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Clinical significance of plasma free DNA concentration and integrity in detection of primary hepatocellular carcinoma

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Abstract

Objective: The purpose of this study was to examine the clinical significance of plasma cell-free DNA (cfDNA) concentration and integrity in the detection of primary liver cancer (PLC).

Methods: Select 96 PLC patients confirmed by the second hospital of Nanjing as the research group and record the basic clinical information of the patients in detail. Another 50 healthy subjects from the same physical examination were included as the control group. After the qRT-PCR method was used to determine the plasma cfDNA concentration of all subjects, the integrity was calculated. Combined with the basic clinical data of PLC patients, the relationship between cfDNA concentration and integrity and the clinical characteristics of patients was analyzed. Receiver operating characteristic (ROC) curves of cfDNA concentration, cfDNA integrity and other tumor markers (AFP, HEPPAR-1, KI-67, GPC-3, CK19 and CD31) were established to analyze the diagnostic efficacy of each indicator for PLC. Kaplan-meier univariate analysis and COX multivariate analysis were used to analyze the factors affecting the recurrence rate of PLC patients, and to compare the relapse-free survival (RFS) of PLC patients with high and low cfDNA expression after treatment.

Results: Before treatment, the plasma cfDNA concentration and integrity of PLC patients were significantly higher than that of healthy people ($P<0.05$). After treatment, the cfDNA concentration and integrity were significantly lower than before treatment ($P<0.05$), but still higher than that of healthy people ($P<0.05$). CfDNA concentration was significantly correlated with tumor diameter, differentiation degree, BCLC stage and AFP level in PLC patients ($P<0.05$), while cfDNA integrity was significantly correlated with differentiation degree, BCLC stage and AFP level in PLC patients ($P<0.05$). ROC curve results showed that the area under the curve (AUC) of cfDNA concentration and integrity was higher than other tumor markers, and had higher sensitivity and specificity. Kaplan-meier univariate analysis showed that tumor size, AFP, BCLC stage, cfDNA concentration and integrity were significant prognostic factors, and COX multivariate analysis further showed that BCLC stage and cfDNA concentration were independent risk factors for recurrence in PLC patients. After treatment, patients with low plasma cfDNA expression had longer RFS and lower recurrence rates.

Conclusion: CfDNA concentration and integrity have better diagnostic efficacy and prognostic evaluation ability, and can be used as a potential marker of PLC.

Keywords: cfDNA concentration; cfDNA integrity; PLC; diagnose; prognosis

Introduction

Primary liver cancer (PLC) is one of the most common malignant tumors. PLC has a high morbidity and mortality rate, accounting for the fifth and second place in the world respectively [1]. Because PLC is not sensitive to radiotherapy and chemotherapy, currently, the main treatment for PLC is surgical resection. Unfortunately, because of the absence of symptoms in early stage liver cancer and the lack of sensitive and convenient screening methods, most patients with liver cancer are not

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diagnosed until late stage and cannot be surgically removed. Therefore, early diagnosis of PLC is very important to improve the survival rate of patients. However, the diagnostic efficacy of traditional tumor markers such as alpha-Fetoprotein (AFP), KI-67 and HeppAR-1 is often not satisfactory [2-3]. Finding an ideal tumor marker that can diagnose PLC sensitively and specifically has always been the focus of researchers.

Cell-free DNA(cfDNA) in the blood refers to fragments of fragmented nucleic acid that exist in the peripheral circulation and originate from apoptotic and necrotic tumor cells [4-5]. This kind of free DNA can not only show the whole picture of tumor genes, but also indirectly reflect the occurrence and development of tumors to a certain extent. If the cfDNA from this type of tumor can be detected and analyzed, it is possible to achieve non-invasive liquid biopsy, which will bring important clinical value to tumor diagnosis, drug screening, prognostic evaluation and real-time treatment. CfDNA has a short half-life, usually only 16 minutes, and can accurately reflect tumor status in real time [6]. At present, a number of research results suggest that cfDNA is highly expressed in peripheral blood of patients with various malignant tumors, such as lung cancer, colorectal cancer, breast cancer and esophageal cancer, and is related to tumor burden and prognosis [7-10]. But in the PLC application value of the research is less. In this experiment, the concentration and integrity of cfDNA in plasma of PLC patients before and after TACE treatment were detected by fluorescence quantitative PCR. The relationship between cfDNA concentration and integrity and the clinical and pathological characteristics of PLC patients was analyzed. The diagnostic efficacy of cfDNA was compared with that of traditional tumor markers. At the same time, single-factor and multi-factor analysis of factors affecting the survival rate of PLC patients, a survival curve was established to compare the high and low expression RFS of cfDNA in PLC patients after treatment, and to explore the possibility of cfDNA as an indicator of efficacy and prognosis for PLC patients.

Methods

Clinical data

96 PLC patients confirmed in the second hospital of Nanjing were selected as research group. There were 49 males with an average age of 56.47±18.14 years, and 47 females with an average age of 61.31± 14.49 years. Another 50 healthy subjects from the same hospital, excluding tumors and other chronic diseases, were included as the control group.

Inclusion criteria: (1) All patients meet the PLC diagnostic criteria proposed in the Code for diagnosis and Treatment of primary Liver cancer [11]. 2. Patients who have not received radiotherapy or chemotherapy. 3. PLC patients who conform to the TACE indications and can be tolerated for treatment. 4. Patients who volunteered to participate in the study.

Exclusion criteria: (1) Patients with coronary heart disease, cardiomyopathy, blood diseases, severe liver and kidney dysfunction, acute cerebrovascular disease, etc. (2) Patients who received transfusion prior to treatment. (3) PLC patients with viral and bacterial infection history within 2 weeks.

Therapeutic schedule

The femoral artery was punctured by Seldinger and insert microcatheter (Beijing Miraitong Medical Equipment Co. LTD, China). Fluorouracil(Nantong Jingjing Pharmaceutical Co. LTD, China) and oxaliplatin (Shenzhen Haiwang Pharmaceutical Co. LTD, China) were injected slowly according to the size, location, number, liver function and other specific conditions of individual patients. Then iodized oil (Garber Pharmaceutical Factory, France) was injected to embolize tumor microvessels, and some patients were embolized with gelatin sponge particles (Hangzhou Ailikang Medical Technology Co. LTD, China).

Plasma cfDNA detection

1. Sample collection and pre-treatment: 2ml venous blood of PLC patients (before and after TACE) and healthy physical examinees was collected and placed in an EDTA anticoagulant tube. Centrifugation was conducted at 1900×g at low speed for 15 min as soon as possible (within 6 h). The plasma layer was placed in a clean microcentrifuge tube, diluted with PBS buffer, and centrifuged at a high speed of 13000×g. The supernatant 50μL was directly used for cfDNA detection or cryopreserved at -80 °C.

2. Extraction of cfDNA from plasma: The QIAamp DNA Blood Mini Kit (Qiagen Germany, 51104) was used, and the specific operation was carried out in strict accordance with the Kit instructions. Primer 1 (97bp): forward primer: 5′-TGGCAC ATATACACCATGGAA-3′, reverse primer: 5′-TGAGAATGATGGTTT C-3′. Primer 2 (300bp): forward primer: 5′-ACAACCTATTCCAA AATTGACCA C-3′, reverse primer: 5′-TTCCCTCTACACACTGCTTTGA-3′. The amplified fragment of internal reference β-action was 186bp. CfDNA integrity index was calculated as the ratio of

QRT-PCR results of LINE 300 bp and LINE 197 bp.

Detection of other tumor markers

The levels of AFP and CK19 in PLC patients before TACE treatment were determined by ELISA. AFP ≤ 200ng/mL is negative (-), AFP>200ng/mL is positive (+). CK 19 ≤ 7ng/mL is negative (-), CK19 >7ng/mL is positive. After puncture tissue samples were collected, the positive rates of heppar-1, KI-67, GPC-3 and CD31 were determined by immunohistochemistry. Both heppar-1 and GPC-3 were clearly located in the cytoplasm. CD31 is localized in the cell membrane and cytoplasm. Semi-quantitative results were used to judge: the absence of uniform brownish yellow granules in cells was negative and the presence of brownish yellow granules was positive. Staining intensity: 0 points: no staining, 1 points: light yellow, 2 points: yellow, 3 points: brownish yellow, 4 points: tan. According to the percentage of staining positive cells, when staining cells <10% were focal, ≧10% were diffuse. Only when the staining intensity is more than 2 points and it is diffuse, it is judged as positive (+). Positive CD31 staining showed brownish or tan staining to the vascular endothelial cell membrane or cytoplasm. The number of vessels stained brown by CD31 in 5 non-recurrent visual fields was counted under 200x visual field, and the mean value was taken as the microvascular density (MVD) within the tumor. The units are 1 /200 field of view. Ki67 is localized to the nucleus, ≤ 10% is negative (-), >10% is positive (+).

Statistical analysis

SPSS 21.0 software was used for statistical analysis of all data. The measurement data were expressed as (x±SD), and t-test and one-way an OVA were used. Count data are represented as n, the $\chi 2$ test was used. The ROC curve was established to analyze the diagnostic effectiveness. Kaplan-meier method was used for univariate analysis.Variables with P<0.05 were analyzed by COX stepwise regression model. P<0.05 was considered statistically significant.

Results

The levels of hePPAR-1, KI-67, GPC-3 and CD31 in PLC patients

The positive hePPAR-1 and GPC-3 in PLC patients were located in the cytoplasm in granular shape, and the positive rates were (67.3±22.7)% and (60.25±21.53)%, respectively. Ki-67 was positively localized to the nucleus in granular shape, and its proliferation index was (30.03±24.16)%. CD31 microvessels showed long strips or branches, and was uniformly distributed. The MVD of PLC patients was 62.75 ± 12.68.

Plasma cfDNA concentration and integrity before and after TACE in healthy people and PLC patients

The plasma cfDNA concentration in healthy subjects was 10.26±0.95ng/mL. Plasma cfDNA concentrations of PLC patients before and after TACE were 18.84±1.45 and 12.34±1.83ng/mL, respectively. The plasma cfDNA integrity of healthy subjects was 3.98±0.62. CfDNA integrity of PLC patients before and after TACE was 5.08±0.69 and 4.23±0.70, respectively.

Relationship between plasma cfDNA concentration and clinical characteristics of PLC patients

The correlation between plasma cfDNA concentration and clinical characteristics of 96 PLC patients before and after TACE treatment was analyzed. The results showed that cfDNA concentration before and after treatment was significantly correlated with tumor diameter, differentiation degree, BCLC stage, and AFP level in PLC patients (P<0.05). (Table 1).

Relationship between plasma cfDNA integrity and clinical characteristics of PLC patients

The correlation between plasma cfDNA integrity and clinical characteristics of 96 PLC patients before and after TACE treatment was analyzed. The results showed that cfDNA integrity before and after treatment was significantly correlated with the differentiation degree, BCLC stage and AFP level of PLC patients (P<0.05). (Table 2).

ROC curve

ROC curve results showed that the AUC of cfDNA concentration and integrity was higher than other tumor markers (AFP, HEPPAR-1, KI-67, GPC-3, CK19, CD31).CfDNA concentration and integrity have higher sensitivity and specificity. (Table 3).

Kaplan-meie univariate analysis and COX multivariate analysis

In this study, the median 11.85ng/mL was taken as the limit of cfDNA concentration, that is, >11.85ng/mL was the high expression population (+), ≤11.85ng/mL was the low expression population (-). 4.18 is the cfDNA integrity limit, that is, >4.18 is the high expression group (+), ≤4.18 is the low expression group. Kaplan-meier univariate analysis showed that tumor size, AFP, BCLC stage, cfDNA concentration and cfDNA integrity were significant factors influencing RFS (Table 4). COX

multivariate analysis further determined that BCLC stage and cfDNA concentration were independent risk factors for PLC recurrence (Table 5).

Discussion

PLC refers to the primary malignant tumor in the liver, the onset of symptoms is relatively insidious, when the patient has obvious symptoms may have progressed to the disease advanced stage, failure in early diagnosis and treatment is the main reason for the high mortality of primary liver cancer patients [12]. In recent years, with the continuous improvement of the medical level, in addition to surgical resection, liver transplantation, liver artery embolization chemotherapy and other means in the clinical treatment of PLC patients have been widely used, so that the prognosis of patients has been improved to some extent. However, the recurrence and metastasis rate of PLC is still high after treatment, so it is very important to predict the recurrence risk of primary liver cancer as early as possible and take reasonable treatment measures in time to improve the prognosis of patients with primary liver cancer [13]. Current diagnostic methods for liver cancer include magnetic resonance imaging, liver biopsy and other imaging tests, as well as non-invasive blood biomarkers. Histopathological biopsy is currently recognized as the most effective diagnostic method in the world, but biopsy is highly invasive and limited by tumor heterogeneity, so it is not suitable for real-time assessment of tumor load. For asymptomatic individuals and patients with symptoms of liver disease, imaging examination is usually unable to quickly find small lesions and timely reflect the changes in tumor load. And some biomarkers have also been found to have certain limitations. For example, the false negative rate of AFP is still very high, about 30%, and is not satisfactory in the diagnosis and prediction of PLC recurrence and prognosis [14]. Some studies have also shown that potential biomarkers include plasma HEPPAR-1, KI-67, GPC-3, CK19 and CD31 as prognostic indicators, but these biomarkers have not been verified by a large number of clinical data or translated into clinical practice. Therefore, the limited availability of liver tissue has created another need to find a non-invasive approach to liver tissue for diagnosis, treatment response monitoring, prognosis, and so on.

CfDNA in human peripheral blood was discovered by Mandel in 1948 [15], but it was not until decades Leon discovered that cancer patients had higher concentrations of cfDNA in their serum and plasma than healthy individuals [16] that cfDNA caught the attention of researchers. In recent foreign studies, blood cfDNA has played a new role in the diagnosis of malignant tumors, and many studies have proved the correlation between cfDNA and PLC. Vasioukhin showed that cfDNA has cancer-like characteristics, and that cancer cells can release DNA into the peripheral blood [17]. Chen[18] and Huang [19] found in their study that the total concentration of cfDNA was significantly higher than that of patients without liver cancer. When evaluating the diagnosis of liver cancer with plasma cfDNA mutation, Iyer[20] found that this method showed good performance in the diagnosis of liver cancer, with specificity of 100% and sensitivity of 65%, and indicated that cfDNA was negatively correlated with the prognosis of PLC, that is, the better the prognosis, the lower the level of cfDNA. Yan et al. [21] recently found that age and cfDNA were independent predictors of PLC through multi-factor analysis. As shown in the above studies, cfDNA level as a clinical tool based on liquid biopsy has broad application prospects in the early diagnosis of liver cancer, and several other studies have shown that cfRNA may be applied in the detection and monitoring of liver cancer [22-24].

In this study, the plasma cfDNA concentration and integrity of PLC patients before treatment were significantly higher than that of healthy people ($P<0.05$), and the cfDNA concentration and integrity of PLC patients after treatment were significantly lower than that before treatment ($P<0.05$), but still higher than that of healthy people. The results showed that the cfDNA concentration and integrity of PLC patients were different from that of healthy people, which could be used as an auxiliary diagnostic indicator of PLC. According to the analysis of different clinical characteristics, cfDNA concentration was found to be significantly correlated with tumor diameter, differentiation degree, BCLC stage and AFP level in PLC patients, while cfDNA integrity was significantly correlated with differentiation degree, BCLC stage and AFP level in PLC patients. Therefore, we inferred that cfDNA concentration and integrity could reflect tumor load to some extent. Further ROC curve results showed that cfDNA concentration and integrity of the area under the curve (AUC) is higher than other tumor markers (AFP, HEPPAR-1, KI-67, GPC-3, CK19, CD31), indicating that cfDNA concentration and integrity have higher sensitivity and specificity. These results suggest that cfDNA concentration and integrity for the diagnosis of PLC has a certain significance. COX multivariate regression analysis further found that BCLC stage and cfDNA concentration were independent risk

factors affecting the survival of PLC patients, indicating that cfDNA concentration affects prognosis and is a potential indicator for prognosis assessment. In summary, plasma cfDNA has potential applications in screening for liver cancer as well as monitoring treatment responses and predicting liver cancer recurrence for real-time diagnosis and follow-up of treatment responses.

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Table 1. **Relationship between plasma cfDNA concentration and clinical characteristics of PLC patients**

clinical features	Case (n)	cfDNA concentration（ng/mL）	
		Before TACE	After TACE
Age（years）			
<60	46	20.59±2.68	12.34±1.83
≥60	50	21.03±2.16	12.40±1.53
P		0.3763	0.8616
Gender			
Female	47	20.82±2.41	12.37±1.79
Male	49	20.69±2.47	12.39±1.54
P		0.7948	0.9519
Tumor size (d/cm)			
<5	42	19.79±2.23	11.45±1.39
≥5	54	21.11±2.08	13.12±1.60
P		**0.0036**	**<0.0001**
Degree of differentiation			
Poor	24	19.35±1.94	13.79±1.41
Medium	31	21.56±1.71	12.37±1.08
High	41	22.12±2.75	10.92±1.53
P		**<0.0001**	**<0.0001**
BCLC Stage			
A	21	18.02±1.49	10.59±1.18
B	27	20.25±2.11	12.22±1.10
C	35	21.56±1.75	13.04±1.57
D	13	22.94±2.10	14.21±1.23
P		**<0.0001**	**<0.0001**
AFP			
+	49	21.76±2.11	12.95±1.54
-	47	19.71±2.32	11.79±1.61
P		**<0.0001**	**0.0005**
Hepatitis			
+	52	20.80±2.24	12.29±1.58
-	44	20.71±2.66	12.42±1.17
P		0.8575	0.7047
heppar-1			
+	50	20.36±2.26	12.28±1.53
-	46	21.13±2.62	12.49±1.83
P		0.1256	0.5422
Ki-67			
+	51	20.31±2.33	12.27±1.58
-	45	21.25±2.47	12.51±1.78
P		0.0582	0.4857
GPC-3			
+	50	21.01±2.42	12.55±1.71
-	46	20.47±2.44	12.19±1.62
P		0.2794	0.2933
CK19			
+	51	20.99±2.44	12.59±1.70
-	45	20.51±2.43	12.16±1.61
P		0.3368	0.2067
CD31			
+	50	20.98±2.29	12.70±1.65
-	46	20.43±2.46	12.64±1.63
P		0.2595	0.0519

Table 2. **Relationship between plasma cfDNA integrity and clinical characteristics of PLC patients**

clinical features	case(n)	cfDNA integrity	
		Before TACE	After TACE
Age（years）			
<60	46	5.46±0.83	4.24±0.69
≥60	50	5.52±0.70	4.36±0.68
P		0.7019	0.3930
Gender			
Female	47	5.47±0.72	4.30±0.65
male	49	5.49±0.82	4.28±0.72
P		0.8994	0.8869
Tumor size (d/cm)			
<5	42	5.31±0.78	4.39±0.67
≥5	54	5.46±0.69	4.19±0.68
P		0.5946	0.1535
Degree of differentiation			
Poor	24	6.11±0.52	4.53±0.76
Medium	31	5.61±0.58	4.50±0.56
High	41	5.02±0.83	3.89±0.60
P		**<0.0001**	**<0.0001**
BCLC Stage			
A	21	4.62±0.56	3.55±0.33
B	27	5.24±0.59	4.08±0.37
C	35	6.05±0.60	4.53±0.40
D	13	6.14±0.34	5.29±0.48
P		**<0.0001**	**<0.0001**
AFP			
+	49	5.84±0.61	4.58±0.64
-	47	5.11±0.74	3.98±0.59
P		**<0.0001**	**<0.0001**
Hepatitis			
+	52	5.51±0.75	4.27±0.74
-	44	5.38±0.76	4.31±0.61
P		0.4025	0.7758
heppar-1			
+	50	5.40±0.70	4.26±0.70
-	46	5.57±0.83	4.32±0.67
P		0.2795	0.6695
Ki-67			
+	51	5.34±0.67	4.24±0.71
-	45	5.59±0.83	4.34±0.66
P		0.2991	0.4784
GPC-3			
+	50	5.56±0.65	4.41±0.75
-	46	5.37±0.85	4.15±0.57
P		0.2195	0.0605
CK19			
+	51	5.57±0.67	4.40±0.74
-	45	5.38±0.85	4.16±0.58
P		0.2259	0.0810
CD31			
+	50	5.60±0.65	4.43±0.74
-	46	5.34±0.86	4.17±0.59
P		0.0964	0.0615

Table 3. **The ROC curve results**

Index	AUC	Sensitivity	Specificity	95% CI
cfDNA integrity	0.8435	81.82%	74.68%	0.7829-0.9040
cfDNA concentration	0.8179	81.82%	74.87%	0.7487-0.8871
AFP	0.7037	75.00%	55.19%	0.6114-0.7961
heppar-1	0.6847	68.83%	54.17%	0.6008-0.7685
Ki-67	0.7198	70.78%	72.92%	0.6275-0.8122
GPC-3	0.6676	66.88%	52.08%	0.5809-0.7543
CK19	0.6760	67.53%	54.17%	0.5903-0.7617
CD3	0.7317	73.38%	60.42%	0.6513-0.8122

Table 4. **Kaplan-meier univariate analysis**

Factor	case(n)	Recurernce rate(%)	χ^2	P
Age（<60/≥60岁）	46/50	34.79/39.98	2.208	0.1369
Gender(Female/ male)	47/49	40.36/34.71	0.082	0.7752
Tumor size (<5/≥5 d/cm)	42/54	20.05/49.76	5.680	**0.0174**
Degree of differentiation（Poor/Medium/High）	24/31/41	54.79/28.57/18.02	6.561	**0.0382**
BCLC Stage(A/B/C/D)	21/27/35/13	8.3/25.0/48.1/61.9	6.440	**0.0423**
AFP(+/-)	49/47	55.12/20.41	8.632	**0.0071**
Hepatitis(+/-)	52/44	40.49/31.17	0.4352	0.5091
heppar-1(+/-)	50/46	36.48/34.68	0.1993	0.6551
Ki-67(+/-)	51/45	37.35/37.81	0.0891	0.7648
GPC-3(+/-)	50/46	38.35/36.46	0.1809	0.6671
CK19(+/-)	51/45	37.87/37.25	0.0149	0.9027
CD31(+/-)	50/46	37.87/38.03	0.0031	0.9846
cfDNA concentration (+/-)	55/41	27.3/9.82	6.129	**0.0047**
cfDNA integrity (+/-)	49/47	55.3/26.5	5.489	**0.0121**

Table 5. **COX multivariate analysis**

Factor	B	SE	Wald	df	Sig.	Exp	95.0% CI for Exp(B)	
							Lower	**Upper**
cdDNA concentration	0.395	0.181	4.782	1	**0.029**	1.484	1.042	2.115
cdDNA integrity	-0.706	0.442	2.558	1	0.11	0.494	0.208	1.173
Tumor size (<5/≥5 d/cm)	-0.266	0.473	0.316	1	0.574	0.766	0.303	1.938
Degree of differentiation	-0.181	0.231	0.611	1	0.435	0.835	0.53	1.313
BCLC	0.717	0.358	3.999	1	**0.046**	2.047	1.014	4.132