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Original Article

Serum cell free DNA by branched DNA in patients with gastric cancer

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Abstract: The study aim was to quantify cell free DNA (cf-DNA) in patients with gastric cancer by a branched DNA (bDNA) assay and to analyze the relationship between serum cf-DNA levels and clinical and pathological features in an attempt to explore the value of early screening and aided diagnosis of cf-DNA in gastric cancer (GC). We measured concentrations of cf-DNA, carcinoembryonic antigen (CEA), carcinoembryonic antigen 199 (CA199), carcinoembryonic antigen 50 (CA50), and carcinoembryonic antigen 724 (CA724) in 99 GC patients, 32 gastric benign tumor patients, and 100 normal controls. The Kruskal-Wallis test, receiver operating characteristic curves (ROC) test and the Fisher's exact test were used in this study. There was a significant difference in concentrations of cf-DNA between GC patients and gastric benign tumor patients or healthy individuals ($P<0.0001$). There was no significant difference in cf-DNA in different subgroups of GC patients with respect to gender, age, tumor sites and pathologic stage. There was no correlation between cf-DNA and CEA ($P=0.736$), CA199 ($P=0.254$), CA50 ($P=0.629$) and CA724 ($P=0.422$). The areas under the ROC curves and sensitivity assessing cf-DNA concentration (0.94, 76.19%) were significantly greater than serum CEA (0.66, 16.67%), CA199 (0.63, 66.67%), CA50 (0.83, 36.67%), and CA724 (0.67, 10.00%) in GC patients. cf-DNA values are significantly higher in GC patients than in the gastric benign tumor patients or healthy controls; this has practical implications in aiding the diagnosis of GC. cf-DNA showed better values than CEA, CA199, CA50, and CA724 by bDNA assay in GC.

Keywords: Cell-free DNA, gastric cancer, branched DNA assay

Introduction

Gastric cancer (GC) is one of the most common malignant cancers worldwide [1], accounting for 12% of all malignant cancer-related deaths. At present, the global cancer mortality rate of GC is second only to lung cancer [2]. So far, although the incidence of GC in some parts of the world has declined, it is still the leading cause of cancer-related deaths worldwide, especially in China and other Asian countries [3]. An estimated 930,000 people worldwide develop GC each year. Despite efforts to advocate improvements in making smart dietary choices, early diagnosis, early treatment, and other measures, at least 700,000 people die from gastric carcinoma every year [4]. Clinical symptoms of gastric carcinoma and precancerous lesions are subtle and not easily detected, making gastric cancer difficult to find in its early stages. In fact, only 5~10% of gastric cancers were diagnosed early in China [5]. Adenocarcinomas account for 90% of gastric malignant tumors and tend to be insensitive to radiation and chemotherapy and thus require surgical intervention. When found early, gastric cancer has a good prognosis. If it only affects the mucous membrane, patients have an excellent prognosis with a postoperative 5-year survival rate of over 90% [6]. If it has invaded the shallow muscularis, the postoperative 5-year survival rate is 50%. If it has invaded the deep muscularis, the postoperative 5-year survival rate drops to 25%. If it has invaded the serosa, the postoperative 5-year survival rate is just 10% [7]. Therefore, current clinical and basic

research has focused on improving methods to detect and diagnosis the disease while it is still in the early stages.

An endoscopic examination is a reliable diagnostic tool in gastric cancer. However it is a costly and invasive approach, one that patients often do not tolerate well. The endoscopic exam is unsuitable for gastric cancer screening. Early diagnosis of gastric cancer relies on detecting specific tumor markers, which is an effective, sensitive, and noninvasive method in addition to conventional endoscopy and imaging. The most common such tumor markers are carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA125), carbohydrate antigen 199 (CAI-99), and carbohydrate antigen 724 (CA724). They are tumor-associated antigens found on the surface of gastrointestinal tumor cells, which have a special meaning in the early diagnosis of gastric cancer. CEA, CA19-9, CA724, and CA125 are serum tumor markers commonly used for the diagnosis of GC, but they are not entirely satisfactory in this regard due to their lack of sufficient specificity and sensitivity. They lead to higher rates of false positive and false negative results. To improve reliability, we must combine these exams together [8]. To improve the monitoring and evaluation of GC patients, there is a need to find a complementary marker that can ensure accurate diagnosis and early detection of recurrence. This study used branch DNA (bDNA) technology to quantitatively determine concentrations of cell free DNA in the serum of gastric cancer patients and to study the relationship between the serum levels of cell free DNA and clinicopathological parameters.

The objective of our study was to assess the diagnostic value of the quantitative determination cell free DNA concentration from the serum of gastric cancer patients compared to gastric cancer screening and other diagnostic methods.

Materials and methods

Clinical information

We recruited 99 patients with GC (76 men and 23 women; median age, 65 years, range, 36-82; quartile age range, 55~69) presenting to the Department of Clinical Laboratory at the Affiliated Cancer Hospital of Nantong University from January 2014 to December 2014. All patients underwent surgical tumor resection, and a diagnosis of gastric cancer was confirmed by histological examination. A 5 ml peripheral blood sample was drawn from each of the 99 patients before radiotherapy and chemotherapy. Grading and staging of tumors was defined according to the American Joint Committee on Cancer (AJCC) 2010 Guidelines (seventh edition). Blood samples were collected from 100 healthy control subjects (69 men and 31 women; age range, 40-78 years). These individuals were visiting at the outpatient department at the Affiliated Cancer Hospital of Nantong University, had no history of autoimmune disease or tissue injury or trauma at the time of examination, and their hematological-biochemical profile was normal. We collected the serum samples of 32 patients with benign stomach tumors between January 2014 to December 2014 at the Affiliated Hospital of Nantong University (15 men and 17 women; median age, 56 years; range, 37-82; quartile age range, 52~73). The 32 cases included 9 cases of gastric polyps adenoma, 2 cases of gastric mucous membrane high-grade intraepithelial neoplasia, 20 cases of gastric stromal tumor, and 1 case of stomach secrete melanoma. This study was conducted in accordance with the declaration of Helsinki. This study protocol was approved by the Independent Ethics Committee of Affiliated Cancer Hospital of Nantong University. Written informed consent was obtained from all participants.

Sample preparation

Blood samples (4-5 ml) for biochemical indicator testing were collected after overnight fasting in the hospital in serum separator tubes containing clot activation additive and a barrier gel (Vacuette, Kremsmunster, Austria). Whole blood was separated at 25°C by centrifugation at 4000 r/min for 10 min. Serum samples were stored in Eppendorf tubes at -80°C. After collection, all samples were analyzed for cell free DNA (cf-DNA) on the same day in order to minimize testing error.

cf-DNA quantification

The serum samples were diluted at 1:20 with steam water heated to 95°C for 5 minutes and then immediately cooled with ice water. Thereafter, 96-well plates were coated with capture

probe and combined with 90 μl WPS (33 μl pyrolysis liquid, 55.3 μl TE buffer, 0.1 μl protease K, 1.0 μl sealing fluid, 0.3 μl capture probe CE, 0.3 μl labeled probe LE) and 10 μl DNA samples (samples, two quality controls, three standard controls). After wells were closed with the foil, they were immediately put into the hybrid furnace at 55°C for 16 to 21 hours. Each well was rinsed three times using 300 μl WPS and then incubated with 100 μl of the pro-magnified probe (diluted 1:1000) at 55°C for 60 min. After the same washing, they were incubated with 100 μl of the magnified probe (diluted 1:1000) at 55°C for 60 min. The plate was rinsed three times and 100 μl labeled probe (diluted 1:1000) was added at 50°C for 60 min. Finally the plate was in the same method and the substrate working liquid was added into every well for 5 to 10 minutes at room temperature. The OD value of every well was measured by luminescence and the average value of each sample was calculated. The concentration of cf-DNA in every well was calculated under 20 multiple diluted cf-DNA concentrations according to the standard curve.

cf-DNA concentrations were determined by HB 100 molecular hybridization instrument (Xinchen Technology Corporation, Nanjing), ECLIA-IIS chemiluminescence assay (Tianzongda Technology Corporation, Xiamen), automatic washing machine (Experimental Apparatus Corporation, Shanghai), multi-function centrifuge (Hitachi, Japan) and the metal thermostat (Lan-bao Experimental Apparatus Corporation, Shanghai).

Quantification of CEA, CA199, CA50, and CA724

CEA, CA199, and CA724 concentrations were tested with E170 chemiluminescence (Roche, Germany). CA50 concentrations were tested with JETLIA-962 chemiluminescence (Yuande Biomedical Engineering Corporation, Beijing). These reagents were provided by Professor Lu-rong Zhang of the University of Florida.

Statistical analysis

Data analysis was performed with SPSS version 13.0 (SPSS Inc., Chicago, USA). The results for these concentrations are presented as the median with the 25th and the 75th percentile values. The difference of cf-DNA concentrations between GC, gastric benign tumor patients and healthy controls was analyzed by the Kruskal-Wallis test.

The association between cf-DNA concentrations and clinicopathological features was analyzed using the Fisher's exact test.

The concentrations of cf-DNA and the values of CEA, CA50, CA199, and CA724 were performed using the Spearman rank correlation test because the data were not normally distributed.

Receiver operating characteristic curves (ROC) were generated to assess the diagnostic accuracy of each parameter, and the sensitivity and specificity of the optimum cut-off point were defined as values that maximized the area under the ROC curve (AUC). The confidence interval of ROC was defined as 95%. cf-DNA, CEA, CA50, CA199, and CA724 concentrations were analyzed by ROC.

All statistical tests were two-sided, and a *P* value lower than 0.05 was considered statistically significant.

Results

Cell-free DNA in patients with GC, gastric benign tumor, and in healthy individuals

The bDNA assay was used to measure the level of cf-DNA. To test whether cf-DNA concentrations differed between GC and gastric benign tumor patients and healthy individuals, blood samples from 99 GC patients and 32 gastric benign tumor patients and 100 controls were investigated. The median concentrations of cf-DNA in patients GC and gastric benign tumor and healthy individuals were 2316.0 ng/mL (interquartile range 1046.4-3737.1 ng/mL), 423.1 ng/mL (interquartile range 256.3-513.6 ng/mL) and 313.5 ng/mL (interquartile range 156.0-489.0 ng/mL), respectively.

There was significant difference between the GC and gastric benign tumor patients or healthy individuals ($P<0.0001$), but there was no statistical difference between the gastric benign tumor patients and healthy individuals ($P=0.2672$) (**Figure 1**).

cf-DNA concentrations and clinicopathological parameters in GC

To explore further the physiological or pathological relation in GC, the clinicopathological data are summarized in **Table 1**. A total of 99 patients with GC were investigated in this study.

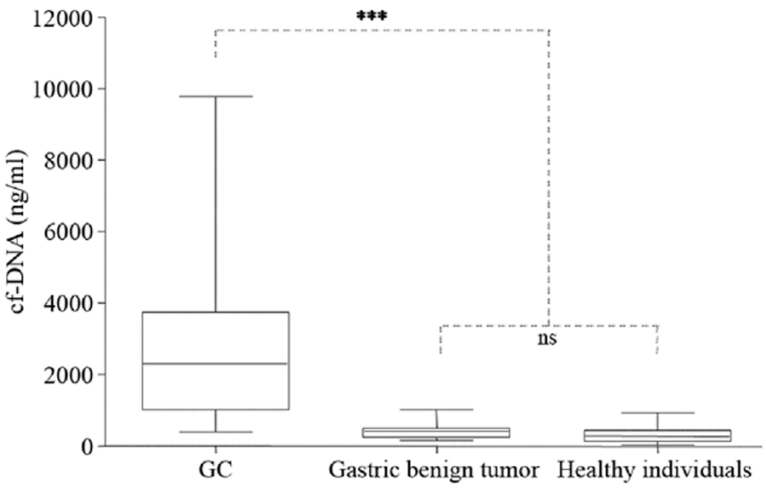

Figure 1. The cf-DNA concentrations in patients with GC and gastric benign tumor and healthy individuals. The serum cf-DNA concentrations of GC patients were higher than gastric benign tumor or healthy individuals (P<0.0001).

Table 1. The concentration of cf-DNA in subgroups of GC patients (n=99)

Parameters	n	Median (interquartile range)	*P*
Age (years)			0.80
≤60	41	2367.35 (1095.76~3813.52)	
>60	58	2256.61 (916.88~3142.08)	
Gender			0.98
Male	76	2265.88 (852.70~3737.14)	
Female	23	2316.05 (1317.35~3624.25)	
Tumor site			0.33
Upper 1/3	37	2368.48 (724.78~3102.45)	
Central 1/3	22	3459.16 (1240.63~4467.68)	
Lower 1/3	35	2068.80 (1290.01~3209.31)	
Total	5	885.47 (770.66~7910.90)	
Histological differentiation			0.87
High	14	2316.27 (1286.73~4002.57)	
Middle	38	2366.22 (870.04~3737.14)	
Low	47	2265.88 (983.55~3156.54)	
AJCC Stages			0.11
I	4	732.69 (409.85~1793.88)	
II	13	1917.84 (1483.01~2431.31)	
III	36	2823.19 (1047.28~3752.05)	
IV	46	2306.78 (1095.76~4226.20)	

Statistical analyses were performed by the Fisher's exact test. *P*<0.05 was considered significant.

Patient characteristics, including sex, age, tumor site and TNM staging are illustrated. 99 patients in the serum samples group included 37 cases upper stomach GC, 22 cases central stomach GC, 35 cases lower stomach GC and 4 cases total stomach GC. Pathologic diagnosis were confirmed by histological examination, including 14 cases well grade, 38 cases mod grade and 47 cases poor grade (7 cases gastric signet ring cell carcinoma, 5 cases mucous adenocarcinoma). There was no statistically significant difference in cf-DNA in these different groups: gender (P=0.98), age (P=0.80), tumor site (P=0.33), Histological grade (P=0.87) and pathologic stage (P=0.11). Major clinical and biologic characteristics of the patients are summarized in **Table 1**.

The quantification of CEA, CA19-9, CA50 and CA724. CEA, CA19-9, CA50, and CA724 concentrations were determined by chemiluminescence method between 99 GC patients and healthy individuals (**Table 2**).

Comparing the diagnostic efficacy of cf-DNA, CEA, CA199, CA50 and CA724 in GC patients

Correlation analysis: Serum cf-DNA concentration in gastric cancer patients was high. To evaluate cf-DNA as a biomarker, Spearman correlation analysis was performed. No significant correlation was observed between cf-DNA and CEA (r=0.196, P=0.196), CA199 (r=0.126, P=0.126), CA50 (r=0.0505, P=0.0505), or CA724 (r=0.0888, P=0.0888) (**Figure 2**).

ROC curve analysis

The ROC curve was plotted to identify a cut-off value that could distinguish between several tests. As the serum CEA and CA19-9 tests are widely used markers for the diagnosis of GC, the ROC curve analyses of cf-DNA (AUC =0.94), CEA (AUC=0.66), CA19-9 (AUC=0.63), CA50 (AUC=0.83), CA724 (AUC=0.67) and were performed between GC patients and healthy individuals (**Figure 3**). Comparisons were also

Table 2. CEA, CA19-9, CA50 and CA724 concentrations in GC patients and normal controls

Marker	GC patients		Healthy individuals		*P*
	Median	Interquartile range	Median	Interquartile range	
CEA (ng/ml)	2.49	1.57~4.39	1.80	1.20~2.85	0.0094
CA199 (U/ml)	11.22	6.11~23.07	7.15	5.13~11.00	0.0401
CA50 (U/ml)	12.26	6.90~24.63	4.25	2.75~7.90	<0.0001
CA724 (U/ml)	3.69	1.98~6.47	2.45	1.60~3.70	0.0072

P<0.05 was considered significant.

performed between patients with GC and healthy individuals in cf-DNA, CEA, CA199, CA50, and CA724. The test showed that there was significant difference in cf-DNA (P<0.0001), CA50 (P<0.0001), while there was slight difference in CEA (P=0.009), CA199 (P=0.0398), and CA724 (P=0.0072) (**Table 3**).

Discussion

It has been recognized recently that cf-DNA has potential clinical value, along with tumor cell biology and molecular biology, in the field of cancer research [9-12]. Convenient and minimally invasive, blood analyses of cf-DNA may have the potential to complement or replace existing blood biomarkers. One crucial factor in the continued development of cf-DNA biomarkers is the standardization of nomenclature and assays. The existing blood biomarkers were restricted by specimen collection, unsuitable for continuous monitoring or follow-up.

The presence of cf-DNA in the human bloodstream was first described in 1948 by Mandel and Metais. Tan et al. demonstrated the presence of DNA in the serum and plasma of patients with systemic lupus erythematosus in 1966. Several years later, cf-DNA was also found in the serum of cancer patients and quantitated by radioimmunoassay by Leon et al. The level of cf-DNA was low and constant in healthy controls but significantly increased in cancer patients. In addition, a partial correlation was noted between the persistence of elevated serum DNA levels and the lack of response to therapy.

In 1989, Stroun et al. first isolated and purified cf-DNA by ^{32}P-DNA technology. They found that blood cf-DNA shares some characteristics with tumor cell DNA, leading some scholars to speculate that parts of cf-DNA in tumor patients were derived from tumor tissue.

One recent study found that a variety of patients had DNA changes in the circulating blood that were consistent with the gene expressed in tumor tissues [13].

Later, it was determined that cell death, through processes such as apoptosis and necrosis, was the primary origin of cf-DNA, in addition to the tumor tissue hyperplasia active in the peripheral blood. The concentration of cf-DNA depends upon the biological characteristics of the tumor DNA content. Correlations with this concentration were identified with the tumor aggressiveness and the extent of metastasis, but none with tumor size or location. Angiogenesis, or the formation of tumor blood vessels, was also considered an important factor for tumor cells releasing free nucleic acid into the blood [14-18].

The origin of cf-DNA is not fully understood, but cell lysis, necrotic death, apoptosis, and active release are possible mechanisms by which DNA is released into the circulating blood. Levels of cf-DNA, which differ among various types of disease [19-22], can be measured by different laboratory techniques; however, all of these methods are laborious, and the accurate and precise quantification of cf-DNA in whole blood is made even more difficult by dependence on the efficiency of cf-DNA extraction and subsequent purification. Here, we described a new bDNA amplification-based Alu repeats method for direct quantification of cf-DNA in whole blood, serum, and plasma.

Alu repeats are the most abundant sequences in the human genome, with a copy number of about 1.4×10^6 per genome. Alu sequences are short interspersed elements (SINEs), typically 300 nucleotides long, that account for >10% of the genome [23, 24].

Two subunits which are homologous but different come from the 7SLRNA gene and comprise the Alu. 7SLRNA exists with deletions and point mutations and has the effect of RNA polymerase III. So 10%-20% of Alu can be transcribed into RNA. Alu with the polyA in the 3' end is mediated by RNA, reverse transcribed

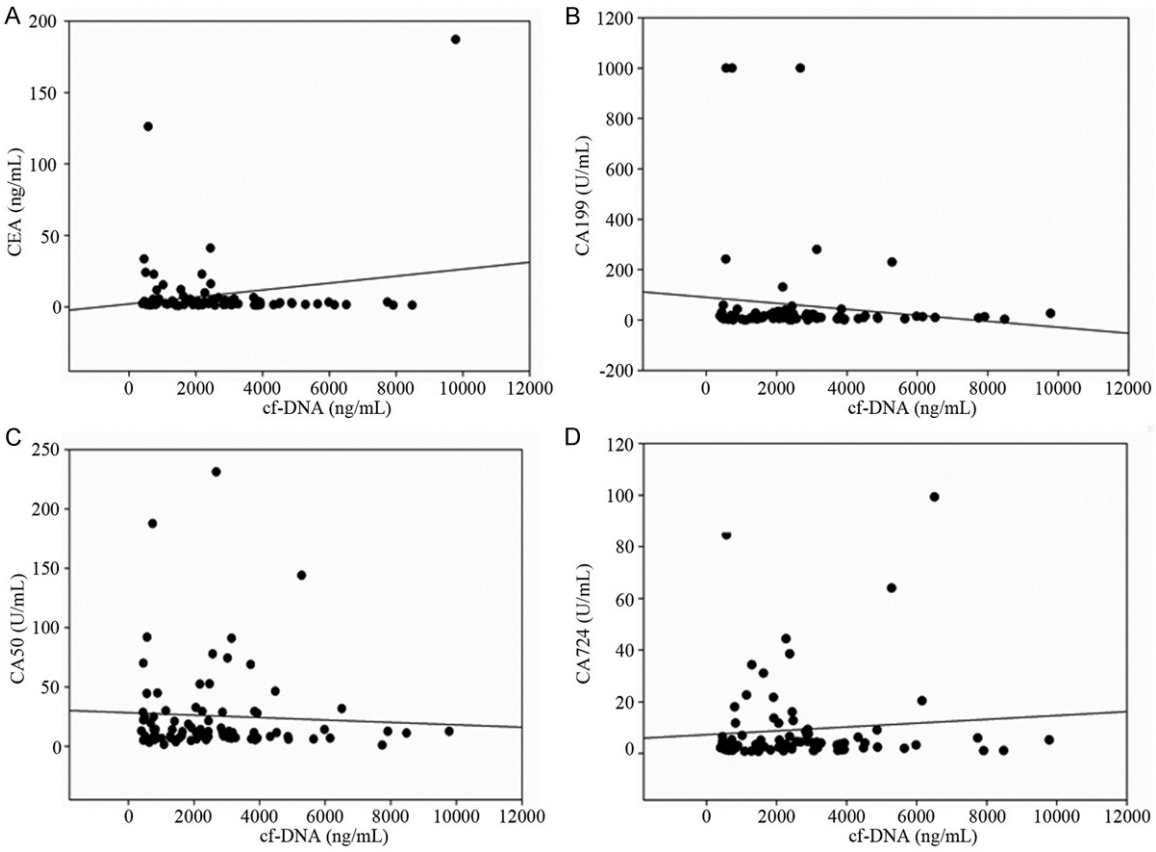

Figure 2. Compared with the correlation of cf-DNA, CEA, CA199, CA50 and CA724 in GC patients. No significant correlation was observed between cf-DNA and CEA (r=0.196, P=0.196), CA199 (r=0.126, P=0.126), CA50 (r=0.0505, P=0.0505), or CA724 (r=0.0888, P=0.0888).

into DNA and integrated into the new position on the genome. Insertion, deletion, or restructuring of the Alu component can lead to cancer, making the Alu family a target of oncology research.

Our research method only requires 10 μl serum, but it has good linearity, sensitivity and accuracy [25]. It is repeatable, easily standardized, and can more truly reflect blood cf-DNA levels after the nucleic acid is extracted.

The method is a kind of signal amplification technology, and does not require cf-DNA extraction and purification, but it improves detection sensitivity by adding labeled probes to copy numbers or enhances signal markers [26]. Therefore, the bDNA-based Alu assay is a good method for the direct quantification of cf-DNA.

In this study, we used serum samples. The concentration of cf-DNA was elevated in serum because of the lysis of white blood cells, which probably occurred during the process of clotting, which releases nuclear fragments into the serum. So we eliminated that effect on the data by comparing the normal control groups and GC patients [25]. In our study, the cf-DNA of GC patients was significantly higher than it was in patients with gastric benign tumors or in healthy individuals. There was significant difference between the GC and gastric benign tumor patients or healthy individuals ($P<0.0001$), but there was no statistical difference between the gastric benign tumor patients and healthy individuals ($P=0.2672$).

These findings strongly suggest that the concentration of cf-DNA in GC patients is much higher than in healthy individuals. Our results are consistent with the results of Sai et al. [27] and Kolesnikova et al. [28]. In summary, cf-DNA quantification may be an important biological marker in the auxiliary diagnosis of gastric cancer.

In this study, we found that there was no statistically significant difference in cf-DNA with regard to the following characteristics: sex, age,

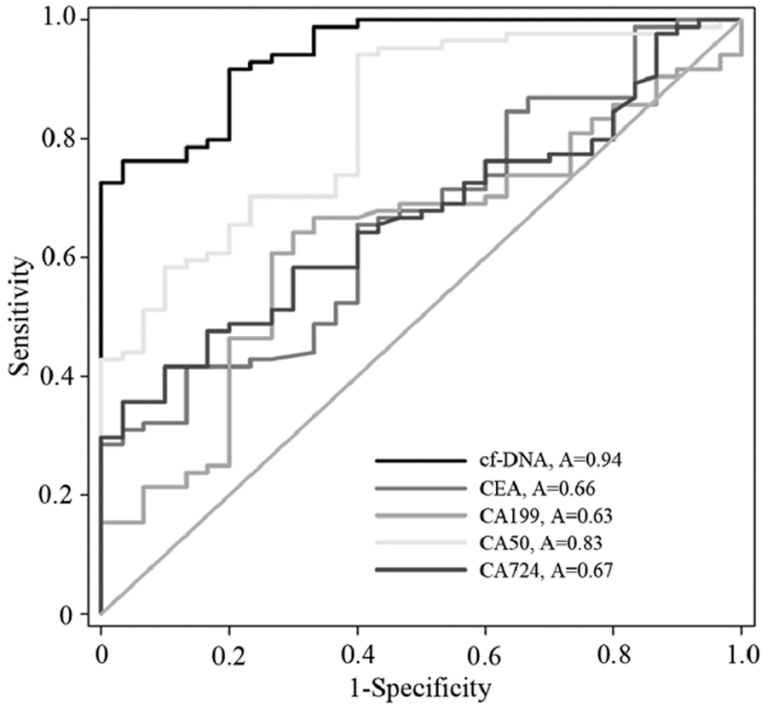

Figure 3. The ROC curves of cf-DNA, CEA, CA199, CA50, CA724. The ROC curve analyses of cf-DNA (AUC=0.94), CEA (AUC=0.66), CA19-9 (AUC=0.63), CA50 (AUC=0.83), CA724 (AUC=0.67) were performed between GC patients and healthy individuals.

Table 3. Compared with the diagnostic efficiency of cf-DNA, CEA, CA199, CA50 and CA724 in GC patients

	AUC	95% CI	*P*	Cut-off	Sensitivity (%)	Specificity (%)
cf-DNA	0.94	0.90~0.98	<0.0001	973.6 ng/ml	76.19	96.67
CEA	0.66	0.55~0.77	0.009	0.91 U/ml	16.67	98.81
CA199	0.63	0.52~0.74	0.0398	7.99 U/ml	66.67	66.67
CA50	0.83	0.75~0.91	<0.0001	3.29 U/ml	36.67	97.62
CA724	0.67	0.57~0.77	0.0072	0.84 U/ml	10.00	98.81

P<0.05 was considered significant.

tumor site, histological grade and pathologic stage. The level of cf-DNA has a tendency to be increased in patients with late-stage cancer and poorly differentiated cancer. Our results are consistent with the results of Frattini et al. [29]. Monitoring serum cf-DNA levels can provide important information for judging the progression and prognosis of GC.

Multiple serum markers including CEA, CA50, CA199, and CA724 have been well recognized as clinical common tumor markers for GC. They are found widely in digestive system tumors in the germ layer. But there is little or none of these markers in normal human serum. Compared with healthy controls, the levels of multiple serum markers are significantly increased in GC patients. If they are found, or they are found to have changed, this may indicate cancer. Although they are good markers for making therapeutic decisions, monitoring the progression of disease, and evaluating prognosis [30], they have no obvious impact on early diagnosis because of their poor specificity and sensitivity [31].

We found 76.19% sensitivity and 96.67% specificity at a cutoff value of 973.60 ng/ml using ROC analysis in GC. The result of ROC analysis showed that cf-DNA is more sensitive and specific than CEA, CA-199, CA50, and CA724.

Compared with the diagnostic efficacy of CA199, CA50 and CA724 in GC patients, the diagnostic efficacy of cf-DNA is much more apparent. Our results are consistent with the results of Boni et al. [32] and Danese et al. [33]. The AUC of cf-DNA for distinguishing patients with GC from healthy controls was 0.94. The AUC of cf-DNA was significantly greater than CEA (0.66), CA50 (0.83), CA199 (0.63), and CA724 (0.67). ROC analysis demonstrated that cf-DNA was a better indicator of diagnostic efficacy.

In summary, the bDNA-based assay is a novel method for quantifying human cf-DNA. The levels of cf-DNA are significantly higher in GC patients than those in normal controls, and they peak at earlier times, which might play a role in the diagnosis of GC. cf-DNA was a better indicator of diagnostic efficacy and may be an important biological marker for GC. Quantitative analysis of serum cf-DNA with bDNA technology is superior to the traditional index of serum tumor markers such as CEA, CA199, CA50 and CA724.

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Disclosure of conflict of interest

None.

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