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Original Research

Cell-free DNA as a biomarker for colorectal cancer: a retrospective analysis in patients before and after surgery

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Abstract: This study was aimed at investigating the potential of cell-free DNA (cfDNA) as a biomarker for colorectal cancer prognosis. Sixty patients with colorectal cancer who had not undergone surgery were enrolled as study group. Their peripheral blood samples were collected, and peripheral blood of 30 healthy volunteers (control) was collected. The cfDNA concentration and integrity were determined using q-PCR so as to ascertain if cfDNA was associated with clinical presentations of the disease. Then, the specificities and sensitivities of cfDNA, CFA and CA199 were determined with ROC curve. The level and integrity of cfDNA in patients with colorectal cancer before surgery were significantly higher than those in patients with colorectal cancer after surgery, and cfDNA concentration of colorectal cancer patients after surgery was also significantly higher than that in healthy control group. However, the integrity was not significantly different from that of control group. There was a significant correlation between cfDNA concentration and TNM stage, differentiation degree and CEA expression, while cfDNA integrity was significantly correlated with TNM stage and degree of differentiation. Moreover, specificity and sensitivity of cfDNA concentration and integrity were higher than those of CEA and CA199. The TNM stage and cfDNA concentration were independent risk factors for progression-free survival (PFS) in colorectal cancer patients. In conclusion, cfDNA concentration and integrity were more sensitive and specific than traditional tumor markers (CA199, CEA). Thus, changes in cfDNA changes can be effectively used to determine the prognosis of postoperative colorectal cancer patients.

Key words: Cell-free DNA; colorectal cancer; CA199; CEA; ROC curve.

Introduction

Colorectal cancer is one of the malignant tumors that seriously threaten human health and life. Its incidence is increasing year by year (1). The onset of colorectal cancer is not easily noticed because it develops slowly. Therefore, early detection is difficult because early symptoms of colorectal cancer are not obvious. Thus, when most patients with colorectal cancer are diagnosed, the tumor cells have already metastasized, so that they miss the opportunity of cure. Although patients with colorectal cancer in the early and middle stages are treated with radical surgery, the degree of recurrence and progression are still high, and the prognosis is not promising (2). Therefore, prognostic evaluation of colorectal cancer is of great significance for improving their quality of life, survival, and progression-free survival.

The detection of tumor markers can be effectively used to monitor the occurrence, progression and prognosis of tumors. It has the advantages of convenience, rapidity and non-invasiveness. The tumor markers cancer antigen 199 (CA199) and cancer embryonic antigen (CEA), as well as fecal occult blood are commonly used markers for the assessment of CRC prognosis. However, their sensitivity and specificity are low on their own, and they have certain limitations in the evaluation of pathology, which do not meet clinical requirements (3). Hence, there is a need for examination methods that are simple, easily accepted by patients, and have high prognostic evaluation value, so as to improve the survival of colorectal cancer patients.

The circulating free DNA (also known as cell-free DNA, cfDNA) in the blood is mainly derived from apoptosis and necrosis of body cells. Numerous reports have shown that the cfDNA level of tumor patients is significantly higher than that of normal people (4-6). With specific reference to colorectal cancer, Spindler *et al.* reported that the level of circulating DNA has a good predictive value for the prognosis of patients with metastatic colorectal cancer (7). In another study,

higher cfDNA levels in plasma of metastatic colorectal cancer patients receiving third-line chemotherapy were significantly associated with poor overall survival, and patients with low cfDNA levels had longer median overall survival (8). These studies suggest that cfDNA may play a role as a tumor marker. Therefore, the present study used qPCR to determine cfDNA levels and integrity in plasma of patients with colorectal cancer before and after surgery, and simultaneously measured multiple tumor markers in plasma of these patients. These were done in order to analyze and compare their correlation with the CRC diagnosis and clinic-pathological features, and to unravel their clinical significance.

Materials and Methods

Sample selection

Sixty primary colorectal cancer patients who had not undergone surgery between June 2018 and November 2018 were selected as study subjects. Thirty-four (34) (56.67%) of the patients were male and 26 (43.33%) were female, with ages ranging from 32 to 77 years (average age = 49.3±4.1 years).

Inclusion criteria: (1) patients diagnosed through histopathological examination, digestive tract barium meal and B-ultrasound; (2) patients with intact case data, and (3) patients with KPS score > 60 points were included in the study.

Exclusion criteria: (1) The excluded patients were those with other malignant tumors at the same time; (2) patients with serious diseases such as heart, liver and kidney diseases; (3) patients with acute or chronic infectious diseases; and (4) those with mental illness who were unable to cooperate with normal medical activities. A set of 30 healthy people during the same period served as control group, including 13 (43.33%) males and 17 (56.67%) females, with ages ranging from 40 to 71 years (mean age = 50.5±2.9 years). Sex and age were comparable between both groups. All study participants signed informed consent prior to enrollment in the study. This clinical trial was performed following approval by the Clinical Research Ethics Committee of Yixing People's hospital.

Plasma separation and cfDNA extraction

All patients underwent radical surgery, and 4ml of peripheral venous blood was collected 10 days before surgery, and 10 days after surgery. The blood was taken in EDTA bottles and centrifuged at 1600g to obtain plasma which were used for extraction of DNA. The extracted DNA samples were kept frozen at -80°C. Prior to purification, the DNA was centrifuged at 10,000g for 3 minutes, and purified using QIAamp Blood DNA Mini Kits (Qiagen, CA) in line with the kit protocols.

Q-PCR

Plasma cfDNA concentrations were determined by amplification of two LINE1 DNA fragments of 97 and 259bps (9). Total plasma DNA was determined with q-PCR using a primer of 97 bp-LINE1, and DNA integrity was expressed as ratio of result of LINE1 259bp to that of LINE1 97bp. A standard curve was established with q-PCR results from seral dilutions of human genomic DNA. Quantitative PCR amplification was carried out using 2µl of the cfDNA obtained from extraction with QIAamp Circulating DNA Extraction Kit, and 2µl of the diluted standard cfDNA (1, 1:10, 1:100, 1:1000, 1:10000). The 20µl q-PCR mixture contained 0.5µl forward primer, 0.5µl reverse primer, 1µl DNA template, 10 µl UltraSYBR Green Mixture (Tiangen, Beijing) and 8µl dd H_2O. The p-PCR was carried out for 1 min at 95°C**,** 95°C for 8sec, and 60°C for 15sec, and repeated for 35 cycles. Each sample was done in triplicate.

Detection of tumor biomarkers

Electrochemiluminescence was used for the determination of tumor biomarkers. Plasma samples were obtained by centrifugation of fasting venous blood, using fully automated Electrochemiluminometer E170and assorted kits (Roche, Switzerland). The reference ranges for each item are given as follows: cancer antigen199 (CA199) < 39 U/ml and carcino-embryonic antigen (CEA) < 3.5 ng/mL.

Statistical analysis

Data on cfDNA levels are expressed as mean ±SD**.** Statistical analysis was done using SPSS 21.0 software. Comparisons amongst groups were done with *t*-test or one-way ANOVA. Comparison of count data was done with chi-square test, while ROC curve was used to evaluate the specificity and sensitivity of cfDNA for colorectal cancer screening. Values of $p < 0.05$ were considered statistically significant.

Results

Clinicopathological features

Table 1 shows the clinical features of colorectal cancer subjects. Sex and age distribution were comparable in the study and control groups. There were no significant differences in the clinicopathological characteristics of patients before or after surgery. These results show that there was comparability between the groups.

Comparison between cfDNA concentration and integrity

As shown in Figure 1, the cfDNA level in healthy people was 6.59 ± 1.51 ng/mL, and the cfDNA integrity was 0.78 ± 0.31. The cfDNA level in colorectal cancer patients before surgery was 26.12±8.59 ng/mL, while cfDNA integrity gave a value of 3.66±1.51. However, after surgery, cfDNA level of patients with colorectal cancer was 13.17±2.74 ng/mL, and cfDNA integrity was 1.38±0.69. Thus, cfDNA level and cfDNA integrity of patients with colorectal cancer before surgery

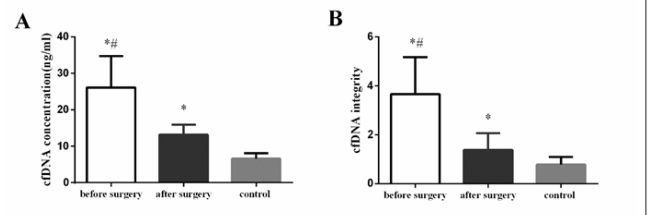

Figure 1. Serum cfDNA concentration and integrity in colorectal cancer patients and healthy people. *p<0.05, compared with the control group; # p<0.05, compared with the after-surgery group.

Table 1. Clinical features of colorectal cancer patients.

Variables	Before surgery	After surgery	Control	*p*
Gender				
Male	28	28	13	0.7647
Female	32	32	17	
Age				
≥65	24	24	10	0.5386
<65	36	36	20	
Tumor site				
Colon	24	24	-	-
Rectum	36	36	-	
TNM Staging				
I/II	26	26	-	-
III/IV	34	34	-	
Differentiation degree				
Low-medium	46	46	-	-
High	14	14	-	
Tumor diameter (cm)				
≤ 5	10	6	-	0.421
> 5	50	54	-	
CA199				
≥39 U/ml	29	15	-	0.008
<39 U/ml	31	45	-	
CEA				
≥3.5 ng/mL	29	17	-	0.024
<3.5 ng/mL	31	43	-	
Lymphatic metastasis				
Yes	10	9	-	0.803
No	50	51	-	

Table 2. Correlation between plasma cfDNA concentration and clinical characteristics.

Variables	Before surgery	After surgery
Gender		
Male	21.16±10.81	11.27±3.89
Female	18.14±6.46	12.95±4.34
p	0.1879	0.1220
Age		
≥65	25.28±13.49	12.05±2.02
<65	29.61±18.74	10.46±3.65
p	0.3337	0.0571
Tumor location		
Colon	18.64±9.33	13.14±3.71
Rectum	24.18±11.27	11.69±2.58
P	0.0509	0.0791
TNM Staging		
I/II	20.20±9.54	15.58±3.67
III/IV	25.19±8.84	19.16±3.21
p	0.0427	0.0002
Differentiation degree		
Low-medium	25.67±11.72	15.84±4.81
High	18.31±10.42	11.61±2.34
p	0.0394	0.0025
Tumor diameter (cm)		
≤ 5	18.91±6.17	13.96±2.87
> 5	23.65±12.48	16.49±3.68
P	0.0734	0.0509
CA199		
≥39 U/ml	30.58±14.34	19.61±6.54
<39 U/ml	26.51±8.64	16.97±3.67
P	0.1849	0.0556
CEA		
≥3.5 ng/mL	32.14±12.97	18.64±3.27
<3.5 ng/mL	24.88±9.47	15.91±3.49
p	0.0157	0.0074
Lymphatic metastasis		
Yes	23.91±6.85	13.16±2.62
No	20.77±8.46	11.54±1.65
p	0.7848	0.6959

were significantly higher than those of colorectal cancer patients after surgery. After surgery, the two indices of colorectal cancer patients were also significantly higher than those of the healthy control group ($p<0.05$).

Analysis of correlation between cfDNA concentration/integrity and clinical features

Table 2 (cfDNA concentration) and Table 3 (cfDNA integrity) show the results of the correlation analyses. There were no significant correlations between cfDNA

concentration/integrity and gender, age, tumor location, tumor diameter and CA199 expressions in patients with colorectal cancer before or after surgery ($p > 0.05$). However, cfDNA was significantly correlated with TNM stage, differentiation degree, and CEA expression ($p<0.05$). Moreover, there was significant correlation between cfDNA integrity, TNM stage and degree of differentiation.

Receiver operator characteristic curve analysis of cfDNA levels in colorectal cancer patients

An ROC curve was established based on serum cfDNA concentration and tumor markers (CA199 and CEA) concentrations in patients with colorectal cancer before and after operation. The results are shown in Figure 2. For colorectal cancer patients before surgery, the AOCs for CA199, CEA and cfDNA concentration were 0.7494 (95% CI: 0.6539 - 0.8449), 0.7330 (95% CI: 0.6621 - 0.8039), and 0.7797 (95% CI: 0.7031 - 0.8562), respectively. The AUC for cfDNA integrity was 0.8058. (95% CI: 0.7487 to 0.8630). For colorectal cancer patients after surgery, AUCs for CA199, CEA and cfDNA level were 0.6942 (95% CI: 0.6239 - 0.7646), 0.7762 (95% CI: 0.6977 - 0.8547), and 0.8640 (95% CI: 0.8154 - 0.9126), respectively, while AUC for cfDNA integrity was 0.8919 (95% CI: 0.8456 -to 0.9382). It can be seen from the above results that specificity and sensitivity of cfDNA concentration and integrity were higher than those of CEA and CA199. From the ROC curve of cfDNA concentration after surgery, the calculated optimal cut-off value of cfDNA concentration was 11.25ng/mL.

Univariate Kaplan-Meier analysis on the clinical characteristics of colorectal cancer

The clinical characteristics of colorectal cancer patients, including gender, age, tumor location, TNM stage, degree of differentiation, tumor diameter, CA199 level, CEA level and cfDNA concentration, were included in a univariate analysis. The results of Kaplan-Meier analysis showed that TNM stage, differentiation degree, CEA level, cfDNA concentration and lymph node metastasis had impacts on the postoperative progression of colorectal cancer patients. The progression-free survival (PFS) curve of cfDNA concentration was drawn, and it was found that PFS of patients with high cfDNA expression was significantly shorter than those of patients with low cfDNA expression.

COX multivariate regression analysis

Following results of univariate analysis on TNM staging, differentiation degree, CEA, lymphatic metastasis and cfDNA concentration, COX multivariate regression analysis was conducted. The results showed that TNM stage and cfDNA concentration were independent risk factors for PFS in colorectal cancer patients.

Table 3. Correlation between integrity of cfDNA and clinical characteristics.

Variables	Before surgery	After surgery
Gender		
Male	4.34±1.61	1.38±0.53
Female	4.73±1.22	1.25±0.55
p	0.2912	0.3568
Age		
≥65	4.35±1.25	1.60±0.25
<65	3.98±0.64	1.29±0.74
P	0.1370	0.0532
Tumor location		
Colon	6.33±1.35	1.54±0.26
Rectum	5.71±0.82	1.76±0.66
p	0.1390	0.1263
TNM Staging		
I/II	6.37±1.28	1.45±0.24
III/IV	4.81±1.52	1.24±0.33
p	<0.01	0.0060
Differentiation degree		
Low-medium	5.27±1.36	2.04±1.20
High	4.30±1.29	1.48±0.75
p	0.0181	0.0396
Tumor diameter (cm)		
≤ 5	2.49±1.08	1.28±0.58
> 5	3.15±1.36	1.62±0.53
p	0.0966	0.1753
CA199		
≥39 U/ml	4.09±1.81	2.58±1.03
<39 U/ml	3.34±1.27	3.18±1.25
p	0.0700	0.0649
CEA		
≥3.5ng/mL	6.11±1.46	4.02±1.26
<3.5ng/mL	5.49±1.54	3.56±1.02
p	0.1148	0.1467
Lymphatic metastasis		
Yes	4.19±1.64	1.65±0.53
No	3.55±0.91	1.24±0.64
p	0.0857	0.0752

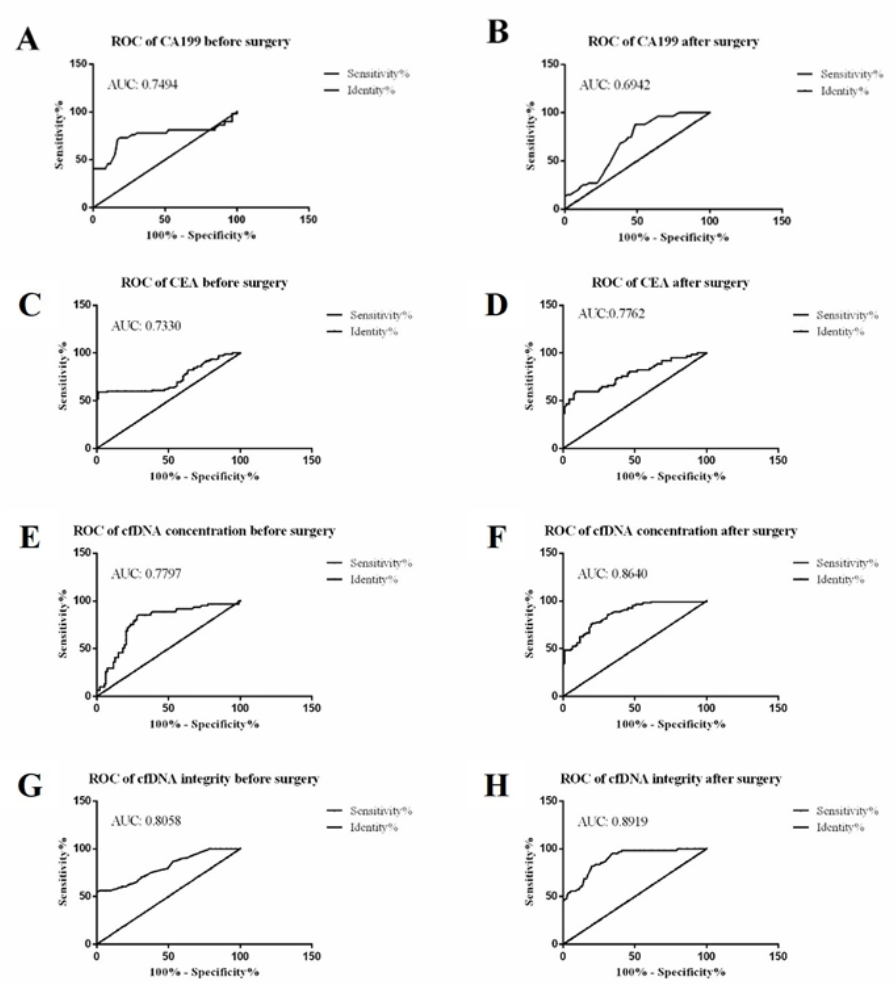

Figure 2. ROC curve analysis of correlation between tumor markers and cfDNA before and after surgery in colorectal cancer patients.

Table 4. Univariate analysis of clinical features affecting PFS in colorectal cancer patients.

	χ^2	df	Sig.
Gender	0.322	1	0.570
Age	3.672	1	0.055
Tumor location	3.781	1	0.052
TNM stage	5.487	1	0.019
Differentiation	4.030	1	0.045
Tumor diameter	0.892	1	0.345
CA199	0.759	1	0.384
CEA	5.043	1	0.025
Lymphatic metastasis	5.564	1	0.018
cfDNA concentration	3.965	1	0.046

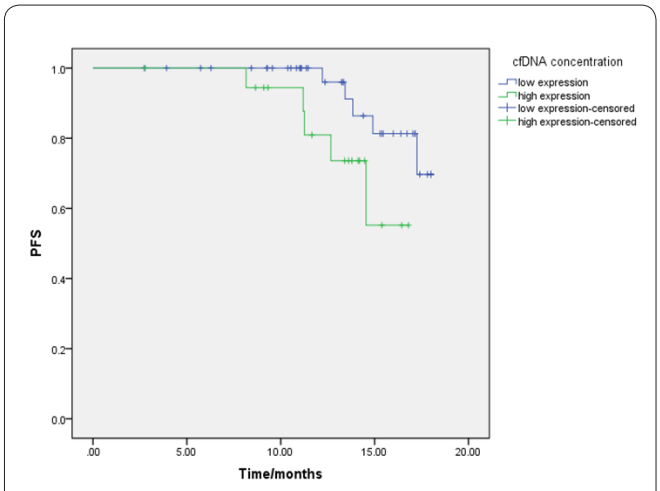

Figure 3. PFS curves of colorectal cancer patients with high and low expressions of cfDNA

Discussion

Cell-free DNA (cfDNA) is a small amount of endogenous and heterologous DNA fragments that is free of extracellular and, including free genomic DNA and free mitochondrial DNA. It is present in various body fluids such as blood, urine, pleural effusion, and in ascites and bronchoalveolar lavage fluid in the form of DNA-protein complexes or free fragments, derived from blood cells, tissue cells, and fat cells. It may also be derived from tumors, grafts or fetuses under certain conditions (11, 12).

The exact mechanism by which cfDNA is produced is currently unclear, but it is thought to result from apoptosis, necrosis, and NETosis (13). NETosis is a newly discovered mode of programmed cell death in-between apoptosis and necrosis, in which neutrophils release their DNA-histone complexes, produce extracellular nets, and kill pathogens. The cfDNA content in humans is below 100 μg/L, with an average of 30 μg/L. The cfDNA content increases in pathological conditions such as infection, inflammation, malignancy, hemodialysis, and organ transplantation. The length of cfDNA in the plasma of the general population is 185-200 bp, while the length of cfDNA in plasma of pregnant women, tumor patients, and organ transplant recipients is shorter than that of the general population (14).

The half-life of cfDNA is 1 to 13 h. Thus, it can be used as an effective biomarker for monitoring rapid

Table 5. COX multivariate regression analysis.

Factor	B	SE	Wald	df	Sig.	Exp	95.0% CI for Exp(B)	
							Lower	Upper
TNM Stage	1.685	0.648	4.375	1	0.047	5.958	1.123	15.11
Differentiation degree	0.322	0.899	0.128	1	0.720	1.380	0.237	8.043
CEA	1.340	0.733	3.346	1	0.067	3.819	0.909	16.05
Lymphatic metastasis	0.782	0.722	1.172	1	0.279	2.185	0.531	8.998
cfDNA concentration	1.822	0.859	4.504	1	0.034	6.185	1.149	33.29

changes in patients (15). The mechanism of cfDNA clearance is not yet understood. Experiments have shown that the liver plays an important role in the clearance of plasma cfDNA, while DNase plays only a part in the degradation of human plasma cfDNA, clearance of fetal cfDNA in maternal plasma, and clearance of DNA injected into mice (16, 17). Kidney metabolism is also one of the possible mechanisms of cfDNA clearance because urine contains plasma-derived cfDNA (15).

In the process of tumor diagnosis and treatment, it is of clinical significance to monitor the size and changes in tumor cell load *in vivo*. The National Cancer Institute (NCI) has given the definition of tumor burden (TB) as the number of cancer cells in the human body, the size of the tumor, or the total number of cancer lesions (18). Studies have shown that the concentration of cfDNA and long-segment DNA in peripheral venous blood indirectly and conveniently reflect changes in TB in humans, and that high specificity and sensitivity are associated with low invasiveness (19, 20). Therefore, TB can be dynamically monitored by measuring cfDNA as a reflection of the biological activity of the tumor.

The free DNA of peripheral blood of healthy people is mostly derived from the normal metabolism of somatic cells, while the free DNA of peripheral blood of tumor patients is derived mainly from tumor cells, and the content of free DNA in peripheral blood of healthy people is lower than that in peripheral blood of tumor patients (21, 22). At present, a large number of studies in China and elsewhere have shown that the total concentration of cfDNA in peripheral venous blood is related to the occurrence and development of tumors. Studies have shown that the cfDNA level of tumor patients is higher than that of normal people, and cfDNA has characteristics of ctDNA such as tumor-related gene mutations, microsatellite changes, methylation abnormalities, and mitochondrial DNA mutations (23, 24).

The combination of peripheral blood cfDNA with tumor markers can improve the accuracy of prognosis assessment of gastric cancer, and the combination of the two has clinical application value for gastric cancer screening (25). The concentration of cfDNA in peripheral blood of patients with cervical cancer was positively correlated with the clinical stage of the patient, level of pathological grade, tumor size, and increases or decreases in serum tumor markers (26). High serum cfDNA levels indicate progression or poor prognosis of cervical cancer.

This study found differences in plasma cfDNA concentration and integrity between colorectal cancer patients and healthy people. Dynamic monitoring may reflect changes in tumor burden *in vivo*. Moreover, cfDNA concentration and integrity were significantly correlated with TNM stage and degree of differentiation in patients, suggesting that plasma cfDNA level and integrity could reflect the degree of tumor malignancy. The AUC of cfDNA was higher than that of CEA and CA199 for colorectal cancer before and after surgery, suggesting that as tumor marker, cfDNA is better than CEA or CA199 in detecting colorectal cancer. Survival analysis results showed that the expression of cfDNA was correlated with patients' PFS. High cfDNA concentration indicated poor prognosis.

This study provides a theoretical basis for the application of cfDNA in the prognosis assessment of colorectal cancer. Since cfDNA is non-invasive and easy to obtain, it may be used as an ideal index for tumor monitoring. It may also be used for dynamic monitoring of prognosis of patients with colorectal cancer. Moreover, it may play an auxiliary role in clinical diagnosis and treatment, timely screening of prognosis of patients, and improvement in progression-free survival of patients.

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Not applicable.

Competing interests
The authors declare no conflict of interest.

Authors' contributions
LC conceived and designed this study. CJ and ZZ performed the experiments and analyzed the data. XZ, JX, CJ and LC collected the patient data. YZ and QZ wrote the manuscript. YZ, SZ and GZ revised the manuscript.

Availability of data and materials
The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All trial participants signed informed consent prior to enrollment in the study. This clinical trial was performed following approval by the Clinical Research Ethics Committee of Yixing People's hospital.

Patient consent for publication
Not applicable.

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