Diagnostic Value of Serum Concentration and Integrity of Circulating Cell-Free DNA in Breast Cancer: A Comparative Study With CEA and CA15-3

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ABSTRACT

Breast cancer (BC) is one of the most common types of malignant neoplasm in women; the incidence of BC increases yearly. In a previous study, a novel and sensitive method for quantitying cell-free DNA (CFD) in human blood was established and tested for its ability to predict which patients harbored tumors. Our objective in this study was to investigate the clinical value of serum concentration and the integrity of circulating free DNA (CFD) as a biomarker for auxiliary diagnosis of BC. The concentration of CFD was quantitated by branched DNA (bDNA)–based Alu assay. Carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3) concentrations were determined via Abbott ARCHITECT I2000 SR testing. We report that the median (quartile interval) values of serum ALU115 and ALU247/115 in patients with BC were significantly higher than those in patients with benign mammary hyperplasia and in healthy control individuals (1083.66 ng/mL [1.81] vs 145.87 ng/mL [0.33] and 228.19 ng/mL [0.48]; P <.001); there was no significant difference between the latter 2 groups (P> .05). The cutoff values of ALU115, ALU247/115, CEA, and CA15-3 were set as 300.96 ng per mL, 0.78, 5 ng per mL, and 31.3 ng per mL, respectively. The area under the receiver operating characteristic (ROC) curve was 0.70 (95% confidence interval [CI], 0.58–0.81), 0.97 (<.001–>.99), 0.75 (0.65–0.86), and 0.89 (0.82–0.96), respectively. Combined detection of the 4 indices significantly improved the diagnostic accuracy of BC, with sensitivity of 97.5% and negative predictive value of 96.4%. Also, serum ALU115 was significantly correlated with lymph-node metastasis (P = .048), and the ALU247/115 index was significantly correlated with tumor stage (P = .001) and lymph-node metastasis (P = .008) in patients with BC. Serum cell-free DNA (CFD) and its integrity may prove to be useful biomarkers for auxiliary diagnosis, grading of malignant neoplasms, and prognostic prediction of BC.

Keywords: breast cancer, cell-free DNA (CFD), ALU sequence, integrity, ALU-qPCR, diagnose

Breast cancer (BC) is one of the most common malignant neoplasms in women; its incidence increases yearly. Early diagnosis and prevention of BC is of great significance in improving the long-term survival and quality of life of patients with the disease. The value of traditional tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3) for BC screening has been generally recognized. However, the low sensitivity of those markers, yield of information that leads to misdiagnosis of early-stage tumors, and lack of ability to distinguish between recurrence and metastasis have limited the wider applications of those markers. Studies.[1,2] have demonstrated that circulating free DNA (CFD) is a type of extracellular DNA that occurs mainly from cell apoptosis and necrosis. Normal tissues may release a small amount of homogenous short-segment DNA through apoptosis, whereas the cell-free DNA (cf-DNA) content in patients with cancer is increased significantly. Some studies reported that CFD could be used for malignant grading, postoperative monitoring, and prognostic prediction in patients with cancer. In this study, we used the ALU–quantitative polymerase chain reaction (qPCR) method to detect the concentration and integrity index of serum CFD in patients with BC,

Abbreviations

BC, breast cancer; CEA, carcinoembryonic antigen; CA 15-3, carbohydrate antigen 15-3; CI, confidence interval; CFD, circulating free DNA; cf-DNA, cell-free DNA; qPCR, quantitative polymerase chain reaction; Ct, cycle threshold; bDNA, branched DNA; CLIA, chemiluminescence immunoassay; ER, estrogen receptor; PR, progesterone receptor; PPV, positive predictive value; NPV, negative predictive value; IQR, interquartile range; AUC, area under the receiver operating characteristic (ROC) curve; ROC, receiver operating characteristic

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patients with benign mammary hyperplasia, and healthy control individuals, to explore the application value of this method in auxiliary diagnosis, grading of malignant neoplasms, and prognostic prediction of BC.

Materials and Methods

Subjects

Included in this study were 40 patients who were pathologically diagnosed with BC between the years 2014 and 2015 in our hospital. They ranged in age from 28 years to 64 years, with a median of 48 years. According to TNM tumor staging, there were 5, 8, 10, 11, and 6 cases of tumors at stages 0 (orthotopic carcinoma), I, II, III, and IV, respectively. Data from 40 patients with benign mammary hyperplasia who were between the ages of 21 years and 68 years (median, 46 years) and 40 healthy women between the ages of 23 years and 64 years (median, 45 years) were compared. Patients with autoimmune diseases and/or tissue injuries were excluded from the study. This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (Nantong, China). Oral informed consent was obtained from all participants.

Reagents and Instruments

Reagents and instruments used in this study included the free DNA Extraction Kit (Xi'an Tianlong Technology Co, Ltd), CEA and CA15-3 (determined by chemiluminescence immunoassay; Abbott Laboratories Inc), human genomic DNA standards (Promega Corporation), Light Cycler 480 SYBR Green I Master (F. Hoffman-La Roche Ltd), and qPCR primers ALU115 and ALU247 (Sangon Biotech Co, Ltd).

Specimen Collection

Fasting venous blood (3 mL) was collected in a negative pressure vacuum tube, tranquillized for 10 minutes until clotted, and centrifuged at 1600*g* for 10 minutes at 4℃. The resulting serum was collected in an autoclaved Eppendorf tube and stored at –80℃ before use.

Serum CFD Extraction

Free DNA extraction and purification were completed according to the free DNA extraction kit instructions (magnetic bead method) and the magnetic beads adsorption principle. The transfer of magnetic beads/nucleic acid was achieved from the adsorption, with the transfer and release of beads performed by use of a special magnetic bar. The eluent solutions were transferred to a clean nuclease-free centrifuge tube and stored at –20℃.

Quantification of CFD

Primer design: Primer P1/P2 (for ALU115) and P3/P4 (for ALU247) were designed using Oligo 6 software. The sequences (5′-3′) were as follows: P1 (forward) 5′-CCTGAGGTCAGGAGTTCGAG-3′, P2 (reverse) 5′-CCCGAGTAGCTGGGATTACA-3′, P3 (forward) 5′-GTGGCTCACGCCTGTAATC-3′, P4 (reverse) 5′-CAGGCTGGAGTGCAGTGG-3′. The final concentration of the primer working solution (50 μmol) was stored at –20℃.

Standard solution preparation: The human genomic DNA solution with a known concentration (180 μg/mL) was diluted 10-fold sequentially as the control substance with the final concentration being 18000, 1800, 180, 18, 1.8, and 0.18 ng per mL. The specimens to be tested and the standards were amplified in the same fluorescence qPCR experiments. The standard curve was generated by cycle threshold (Ct) values of standards; concentrations of specimens were calculated by use of the standard curve.

Specimen loading and amplification: To determine the concentration of serum CFD and evaluate its integrity, the long (247-bp) and short (115-bp) sequences were amplified based on ALU sequence. The ratio of long and short fragments was an indicator of serum CFD integrity. PCR reaction conditions were as follows: 95°C for 10 minutes, 95°C for 15 seconds, and 64°C for 30 seconds, during 35 cycles. The melting curve of 64°C to 95°C fluorescence signals was used to verify the specificity. Distilled water was used as the blank control substance. The experiment was performed 3 times, and the mean of the 3 results was considered as 1 measurement.

Detection of Serum CEA and CA15-3

CEA and CA153 levels in the specimens were measured using the chemiluminescence method. (Chemiluminescence-method detection involves use of a kind of immunoassay that is directly labeled as antibody or antigen by emitting light material instead of radionuclides or enzymes.)

Statistical Analysis

We performed statistical analysis using SPSS software, version 17.0 (IBM). The CFD concentration and completeness of each group were expressed as median (quartile interval). We used Mann-Whitney *U* testing to compare the specimens between groups. The Fisher exact probability method was used to analyze the relationship between the clinical pathological parameters and the serum CFD concentration and integrity. We used ROC-curve calculation to evaluate the value of CFD detection as the auxiliary diagnosis of BC, with statistical significance when $P < .05$.

Results

Detection of Serum Levels of ALU115 and ALU247/115 Index

The results of Mann-Whitney *U* testing (**Figure 1**) showed that the median serum ALU115 level and ALU247/115 index of the patients newly diagnosed with BC were 1083.66 ng per mL (342.87 to 2248.42 ng/mL) and 1.81 ng per mL (0.52 to 3.95 ng/mL), respectively. These values were significantly higher than those in patients with benign mammary hyperplasia: 145.87 ng per mL (87.45 to 357.92 ng/mL) and 0.33 ng per mL (0.26 to 0.55 ng/mL) and healthy controls: 228.19 ng per mL (123.47 to 597.1 ng/mL) and 0.48 ng/mL (0.21 to 0.55 ng/mL) (both $P < .001$). There was no significant difference between the latter 2 groups ($P > .05$).

Evaluation of ROC Curves of Serum ALU115 and ALU247/115 Index for BC Diagnosis

In the 40 patients with BC and 40 healthy controls, the serum ALU115 and ALU247/115 indices were quantitated by branched DNA (bDNA)–based Alu assay; CEA and CA15-3 levels were detected by chemiluminescence immunoassay (CLIA). The cutoff value of ALU115 level, ALU247/115 index, and CEA and CA15-3 concentrations was set as 300.96 ng per mL, 0.78, 5 ng per mL, and 31.3 ng per mL, respectively. The area under the ROC curve was 0.70 (95% CI, 0.58–0.81; $P = .002$), 0.97 (<.001–>.99; $P < .001$), 0.75 (0.65–0.86; $P < .001$), and 0.89 (0.82–0.96, $P < .001$), respectively. Data are shown in **Figure 2** and **Table 1**.

The Value of Combined Detection of Serum ALU115, ALU247/115 Index, CEA, and CA15-3 in Patients With BC

When we tested the 4 indices separately, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and total efficiency of the serum ALU 247/115 index in patients with BC were 92.5%, 87.5%, 88.1%, 92.1%, and 90%, respectively. When the 4 indices were tested in combination, the sensitivity increased to 97.5%, and the NPV increased to 96.4% (**Table 2**).

Correlations of Serum ALU115 and ALU247/115 Index With Clinicopathological Parameters of Patients With BC

According to the median value of ALU115 and ALU247/115 index, we categorized patients as being in the high-level group (n = 20) or the low-level group (n = 20). The results showed that high levels of serum ALU115 were significantly correlated with lymph-node metastasis ($P = .048$), but not with the tumor stage and the positive rate of estrogen receptor (ER), progesterone receptor (PR), and CerbB-2 (HER2/neu) ($P > .05$). Meanwhile, a high ALU247/115 index was significantly correlated with lymph-node metastasis ($P = .008$) and tumor stage, but not with the positive rate of ER, PR, and CerbB-2 ($P > .05$) (**Table 3**).

Discussion

CFD is a type of decellularized extracellular DNA, mainly derived from apoptosis and necrosis. Normal tissues may release a small amount of homogenous short-segment (185 bp–200 bp) DNA through apoptosis. The concentration and composition of CFD may vary with the pathological state. In the process of tumor-cell necrosis, the CFD level in peripheral blood is abundant so that they can not be digested by deoxyribonuclease completely. Therefore, the genomic DNA fragments show a variety of lengths, with long DNA fragments predominating.[3–5] In recent years, CFD detection and clinical application have attracted increased attention. Various methods of detecting CFD have emerged, including some with high sensitivity and throughput.[6,7]

Umetani et al[8] established a qPCR method based on the ALU sequences. Other researchers[9–12] have

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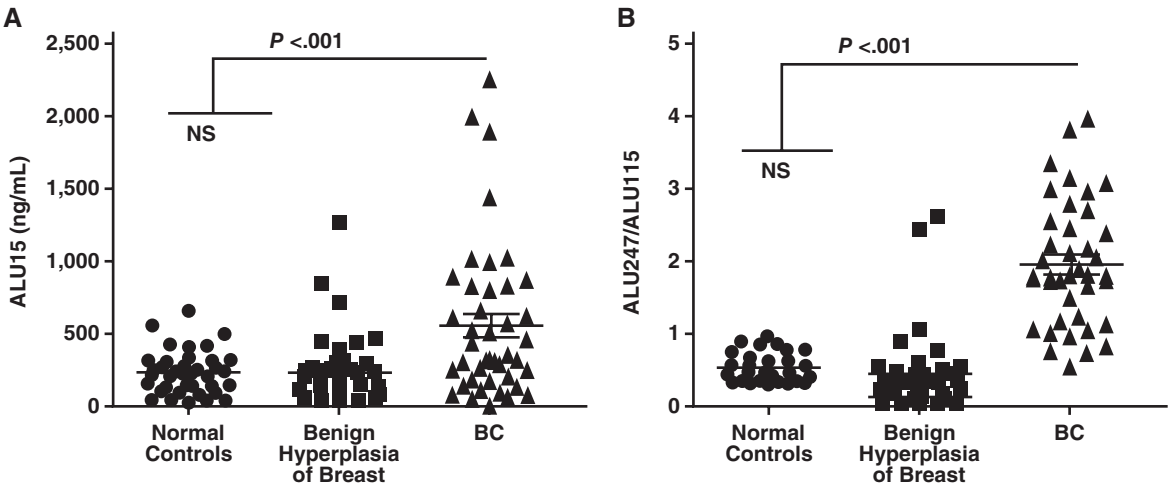

Figure 1

Comparison of ALU115 and ALU247/115 index between 3 different groups: serum ALU115 level (**A**) and integrity index ALU247/115 (**B**) of patients newly diagnosed with breast cancer (BC; n = 40), benign mammary hyperplasia group (n = 40), and healthy controls (n = 40) detected by ALU–quantitative polymerase chain reaction (qPCR). NS indicates no statistically significant difference.

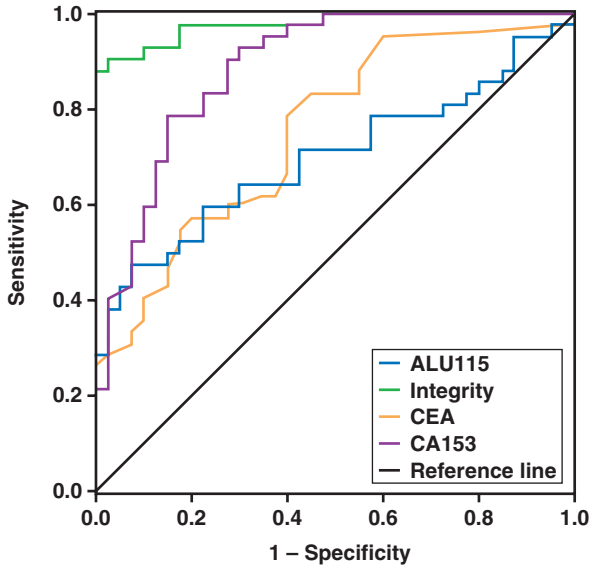

Figure 2

Receiver operating characteristic (ROC) curves for the evaluation of all the indexes for the diagnosis of breast cancer.

conducted systematic research on the ALU concentration and integrity of CFD in various malignant diseases, such as colorectal cancer, melanoma, prostate cancer, and malignant pleural effusion, showing that the CFD concentration and integrity can be used as potential biomarkers for early diagnosis, staging and grading of tumors, and monitoring radiotherapy and chemotherapy.

In this study, we examined the serum levels of ALU115, ALU247/115, CEA, and CA15 in 40 patients newly diagnosed with BC, 40 patients with benign mammary hyperplasia, and 40 healthy controls, and evaluated the diagnostic usefulness of those serum-level values in BC. The results showed that serum ALU115 level and ALU247/115 index were significantly higher in patients with BC than those in healthy controls, but there was no significant difference between the latter 2 groups, indicating that the increased serum ALU115 concentration and ALU247/115 index had some correlation with the increased degree of breast lesions and may prove to be an objective indicator of BC auxiliary diagnosis. ROC-curve analysis showed that the area under the curve of ALU247/115 was the highest, reaching 0.97 (95% CI, <.001–>.99). When the 4 indices were tested separately, the serum ALU247/115 index was strongest in sensitivity, specificity, PPV, NPV, and total efficiency of BC diagnosis, suggesting that the ALU247/115 index was stronger than ALU115, CEA, and CA15 in diagnosing BC. When the 4 indices were detected in combination, the sensitivity (97.5%) and the NPV (96.4%) were significantly improved. These results suggest that the combination of

Table 1. Values of All Studied Markers for the Diagnosis of Breast Cancer

Indices: IQR, 25–75	Patients Newly Diagnosed with Breast Cancer (n = 40), No. (IQR)	Healthy Controls (n = 40), No. (IQR)	Cutoff Value	AUC	*P* Value
ALU115 (ng/mL)	1083.66 (342.87–2248.42)	228.19 (123.47–597.1)	300.96	0.70	.002
ALU247/115	1.81 (0.52–3.95)	0.48 (0.21–0.55)	0.78	0.97	<.001
CEA (ng/mL)	4.25 (1.53–4.70)	4.2 (1.20–2.80)	5.00	0.75	<.001
CA15-3 (ng/mL)	21.15 (11.83–25.45)	3.55 (3.68–10.53)	31.30	0.89	<.001

IQR, interquartile range; AUC, area under the receiver operating characteristic (ROC) curve; CEA, carcinoembryonic antigen; CA15-3, cancer antigen 15-3.

Table 2. Diagnostic Values for Single and Combined Detections

Index	No. (%)				
	Sensitivity	Specificity	PPV	NPV	Efficiency
ALU115	26/40 (65.0%)	28/40 (70.0%)	26/38 (68.4%)	28/42 (66.7%)	54/80 (67.5%)
ALU247/115	37/40 (92.5%)	35/40 (87.5%)	37/42 (88.1%)	35/38 (92.1%)	72/80 (90.0%)
CEA	21/40 (52.5%)	32/40 (80.0%)	21/29 (72.4%)	32/51 (62.7%)	53/80 (66.3%)
CA15-3	31/40 (77.5%)	34/40 (85.0%)	31/37 (83.8%)	34/43 (79.1%)	65/80 (81.3%)
Combined	39/40 (97.5%)	27/40 (67.5%)	39/52 (75.0%)	27/28 (96.4%)	66/80 (82.5%)

PPV, positive predictive value; NPV, negative predictive value; CEA, carcinoembryonic antigen; CA15-3, cancer antigen 15-3.

Table 3. Correlation Between CFD and Various Clinicopathological Parameters

Clinicopathological Parameters	Grouping	Total	Serum ALU115		*P* Value	Serum ALU247/115		*P* Value
			High (n = 20)	Low (n = 20)		High (n = 20)	Low (n = 20)	
Tumor staging	Tis+I+II	23	10	13	.52	7	16	.001
	III + IV	17	10	7		13	4	
Lymph-node metastasis	Yes	15	11	4	.048	12	3	.008
	No	25	9	16		8	17	
ER	Positive	30	17	13	.27	18	12	.06
	Negative	10	3	7		2	8	
PR	Positive	27	16	11	.18	15	12	.50
	Negative	13	4	9		5	8	
CerbB-2	Positive	24	14	10	.33	15	9	.10
	Negative	16	6	10		5	11	

CFD, cell-free DNA; ER, estrogen receptor; PR, progesterone receptor , Tis, tumor in situ, I and II stage.

the 4 indices had a certain reference value for the clinical diagnosis of BC, choice of treatment, and monitoring of treatment course.

Also, we explored the correlation of serum ALU115 and ALU247/115 with the clinicopathological parameters of the patients with BC and discovered that that serum ALU115 level was correlated with lymph-node metastasis in patients with BC, whereas the ALU247/115 index was closely correlated with tumor stage and lymph-node metastasis. Umetani et al[2] report that serum DNA integrity (ALU247/115 index) in patients with BC was significantly higher than that in healthy women and was highly correlated with tumor size, lymphatic-vessel invasion, lymph-node metastasis, and tumor stage, suggesting that this value could be used as a prognostic indicator. Their results are consistent with our findings, suggesting that the serum ALU115 and ALU247/115 indices have potential reference value for assessing disease status, malignant-neoplasm grade, and prognosis in patients with BC.

Our results have demonstrated a definite value of serum ALU247/115 in the clinical diagnosis of BC. With the deepening of research and the rapid development of medical transformation, the value of CFD in the process

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of BC treatment should draw more widespread attention. However, due to the lack of standardized quantification of CFD, testing to detect CFD has not been currently performed on a large scale in clinical practice. Therefore, more large-sample and longer-term clinical trials are required to further validate the clinical application value of the CFD concentration and integrity in prognostic prediction and postoperative disease monitoring of patients with BC. **LM**

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