



Plasma cell-free DNA integrity: a potential biomarker to monitor the response of breast cancer to neoadjuvant chemotherapy

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Background: Although the clinical significance of neoadjuvant chemotherapy (NACT) is widely recognized, there is still no effective means to monitor the therapeutic response in real time. The present study aimed to investigate the significance of the cell-free DNA (cfDNA) concentration and integrity (cfDI) to monitor the response of breast cancer to NACT.

Methods: Twenty-nine patients with breast cancer receiving NACT were included in this study. Patients' peripheral blood was drawn before, in the mid-term, and at the end of chemotherapy. The cfDNA concentration and cfDI were assessed using absolute quantitative PCR.

Results: The results showed that the cfDNA concentration and cfDI pre-NACT were not obviously correlated with the patients' clinical characteristics. The mean cfDI value increased significantly when the patients received NACT ($P < 0.05$), and an increasing cfDI was associated with tumor shrinkage and reduced Ki67 levels ($P < 0.05$). In addition, the cfDI after NACT was inversely correlated with the number of metastatic lymph nodes, and the cfDI value of patients with a pathologically complete response was significantly higher than that of patients with distant metastasis after surgery.

Conclusions: This study suggested that cfDI could be used as an indicator to monitor the therapeutic response to NACT; however, more research is needed to confirm this conclusion.

Keywords: Breast neoplasms; neoadjuvant therapy; biomarkers; cell-free nucleic acids

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Introduction

Breast cancer has become the most frequently diagnosed cancer in women worldwide (1,2). Surgery, systemic therapy, and radiotherapy, as the main treatment modalities, have significantly improved the prognosis of breast cancer (3). In particular, neoadjuvant chemotherapy (NACT), with the advantage of downsizing the tumor before surgery, provides a therapeutic alternative for patients with locally advanced breast cancer (4). However, although some patients that respond to NACT have good prognosis, a considerable

proportion of patients gain little or no benefit from such treatment. More seriously, these patients are exposed to the deleterious effects of cytotoxic drugs (5). Therefore, it is particularly important to monitor treatment responses during NACT. Currently, treatment-monitoring methods are limited and the most common evaluation method is radiographic inspection (6). However, radiological imaging cannot properly reflect the state of the disease in real time. Therefore, it is necessary to identify a predictive biomarker to monitor the disease response to NACT, which will be

useful for the individualized breast cancer management and the more effective administration of chemotherapeutics (7).

Blood-based biomarkers have some advantages over imaging or histological examination because they can be accessed using minimally invasive procedures, and multiple samples can be obtained over a specific period (8). However, traditional circulating biomarkers, such as carcinoembryonic antigen and carbohydrate antigen 153 (*CA153*) have low sensitivity and specificity (9). Recently, cell-free DNA (cfDNA) in blood has attracted research 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 studied extensively (10). Furthermore, cfDNA integrity (cfDI), which measures the extent of cfDNA fragmentation, has also been exploited as a diagnostic and prognostic biomarker in cancer (11). As a biomarker, cfDI has practical advantages. For example, serum can be obtained easily, and the fast and well-established quantitative real-time PCR (qPCR) method can be used, which requires small amounts of blood and is a relatively cost-efficient technology (12). In circulation, DNA has a short half-life, ranging from 15 min to several hours, which can represent the real-time status of a tumor (13,14). Although cfDI could serve as an attractive prognostic marker for patients with metastatic breast cancer at baseline and during systemic therapy (15), results from different studies have been inconsistent. Furthermore, few studies have focused on the clinical significance of cfDI for patients receiving NACT.

In the present study, the cfDNA concentration and cfDI of patients with locally advanced breast cancer were detected during NACT. The aim was to investigate whether the dynamics of the cfDNA concentration and cfDI reflect the therapeutic effect and whether they could be used as biomarkers for real-time monitoring of NACT.

Methods

Subjects and plasma sample preparation

Female patients with locally advanced breast cancer that were clinically diagnosed using tissue biopsy and radiological imaging were included in this study. The basic information of the enrolled patients and their chemotherapy regimens were collected. All patients comprised individuals with no other clinically diagnosed malignancies, autoimmune diseases, or infections. At the time of definitive

diagnosis, blood was collected from each patient before they underwent any therapy. The second blood sample was drawn approximately half way through the NACT procedure. All patients were evaluated systematically every two cycles, and we collected peripheral blood during each evaluation period. For patients receiving three to four cycles of NACT, the intermediate time point referred to the assessment period after the second cycle. For patients receiving eight cycles of NACT, the intermediate time point referred to the assessment period after the fourth cycle. The last blood sample was collected before surgery or at the termination of treatment. The histopathological findings at surgery were compared with the diagnostic biopsies.

Peripheral blood (10 mL) was collected and shipped at room temperature within 2 h to the laboratory for immediate processing using Ficoll density gradient centrifugation (STEMCELL Technologies, Vancouver, Canada), according to the manufacturer's protocol. The supernatant plasma was centrifuged again at 2,000 $\times g$ for 10 min at 4 °C to minimize any contamination from blood cells or cell debris. All samples were stored at -80 °C until further use. This study was approved by the ethical and scientific committee of our institution (Ethics Approval Number: 2010-SR-091. A1, Ethics Committee of the First Affiliated Hospital of Nanjing Medical University) and informed consent was obtained from all participants.

DNA extraction from plasma

DNA was extracted from 200 μL of plasma using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and the final eluate was collected and stored at -20 °C. Samples were extracted together to avoid batch effects.

Estimation of the cfDNA concentration and cfDI using repetitive elements

The cfDNA concentration and cfDI were derived by analyzing two repetitive elements, Alu and LINE1. These two repetitive elements have a high copy number and are distributed throughout human genomic DNA. They were independently analyzed in parallel for each sample, which ensured the generation of accurate cfDI estimates. For each of the targets, short (Alu =111 bp, LINE1 =97 bp) and long (Alu =260 bp, LINE1 =266 bp) fragments were measured in triplicate using qPCR with an Absolute SYBR Green assay and the Step One Plus Real-Time PCR System

(Applied Biosystems, Waltham, MA, USA). Primers were designed according to a previous report (*Table S1*) (16). PCR was performed using the FastStart Universal SYBR Green Master (Rox, Hofstetten, Germany) according to the manufacturer's instructions, in a final volume of 20 μ L containing 10 μ L of 2 \times SYBR Green, 0.2 μ L of 10 μ M PCR forward primer, 0.2 μ L of 10 μ M PCR reverse primer, 1 μ L of DNA template, and 8.6 μ L of distilled water (dH₂O). The thermal cycling conditions were 10 min at 95 °C; followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and extension at 95 °C for 15 s. A known-concentration DNA standard was divided into five copies and diluted to 1, 10, 100, 1,000, and 10,000 ng/mL. DNA concentration-Ct value standard curves were constructed. The respective absolute concentrations of the long and short fragments were calculated and the cfDI was subsequently calculated as the ratio of the long fragment concentration to the short fragment concentration. The total cfDNA concentration of a sample was derived using the short fragment concentration.

Data analysis

Statistical analysis was performed using SPSS 20 (IBM Corporation, Armonk, NY, USA). Comparisons between two groups were made using a paired T test and between three groups was performed using one-way analysis of variance (ANOVA). Correlation coefficients of two variables were calculated using Pearson analysis. Multivariate analysis was performed using multiple regression analysis. In all figures, asterisks denote significance levels as follows: *P<0.05, **P<0.01.

Results

Study inclusion and patient characteristics

Twenty-nine patients with locally advanced breast cancer were included in this study. All subjects were female. Their median age was 44 years old (range: 29 to 67 years old). Twenty-two patients (75.8%) were pre-menopausal. All tumors were diagnosed as invasive ductal carcinoma (IDC). Immunohistochemical analysis showed the marker of proliferation Ki-67 (Ki67) index in four patients was less than 14%. Luminal A [estrogen receptor (ER) and/or progesterone receptor (PR) positive, human epidermal growth factor receptor 2 (HER-2) negative, Ki67 <14%],

luminal B (ER and/or PR positive, HER-2 negative, Ki67 high; or ER and/or PR positive, Any Ki67, HER-2 overexpressed or amplified), HER-2 positive (HER-2 over-expressed or amplified, ER and PR absent), and triple negative (ER and PR absent, HER-2 negative) breast cancer accounted for 3.4%, 44.8%, 41.4%, and 10.4%, respectively.

NACT regimens in this study included docetaxel + epirubicin + cyclophosphamide; epirubicin + cyclophosphamide followed by paclitaxel every 2 weeks; epirubicin + cyclophosphamide followed by paclitaxel plus trastuzumab; fluorouracil + epirubicin + cyclophosphamide; and docetaxel + carboplatin + trastuzumab. *Table S2* lists the clinical and histological data of the patients.

Standard curve setting

We constructed standard curves for Alu and LINE1. 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%, LINE1_(97 bp): 74.05%, LINE1_(266 bp): 80.29%]. Primer specificity was confirmed by melt curve analysis, and no multiple peaks were found. The concentration-Ct value formula of Alu was: $Y_{(111 bp)} = -3.62X + 25.41$, $Y_{(260 bp)} = -4.266X + 29.993$. The formula of LINE1 was: $Y_{(97 bp)} = -4.155X + 34.243$,

$$Y_{(266 bp)} = -3.907X + 28.69 \text{ (Figure S1).}$$

DNA concentration and cfDI pre-NACT had no obvious correlation with the patients' clinical characteristics

Our results showed that the mean concentration of the short Alu fragment was 75.89 \pm 71.18 ng/mL (range: 9.23 to 313.50 ng/mL), and the mean cfDI value of Alu was 0.51 \pm 0.26 (range: 0.09 to 0.95) before NACT. The mean concentration of the short LINE1 fragment was 82.47 \pm 132.93 ng/mL (range: 1.51 to 727.60 ng/mL), and the mean cfDI value of LINE1 was 0.23 \pm 0.19 (range: 0.01 to 0.67). We attempted to determine whether the cfDNA concentration or cfDI correlated with the clinical characteristics of the patients. The results showed that the cfDNA concentration or cfDI had no significant correlation with most of the clinical characteristics of the patients, except for the cfDI value of Alu, which correlated positively with the expression of PR (P=0.04). In addition, *CA153* levels were investigated, but no positive result was obtained (*Table S3*).

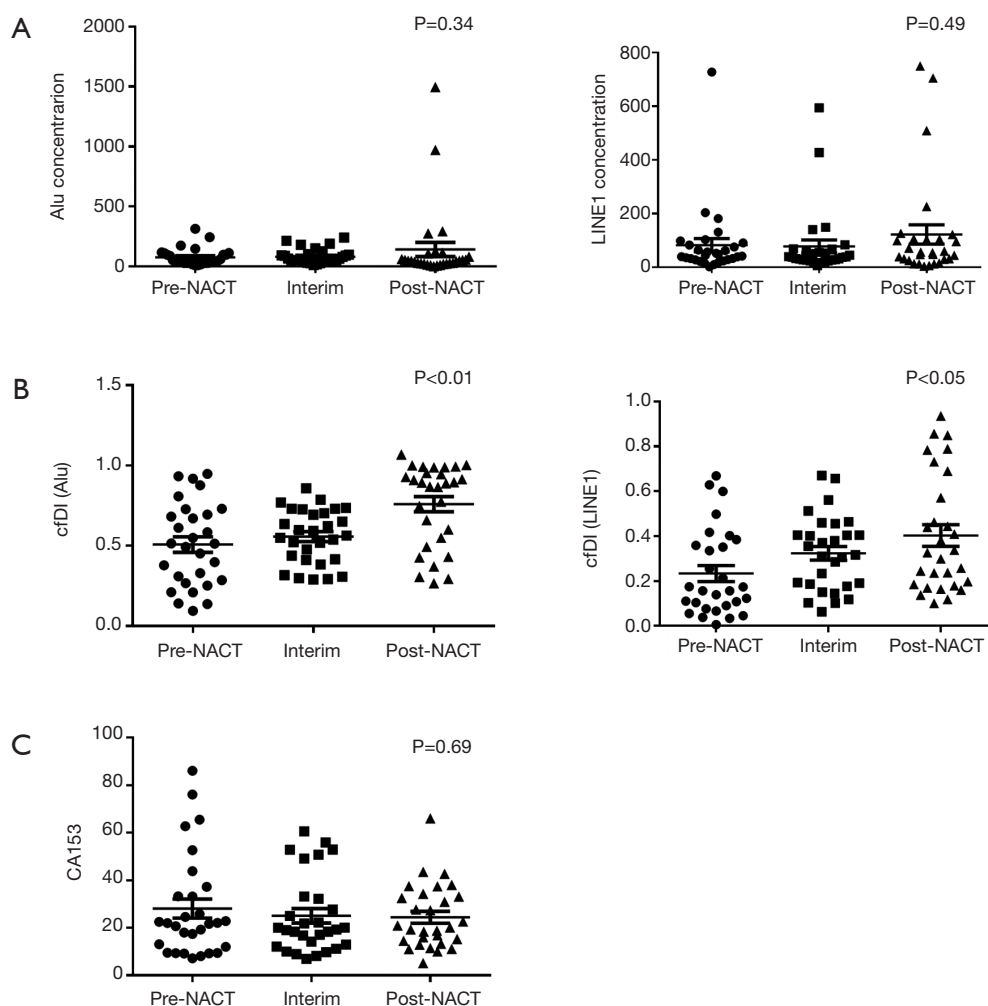


Figure 1 Comparisons of different hematological biomarkers during neoadjuvant chemotherapy (NACT). (A) Comparisons of plasma cell-free DNA (cfDNA) concentrations at three time points; (B) comparisons of cell-free DNA integrity (cfDI) at three time points; (C) comparisons of carcinoembryonic antigen and carbohydrate antigen 153 (CA153) levels at three time points.

Breast cancer patients receiving NACT have increasing cfDI values

We sampled peripheral blood from each patient before, in the middle, and at the end of the NACT. CfDNA was extracted from the blood and the cfDNA concentration and cfDI were measured. Our results showed that the cfDI value increased gradually with the progression of NACT. At each checkpoint, the mean cfDI values of Alu were 0.51 ± 0.26 , 0.56 ± 0.03 , and 0.76 ± 0.26 ($P < 0.01$), respectively. The mean cfDI values of LINE1 were 0.23 ± 0.03 , 0.32 ± 0.03 , and 0.40 ± 0.26 ($P < 0.05$), respectively. Although the mean cfDNA concentration increased when patients finished their NACT, there was no statistical

difference between the concentrations pre and post NACT, whether for Alu (75.89, 80.69, 142.26 ng/mL) or LINE1 (82.47, 77.89, 122.25 ng/mL) (Figure 1). In addition, CA153 expression before and after NACT was also assessed. The results showed that the average values of CA153 were 28.13, 25.11, and 24.46 ng/mL respectively, and there was no statistical difference.

Increased cfDI was associated with tumor shrinkage and Ki67 decline

We measured the maximum diameter of each tumor pre and post NACT using ultrasound and found that the mean

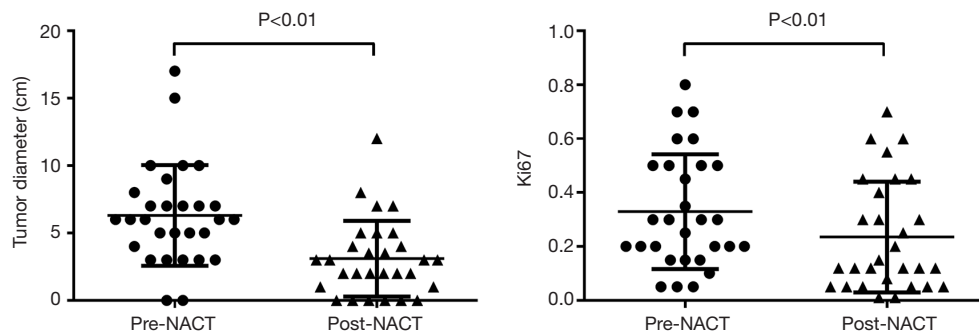


Figure 2 Comparisons of tumor diameter and the Ki67 index before and after neoadjuvant chemotherapy (NACT).

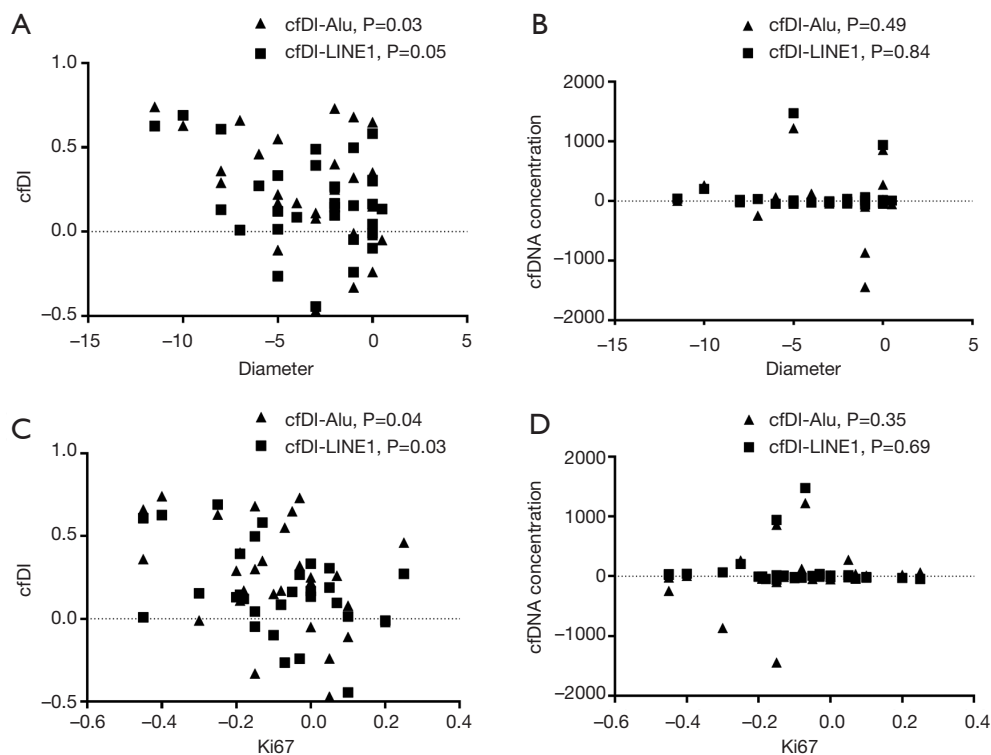


Figure 3 Scatter plots of the correlation analysis between cell-free DNA integrity (cfDI)/cell-free DNA (cfDNA) concentration and tumor diameter/Ki67. (A) Correlation analysis between cfDI and tumor diameter; (B) correlation analysis between the cfDNA concentration and tumor diameter; (C) correlation analysis between cfDI and Ki67; (D) correlation analysis between the cfDNA concentration and Ki67.

tumor diameter decreased significantly (from 6.31 ± 3.73 to 3.10 ± 2.80 cm, $P < 0.01$). In addition, immunohistochemical analysis showed reduced Ki67 levels post NACT, which was expected because of the reduction in cellularity caused by NACT (from $32.93\% \pm 21.28\%$ to $23.52\% \pm 20.52\%$, $P < 0.01$) (Figure 2).

Correlation analysis showed that tumor shrinkage correlated positively with the increase in the cfDI value of

Alu ($P=0.03$) and had a near statistical correlation with the cfDI value of LINE1 ($P=0.05$). However, the increase in the cfDNA concentration was not associated with tumor shrinkage. Similar results were obtained in the analysis of Ki67 levels. Ki67 levels correlated negatively with the cfDI values (Alu: $P=0.04$; LINE1: $P=0.03$); however, the relationship between the cfDNA concentration and Ki67 was not significant (Figure 3).

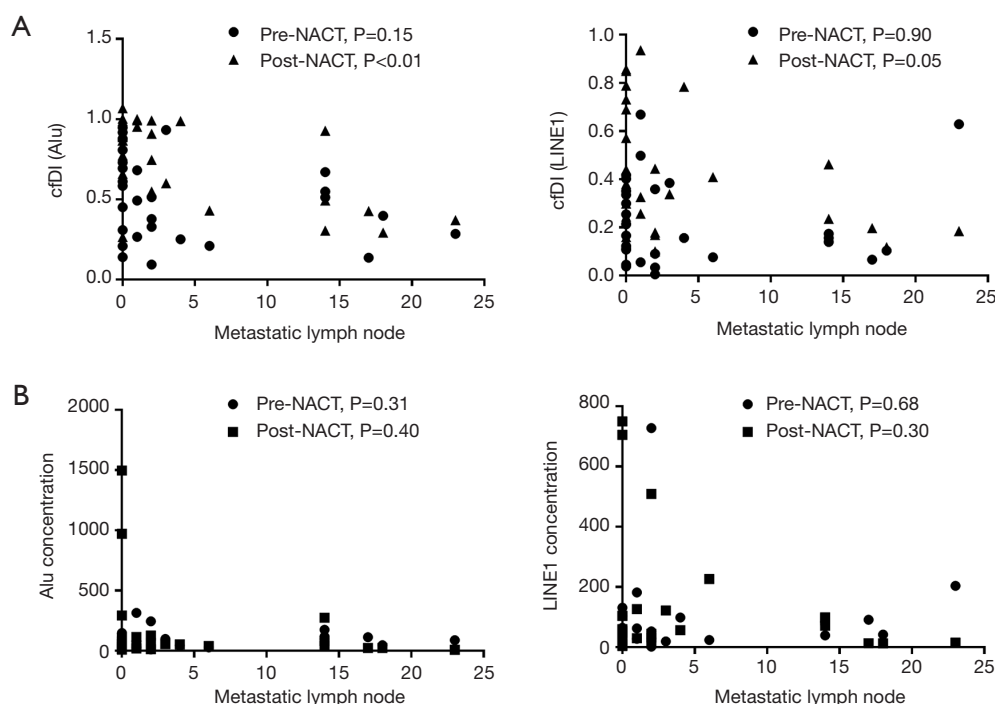


Figure 4 Scatter plots of the correlation analysis between cell-free DNA (cfDNA) concentration/cell-free DNA integrity (cfDI) and the number of metastatic lymph nodes. (A) Correlation analysis between cfDI and the number of metastatic lymph nodes; (B) correlation analysis between the cfDNA concentration and the number of metastatic lymph nodes.

CfDI after NACT was inversely correlated with the number of metastatic lymph nodes

All patients eventually underwent surgery, and excised lesions and lymph nodes were examined pathologically. The results of the pathological examination showed that five patients had no residual tumor cells in their primary lesions. The average number of lymph nodes removed was 18.90 (range: 0 to 34), and the average number of positive lymph nodes was 3.79 ± 6.54 (range: 0 to 23). No positive lymph nodes were found in 14 patients. In brief, four patients reached a pathologically complete response (pCR). No clear relationship was found between the pCR and the cfDNA concentration or cfDI. However, we found that the cfDI of Alu after NACT was significantly inversely correlated with the number of metastatic lymph nodes ($P < 0.01$) and there was a near-significant inverse relationship between LINE1 cfDI and the number of metastatic lymph nodes ($P = 0.05$) (Figure 4).

Patients with increasing cfDI tended to obtain better NACT responses

In this study, none of the four patients with pCR showed tumor recurrence during follow-up, while the other four patients showed distant metastasis within 6 months after surgery. We compared the difference in cfDI values between patients who achieved a pCR and those with metastasis. We found that patients who achieved a pCR had gradually increasing cfDI values during NACT; however, those showing disease progression after surgery demonstrated no significant change in cfDI values. Furthermore, at the end of the NACT, the mean cfDI value in the pCR group was significantly larger than that in the metastatic group (Figure 5).

Discussion

In the present study, we assessed the cfDNA concentration and cfDI of Alu and LINE1 genomic elements in patients

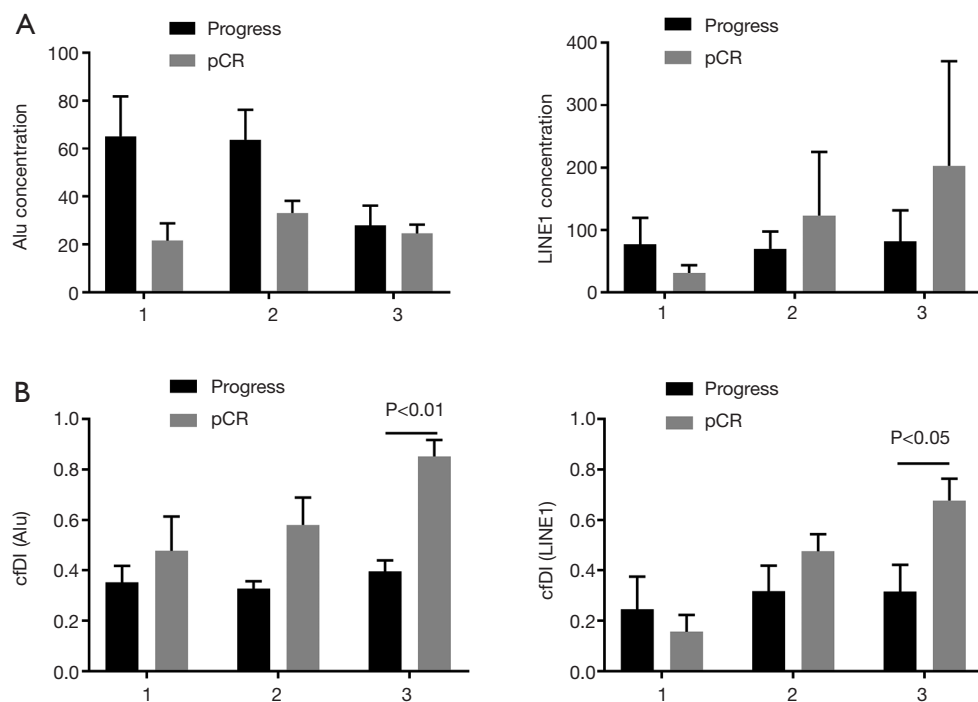


Figure 5 Comparisons of biomarkers between patients with a pathologically complete response (pCR) and those with distant metastasis after surgery. (A) Comparisons of cell-free DNA (cfDNA) concentrations between patients with a pCR and those with distant metastasis after surgery; (B) comparisons of cell-free DNA integrity (cfDI) between patients with a pCR and those with distant metastasis. Arabic numerals represented three assessment time points. The first time point was before the first chemotherapy, and the last time point was after the last chemotherapy. The intermediate time points were selected according to the neoadjuvant chemotherapy (NACT) duration. For patients receiving three to four cycles of NACT, data after the second cycle were used, and for patients receiving eight cycles of NACT, data after the fourth cycle were used.

with breast cancer receiving NACT. We found that these indicators had no significant correlation with the clinical and pathological characteristics of patients before NACT. The cfDI value increased significantly after treatment; however, the changes in the cfDNA concentration and *CA153* were not significant. When patients received NACT, both tumor volumes and proliferative capacities decreased significantly. Correlation analysis showed that tumor shrinkage and reduced Ki67 levels correlated positively with an increased cfDI value. The results also suggested that the number of pathologically detected metastatic lymph nodes after surgery correlated inversely with the cfDI measured after NACT. Our results also suggested that a gradual increase in the cfDI during NACT might suggest a better treatment response. Conversely, if a patient's cfDI did not change significantly, it might indicate a poor response and eventual recurrence.

Recently, many studies have been carried out to

investigate the potential of the cfDNA concentration and cfDI as new tumor biomarkers. However, previous studies formed inconsistent conclusions concerning these two markers in patients with cancer (16-18). There are several partial explanations for this contradiction. First, the source of cfDNA is unclear. Some studies believed that an 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 (19,20). Apoptotic cells release DNA fragments that are usually 185–200 bp long, while DNA fragments from necrotic cells vary in size and can even be several kilobase pairs (21). Different sources have been proposed as the cause of the increased cfDI observed in patients with cancer (22). However, an increasing number of reports have shown that cfDNA in patients with cancer is highly variable and mainly comprises short DNA molecules (<200 bp), which preferentially carry tumor-associated gene aberrations (23).

Our previous study also confirmed the presence of short DNA fragments, as well as decreased cfDI values, in patients with breast cancer compared with those with benign breast tumors (24).

Although the significance of the cfDNA concentration and cfDI in the diagnosis of cancer has been widely studied, the predictive and prognostic values of these biomarkers in patients receiving NACT have only been investigated in a small number of studies. Previous studies mainly identified a decreasing cfDNA level as a marker for early treatment response, e.g., in lung cancer (25) or rectal cancer during neoadjuvant therapy (26); however, cfDI has not yet been addressed for this indication. Lehner *et al.* reported that the kinetics of Alu levels during NACT showed decreases in patients with complete remission, while in patients who showed no change in their disease, increased Alu levels were observed ($P=0.033$). The authors also demonstrated that cfDI and *CA153* were not informative for therapy outcome (27). The findings of the present study showed contradictory results to those of previous studies. Although there was no statistically significant change in the cfDNA concentration, it showed an increasing trend after NACT. We speculated that this could partly be attributed to the fact that the tumors destroyed by cytotoxic drugs could release large amounts of DNA molecules into circulation that exceeded the degradation rate of deoxyribonucleases. Besides, DNA molecules released by damaged or necrotic tumor cells trended to be long fragments, which would explain why the cfDI increased significantly after NACT. In addition, the number of metastatic lymph nodes correlated inversely with the cfDI in the blood of patients after NACT in this study. This phenomenon suggested that patients with breast cancer with lymph node metastasis often have a greater tumor burden, which might lead to an increase in shorter DNA fragments in blood and consequently lower cfDI values. However, this speculation requires confirmation by further research.

To some extent, our results confirmed the potential of cfDI to monitor the therapeutic response of breast cancer to NACT. However, some limitations were present. First, the sample size of this study was small, and it was a retrospective study. Randomized controlled studies with larger sample sizes are needed to further confirm the significance of cfDI in the background of NACT. Second, we did not further explore whether NACT regimens had effects on the cfDNA concentration or cfDI because of the small sample size. Finally, this was a preliminary study and the follow-up time was short. Although we showed some positive findings, the

results were not sufficiently persuasive.

In summary, this study demonstrated that cfDI had the potential to be used as an indicator to monitor the therapeutic response of breast cancer to NACT. However, because of the limitations of this study, more research is needed to explore the clinical application of cfDI in the context of NACT.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.08.05>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethical and scientific committee of the institution (2010-SR-091). Informed consent was obtained from all participants.

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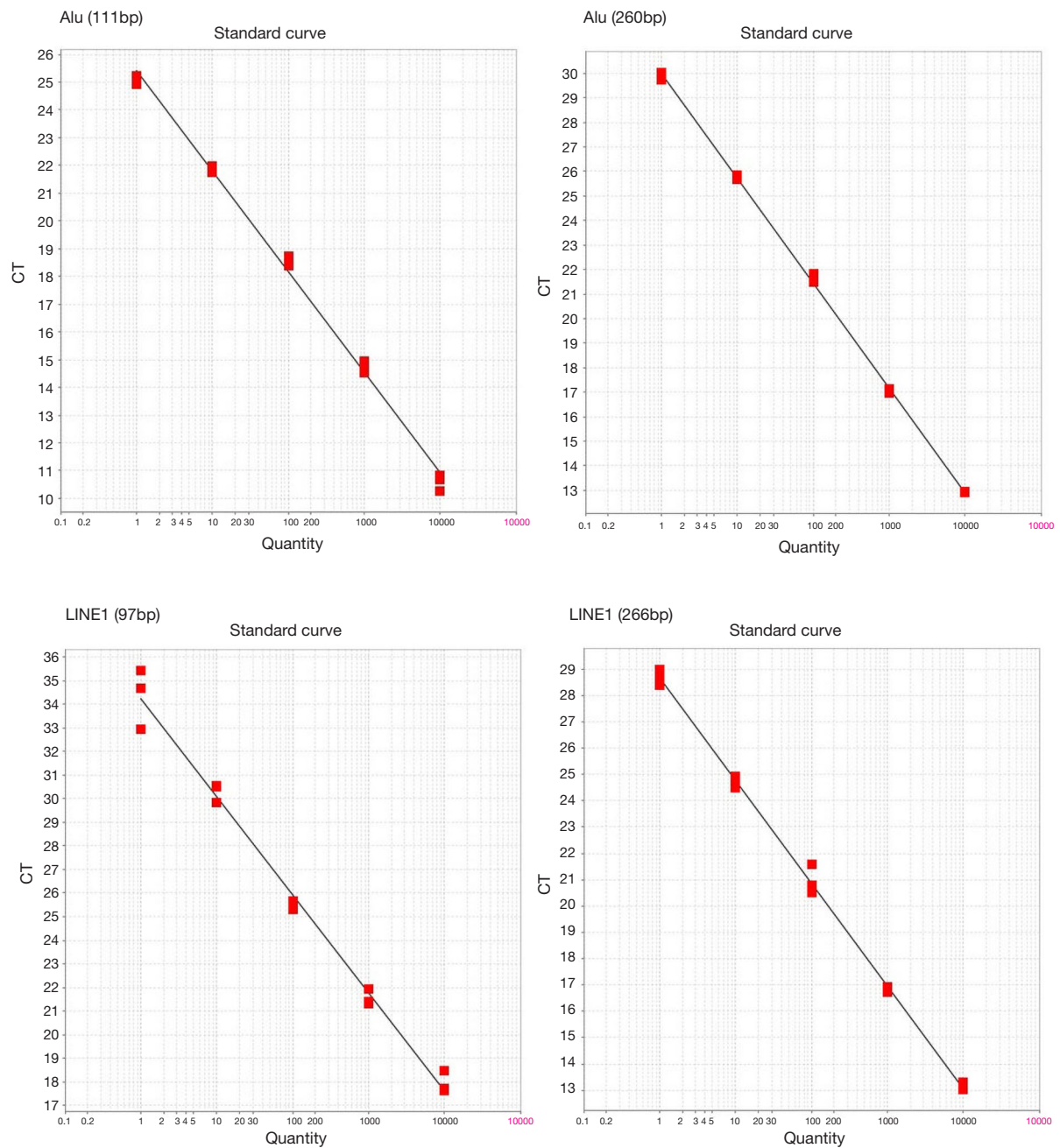


Figure S1 Standard curves of Alu and LINE1.

Table S1 Primer sequences

Gene	Sequence (5'-3')
Alu (111 bp)	Forward: CTGGCCAACATGGTGAAAC, reverse: AGCGATTCTCCTGCCTCAG
Alu (260 bp)	Forward: ACGCCTGTAATCCCAGCA, reverse: CGGAGTCTCGCTCTGTCTG
LINE1 (97 bp)	Forward: TGGCACATATACACCATGGAA, reverse: TGAGAATGATGGTTTCCAATTTC
LINE1 (266 bp)	Forward: ACTTGGAACCAACCCAAATG, reverse: CACCACAGTCCCCA GAGTG

Table S2 Clinical and histological characteristics of patients

Number	Age (year)	Menopause	Tumor size (cm)	Axillary lymph node status	ER	PR	HER-2	Ki67	CA153 (ng/mL)	NACT regimen
1	43	No	9	multiple suspicious lymph nodes	(+)	(+)	(+)	20%	62.82	TEC ×3
2	41	No	3	1 suspicious lymph nodes, 2.0 cm × 2.0 cm	(+)	(−)	(−)	60%	19.28	EC ×4-T ×4 (14 days)
3	43	No	6	2 suspicious lymph nodes, 1.5 cm × 1.5 cm, 2.0 cm × 1.5 cm	(−)	(−)	(+)	35%	52.74	EC ×4-T ×4 (14 days)
4	43	No	5	no obvious abnormal lymph nodes	(+)	(+)	(−)	15%	22.55	EC ×4-T ×4 (14 days)
5	42	No	15	1 suspicious lymph nodes, 1.8 cm × 1.5 cm	(−)	(−)	(−)	70%	9.35	EC ×4-T ×4 (14 days)
6	32	No	10	2 suspicious lymph nodes, 2.0 cm × 1.5 cm, 1.8 cm × 1.5 cm	(−)	(−)	(+)	50%	37.25	TCH×3
7	60	Yes	10	1 suspicious lymph nodes, 3.0 cm × 2.0 cm	(−)	(−)	(+)	80%	86.15	TEC ×4
8	29	No	3	no obvious abnormal lymph nodes	(−)	(−)	(+)	10%	9.55	EC ×4-TH×4
9	47	No	17	1 suspicious lymph nodes, 2.0 cm × 1.5 cm	(−)	(−)	(+)	20%	9.18	EC ×4-TH×4
10	44	No	5	multiple suspicious lymph nodes	(−)	(−)	(+)	60%	25.88	TEC ×3
11	41	No	4	1 suspicious lymph nodes, 2.0 cm × 1.0 cm	(+)	(+)	(+)	25%	33.21	TEC ×3
12	52	Yes	10	no obvious abnormal lymph nodes	(−)	(−)	(+)	30%	20.68	EC ×4-T ×4 (14 days)
13	64	Yes	7	no obvious abnormal lymph nodes	(−)	(−)	(−)	50%	18.07	FEC ×3
14	49	No	5	2 suspicious lymph nodes	(−)	(−)	(−)	5%	65.55	FEC ×4
15	38	No	7	no obvious abnormal lymph nodes	(−)	(−)	(+)	30%	43.89	TEC ×3
16	36	No	6	no obvious abnormal lymph nodes	(+)	(+)	(+)	15%	17.48	TEC ×3
17	30	No	8	no obvious abnormal lymph nodes	(−)	(−)	(+)	50%	22.05	EC ×4-T ×4 (14 days)
18	47	No	6	1 suspicious lymph nodes, 2.0 cm × 1.0 cm	(+)	(+)	(−)	5%	21.69	FEC ×3
19	49	No	3	no obvious abnormal lymph nodes	(+)	(+)	(+)	20%	8.15	FEC ×3
20	33	No	7	no obvious abnormal lymph nodes	(−)	(−)	(+)	20%	33.25	TEC ×3
21	61	Yes	7	multiple suspicious lymph nodes	(+)	(+)	(+)	45%	76.16	EC ×4-TH×4
22	45	No	6	1 suspicious lymph nodes, 1.5 cm × 1.2 cm	(+)	(+)	(−)	70%	9.42	EC ×4-TH×4
23	30	No	3	no obvious abnormal lymph nodes	(+)	(+)	(+)	5%	9.29	EC ×4-TH×4
24	34	No	Diffuse mass	2 suspicious lymph nodes, 1.6 cm × 1.2 cm, 1.0 cm × 1.0 cm	(+)	(−)	(+)	30%	22.87	TEC ×3
25	67	Yes	5	1 suspicious lymph nodes, 2.2 cm ×1.8 cm	(−)	(−)	(+)	30%	22.11	EC ×4-TH×4
26	52	No	Diffuse mass	multiple suspicious lymph nodes	(−)	(−)	(+)	50%	7.22	FEC ×3
27	59	Yes	7	multiple suspicious lymph nodes	(+)	(+)	(−)	15%	24.65	TEC ×8
28	52	No	3	2 suspicious lymph nodes, 2.8 cm × 2.0 cm, 1.8 cm × 1.5 cm	(+)	(+)	(−)	20%	12.04	TEC ×8
29	55	Yes	6	no obvious abnormal lymph nodes	(+)	(+)	(+)	20%	13.13	FEC ×3

TEC ×3, TEC ×4, TEC ×8: 3, 4 or 8 cycles of docetaxel/epirubicin/cyclophosphamide; EC ×4-T ×4 (14 days): 8 cycles of epirubicin/cyclophosphamide followed by paclitaxel every 2 weeks; EC ×4-TH ×4: 8 cycles of epirubicin/cyclophosphamide followed by paclitaxel plus trastuzumab; FEC ×4, FEC ×3: 3 or 4 cycles of fluorouracil/epirubicin/cyclophosphamide; TCH ×3: 3 cycles of docetaxel/carboplatin/trastuzumab. All patients were expected to complete all chemotherapy before surgery. However, some patients strongly required early operation only after 3–4 cycles due to excessive psychological pressure; Some patients were reluctant to continue chemotherapy because of severe side effects; and since this is a retrospective study, it was unknown exactly why individual patients did not complete standard neoadjuvant chemotherapy. But look at the medical records, we found that most of the patients had completed the rest of the chemotherapy after the surgery.

Table S3 Multivariate analysis between cfDNA concentration or cfDI and clinical characteristics

Variable	Alu conct		cfDI-Alu		LINE1 conct		cfDI-LINE1		CA153	
	95% CI	P	95% CI	P	95% CI	P	95% CI	P	95% CI	P
Age	−3.82 to 6.45	0.60	−0.02 to 0.01	0.72	−6.42 to 12.60	0.51	−0.02 to 0.00	0.41	−1.21 to 1.88	0.66
Menopause	−80.33 to 155.67	0.51	−0.36 to 0.37	0.97	−129.69 to 307.35	0.41	−0.25 to 0.29	0.87	−39.79 to 31.31	0.81
ER	−177.47 to 78.88	0.43	−0.76 to 0.03	0.07	−251.67 to 223.04	0.90	−0.41 to 0.18	0.42	−42.58 to 34.65	0.83
PR	−108.61 to 158.29	0.70	0.04 to 0.86	0.04	−174.24 to 320.02	0.55	−0.12 to 0.49	0.23	−37.72 to 42.70	0.90
HER-2	−66.34 to 80.97	0.84	−0.24 to 0.22	0.94	−64.29 to 208.50	0.28	−0.32 to 0.01	0.07	−12.57 to 31.81	0.38
Ki67	−250.68 to 79.42	0.29	−0.66 to 0.36	0.54	−353.21 to 258.08	0.75	−0.31 to 0.45	0.70	−37.26 to 62.19	0.61
Tumor size	−8.44 to 11.81	0.73	−0.05 to 0.00	0.14	−10.62 to 26.88	0.38	−0.03 to 0.02	0.85	−3.15 to 2.95	0.95

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; conct, concentration.