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Plasma cell-free DNA for screening patients with benefit-assisted neoadjuvant chemotherapy for advanced gastric cancer

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ABSTRACT: The purpose of this study was to explore the reliability of cell-free DNA (cfDNA) as an indicator for screening patients who benefit from neoadjuvant chemotherapy (NACT) in advanced gastric cancer (GC). 70 GC patients with TNM (Tumor, Lymph Node, Metastasis) stage II-III were enrolled. Plasma specimens of GC patients before and after NACT and of 50 healthy volunteers were collected. The concentration and integrity of cfDNA were detected by qRT-PCR. cfDNA concentration and integrity of different groups were analyzed to explore its relationship with clinical characteristics of gastric cancer patients. ROC (Receiver operating characteristic) curve was established to compare the cfDNA sensitivity and specificity with cancer antigen 724 (CA724), carcinoembryonic antigen (CEA), cancer antigen 199 (CA199) and alpha-fetoprotein (AFP). Factors affecting the prognosis of advanced GC patients were analyzed by COX univariate/multivariate analysis. The result showed that plasma cfDNA concentration and integrity of advanced GC patients before NACT were significantly higher than those of normal people. After receiving NACT, cfDNA concentration and integrity were significantly decreased ($p < 0.05$). There was a significant correlation between cfDNA concentration and TNM stage ($p < 0.05$). The values of area under curve (AUC) of ROC curve for cfDNA concentration and integrity were greater than those of CEA, CA724, CA199 and AFP. COX analysis showed that the tumor differentiation degree and cfDNA concentration were independent risk factors for the advanced GC patients prognosis. In conclusion, cfDNA can be used to predict the prognosis of advanced GC patients, and as a reliable indicator to evaluate for further NACT in advanced GC patients.

KEYWORDS: advanced gastric cancer, NACT, cell-free DNA, carcinoembryonic antigen

INTRODUCTION

According to the latest research, GC's mortality and incidence are ranked in fifth and third places of global malignant tumors [1]. Due to the lack of awareness of early screening in Chinese patients with GC, most patients are already in local progression at the time of diagnosis [2, 3]. Surgical resection is the main treatment for GC, but the resection rate of locally advanced GC patients is low. The prognosis is poor after single operation, and the 5-year survival rate is only 20–30% [4]. With the development of medical technology, the treatment mode of GC has changed from simple surgical resection to multidisciplinary collaborative treatment mode, and NACT has become an important part of advanced GC treatment. NACT is originally used to treat solid tumors, such as bone tumors, breast cancer, and neck cancer. In 1989, Willke et al [5] first reported NACT for GC treatment. With the successful report of MAGIC test [6] and FNCLCC/FFCD test [7], it was confirmed that NACT has a significant positive effect on the pathological remission rate, radical surgical resection and survival benefit of patients with locally advanced GC. Although there is still controversy about how to choose beneficiaries and the best drug treatment options, the concept of applying NACT in locally advanced GC is still recognized by internal and external gastrointestinal tumor surgeons, and some patients have access to radical surgery and long-term survival [8]. However, due to the difficulty in accurately judging the efficacy of NACT for GC, some patients who are considered having a stable disease have not benefited from NACT, and have

even failed to undergo radical surgery [9]. The treatment strategy of locally advanced GC is a comprehensive treatment centered on radical surgery. It is extremely important to guide the postoperative chemotherapy strategy according to the effect of preoperative chemotherapy [10]. Therefore, a clear judgment of preoperative chemotherapy is the first step in screening patients. Monitoring the efficacy of NACT is critical to preventing unnecessary side effects, avoiding continuing ineffective treatment, and determining the benefits of new therapies. Clinically, it is particularly in urgent need to screen markers for advanced GC that can benefit from NACT.

Molecular marker detection plays an important role in the field of NACT for GC. The subject of this trial is cfDNA. As a nucleic acid fragment, cfDNA is found in extracellular plasma, serum, and other body fluids [11]. Studies have shown that the cfDNA concentration in peripheral blood of patients with malignant tumors was higher than that of normal people [12]. The long-segment DNA concentration was representative and had the characteristics of tumor cells. It was considered that the abnormal changes of cfDNA concentration and fragment length were closely related to tumors [13, 14]. cfDNA is expected to be an effective molecular marker for assessing the efficacy or prognosis of tumor patients. There are no reports on the detection of cfDNA as a molecular marker in advanced GC patients with NACT. This study was conducted to detect the concentration and fragment integrity of cfDNA in plasma of patients with advanced GC, and examined its correlation with NACT efficacy and clinicopathological features. The ROC curve was established to explore the reliability of the screening index as a beneficiary crowd of NACT for GC patients, and to provide a scientific basis for further clinical development of treatment strategies.

MATERIALS AND METHODS

Clinical information

70 patients with stage II-III GC who were admitted to Affiliated Hospital of Nanjing University of Chinese Medicine between October 2016 and October 2017 were selected as study subjects. Inclusion criteria: (1) clear diagnosis of patients with advanced GC; (2) case data were recorded intactly; (3) none of them were treated with other chemotherapy drugs; (4) no chemotherapy contraindications; (5) liver function, kidney function and cardiopulmonary function are normal. Exclusion criteria: (1) those with other malignant tumors at the same time; (2) those suffer from serious organic diseases such as liver, heart and kidney; (3) those with mental illness who were unable to cooperate with normal medical activities. Of the 70 cases, 50 were male and 20 were female, their age ranged 28–76 years, with an average age of (57.21 ± 11.61) years. Disease grading was performed for all enrolled patients on the basis of the World Health Organization grading standard for GC, 29 patients were staged II and 41 patients were staged III. There were 29 cases belonged to poorly differentiated adenocarcinoma, 23 cases belonged to medium differentiated adenocarcinoma, 11 cases belonged to signet ring cell carcinoma, 7 cases belonged to mucinous adenocarcinoma. Tumor location: 20, 17 and 33 cases occurred in the stomach fundus, stomach corpus and stomach antrum, respectively. Borrmann classification: 12, 27, 21 and 10 cases of type I, type II, type III and type IV, respectively. Another 50 healthy people during the same period served as a control group, including 31 males and 19 females, age ranged between 39–72 years, with an average age of (53.15 ± 9.91) years. The experiment has been approved by the ethics committee of Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, China.

Chemotherapy regimens

Epirubicin (Manufacturer: Zhejiang Haizheng pharmaceutical co., Ltd. Batch, Zhejiang, China) 50 mg/m^2 intravenous injection, day 1. Oxaliplatin (Manufacturer: Jiangsu Aosaikang pharmaceutical co., Ltd., Batch, Jiangsu, China) 85 mg/m^2 intravenous infusion for 2 h, day 1. Oral Capecitabine (Manufacturer: Shanghai roche pharmaceutical co., Ltd. Batch, Shanghai, China) 625 mg/m^2, twice daily, throughout treatment. One chemotherapy cycle was 3 weeks, and 2 cycles were completed.

Efficacy judgment standard

The efficacy of NACT was evaluated by tumor regression score (TRG) and microscopically determined by the scale proposed by Mandard's et al [15]. Regression grade [16] was as shown in Table 1. Post-chemotherapy pathological response rate (PRR) $=$ [(TRG3 + TRG4)/total number of cases] $\times$ 100%.

Plasma cfDNA assay

Plasma sample collection and processing: collected 10 ml of peripheral venous blood of the test subject

Table 1 TRG grading basis for advanced GC patients.

TRG	Regression	Fibrosis
TRG0	no regression	fibrosis was completely absent
TRG1	minor regression	dominant tumor mass with obvious fibrosis in ⩽ 25% of the tumor mass
TRG2	moderate regression	dominant tumor mass with obvious fibrosis in 26%–50% of the tumor mass
TRG3	good regression (more than 50%)	dominant fibrosis outgrowing the tumor mass
TRG4	total regression	no viable tumor cells; only fibrotic mass

TRG = tumor regression score.

in an EDTA anticoagulation tube, centrifuged at $1600 \times g$ for 10 min at room temperature, the pale yellow plasma supernatant was aspirated into a new centrifuge tube carefully, and then centrifuged at $16\,000 \times g$ for 10 min at 4 °C, the residual cell debris was carefully removed to obtain a plasma sample. The plasma samples were diluted 10-fold with Tris-EDTA buffer and used as a direct amplification template for plasma. In dilution of the standard, the Human DNA Standard (100 ng/ml, Wei Yin biotechnology co., Ltd., Shanghai, China) was first diluted with DNA Diluent to get the stock at 20 ng/ml, and then diluted again to 1.0 ng/ml. The obtained standard solution was diluted 6 times successively to get 7 different concentrations of standards at concentrations ranging from 1.0 to 0.0002 ng/ml. Human β-myosin (β-globin) sequence was used as the target gene for amplification and detected by fluorescent quantitative PCR kit (Syme fisher technology co. Ltd., Shanghai, China). In the qRT-PCR reaction system preparation and reaction, to determine plasma cfDNA concentration, repetitive LINE 1 (Long interspersed nuclear element 1) 97 bp and LINE1 300 bp DNA fragments were amplified, respectively [17]. Primer 1 (97 bp): forward: 5′-TGGCACATATACACCATGGAA-3′, reverse: 5′-TGAGAATGATGGTTTC-3′; Primer 2 (300 bp): forward: 5′-ACAACCTATTCCAAAATTGACCAC-3′, reverse: 5′-TTCCCTCTACACACTGCTTTGA-3′. Cycling conditions were 1 min at 95 °C, 8 s at 95 °C, 15 s at 60 °C, 35 cycles in total. The ratio of LINE 1 300 bp and 97 bp was used to represent the DNA integrity index. The cfDNA concentration reference value was 0–10.32 ng/ml, the cfDNA integrity reference value was 0–1.03.

Detection of tumor biomarkers

Electrochemiluminescence was used for the detection of carcinoembryonic antigen (CEA), cancer antigen 724 (CA724), cancer antigen 199 (CA199), and alpha-fetoprotein (AFP). Serum were obtained by centrifugation of fasting venous blood, using fully automated electrochemiluminometer E170 (Roche Diagnostics Ltd. Shanghai, China) and assorted kits (Roche Diagnostics Ltd. Shanghai, China). The reference ranges for each item are given as follow: CEA < 3.5 ng/ml, CA724 < 6.9 U/ml, CA199 $<$ 39 U/ml and AFP < 7 ng/ml.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. Measurement data were expressed as ($x \pm$ SD), t-test was used for comparison between two groups, and one-way analysis of variance was used for comparison between multiple groups; count data were analyzed by Fisher's exact test or Chi-square test. Patients' prognostic univariate/multivariate correlation analysis used COX proportional hazard regression model. Kaplan-Meier analysis was used to establish progression-free survival (PFS) curve. $p < 0.05$ was considered as statistical significance.

RESULTS AND DISCUSSION

Efficacy of NACT in patients with advanced GC

After 70 patients with advanced GC were treated with two courses of neoadjuvant chemotherapy, 7, 10, 14, 37 and 2 cases of TRG0, TRG1, TRG2, TRG3 and TRG4 were found, with the pathological response rate (PRR) of 55.71%. All patients showed no disease progression during chemotherapy. Two patients (2.8%) had complete pathological remission.

Plasma cfDNA concentration and integrity in healthy individuals and advanced GC patients

The average cfDNA concentration in healthy people, advanced GC patients before NACT and after NACT were 5.93 ± 2.11, 23.41 ± 8.26 and 15.39 ± 7.28 ng/ml, respectively. As for cfDNA integrity, the average values for healthy individuals, advanced GC patients before NACT and after NACT were 0.96 ± 0.32, 5.13 ± 1.19 and 2.57 ± 1.04 ng/ml, respectively. (Fig. 1A-B).

The cfDNA concentration and integrity of patients in the TRG0, TRG1, TRG2, TRG3, and TRG4 groups before and after NACT were analyzed (Fig. 1C-D). The results showed that the cfDNA concentration and integrity of patients with advanced GC before NACT were significantly higher than those in healthy people, and both indexes of advanced GC patients after NACT were significantly lower than the patients with advanced GC before NACT, all $p < 0.05$ (Fig. 1A-B). Grouped by

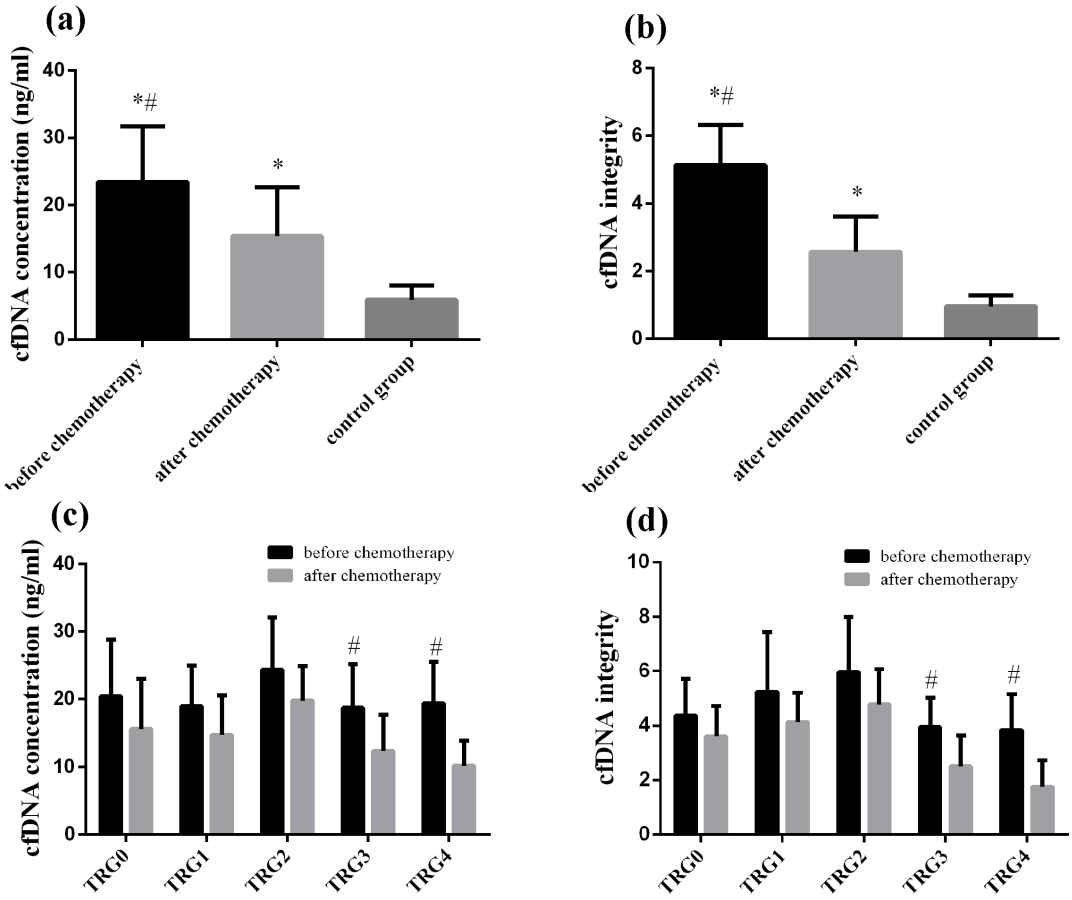

Fig. 1 Concentration and integrity of cfDNA in plasma of patients with advanced GC. * indicates $p < 0.05$ compared with the control group; # indicates $p < 0.05$ compared with the after chemotherapy group. Comparison of (A) plasma cfDNA concentrations and (B) plasma cfDNA integrity, between healthy volunteers and GC patients before/after chemotherapy, each dot represents the value from the individual patient (total of 70) or healthy volunteer (total of 50); Comparison of (C) cfDNA concentration and (D) cfDNA integrity, before and after chemotherapy in GC patients with different curative effects, the values are averages from each group of patients or from healthy group.

different TRG grades after chemotherapy, cfDNA concentration and integrity were analyzed before and after chemotherapy in each group. It was found that cfDNA concentration and integrity of TRG3 and TRG4 were significantly different before and after chemotherapy, all $p < 0.05$ (Fig. 1C-D). This suggests that the better chemotherapy effect on the patient, the greater amplitude of reduction in cfDNA concentration/integrity.

Relationship between plasma cfDNA concentration and clinical characteristics in patients with advanced GC

Correlations between plasma cfDNA concentration and clinical features in 70 patients with GC before and after NACT were analyzed. The results showed that there was no significant correlation between cfDNA concentration and age, gender, tumor location, size, tumor differentiation, Borrmann classification, lymph node metastasis, infiltration depth, CEA, CA724, CA199 and AFP expressions in patients with GC before and after NACT ($p > 0.05$), but significantly related to TNM staging ($p < 0.05$, Table 2).

Relationship between plasma cfDNA integrity and clinical features of advanced GC patients

Correlation between plasma cfDNA integrity and clinical features in 70 patients with advanced GC before and after NACT were analyzed. We found no significant correlation between cfDNA integrity and age, gender, TNM staging, tumor location, tumor

Table 2 Correlation between plasma cfDNA concentration and clinical characteristics.

Clinicopathological parameter	Case (n)	cfDNA concentration (ng/ml) Before NACT	After NACT	Clinicopathological parameter	Case (n)	cfDNA concentration (ng/ml) Before NACT	After NACT
Age/year				Borrmann classification			
< 60	37	21.57 ± 9.06	14.49 ± 5.22	III	21	27.84 ± 13.01	18.57 ± 9.61
$\geqslant 60$	33	25.77 ± 10.35	17.12 ± 7.54	IV	10	21.94 ± 9.84	14.68 ± 6.55
p		0.0746	0.0915	p		0.4370	0.1615
Gender				Lymph node metastasis			
Male	50	20.81 ± 9.50	14.45 ± 4.81	N_0	23	29.57 ± 13.85	18.49 ± 6.51
Female	20	24.66 ± 12.21	16.85 ± 6.39	N_1	15	23.01 ± 8.18	15.98 ± 5.69
p		0.1634	0.0915	N_2	11	20.59 ± 8.51	12.84 ± 4.11
TNM Stage				N_3	21	26.54 ± 10.97	17.26 ± 7.67
II	29	19.98 ± 10.29	10.75 ± 3.13	p		0.1225	0.1182
III	41	26.72 ± 11.91	15.17 ± 7.56	Depth of infiltration			
p		0.0163	0.0041	T1–T2	25	20.71 ± 8.32	12.91 ± 4.17
				T3–T4	45	25.03 ± 10.12	16.01 ± 7.93
Cardia	20	24.62 ± 12.03	12.94 ± 5.02	p		0.0734	0.0738
Gastric body	17	26.33 ± 11.14	15.89 ± 6.12	CEA (ng/ml)			
Gastric antrum	33	21.57 ± 9.85	16.35 ± 6.22	< 3.5	52	21.56 ± 5.21	13.79 ± 4.06
p		0.3042	0.1164	$\geqslant 3.5$	18	25.28 ± 10.59	16.44 ± 7.52
Tumor size (d/cm)				p		0.0546	0.0641
< 5	42	19.07 ± 8.62	13.78 ± 4.92	CA724 (U/ml)			
$\geqslant 5$	28	23.92 ± 13.38	16.13 ± 8.05	< 6.9	49	18.67 ± 8.15	12.92 ± 5.54
p		0.0692	0.1339	$\geqslant 6.9$	21	22.66 ± 10.74	16.05 ± 7.05
Degree of differentiation				p		0.0934	0.0504
Poor	29	30.16 ± 10.01	16.02 ± 7.43	CA199 (U/ml)			
Medium	23	25.57 ± 9.66	14.03 ± 3.82	< 39	46	26.60 ± 10.95	16.01 ± 7.69
Signet-ring cell carcinoma	11	23.19 ± 11.58	15.64 ± 5.18	$\geqslant 39$	24	21.46 ± 11.23	12.99 ± 5.54
Mucinous cell carcinoma	7	22.14 ± 10.14	11.53 ± 3.84	p		0.0689	0.0929
p		0.1058	0.2575	AFP (ng/ml)			
Borrmann classification				< 7	67	23.03 ± 9.39	17.33 ± 7.01
I	12	22.43 ± 12.67	12.36 ± 4.49	$\geqslant 7$	3	18.01 ± 7.13	14.28 ± 6.12
II	27	26.19 ± 10.34	17.07 ± 8.15	p		0.3652	0.4619

AFP, alpha-fetoprotein; CEA, carcinoembryonic antigen; CA724, cancer antigen 724; CA199, cancer antigen 199.

Table 3 Correlation between integrity of cfDNA and clinical characteristics.

Clinicopathological parameter	Case (n)	cfDNA concentration (ng/ml) Before NACT	After NACT	Clinicopathological parameter	Case (n)	cfDNA concentration (ng/ml) Before NACT	After NACT
Age/year				Borrmann classification			
< 60	37	5.54 ± 1.76	3.06 ± 1.92	III	21	7.11 ± 4.76	4.39 ± 1.05
$\geqslant 60$	33	4.87 ± 1.18	2.41 ± 0.98	IV	10	4.64 ± 2.82	3.58 ± 1.15
p		0.0691	0.0845	p		0.2738	0.0567
Gender				Lymph node metastasis			
Male	50	5.97 ± 2.57	2.97 ± 1.94	N_0	23	6.54 ± 3.20	4.65 ± 2.57
Female	20	4.98 ± 1.59	2.16 ± 1.73	N_1	15	4.26 ± 1.75	3.08 ± 1.49
p		0.1141	0.1087	N_2	11	4.93 ± 1.81	3.19 ± 1.01
TNM Stage				N_3	21	7.14 ± 4.98	4.23 ± 2.61
II	29	5.05 ± 2.82	3.98 ± 1.28	p		0.064	0.1104
III	41	6.48 ± 3.59	4.69 ± 2.06	Depth of infiltration			
p		0.0781	0.1049	T1–T2	25	4.71 ± 1.52	1.82 ± 0.84
Tumor location				T3–T4	45	6.01 ± 3.12	2.21 ± 1.03
Cardia	20	6.30 ± 3.05	3.09 ± 2.32	p		0.0548	0.1106
Gastric body	17	5.25 ± 2.87	2.89 ± 1.45	CEA (ng/ml)			
Gastric antrum	33	4.91 ± 1.95	2.21 ± 1.03	< 3.5	52	4.86 ± 1.51	2.74 ± 1.05
p		0.1327	0.1177	$\geqslant 3.5$	18	6.05 ± 3.66	3.18 ± 1.32
Tumor size (d/cm)				p		0.0572	0.1567
< 5	42	4.84 ± 2.32	3.43 ± 1.62	CA125 (U/ml)			
$\geqslant 5$	28	6.12 ± 3.83	4.16 ± 2.55	< 35	49	4.37 ± 1.35	2.59 ± 1.06
p		0.086	0.1472	$\geqslant 35$	21	5.19 ± 2.04	3.05 ± 1.10
Degree of differentiation				p		0.0513	0.1045
Poor	29	7.86 ± 5.03	5.11 ± 3.03	CA199 (U/ml)			
Medium	23	7.25 ± 3.36	4.79 ± 2.82	< 39	46	5.49 ± 2.25	3.49 ± 1.68
Signet-ring cell carcinoma	11	6.14 ± 3.78	3.14 ± 1.38	$\geqslant 39$	24	4.63 ± 1.63	2.76 ± 1.14
Mucinous cell carcinoma	7	5.01 ± 1.59	3.02 ± 0.94	p		0.1021	0.0605
p		0.3378	0.0821	AFP (ng/ml)			
Borrmann classification				< 7	67	5.31 ± 2.13	3.55 ± 1.23
I	12	6.18 ± 1.67	3.42 ± 0.97	$\geqslant 7$	3	4.03 ± 1.47	2.14 ± 1.12
II	27	7.03 ± 3.34	4.15 ± 1.17	p		0.3084	0.0556

AFP, alpha-fetoprotein; CEA, carcinoembryonic antigen; CA125, cancer antigen 125; CA199, cancer antigen 199.

size, tumor differentiation, Borrmann classification, lymph node metastasis, infiltration depth, CEA, CA724, CA199 and AFP expressions in patients with GC before and after NACT ($p > 0.05$, Table 3).

ROC curve of cfDNA and traditional tumor markers

The ROC curves of cfDNA, CEA, CA724, CA199 and AFP were as shown in Fig. 2. They were used to compare the sensitivity and specificity of tumor markers, and to determine the best cut off cfDNA concentration with a total progression-free survival (PFS) less than one and a half years. The area under curve (AUC) of cfDNA concentration and cfDNA integrity were 0.8113 (95% CI: 0.7438–0.8789) and 0.8089 (95% CI: 0.7396–0.8782), respectively. The AUC of CEA, CA724, CA199 and AFP were 0.6312 (95% CI: 0.5453–0.7171), 0.7910 (95% CI: 0.7112–0.8709), 0.6186 (95% CI: 0.5301–0.7071) and 0.6830 (95% CI: 0.6003–0.7658), respectively. The AUC values of cfDNA concentration and integrity were higher than the traditional tumor markers CEA, CA724, CA199 and AFP, indicating their greater sensitivity and specificity than traditional tumor biomarkers. In order to facilitate the practical application of our results, the ROC value of 11.24 ng/ml was used to determine the optimal cutoff value of cfDNA concentration of PFS within one and a half years.

Univariate and multivariate analyses of PFS in patients with advanced GC

GC patients were followed up until April 2019. The median follow-up time was 12.24 months. COX univariate and multivariate regression analyses were performed on 70 advanced GC patients with age, gender, TNM stage, tumor location, tumor size, degree of differentiation, Borrmann classification, lymph node metastasis, depth of infiltration and cfDNA concentration at high expression (cfDNA concentration > 11.24 ng/ml). The results showed that degree of differentiation ($p = 0.011$) and cfDNA concentration at high expression ($p = 0.035$) were independent risk factors for PFS of GC (Table 4, Table 5).

Comparison of postoperative PFS after NACT in patients with advanced GC

Further grouping by cfDNA concentration showed that postoperative PFS of GC patients with low expression of cfDNA (cfDNA concentration < 11.24 ng/ml) were longer than those of patients with high expression of cfDNA (cfDNA concentration > 11.24 ng/ml), $p = 0.038$ (Fig. 3).

DISCUSSION

NACT is an important method for clinical treatment of patients with advanced GC, and real-time evaluation of NACT is currently the focus of researchers [18]. Histopathological biopsy is the "gold standard" for the diagnosis of GC. However, due to the large trauma of tissue puncture, low success rate, and limited tumor heterogeneity, it is not an ideal tool for assessing tumor burden in real time. Image examination is incapable of microscopic lesions, which cannot reflect the tumor load changes in time, and radiation cancer is not suitable for long-term use [19]. In recent years, body fluid based tumor detection–"liquid biopsy", has received extensive attention due to its simple operation, small trauma, and resampling for dynamic monitoring [20]. Moreover, since the tumor cells are heterogeneous cell population, liquid biopsy can provide more comprehensive molecular information than traditional methods that only draw on a one part of the tumor [21, 22].

The detection object of "liquid biopsy" is a tumor marker which is present in or produced by tumor cells. Normal cells can also express tumor markers, but at a very smaller amount compared to tumor cells. Currently, they are mainly used for the diagnosis of malignant tumors, evaluation of efficacy, recurrence and prognosis [23]. For many years, medical researchers have been studying the use of abnormal changes in tumor markers as a clue to carry out remedial treatment in advance before the occurrence of clinical symptoms of tumor recurrence, that is, when the tumor markers are abnormally increased and the imaging examination has not found metastasis, the intervention is started [24, 25]. The commonly used tumor markers for the diagnosis of GC are CEA, CA199, CA724 and AFP, but the sensitivity and specificity of the above indicators are low. The sensitivity of CEA, CA724 and CA199 in the GC diagnosis alone was 20.1–27.6%, and increased to 48.2% when combined [26]. Therefore, biomarkers for GC load detection with high sensitivity and specificity and real-time monitoring are urgently needed.

With the development of research in recent years, the results repeatedly confirmed that cancer patients have higher concentrations of cfDNA than healthy people. This led to more and more attention from researchers in early tumor screening and diagnosis [27, 28]. cfDNA is rapidly cleared in

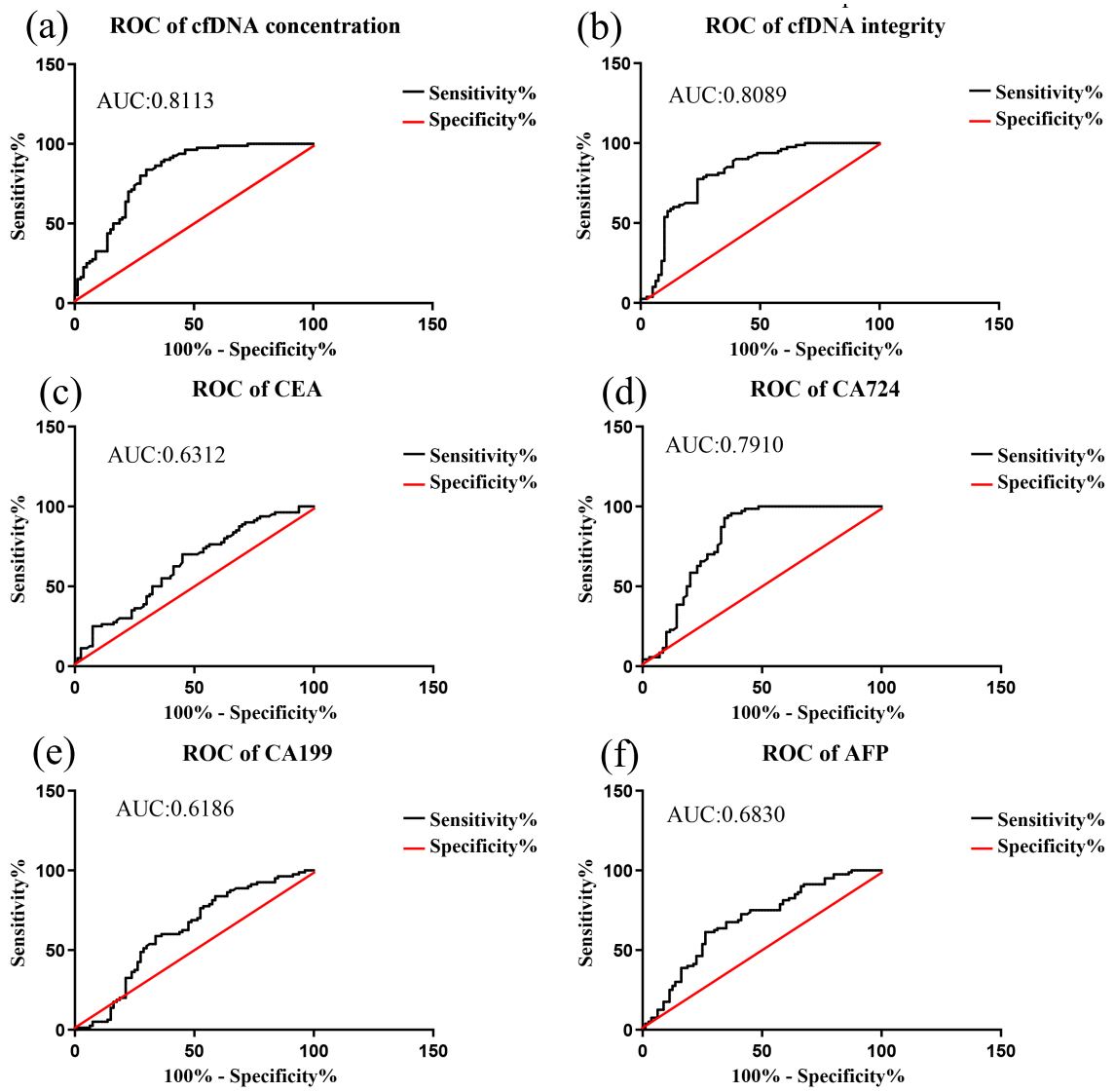

Fig. 2 ROC curve analysis of traditional tumor markers and cfDNA in advanced GC patients.

Table 4 COX univariate regression analysis.

Factor	B	SE	Wald	df	Sig.	Exp	95.0% CI for Exp(B)	
							Lower	Upper
Age/year	−0.004	0.013	0.098	1	0.754	0.996	0.972	1.021
Gender	0.424	0.266	2.537	1	0.111	1.528	0.907	2.575
TNM Stage	−0.458	0.266	2.971	1	0.085	0.632	0.375	1.065
Tumor location	−0.080	0.157	0.258	1	0.612	0.923	0.679	1.256
Tumor size	−0.334	0.269	1.538	1	0.215	0.716	0.423	1.214
Degree of differentiation	−0.281	0.124	5.176	1	0.023	0.755	0.592	0.962
Borrmann classification	−0.050	0.122	0.170	1	0.680	0.951	0.749	1.208
Lymph node metastasis	−0.031	0.110	0.080	1	0.777	0.969	0.782	1.202
Depth of infiltration	−0.035	0.266	0.018	1	0.895	0.965	0.574	1.625
cfDNA (negative/positive)	0.553	0.262	4.453	1	0.035	1.738	1.040	2.905

B, partial regression coefficient; SE, standard error; Wald, test statistic; df, degree of freedom; Sig., significance; Exp, hazard ratio; CI, confidence interval.

Table 5 COX multivariate regression analysis.

Factor	B	SE	Wald	df	Sig.	Exp	95.0% CI for Exp(B)	
							Lower	Upper
TNM Stage	−0.526	0.275	3.675	1	0.055	0.591	0.345	1.012
Degree of differentiation	−0.328	0.129	6.444	1	0.011	0.720	0.559	0.928
cfDNA (negative/positive)	−0.556	0.264	4.444	1	0.035	0.573	0.342	0.962

All abbreviations are the same as in Table 4.

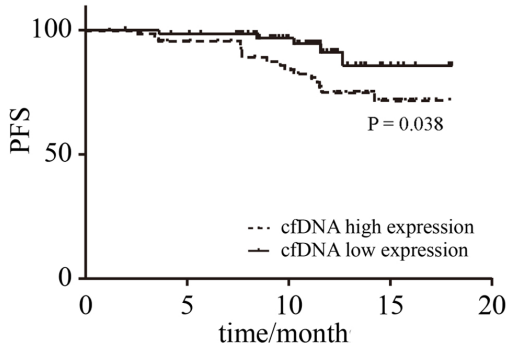

Fig. 3 The PFS curve of patients with advanced GC. cfDNA concentrations were analyzed by Kaplan-Meier.

peripheral blood with a half-life of 15 minutes to several hours, which made cfDNA become a real-time marker.

There are two main sources of cfDNA in plasma, they are derived from the process of apoptosis and the passive lysis of cells (e.g. tissue necrosis) [29]. The production and clearance of cfDNA in normal people is in a state of dynamic equilibrium, so the concentration of cfDNA is low. However, when cancer or inflammation occurs in the body, the production of cfDNA is greatly increased, and the homeostasis is interfered, so that the plasma cfDNA concentration rises rapidly and exceeds the normal level. The inclusion of cfDNA integrity indicators can further improve the accuracy of cfDNA detection. The DNA fragment derived from cell necrosis is larger than the fragment derived from apoptosis, and this feature is used to estimate the proportion of tumor source in cfDNA [30]. Recent studies have shown that cfDNA absolute concentrations and cfDNA integrity may be candidate biomarkers for the diagnosis and prognosis of malignant tumors [31]. In malignant solid tumors, DNA integrity index is associated with tumor burden and is expected to become a molecular marker for clinical diagnosis. Many investigators have studied the prognostic value of cfDNA for tumor recurrence and patient survival in patients with cancer at lung, breast, colon, uterine, prostate, and melanoma, as well as the monitoring value of response to treatment. High concentrations of cfDNA in most cases confirmed that cfDNA was an independent risk factor for disease survival and could be used for detection management and prognosis of tumor response [32–35]. Normando et al [36] had reported that cfDNA concentration in advanced GC patients was significantly higher than that in the normal control group ($p < 0.01$). Yu et al [37] reported that in patients with chemotherapy of colorectal cancer, the concentration of cfDNA increased (earlier and with higher specificity and sensitivity than responses in CEA or image information) after surgery which usually indicated early recurrence. Miao et al [27] demonstrated that there was a certain amount of cfDNA in the blood of patients with breast cancer before surgery. After radical surgery, the cfDNA content of the patients was reduced to different extents due to the removal of the primary tumor and the surrounding blood vessels and lymphatic vessels. When the tumor recurred, the cfDNA content increased. Therefore, it is speculated that cfDNA can be used to evaluate the effect of breast cancer surgery and the prognosis of patients, and to predict the postoperative tumor recurrence in advance. This study used the same idea to analyze the concentration and integrity of cfDNA in healthy and advanced GC patients before and after NACT. We found that cfDNA concentration and fragment integrity of patients with advanced GC before NACT were significantly higher than healthy people. After receiving NACT, cfDNA concentration and integrity were significantly reduced, and the reduction is related to the patient's efficacy, that is, the better the patient's efficacy, the greater the reduction. The concentration and integrity of cfDNA after chemotherapy in patients with TRG3-4 was significantly lower than before chemotherapy. Therefore, we speculated that plasma cfDNA is mainly derived from necrosis of GC cells. After receiving NACT, the patient's condition was relieved and tumor cell necrosis was reduced, thus reducing the concentration and integrity of plasma cfDNA.

The analysis showed that cfDNA concentration was significantly correlated with TNM stage, thus reflected the activity of tumor growth.

After clarifying the difference in cfDNA concentration and integrity in patients with different efficacy, we established the ROC curve and calculated the AUC. The results confirmed that the AUC of cfDNA concentration/integrity was greater than those of CEA, CA724, CA199 and AFP. The higher cfDNA sensitivity and specificity than the mentioned biomarkers can be used as a reliable auxiliary index for detecting the prognosis of NACT in patients with advanced GC. In order to enhance the persuasiveness of cfDNA as a prognostic indicator for GC patients, we performed COX analysis on factors affecting patient prognosis. COX univariate and multivariate analyses showed that cfDNA was an independent risk factor for prognosis, and elevated cfDNA levels predicted poor prognosis.

In summary, the concentration and integrity of cfDNA in patients with advanced GC were significantly higher than those in normal subjects. After receiving NACT, the concentration and integrity of cfDNA were significantly down-regulated. cfDNA sensitivity and specificity were higher than CEA, CA724, CA199 and AFP biomarkers, and cfDNA was an independent risk factor for the prognosis of patients with advanced GC. Therefore, we believe that cfDNA concentration and integrity can be used as a real-time monitoring indicator of the efficacy of NACT in patients with advanced GC. Plasma cfDNA concentration and integrity were measured before NACT chemotherapy and after the second times NACT chemotherapy to assess the patients' benefit from chemotherapy. If the patient's plasma cfDNA concentration and integrity are significantly reduced, then the patient can be identified as a beneficiary of NACT, and the third course of treatment can continue. If there is no significant change in cfDNA concentration and integrity after the second course of treatment, it is considered that NACT does not control the patient's condition, and patients should be treated immediately by surgery to avoid tumor progression and reduce the risk of non-radical treatment. cfDNA has the advantages of high sensitivity and wide application in the detection of gastric cancer patients, which can significantly improve the sensitivity of gastric cancer monitoring and satisfy the clinical monitoring of the efficacy of NACT chemotherapy for gastric cancer patients.

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