast cancer.

Assessment of diagnostic capability of cfDI, cfDNA concentration, CTCs, and CA153. In addition to testing the plasma DNA concentration and cfDI in all cases, we also studied the CTCs in 51 no-MBC and 28 BBT cases. In the 51 no-MBC cases, the positive rate of CTCs was 43.14% (22/51, Table 3), which was lower than the cfDI [72.55% (37/51)] when the cfDI cut-off threshold was set to 0.74 (meeting the best diagnostic sensitivity and specificity). However, the false positive rate of cfDI (50%, 14/28) was higher than that of the CTCs (21.43%, 6/28). The cfDI and CTCs both were positive in 37.25% (19/51) of no-MBC cases and in 10.71% (3/28) of BBT cases. McNemar's test suggested that cfDI had stronger diagnostic power than CTCs, cfDNA concentration, and CA153 ($p < 0.001$, Table 4). Spearman's rho showed that the correlation coefficient between cfDI and CTCs was 0.203 ($p = 0.062$) in all cases, but was 0.278 ($p = 0.04$) in the no-MBC cases.

ROC curve analysis showed that cfDI had a larger AUC than cfDNA concentration (cfDI = 0.67, cfDNA concentration = 0.47), CTCs (cfDI = 0.66, CTCs = 0.61), or CA153 (cfDI = 0.67, CA153 = 0.64). When the conjoint analysis of cfDI and CTCs was performed, the specificity and sensitivity could be improved (AUC = 0.680, Figure 1).

Detection of plasma DNase I levels. We found no remarkable difference between plasma DNase I levels in the patients with no-MBC and the patients with BBT ($p = 0.217$, Figure 2). Correlation analysis showed remarkable positive correlation between cfDI and DNase I levels ($r = 0.416$, $p < 0.001$), but not between DNase I levels and cfDNA concentration ($r = 0.158$, $p = 0.143$, Figure 3).

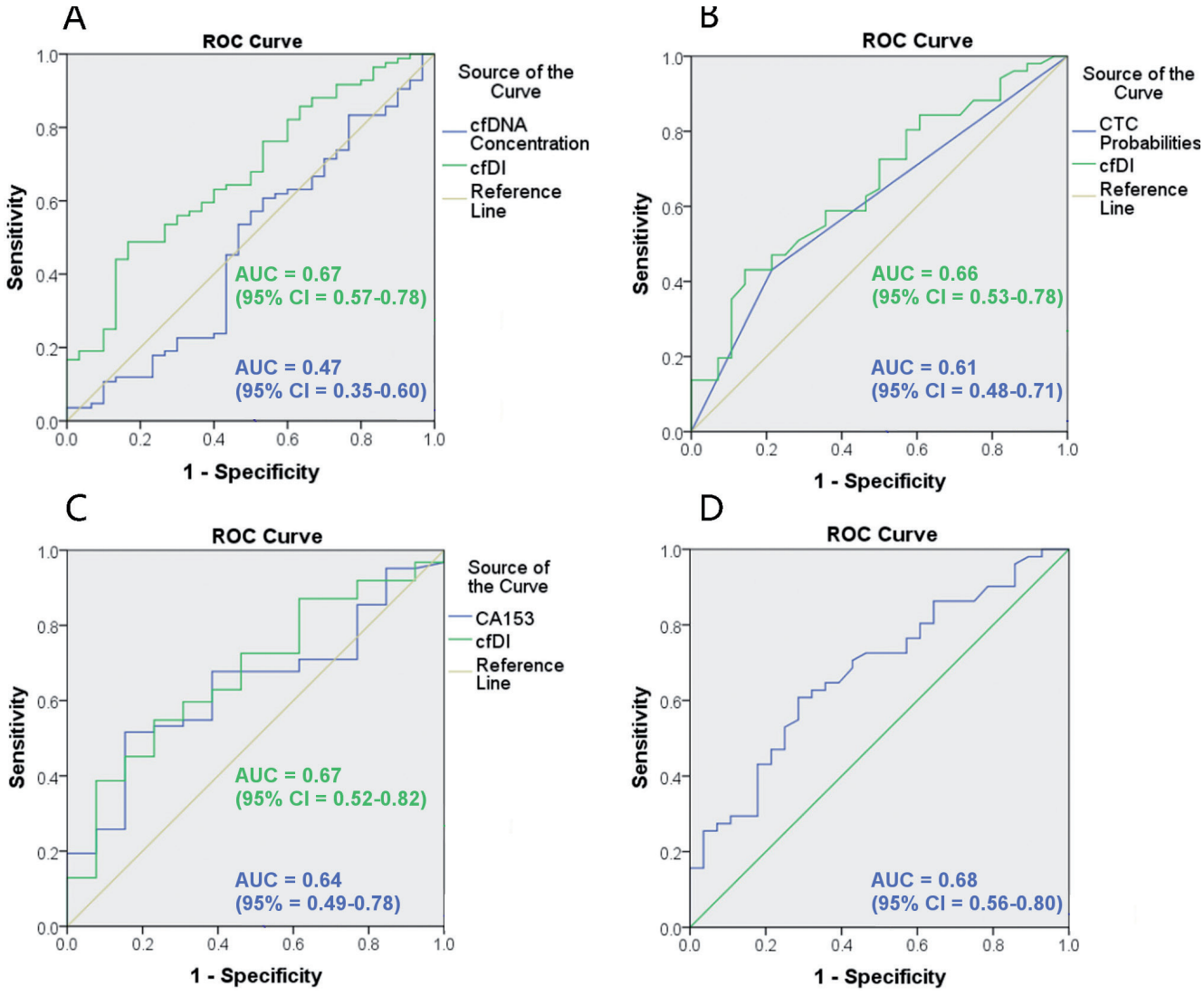

Figure 1. ROC curves of cfDI, cfDNA concentration, CTCs, and CA153. A. Comparison of AUC between cfDI and cfDNA concentration; B. cfDI and CTCs; C. cfDI and CA153; D. Conjoint analysis of cfDI and CTCs.

Discussion

In the present study, the mean cfDI value of the no-MBC group was significantly lower than that of the BBT group. Subgroup analysis showed that decreased cfDI appeared to be associated with high risk factors of prognosis, such as age < 45 years, TNBC, Ki67 > 14%, tumor size > 2 cm, and positive lymph node status but had no statistical significance. Moreover, cfDI had stronger diagnostic power than CTCs, cfDNA concentration, and CA153. Correlation analysis showed that cfDI correlated positively with CTCs in the no-MBC group as well as with DNase I level in all patients. ROC analysis suggested that the cfDI AUC was greater than that of cfDNA concentration, CTCs, and CA153. However, cfDI had a higher false positive rate (50%). If cfDI and CTCs were considered together, the AUC value reached 0.68, and the false positive rate dropped to 10.71%. We demonstrate that cfDI plus CTCs could be a promising diagnostic biomarker in the differential diagnosis of no-MBC and BBT. However, the sample size in this study was small and the survival data were not available because of the short study duration. Therefore, more studies should be done to confirm the clinical significance of cfDI.

Our findings are the opposite of that of previous studies that reported increased cfDI among patients with cancer. This contradiction could be partly due to the following reasons. First, the source of cfDNA is unclear. Some studies believed that the increased cfDI is associated with cfDNA derived from apoptotic and necrotic cells in patients with cancer, while it is derived only from apoptotic cells in healthy individuals [26, 27]. Apoptotic cells release DNA fragments that are usually 185–200-bp in length, while DNA fragments from necrotic cells vary in size and can even be several kilobase pairs [28].

Table 3. Detection of CK19, hMAM and SBEM

	Benign tumor	Breast cancer
RGE of CK19		
N	28	51
No. of patient>cut-off[a]	2	8
Positive rate (%)	7.14	15.69
RGE of hMAM		
N	28	51
No. of patient>cut-off[a]	2	13
Positive rate (%)	7.14	25.49
RGE of SBEM		
N	28	51
No. of patient>cut-off[a]	2	3
Positive rate (%)	7.14	5.88
Two or three marker positive rate (%)	0	3.92 (2/51)
Total positive rate (%)	21.42	43.14

β-actin = beta-cytoplasmic actin 2; CK19 = cytokeratin 19; hMAM = human mammaglobin; SBEM = small breast epithelial mucin; RGE = relative gene expression.

[a]The cut-off was set for each gene marker at three sd from the mean expression in BBT samples.

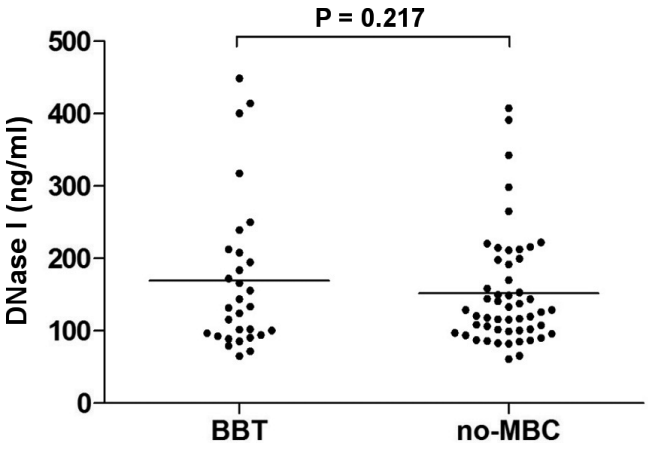

Figure 2. Comparison of DNase I level in the plasma of no-MBC cases and BBT controls. BBT = benign breast tumor; no-MBC = no distant metastatic BC.

Different sources have been proposed as the cause of the increased cfDI observed in patients with cancer [29]. However, this inference has not been confirmed by experimental proof showing that the majority of cfDNA from patients with cancer is indeed from necrotic and apoptotic cells and that cfDNA from healthy subjects is from apoptotic cells.

Table 4. Comparisons between cfDI with CTCs, cfDNA concentration, or CA153

	cfDI (n)		
	<0.74	≥0.74	Total
CTCs (n)			
Positive (any marker above the cut-off threshold)	19	3	22
Negative (all markers below the cut-off threshold)	18	11	29
Total	37	14	51
cfDI positive rate, 72.55% (37/51)			
CTC positive rate, 43.14% (22/51)			
p < 0.001			
cfDNA concentration (n)			
Abnormal (above the cut-off threshold)	30	10	40
Normal (below the cut-off threshold)	32	12	44
Total	62	22	84
cfDI positive rate, 73.81% (62/84)			
cfDNA positive rate, 47.62% (40/84)			
p < 0.001			
CA153 (n)			
Abnormal (≥25 U/ml)	3	1	4
Normal (<25 U/ml)	41	17	58
Total	44	18	62
cfDI positive rate, 70.97% (44/62)			
CA153 positive rate, 6.45% (4/62)			
p < 0.001			

cfDI = cell-free DNA integrity; cfDNA = cell-free DNA; CTCs = circulating tumor cells.

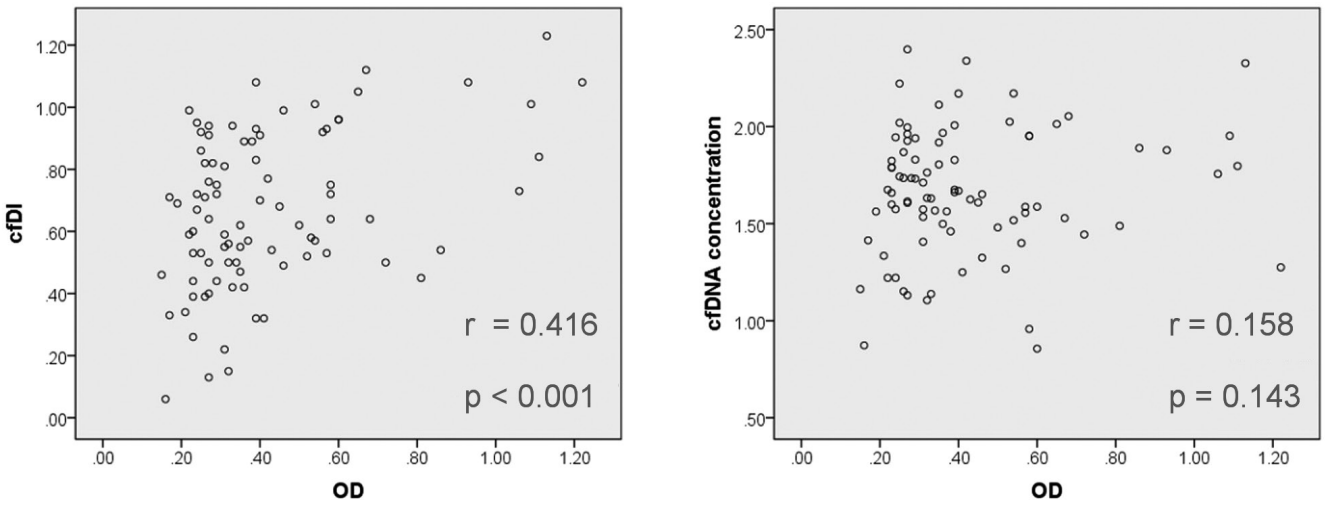

Figure 3. Correlation of DNase I level and cfDI or cfDNA concentration.

An increasing number of reports have shown that cfDNA in patients with cancer is highly variable and mainly comprised short DNA molecules (<200 bp), which preferentially carry tumor-associated gene aberrations [30]. Our study supports the premise of the shorter feature of cfDNA and lower cfDI of patients with no-MBC as compared to that of patients with BBT. Second, cfDNA differs if isolated from different types of blood samples (serum or plasma) [31]. Plasma samples are preferred over serum samples despite studies showing that cfDNA concentrations tend to be higher in the latter, as the coagulation process affects the spectrum of circulating nucleic acids in serum and thereby contributes to higher variability [32, 33]. Lastly, the amount of isolated DNA varies greatly between different extraction kits. In the present study, cfDNA was isolated strictly according to the protocol of a commercial kit to reduce the variability as much as possible. Additionally, the properties of the primer pairs and amplicon lengths might have influenced the results. As qPCR-based methods produce indirect estimates of true biological values, inconsistencies in the real-time amplification or differences in the PCR efficiencies of the long and short amplicons can influence cfDI estimation [34].

The DNA clearance rate of patients could directly contribute to the absolute plasma DNA level. Most cfDNA is degraded by DNase I, which is a secreted enzyme whose function has been presumed to control "waste management" in the human system and is eventually cleared from the blood by the liver and kidneys [35]. DNase I activity is mainly influenced by Ca^{2+} and Mg^{2+} in the blood. Some have reported significantly decreased DNase I activity in the blood of patients with cancer as compared to healthy controls [23]. However, no study has compared the DNase I activity between patients with no-MBC and patients with BBT. In addition, it has been speculated that cfDI is not significantly influenced by the DNA clearance rate, as both the amounts of longer and shorter DNA fragments should be similarly affected [14], but no reports confirm this hypothesis. In the present study, considering all cases were in the early phase of the disease and had no obvious electrolyte disorder, we only tested the DNase I concentration with the sensitive enzyme-linked immunosorbent assay. We found that the average plasma DNase I level of the patients with no-MBC was slightly lower than that of the patients with BBT, but it had no statistical significance. Correlation analysis showed that cfDI had a remarkable positive correlation with DNase I levels, but cfDNA concentration did not.

In conclusion, our findings show that cfDI plus CTCs is a potential diagnostic biomarker in the differential diagnosis of no-MBC and BBT.

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Supplementary table 1. Primer sequences

Gene	Sequence (5'–3')
CK19	Forward: TCCGAACCAAGTTTGAGACG
	Reverse: CCCTCAGCGTACTGATTTCCT
hMAM	Forward: ATGAAGTTGCTGATGGTCCTCAT
	Reverse: GTCTTAGACACTTGTGGATTGATTGTCT
SBEM	Forward: GTATCCAGCTACTGGTCCTGCT
	Reverse: CAATTGCAGAAGACTCAAGCTG
β-actin	Forward: GCTGTGCTATCCCTGTACGC
	Reverse: TGCCTCAGGGCAGCGGAACC
ALU(111bp)	Forward: CTGGCCAACATGGTGAAAC
	Reverse: AGCGATTCTCCTGCCTCAG
ALU (260bp)	Forward: ACGCCTGTAATCCCAGCA
	Reverse: CGGAGTCTCGCTCTGTCG

Abbreviations: β-actin = beta-cytoplasmic actin 2; CK19 = cytokeratin 19; hMAM = human mammaglobin; SBEM = small breast epithelial mucin.

Supplementary Figure 1. Standard curves of ALU (111bp) and ALU (260bp).