Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Clinical value of plasma cfDNA concentration and integrity in breast cancer patients

Yajun Miao[1], Yingrui Fan[2], Liang Zhang[1], Tingting Ma[2], Rong Li[2*]

[1] Department of Oncology, the First People's Hospital of Nantong. No.6, Haierxiang North Road, 226001 Nantong, Jiangsu Province, China
[2] Department of Oncology, TaiKang XianLin Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School. No.188, Lingshan North Road, Qixia District, 210046 Nanjing, Jiangsu Province, China

*Correspondence to: sahselek@gmail.com

Received April 16, 2019; **Accepted** July 30, 2019; **Published** July 31, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.6.11

Abstract: Breast cancer is a malignant tumor that occurs in the glandular epithelial tissues of the breast. It is one of the most common malignant tumors in women. This study was aimed at investigating the role of cell-free DNA (cfDNA) as a potential biomarker for breast cancer diagnosis. Patients with primary breast cancer (n =110) were enrolled in the experimental group, 95 patients with benign breast tumors were in control group 1, while 90 healthy volunteers were in control group 2. Quantitative PCR was used to determine cfDNA concentration and integrity in each group. The cfDNA levels in different groups and their relationship with clinical features of breast cancer patients were analyzed. Receiver operational curves were established to analyze sensitivity and specificity of cfDNA concentration, cfDNA integrity, CEA, CA125 and CA15-3. The cfDNA concentration and cfDNA integrity of the experimental group were significantly higher than those of control groups 1 and 2. The cfDNA concentration and integrity in plasma of experimental group after chemotherapy were significantly lower than those before chemotherapy. While CEA and CA15-3 expressions were significantly correlated with cfDNA concentration, CA125 expression was significantly correlated with cfDNA integrity. Results from ROC curve analysis showed that the sensitivity and specificity of cfDNA concentration and integrity were higher than those of traditional tumor biomarkers. These results indicate that cfDNA concentration and integrity are significantly higher in primary breast cancer patients than in benign breast tumor patients and healthy people. Thus, cfDNA may serve as a potential biomarker of breast cancer.

Key words: Breast cancer; cfDNA; Receiver operational curve; qRT-PCR.

Introduction

Breast cancer is a malignant tumor that occurs in the glandular epithelial tissues of the breast. It is one of the most common malignant tumors in women. According to the latest cancer data from the 2017 China Cancer Registry Annual Report published by the National Cancer Center, the incidence of breast cancer ranks first in female cancer patients. The incidence and mortality of breast cancer are increasing year by year (1). This trend is due to lack of effective early screening methods (2). Most patients are diagnosed with cancer after the symptoms become obvious, by which time the tumors have become advanced. Early diagnosis and treatment of breast cancer patients enhance their survival. Therefore, screening molecular markers for early diagnosis and treatment of breast cancer patients has become a major research focus aimed at reducing breast cancer mortality (3). At present, there are many methods for clinical diagnosis of breast cancer. Determination of blood tumor markers has been widely used in the diagnosis of breast cancer because of its advantages of non-invasiveness and ease of operation, and its usefulness in the early diagnosis and screening of breast cancer. A tumor marker is a substance that is characteristically present in a malignant tumor cell or is abnormally produced by a malignant tumor cell. It is a reflection of tumorigenesis, and it is used to monitor tumor response to treatment. Tumor markers exist in tissues, body fluids, and feces of tumor patients and has important reference value for the diagnosis of tumors, screening for high-risk populations, and monitoring of tumor prognosis (4, 5). They can be assayed using immunohistochemistry, biology and chemical methods. With deep understanding of breast cancer and the continuous improvements in laboratory technology, the role of tumor markers in the occurrence, development and spread of breast cancer, and their impact on prognosis are receiving more and more attention. Moreover, new ideas and methods for the treatment of breast cancer have been proposed; and tumor markers can be used to effectively evaluate the therapeutic efficacy on breast cancer patients , and even predict their prognosis (6). The presence of tumor markers, and quantitative changes in their levels may reflect the nature of the tumor, and may assist in understanding tumor tissue formation, cell differentiation and cell function. Tumor markers are helpful in the diagnosis, classification, prognosis and treatment of tumors.

The currently used tumor markers for diagnosis of breast cancer include serum carcinoembryonic antigen CEA, carbohydrate antigen 125 (CA125), and carbohydrate antigen 153 (CAl53) (7, 8). Studies have found that the tumor markers CAl53, CEA, CAl25, and CAl99 are used to predict the recurrence and metastasis of breast cancer, and for monitoring the prediction of its prognosis. One of the tumor markers, CAl53 is currently

recognized as a tumor marker for the diagnosis of breast cancer (9). The traditional detection of these tumor markers is useful in the clinical diagnosis and treatment of breast cancer. Diagnostic performance based on combination of multiple markers is better and more reliable than single index detection. However, their sensitivity is too low for early diagnosis, effective treatment decision and prognosis monitoring.

Cell-free DNA (cfDNA) is a novel cancer marker that exists in the blood in cell-free form. Studies have shown that increased cfDNA levels can be detected in the plasma of patients with colon cancer, lung cancer, head and neck cancer, and ovarian cancer, while in some cases, elevated cfDNA levels are considered poor prognostic factor (10, 11). Studies on cfDNA in breast cancer in China are limited. The present study was carried out to investigate the value of cfDNA in the diagnosis and treatment of breast cancer, relative to traditional tumor markers, with a view to determining the potential of cfDNA as a biomarker for early breast cancer diagnosis and prognosis.

Materials and Methods

Patients

The study subjects included selected female cases from Chengde City Center Hospital from October 2016 to October 2018, including 110 patients with primary breast cancer, 95 patients with benign breast tumors and 90 healthy women who served as control. The experimental group was made up of 110 patients with primary breast cancer, aged 23 to 70 years (mean age = 35.2±10.3 years). *Inclusion criteria*: (1) women with primary breast cancer treated with chemotherapy; (2) patients with clear pathological diagnosis; (3) breast cancer patients without trauma, acute inflammation or dehydration **;** (4) patients who voluntarily accepted to take part in the study, and who signed informed consent. A total of 95 patients with benign breast tumors were enrolled in the control group 1, with ages ranging from 22 to 69 years (mean age = 33.8±8.6 years). *Inclusion criteria*: (1) patients with benign breast tumors treated with chemotherapy in the same period as the experimental group; (2) patients with clear pathological diagnosis of benign breast cancer; (3) breast cancer patients without trauma, acute inflammation or dehydration; (4) benign breast cancer women whose ages and baseline data were comparable with those of the experimental group; (5) patients who voluntarily accepted to take part in the study, and who signed informed consent. A group of 90 healthy women served as control group 2. Their ages ranged from 20 to 71 years (mean age = 31.9±7.6 years). *Inclusion criteria*: (1) healthy women who had a physical examination at the same time as the experimental group; (2) women who had no tumor based on physical examination and at least 3 months after the physical examination; (3) women who were without trauma, acute inflammation or dehydration ; (4) women with ages and baseline data comparable with those of the experimental group; (5) those who voluntarily accepted to participate in the study, and signed informed consent. *Exclusion criteria*: (1) pregnant women; (2) patients with autoimmune disease e.g. systemic lupus erythematosus; (3) patients undergoing organ transplantation (including bone marrow); (4) patients who had stroke, cerebral hemorrhage or cerebral infarction patients. The study was approved the Ethics Committee of TaiKang XianLin Drum Tower Hospital, and was carried out in accordance with Declaration of Helsinki.

Chemotherapy regimens

A 3-hour intravenous infusion of paclitaxel (Italy Corden Pharma Latine S.P.A.; lot number: 150922, 160310, 160917) was given at a dose of 135 mg/m^2 on the first day, and on the second day, carboplatin (China Qilu Pharmaceutical Co., Ltd.; lot number: 151115, 160523, 161025) was added at a dose of 300 mg/m^2. One course of treatment was given for 3 weeks, and 3 continuous treatment courses were used.

Plasma separation and cfDNA extraction

Blood was taken from the experimental group 1 week before chemotherapy and 4 weeks after chemotherapy for preparation of plasma, while blood was collected 1 week before chemotherapy in control group for plasma preparation. In control groups 1 & 2, blood for plasma preparation was taken at the same time. In each case, 5 ml of venous blood was collected in EDTA tubes, and the blood sample was centrifuged at 1600g for 10 min at room temperature. Then, 1.5 mL of the supernatant was carefully pipetted into an Eppendorf tube, and centrifuged at 16000 g for 10 minutes at 4 ℃. The plasma sample to be tested was diluted 10-fold with Tris-EDTA buffer and used as a direct amplification template for plasma (to ensure the accuracy of sampling dilution, the minimum sampling volume was 10 ul. Human DNA Standard (100 ng/ul) was first diluted 5 times with SDB to a final stock concentration of 20 ng/ul; then 7 different concentrations of the standard were prepared via serial dilution of the standard (Table 1).

Table 1. Dilution of **s**tandard DNA.

Standard sample	Concentration (ng/ul)	Minimum dilution volume (µL)
Stock	20	10 (100ng/ul Human DNA Standard+90 SDB
Std.1	1	10 (20 ng/uL stock) + 190 TE
Std.2	0.25	20 (Std. 1) + 60 TE
Std.3	0.0625	20 (Std. 2) + 60 TE
Std.4	0.015625	20 (Std. 3) + 60 TE
Std.5	0.00390625	20 (Std. 4) + 60 TE
Std.6	0.000976563	520 (Std. 5) + 60 TE
Std.7	0.000244141	20 (Std. 6) + 60 TE

Quantitative Polymerase Chain Reaction

Quantitative real time polymerase chain reaction (qRT-PCR) was performed on a Light Cycler LC480 PCR machine (Roche Molecular Systems, Inc. Pleasanton, CA, USA). To measure the concentration of plasma cfDNA, the long interspersed nuclear element 1 (LINE1) 97bp and LINE1 259bp DNA fragments were amplified according to the method described by Diehl (12) The LINE1 97bp primer amplified apoptotic and non-apoptotic DNA fragments, while the LINE1 259bp primer amplified non-apoptotic DNA fragments only. The total amount of plasma DNA was represented by the result of QPCR with LINE1 97bp primer. The DNA integrity index was calculated as the ratio of results of LINE1 259 and LINE1 97 QPCR. Serial dilutions of standard solution of human genomic DNA (Thermo Fisher Scientific, Waltham, MA, USA) were used for construction of a standard curve. The concentration of cfDNA in each sample was calculated from the standard curve. The qRT-PCR reaction was performed in triplicate and mean values of triplicate determinations were used for further analysis. The qRT-PCR reaction mixture (16μl) volume contained 1μl DNA template, 0.5 μl each of forward and reverse primers (LINE1 97 or LINE1 259), 10μl UltraSYBR Mixture (Cwbiotech, Beijing, China), and 4μl double-distilled water. Each plate consisted of a plasma DNA sample, a negative control (water template) and 7 serially diluted standard DNA solutions. Cycling conditions were 1 minute at 95°C, and 35 cycles of 95°C for 8 seconds, followed with 60°C for 15 seconds.

Determination of tumor biomarkers

Plasma samples were obtained by centrifugation of fasting venous blood, using fully automated Electrochemiluminometer E170 and assorted kits (Roche, Switzerland). The tumor biomarkers were detected using electrochemiluminescence. The reference values for each indicator were: CEA<3.5 ng/mL, CA15-3 < 25 U/ml, CA125< 35 U/ml, and CA199 < 39 U/ml.

Statistical analysis

The results of cfDNA quantification are expressed as mean ± standard deviation ($\bar{x}$ ± SD). Kruskal-Wallis rank sum test was used for comparison between groups. Count data were compared using *r*-test, while measurement data were compared using *t*-test. The ROC curve was used to assess cfDNA quantification as a screening tool for patients with breast cancer, and the area under the ROC curve was used to calculate the accuracy in differentiating between two different diseases for different critical values. All statistical analyses were done with SPSS 21.0 software. Values of $p < 0.05$ were considered statistically significant.

Results

Patient's clinical pathological features

The clinical characteristic of the patients are shown in Table 2. The mean body mass index (BMI) of the 110 female patients with primary breast cancer was 22.7 kg/m^2; 68 patients had BMI ≤ 24 kg/m^2, while 42 patients had BMI >24 kg/m^2. There were 72 breast cancer women with tumor diameters 5cm, while 38 women had tumor diameters > 5cm. All enrolled patients were clinically staged according to the TNM staging of the American Joint Committee on Cancer (AJCC). The staging showed that 42 patients were in stages I/II, while 68 patients were in stages III/IV. There were 73 cases of invasive ductal tumor, and 31 cases of non-invasive ductal tumor. The mean BMI of the 95 patients with benign breast tumors was 21.5 kg/m^2; 52 patients had BMI ≤ 24 kg/m^2, and 43 patients had BMI >24 kg/m^2. There were 54 breast cancer women with tumor diameters ≤

Table 2. Clinicopathological features of breast cancer patients.

Factor	Primary breast cancer patients (n)	Benign breast cancer patients (n)	P
Age			
≤35	45	36	0.6598
>35	65	59	
BMI			
≤24Kg/m^2	68	52	0.3048
>24Kg/m^2	42	43	
Tumor diameter			
≤5cm	72	54	0.2064
>5cm	38	41	
TNM stage			
I-II	42	32	0.5038
III-IV	68	63	
Pathological type			
Invasive ductal tumor	79	61	0.2431
Non-invasive ductal tumor	31	34	
CEA			
<3.5 ng/mL	29	38	0.0379
≥3.5 ng/mL	81	57	
CA15-3			
< 25 U/ml	25	35	0.0268
≥25 U/ml	85	60	
CA125			
< 35 U/ml	31	40	0.0367
≥ 35 U/ml	79	55	
CA199			
< 39 U/ml	40	46	0.0811
≥ 39 U/ml	70	49	

5cm, while 41 patients had tumor diameter > 5cm. staging revealed that 32 of the patients were in stages I/II, 63 cases were in stages III/IV, 61 patients had invasive ductal tumor, while 34 patients had non-invasive ductal tumor. There were no statistically significant differences in the clinicopathological features between primary tumor patients and those with benign breast tumor (p > 0.05), indicating that the two groups were comparable.

Results from assay of the 4 tumor biomarkers in the 110 patients with primary breast cancer showed that 29 patients were negative for CEA and 81 cases were CEA-positive. There were 25 patients with negative expression of CA15-3, while 85 patients had positive expression of CA15-3; 31 patients were CA125-negative, while CA125 expression was positive in 79 patients. There was negative expression of CA199 in 40 patients, while 70 patients had positive expression. In the benign breast tumors, there were 38 cases with negative expression of CEA, and 57 cases with positive expression; 35 patients had negative expression of CA15-3, while 60 patients were CA15-3-positive. Negative expression of CA125 was seen in 40 patients, while 55 patients were CA125-positive. The expression of CA199 was negative in 46 patients, while 49 patients were CA125-positive. There were statistically significant differences in the expressions of plasma CEA, CA15-3 and CA125 between the two groups, but there was no significant difference in the expression of CA199.

Concentration and integrity of cfDNA in plasma of healthy women and women with breast cancer

The cfDNA concentration of the experimental group before chemotherapy was 11.97±10.67ng/mL, and the integrity was 4.89±2.84. The cfDNA concentration of the experimental group with breast cancer after chemotherapy was 7.65±3.10 ng/mL, and the cfDNA integrity was 2.39±2.44. The cfDNA concentration of control group 1 was 3.71±1.24 ng/mL, and the integrity was 1.51±1.38. The cfDNA concentration of control group 2 was 3.17±1.50ng/mL, and the integrity was 0.97±1.40. The concentration and integrity of cfDNA before chemotherapy in the experimental group were significantly higher than the corresponding values in control group 1 and control group 2 (p < 0.01). The concentration and integrity of cfDNA before chemotherapy in the experimental group were significantly lower than those before chemotherapy (p <0.05). However, cfDNA concentration after chemotherapy of the experimental group was significantly higher those of control group 1 and control group 2 (p <0.01). There was no significant difference in cfDNA integrity between the experimental group after chemotherapy and control group 1 (p >0.05), but cfDNA of the experimental group was higher than that of control group 2 (p <0.01). There were no significant differences in cfDNA concentration and integrity between control group 1 and control group 2 (p >0.05). The results of quantification of cfDNA concentration and integrity using qRT-PCR are shown in Table 3, Table 4, and Figure 1.

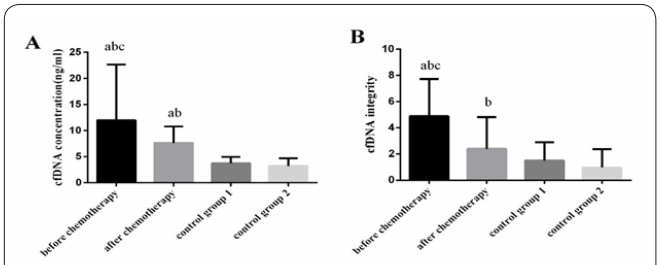

Figure 1. qRT-PCR determination of cfDNA concentration (A), and integrity (B). [a]p <0.05, compared to control group 1; [b]p <0.05, compared to control group 2, [c]p <0.05, compared to experimental group after chemotherapy.

Relationship between cfDNA concentration/integrity and clinical features of breast cancer patients

Analysis of the correlation between cfDNA and clinical features of breast cancer patients was performed after measuring cfDNA concentration and integrity. The results of the correlation analysis are shown in Table 5 (cfDNA concentration) and Table 6 (cfDNA integrity). There were no significant correlations between cfDNA concentration/integrity and age, BMI, tumor diameter, TNM stage, pathological type and CA199 expression in patients with breast cancer before or after chemotherapy (p > 0.05). However, there was a significant correlation between the expression levels of CEA and CA15-3, and cfDNA concentration (p < 0.05), but they were not significantly correlated with cfDNA integrity (p > 0.05). The expression level of CA125 was not significantly correlated with cfDNA concentration (p > 0.05), but it was significantly correlated with cfDNA integrity (p < 0.05).

Table 3. Plasma cfDNA concentrations.

Grouping	cfDNA concentration (ng/mL)	t	*p*
Before chemotherapy	11.97±10.67	4.41	<0.01
Control group 1	3.71±1.24		
Before chemotherapy	11.97±10.67	4.63	<0.01
Control group 2	3.17±1.50		
Before chemotherapy	11.97±10.67	2.30	0.0245
After chemotherapy	7.65±3.10		
After chemotherapy	7.65±3.10	6.98	<0.01
Control group 1	3.71±1.24		
After chemotherapy	7.65±3.10	7.70	<0.01
Control group 2	3.17±1.50		
Control group 1	3.71±1.24	1.64	0.1053
Control group 2	3.17±1.50		

Table 4. cfDNA integrity.

Grouping	cfDNA integrity	t	P
Before chemotherapy	4.89±2.84	6.33	<0.01
Control group 1	1.51±1.38		
Before chemotherapy	4.89±2.84	7.30	<0.01
Control group 2	0.97±1.40		
Before chemotherapy	4.89±2.84	3.95	<0.01
After chemotherapy	2.39±2.44		
After chemotherapy	2.39±2.44	1.86	0.0676
Control group 1	1.51±1.38		
After chemotherapy	2.39±2.44	2.99	<0.01
Control group 2	0.97±1.40		
Control group 1	1.51±1.38	1.63	0.1088
Control group 2	0.97±1.40		

Table 5. Correlation between cfDNA concentration and clinical features.

Variable	Before chemotherapy	After chemotherapy
Age		
≤35	13.26±11.34	7.81±4.05
>35	10.78±10.39	7.10±3.67
P	0.5140	0.5981
BMI		
≤24Kg/m^2	10.46±8.32	6.26±4.29
>24Kg/m^2	14.16±10.13	7.78±5.33
P	0.2440	0.3565
Tumor diameter		
≤5cm	9.51±6.26	5.34±2.51
>5cm	13.17±10.54	7.37±4.24
P	0.2057	0.0839
TNM stage		
I-II	10.06±9.14	4.24±2.60
III-IV	14.61±10.53	7.16±5.29
P	0.1904	0.0582
Pathological type		
Invasive ductal tumor	11.15±9.64	6.39±3.56
Non-invasive ductal tumor	12.39±10.61	8.28±5.26
P	0.7670	0.2626
CEA		
<3.5 ng/mL	7.86±5.65	4.33±1.96
≥3.5 ng/mL	14.33±9.68	9.64±5.32
P	0.0482	0.0031
CA15-3		
< 25 U/ml	8.32±7.14	4.69±2.58
≥25 U/ml	15.94±11.34	9.46±6.49
P	0.0326	0.0136
CA125		
< 35 U/ml	9.06±8.22	6.13±4.02
≥ 35 U/ml	13.44±11.61	8.65±5.26
P	0.2418	0.1466
CA199		
< 39 U/ml	10.21±9.37	6.74±4.16
≥ 39 U/ml	13.54±11.26	9.24±8.01
P	0.3599	0.2793

ROC curve analysis of cfDNA in breast cancer patients

The cfDNA concentration in the plasma of patients with breast cancer before and after chemotherapy and the contents of tumor biomarkers (CEA, CA153 and CA125), as well as their specificity and sensitivity in breast cancer diagnosis were calculated, and the corresponding ROC curves were drawn. The results are shown in Figure 2. For breast cancer patients before chemotherapy, the area under curve (AUC) of CEA was 0.7132 (95% CI: 0.6381 - 0.7883); the AUC of CA15-3 was 0.7434 (95% CI: 6681 - 0.8188); the AUC for CA125 was 0.7066 (95% CI: 0.6265 - 0.7868); the AUC for cfDNA concentration was 0.8254 (95% CI: 7567 - 0.8941), and the AUC of cfDNA integrity was 0.8110 (95% CI: 0. 0.7446 - 0.8774). For breast cancer patients after chemotherapy, the AUC of CEA was 0.6940 (95% CI: 0.6097 -0.7783), the AUC of CA15-3 was 0.7394 (95% CI: 0.6634 - 0.8153), the AUC for CA125 was 0. 6892 (95% CI: 0.6055 - 0.7729), the AUC for cfDNA concentration was 0.8180 (95% CI: 7610 - 0.8749), and the AUC of cfDNA integrity was 0.8040 (95% CI: 0.7344 - 0.8736). These results suggest that cfDNA concentration and integrity are reliable tumor screening methods, with sensitivity and specificity higher than those of traditional tumor biomarkers.

Discussion

In recent years, with the rapid development of mole-

Table 6. Correlation between cfDNA integrity and clinical features.

Variable	Before chemotherapy	After chemotherapy
Age		
≤35	4.64±2.17	2.13±1.69
>35	5.16±2.39	2.88±2.01
P	0.5248	0.2673
BMI		
≤24Kg/m^2	4.01±2.31	2.06±2.29
>24Kg/m^2	5.19±2.44	3.11±2.23
P	0.1537	0.1839
Tumor diameter		
≤5cm	4.52±2.26	1.74±1.51
>5cm	6.19±3.41	2.51±2.04
P	0.0899	0.2081
TNM stage		
I-II	3.76±1.14	1.24±1.20
III-IV	6.09±4.53	2.46±2.16
P	0.0610	0.0576
Pathological type		
Invasive ductal tumor	4.29±2.49	2.04±1.97
Non-invasive ductal tumor	5.83±3.16	3.28±2.26
P	0.1743	0.1569
CEA		
<3.5 ng/mL	3.36±2.15	1.63±1.06
≥3.5 ng/mL	5.33±3.64	2.54±2.32
P	0.1066	0.2254
CA15-3		
< 25 U/ml	3.15±1.74	1.50±1.12
≥25 U/ml	5.14±3.41	2.85±2.33
P	0.0528	0.0525
CA125		
< 35 U/ml	2.46±1.32	1.13±0.98
≥ 35 U/ml	5.43±4.12	2.94±2.26
P	0.0171	0.0102
CA199		
< 39 U/ml	4.21±2.37	1.74±1.21
≥ 39 U/ml	5.61±4.06	2.84±2.01
P	0.2427	0.0695

cular biology technology, the application of cfDNA in the diagnosis and treatment of breast cancer has attracted a lot of interest. The cfDNA is relatively stable and easy to extract. It plays an important role in the early diagnosis, efficacy evaluation, and monitoring of recurrence and metastasis of breast cancer. Thus, cfDNA is considered a new method for tumor molecular diagnosis with broad application prospects. It can be used for non-invasive molecular diagnosis of cancer, in addition to circulating tumor cells (CTC). Cell-free DNA was first discovered in 1948 by French scientists (13). In 1977, Leon et al detected cfDNA at levels of 0-2μg/ml in plasma of breast cancer patients, and found that changes in cfDNA concentration were associated with disease status (14). There are three main sources of cfDNA in the blood, one of which is apoptosis. Apoptosis is characterized by nuclear pyknosis and rupture, and the chromosomal DNA released by the nucleus is eventually degraded into fragments of 180-200 bp (15). Studies on cancer patients have found that the cfDNA concentration is significantly higher than that of healthy people before tumor tissue shows obvious necrosis (16). It has been reported that i*n vitro,* induced apoptosis of tumor cells increased the concentration of extracellular DNA (17). This suggests that cfDNA may be derived from cell apoptosis. Studies on cfDNA fragments derived from apoptosis have found that the length of cfDNA in cancer patients was in the range of 160-180 bp or an integral multiple thereof, which is similar to the electrophoresis results of apoptotic cell nucleosome DNA (18). In addition, the sequencing results of cfDNA in liver cancer patients showed that the length of DNA fragments carrying tumor-specific mutations was about 160 bp (19), which further indicates that the cfDNA derived from tumor cells is not randomly generated. Instead, it is a fragment of a certain length characterized by nuclease cleavage of the nucleosome DNA sequence during apoptosis. The second source of cfDNA is cell necrosis or release after phagocytosis. During tumor growth, local necrosis may occur due to hypoxia, insufficient blood supply, or phagocytosis and destruction by the immune cells. The non-programmed death of the cells causes the DNA to enter the bloodstream before being sheared and degraded, so the released DNA fragments are highly variable and are mostly long fragments (>1000 bp) (20). The third source is active cell secretion. It has been found that the cfDNA concentration in the supernatant of tumor cell culture medium ias directly proportional to the proliferation of cancer cells, but has no significant correlation with cell death. It is believed that a part of cfDNA is derived from living cells (21).

Studies have reported that the average concentration of cfDNA in the blood of healthy people is 30 ng/ml, but in cancer patients, the cfDNA level is 6 times higher, that is 180 ng/ml (22). Other studies have found that the cfDNA concentration in the blood of patients with lymphoma, colorectal cancer, lung cancer and breast cancer is significantly higher than that of healthy people. This indicates that cfDNA can be used for evaluation of tumor treatment and prognosis of tumor patients. Therefore, when the concentration of cfDNA is significantly increased, it is likely to imply tumorige-

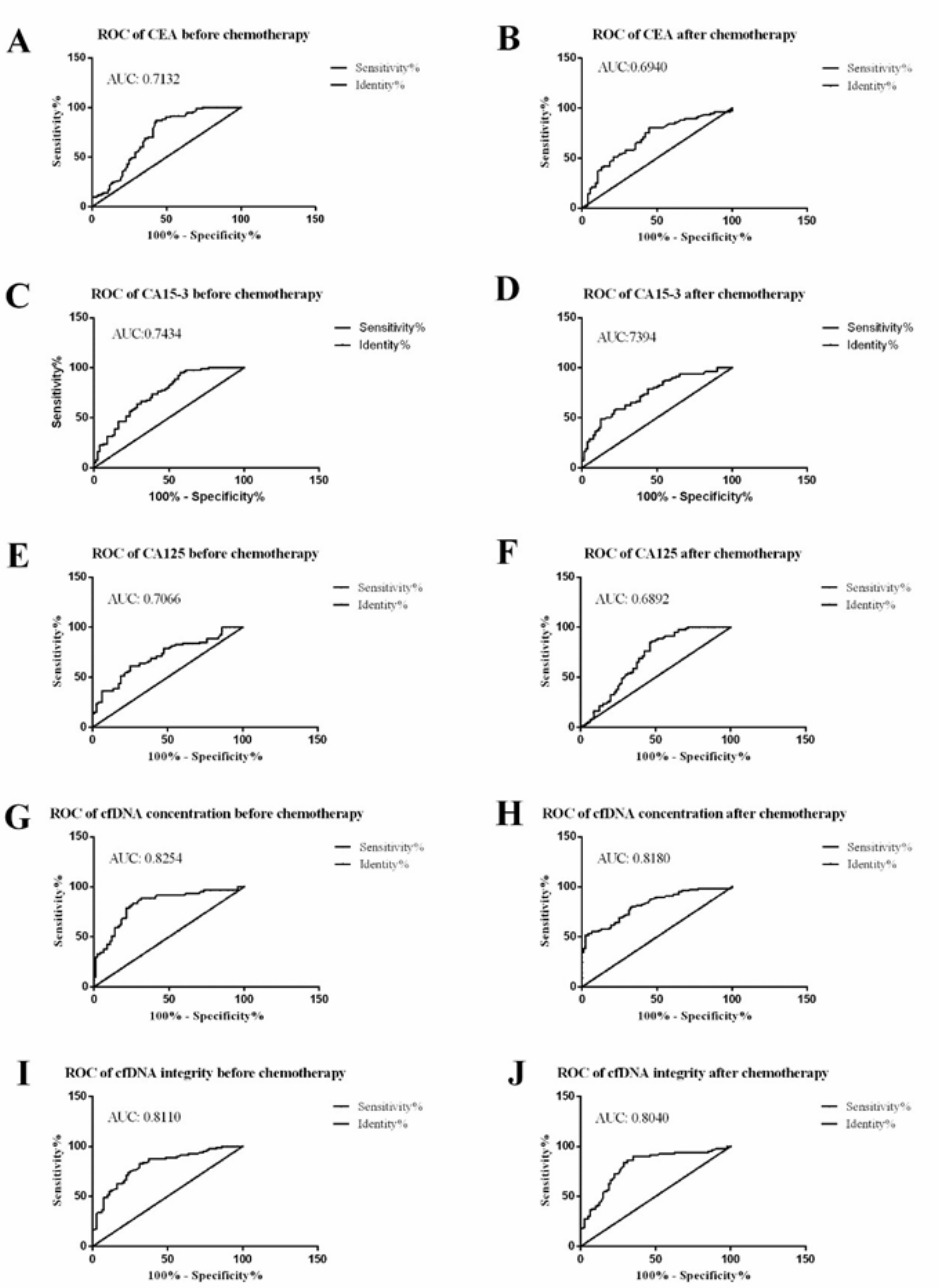

Figure 2. ROC curve analysis of tumor markers and cfDNA in patients with breast cancer before and after chemotherapy.

nesis (23). However, the concentration of cfDNA varies greatly among individuals, especially between benign and malignant tumors. Many studies have shown that the inclusion of cfDNA integrity indicators can further improve the accuracy of cfDNA detection (24-27). The integrity of cfDNA is a relative value that reflects the degree of cfDNA fragmentation. The integrity of cfDNA is determined using real-time quantitative PCR to detect long fragments (259 bp) and short fragments (97 bp) of the widely distributed repeat sequence LINE1 in the genome, and result is expressed as the ratio of LINE1 259bp to 97bp. A significant feature of the integrity of cfDNA in the blood of cancer patients and non-tumor population is that there is no consistency in the lengths of the DNA fragments. The lengths of cfDNA fragments in healthy and benign tumor patients are in the range of 185-200 bp, while the lengths of cfDNA fragments in tumor patients are within the range of 180-800 bp, indicating that cfDNA integrity index is associated with tumor burden. The concentration and integrity of cfDNA reflect the activity of tumor cells in real time, and their detection aids in evaluating the efficacy and prognosis of cancer patients.

Studies have shown that breast cancer patients have higher cfDNA concentrations than healthy people, and the postoperative cfDNA concentration is significantly reduced, suggesting that cfDNA concentration may be related to tumor burden (28, 29). Researchers believe that the cfDNA integrity of cancer patients is higher than that of healthy people. The theoretical basis is that healthy human cfDNA is derived mainly from apoptotic cells, and cancer cells release more long DNA fragments than normal cells, due to pathological processes such as necrosis and autophagy. The present study compared the concentration and integrity of cfDNA of women with benign breast tumors and breast cancer patients, with those of healthy individuals. The study also investigated changes in cfDNA concentration and integrity before and after chemotherapy in breast cancer patients. The results showed that the concentration and integrity

of cfDNA in breast cancer patients were significantly higher than those in benign breast cancer patients and healthy people. In healthy human blood circulation, cfDNA concentration is maintained at a low level due to its clearance by macrophages themselves (30). However, when the DNA released by necrosis and proliferation of cancer cells in breast cancer patients exceeds the ability of macrophages to clear it, the cfDNA concentration of breast cancer patients becomes elevated. Since the DNA fragments released due to cancer cell necrosis are long, the cfDNA integrity in the blood of breast cancer patients is higher than that of healthy people. After chemotherapy, it was found that breast cancer patients had significantly lower cfDNA concentration and integrity than before chemotherapy, demonstrating that cfDNA concentration and integrity are associated with tumor burden. This indicates that cfDNA is a good indicator for recurrence and metastasis, and can be used for monitoring treatment efficacy and evaluation of prognosis of breast cancer patients.

The traditional markers of breast cancer in clinical practice are CEA, CA199, CA125 and CA15-3. The results of systematic analysis of the clinical features of breast cancer before and after chemotherapy showed that plasma cfDNA concentration of breast cancer patients were significantly correlated with the expression levels of CEA and CA15-3, while the cfDNA integrity was significantly correlated with CA125 expression levels. To further evaluate the potential of cfDNA in clinical breast cancer screening, this study calculated the sensitivity and specificity of cfDNA and established ROC curve analysis to compare it with markers of CEA, CA125, and CA15-3. The ROC curve analysis confirmed that the AUC of cfDNA in breast cancer patients before and after chemotherapy was greater than CEA, CA125 and CA15-3, indicating that cfDNA was better than CEA, CA125 or CA15-3 as diagnostic criterion for breast cancer. Thus, cfDNA is a reliable indicator for the diagnosis of breast cancer.

It has been shown that since cfDNA determination is a non-invasive, reproducible, sensitive and specific method, it has important application value in diagnosis, monitoring of treatment response, and assessment of prognosis of tumors (31). Cell-free DNA (cfDNA) is an effective marker for diagnosis of breast cancer and evaluating the prognosis of breast cancer, and can provide more effective information for breast cancer treatment decisions. However, the clinical application of cfDNA is an emerging research field, and its use as a routine testing method in clinical practice is still fraught with numerous challenges. Multi-center, prospective large-scale clinical studies are still needed to further validate the conclusions and provide more scientific evidence for promoting the clinical application of liquid biopsy techniques in breast cancer.

Acknowledgements
The authors thank Chengde Traditional Chinese Medicine Hospital, Langxi County People's Hospital, The First Affiliated Hospital of Bengbu Medical College, and The Second People's Hospital of Wuhu for providing samples and technical supports.

Conflicts of interest
The authors declare that no conflict of interest are associated with this study.

Consent for publication
Not applicable.

Authors' Contributions
All work was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Rong Li. Yajun Miao, Yingrui Fan, Liang Zhang, Tingting Ma and Rong Li collected and analyzed the data; Yajun Miao wrote the text. All authors have read and approved the text prior to publication.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin 2017; 67: 7-30.
2. Simard S, Savard J. Screening and comorbidity of clinical levels of fear of cancer recurrence. J Cancer Surviv 2015; 9: 481-491.
3. Bayo J, Castano MA, Rivera F, Navarro F. Analysis of blood markers for early breast cancer diagnosis. Clin Transl Oncol 2018; 20: 467-475.
4. Wang G, Qin Y, Zhang J, Zhao J, Liang Y, Zhang Z, et al. Nipple discharge of CA15-3, CA125, CEA and TSGF as a new biomarker panel for breast cancer. Int J Mol Sci 2014; 15: 9546-9565.
5. Tang S, Wei L, Sun Y, Zhou F, Zhu S, Yang R, et al. CA153 in Breast Secretions as a Potential Molecular Marker for Diagnosing Breast Cancer: A Meta Analysis. PLoS One 2016; 11: 0163030.
6. Duffy MJ, Walsh S, McDermott EW, Crown J. Biomarkers in Breast Cancer: Where Are We and Where Are We Going? Adv Clin Chem 2015; 71: 1-23.
7. Wang W, Xu X, Tian B, Wang Y, Du L, Sun T, et al. The diagnostic value of serum tumor markers CEA, CA19-9, CA125, CA15-3, and TPS in metastatic breast cancer. Clin Chim Acta 2017; 470: 51-55.
8. Zhao S, Mei Y, Wang Y, Zhu J, Zheng G, Ma R. Levels of CEA, CA153, CA199, CA724 and AFP in nipple discharge of breast cancer patients. Int J Clin Exp Med 2015; 8: 20837-20844.
9. Kazarian A, Blyuss O, Metodieva G, Gentry-Maharaj A, Ryan A, Kiseleva EM, et al. Testing breast cancer serum biomarkers for early detection and prognosis in pre-diagnosis samples. Br J Cancer 2017; 116: 501-508.
10. Gautschi O, Bigosch C, Huegli B, Jermann M, Marx A, Chasse E, et al. Circulating deoxyribonucleic Acid as prognostic marker in non-small-cell lung cancer patients undergoing chemotherapy. J Clin Oncol 2004; 22: 4157-4164.
11. Hamam R, Hamam D, Alsaleh KA, Kassem M, Zaher W, Alfayez M, et al. Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers. Cell Death Dis 2017; 8: 3045.
12. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008; 14: 985-90.
13. Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 1948; 142(3-4):241-3.
14. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977; 37: 646-50.
15. Schwarzenbach H. Circulating nucleic acids as biomarkers in breast cancer. Breast Cancer Res 2013; 15: 211.
16. van der Vaart M, Pretorius PJ. Circulating DNA. Its origin and

fluctuation. Ann N Y Acad Sci 2008; 1137: 18-26.
17. Spellman PT, Gray JW. Detecting cancer by monitoring circulating tumor DNA. Nat Med 2014; 20: 474-475.
18. Morozkin ES, Babochkina TI, Vlassov VV, Laktionov PP. The effect of protein transport inhibitors on the production of extracellular DNA. Ann N Y Acad Sci 2008; 1137: 31-35.
19. Jiang P, Chan CW, Chan KC, Cheng SH, Wong J, Wong VW, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci U S A 2015; 112: 1317-1325.
20. Mouliere F, Thierry AR. The importance of examining the proportion of circulating DNA originating from tumor, microenvironment and normal cells in colorectal cancer patients. Expert Opin Biol Ther 2012; 12: 209-215.
21. Canzoniero JV, Park BH. Use of cell free DNA in breast oncology. Biochim Biophys Acta 2016; 1865: 266-274.
22. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011; 11: 426-437.
23. Babayan A, Pantel K. Advances in liquid biopsy approaches for early detection and monitoring of cancer. Genome Med 2018; 10: 21.
24. Kamel AM, Teama S, Fawzy A, El Deftar M. Plasma DNA integrity index as a potential molecular diagnostic marker for breast cancer. Tumour Biol 2016; 37: 7565-7572.
25. Huang A, Zhang X, Zhou SL, Cao Y, Huang XW, Fan J, et al. Plasma Circulating Cell-free DNA Integrity as a Promising Biomarker for Diagnosis and Surveillance in Patients with Hepatocellular Carcinoma. J Cancer 2016; 7: 1798-1803.
26. Yoruker EE, Ozgur E, Keskin M, Dalay N, Holdenrieder S, Gezer U. Assessment of circulating serum DNA integrity in colorectal cancer patients. Anticancer Res 2015; 35: 2435-2440.
27. Cheng J, Cuk K, Heil J, Golatta M, Schott S, Sohn C, et al. Cell-free circulating DNA integrity is an independent predictor of impending breast cancer recurrence. Oncotarget 2017; 8: 54537-54547.
28. Lin Z, Neiswender J, Fang B, Ma X, Zhang J, Hu X. Value of circulating cell-free DNA analysis as a diagnostic tool for breast cancer: a meta-analysis. Oncotarget 2017; 8: 26625-26636.
29. Uehiro N, Sato F, Pu F, Tanaka S, Kawashima M, Kawaguchi K, et al. Circulating cell-free DNA-based epigenetic assay can detect early breast cancer. Breast Cancer Res 2016; 18: 129.
30. Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. Cell 2010; 140: 619-630.
31. Cheng F, Su L, Qian C. Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer. Oncotarget 2016; 7: 48832-48841.