Circulating cell-free mtDNA content is associated with outcome of HCC patients receiving TACE combined with traditional Chinese medicine treatment

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Abstract

Objectives: Hepatocellular carcinoma (HCC) accounts for 70%-85% of liver cancer, and about 85% of HCC are hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) in china. Most patients are already in the middle or late stages of the disease at the time of diagnosis, trans-hepatic arterial chemoembolization (TACE) combine with traditional Chinese medicine (TCM) have been reported as an effective treatment, and effective prognostic molecular markers are helpful to predict therapeutic efficacy. In this study, we aim to explore whether circulating cell-free mitochondrial DNA (ccf-mtDNA) content is associated with the outcome for HCC patients receiving TACE combine with TCM treatment.

Method: Retrospective analysis was conducted in a cohort with 141 HBV-HCC patients. Univariate and multivariate analysis was conducted with Cox proportional risk regression model to explore the correlation between ccf-mtDNA content and patient prognosis. Kaplan-Meier method was used to draw the survival curve of ccf-mtDNA content and the survival prognosis of patients.

Results: (1) high content of serum ccf-mtDNA is an independent risk factor for the prognosis of HBV-HCC patients treated with TACE combined with TCM adjuvant therapy (HR=4.010, 95%IC=1.252-12.844, P = 0.019). (2) K-M survival analysis showed that patients with high ccf-mtDNA content had poor prognosis (Log Rank P=0.027).

Conclusions: Our findings suggest that ccf-mtDNA is a potential novel non-invasive biomarker for prognosis of HCC patients receiving TACE combine with TCM treatment.

Introduction

Hepatocellular carcinoma (HCC) is a second leading cause of cancer death, and Chronic hepatitis B virus (HBV) infection accounts for at least 50% cases of HCC worldwide[1]. Notably, HBV-related HCC accounts for about 85% of HCC cases in China[2]. The vast majority of HCC patients are diagnosed in middle or later stages, and curative treatments like surgical resection are not suitable for this patients[3]. Trans-arterial chemoembolization (TACE) is the standard of care for patients with intermediate HCC according to The barcelona clinic liver cancer (BCLC) staging system, and studies indicate TACE combined with traditional Chinese medicine (TCM) regimen displayed high efficacy in treating advanced HCC[4].

Mitochondria are ubquitous eukaryotic cell organelles which playing an important role in energy production, cell proliferation as well as apoptosis[5]. The circular genome of mitochondrial (mtDNA) encodes for proteins essential in the oxidative phosphorylation system and the tRNA and rRNA molecules of the mitochondrial translation apparatus. Mitochondrial dysfunction plays an important role in the occurrence and development of liver cancer[6, 7], and when mitochondrial dysfunction causes mtDNA damage, mtDNA fragments can escape from the matrix and enter the cytosol or systemic circulation[8]. And changes in mtDNA content of tumor specimens have been reported[9], the alteration of mtDNA content or sequence mutations has evitable relationship with oncogenesis and becomes potential biomarkers for

certain types of cancers[10], Alterations of mtDNA are generally accepted as independent biomarker for predicting risk and prognosis in various cancers, Bao et al found mtDNA copy number are associated with overall survival in hepatocellular carcinoma patients treated with transarterial chemoembolization[11]. mtDNA also may be released at low levels into the circulation from mitochondria under cellular stress, resulting in circulating cell-free mtDNA (ccf-mtDNA) detectable in plasma[12]. Recently circulating mtDNA have been targeted due to its specific and unique characteristics, which could potentially used as a novel biomarker for diagnostic and prognostic of cancer[13, 14]. Studies on the correlation between ccf-mtDNA content and the risk of HCC showed that the serum ccf-mtDNA content of HBV-HCC patients was significantly lower than that of the control group (non-HCC patients with HBV infection); and HBV infection with higher mtDNA content compared to patients with lower mtDNA content have a significantly higher risk of developing HCC[13]. However, the association of mtDNA content with clinical outcomes of HCC patients remains largely unknown.

In this study, using real-time quantitative PCR method, we measured ccf-mtDNA content in a hospital-based cohort of HCC patients, and further evaluated the association of ccf-mtDNA content with clinical outcomes of HCC patients receiving TACE combined with TCM treatment.

Material And Methods

Patients

Informed consent was obtained from each patient according to the protocols approved by the ethics committees of the Affiliated Fifth People's Hospital of Ganzhou in Gannan Medical University. A total of 148 HBV-HCC patients were enrolled at the Department of Hepatology, Affiliated Fifth People's Hospital of Ganzhou in Gannan Medical University between January 2015 and October 2018. Each patient has two or more serum samples with different blood collection times, and the first serum was selected as the experimental sample after the patient was diagnosed with HCC. 7 patients who had been diagnosed with HCC before admission were excluded, and the final study included a total of 141 patients with HBV-HCC. In the subsequent statistical analysis of correlation of ccf-mtDNA content and prognosis, the study population was adjusted to 50 patients with HBV-HCC treated with TACE combined with TCM treatment, and the details were summarized in Figure 1.

Serum Sampling

Serum sample from each patient was collected in non-anticoagulated blood collection tubes, and then transferred to the laboratory on ice for processing within 6 hours. Serum samples were collected by two-step centrifugation: the serum sample was extracted from the whole blood after centrifuged with 1500rpm for 5min, and then remove the supernatant to a clean tube and re-centrifuged with 12000rpm for another 5min, the supernatant was collected into a 1.5mL tube, and stored at -80℃ for use.

Measurement of serum mtDNA content

First, QIAamp DNA Blood Mini kit (Qiagen, Carlsbad, CA) was used to isolate the circulating DNA from 200μL serum sample according to the manufacturer's protocol, and mtDNA content was measured by real-time qPCR with modified protocol described by previous studies[15, 16]. Briefly, the primer of ND1 gene was used for mtDNA amplification with the primer sequences as follows: ND1-F: 5'-CCCTAAAACCCGCCACATCT-3' and ND1-R: 5'-GAGCGATGGTGAGAGCTAAGGT-3'. The primer of 36B4 gene was used as housekeeping gene control in all reaction, and the primer sequences were as follows: 36B4-F: 5'-CAGCAAGTGGGAAGGTGTAATCC-3' and 36B4-R: 5'-CCCATTCTATCATCAACGGGTACAA-3'. The 20μL qPCR reaction for ND1 and 36B4 consists of 1X TB Green fast qPCR Mix (2X) (Takara), 0.2μM each primer, and 2μL of purified serum DNA sample. The thermal cycling conditions were at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15s and 60 ℃ for 60 s with signal acquisition. All samples were assayed in duplicate on a 96-well plate using QuantStudio™ 7 Flex qRT-PCR system (Applied Biosystems, USA). The same negative control and calibrator DNA samples were incorporated into each plate for quality control and calibration of PCR efficiency. A reference DNA sample was used to construct a standard curve for mtDNA measurement in each palate. For each standard curve, the reference DNA sample was serially diluted by 1:3 to produce a six-point standard curve. The R^2 for each standard curve was ≥0.99.

Statistical analyses

Use IBM SPSS 20.0 statistical software to analyze the collected clinical data and experimental data. Continuous variables are represented by average and standard deviation (mean±SD) or median and interquartile ranges according to specific types and categorical variables are represented by frequency and percentage. Cox proportional hazards regression model was used for univariate and multivariate analysis. Kaplan-Meier method was used to draw survival curves, and Log-Rank test and Breslow test were used for comparison between groups. The $P< 0.05$ was considered as the threshold of statistical significance.

Results

Population characteristics of HBV-HCC patients

In order to study the correlation between ccf-mtDNA content and the prognosis of HBV-HCC patients, the epidemiological and clinical characteristics of 141 HBV-HCC patients were analyzed. The results were shown in Table 1, the average age of the study population is 56.96±11.34, the male to female ratio is about 5:1, and most of the patients were with no smoking (81.9%), drinking (80.9%), and family history of cancer (73%). There were 45(31.9%) patients developed liver cirrhosis, and 117 (83.0%) patients were with B or C Child-Pugh classes. Among these 141 HBV-HCC patients, 70 (49.6%) patients have been treated with TACE, 59 (41.8%) patients have been treated with Chinese medicine, and 50 (35.4%) of them have been treated with TACE combined with TCM. During the follow-up period, there were 88 (62.4%) patients died, and the median survival time is 11 months (Inter-quartile range, 7.5-22 months) with the maximum survival is 44 months.

Correlation between ccf-mtDNA content and prognosis of HBV-HCC patients

The median and tertile values of ccf-mtDNA content were used to group patients, and the correlation between ccf-mtDNA content and the prognosis of HBV-HCC patients was evaluated using univariate and multivariate analysis. As the data shown in Table 2, there is no statistical significance between the ccf-mtDNA content and the overall survival of HBV-HCC patients in the univariate analysis. However, in the multivariate analyses, a bordline statistical significance association between ccf-mtDNA and HCC overall survival was observed in patients with higher ccf-mtDNA content by both median anaysis with HR of 1.49 (95%CI, 0.94-2.34, p = 0.09) and tertiel analysis with HR of 1.76 (95%CI, 1.00-3.11, p = 0.05) in third tertile group, comparing to patients with lower ccf-mtDNA, with adjusting for age, gender, drinking and smoking status, history of family cancer, liver cirrhosis, and TACE treatment. And the bordling significance effect of ccf-mtDNA content was remained in the tertile analysis with HR of 1.66 (95%CI, 0.93-2.94, p = 0.08) in the third tertile group, when further multivariate analysis with above variables plus adjuvant treatment with Chinese medicine.

The Kaplan-Meier analysis method is used to draw the survival curve of HBV-HCC patients with different ccf-mtDNA content (as shown in Figure 2). Results showed that there was no statistically significant difference in the overall survival rate between the low-content group and the high-content group of ccf-mtDNA (Log Rank p =0.732).

The correlation between ccf-mtDNA content and the prognosis of HBV-HCC patients receiving TACE combined with TCM treatment

We further evaluate whether the ccf-mtDNA content correlate with the prognosis of HBV-HCC patients receiving TACE combined with TCM treatment. The epidemiological and clinical characteristics of 50 HBV-HCC patients with TACE combined with TCM treatment were analyzed. Results show (as shown in Table 3) the average age of the study population is 56.48±8.175, the male to female ratio is about 6:1, and most of the patients were with no smoking (70%), drinking (70%), and family history of cancer (73%). There were 34 (68%) patients developed liver cirrhosis, and 36 (72%) patients were with B or C Child-Pugh classes. During the follow-up period, there were 23 (46%) patients died, and the median survival time is 20 months (95%CI: 12, 29 months), the maximum survival time is 44 months.

The patients were divided into groups by the median and tertile values of ccf-mtDNA content, and the correlation between ccf-mtDNA content and prognosis of HBV-HCC patients receiving TACE combined with TCM treatment was evaluated through univariate and multivariate analysis. It showed that the high content of ccf-mtDNA is an independent risk factor for the prognosis of HBV-HCC patients with TACE combined with TCM treatment (as shown in Table 4). And in the multivariate analysis, a significance association between the ccf-mtDNA content and the overall survival of HBV-HCC patients were observed in patients with high content by median analysis with HR of 4.010 (95%CI, 1.252-12.844, p=0.02) and tertile analysis with HR 4.554 (95%CI, 1.159-17.811, P=0.03), 5.322 (95%CI, 1.053-26.909, p=0.04) in second and third tertile group, comparing to patients with lower ccf-mtDNA, with adjusting for gender,

age, cirrhosis, liver function, history of drinking, smoking history, family cancer history, tumor number, tumor size.

The Kaplan-Meier analysis method is used to draw the survival curve of HBV-HCC patients receiving TACE combined with TCM treatment according to the content of ccf-mtDNA (as shown in Figure 3A). Results showed the prognosis of patients with high content of ccf-mtDNA was worse, but the difference in overall survival rate between the two groups is not statistically significant (Log Rank P=0.097). Further analysis found that 3 patients in the study died less than six months after admission, and this may affect the accuracy of the survival analysis results. The analysis was repeated after excluding these 3 abnormal samples, and results showed that (as shown in Figure 3B) the prognosis of the patients in the group with high content of ccf-mtDNA content was worse, and the overall survival rate difference between the two groups was statistically significant (Log Rank P=0.027).

Discussion

In this retrospective study, we measured ccf-mtDNA content in HCC and demonstrate that patients with low ccf-mtDNA content have increased benefit from TACE combined with TCM treatment. In the multivariate analysis of 50 HBV-HCC patients, the results obtained by the median and tertile analysis showed that the prognosis of patients with different ccf-mtDNA content was significantly different, and both were statistically significant with p value of 0.02 by median analysis and p value of 0.04 in third group by tertile analysis. Our data indicated that the high content of ccf-mtDNA is an independent risk factor for the prognosis of HBV-HCC patients with TACE combined with TCM treatment. Kaplan-Meier survival analysis after excluding 3 abnormal samples showed that the prognosis of patients in the group with high content of ccf-mtDNA content was worse, and the difference in overall survival rate between the two groups was statistically significant (Log Rank P = 0.027).

Circulating cell-free mitochondrial DNA (ccf-mtDNA) is that mtDNA fragments are released outside the cell and into the circulation by cell necrosis and secretion. In the last decade, the roles ccf-mtDNA as potential noninvasive biomarkers have been demonstrated in numerous different types of disease, including cancer. Several studies[13, 17, 18] show that ccf-mtDNA in plasma or serum could be used as a new and effective diagnostic and prognostic marker in many solid tumors. Ellinger's research showed that the ccf-mtDNA content in the serum of patients with urinary system malignancies increased significantly[17], and Mahmoud's research showed that ccf-mtDNA has a good prognostic value in breast cancer[18]. Meng et al. reported that increasing levels of ccf mtDNA has a significant association with epithelial ovarian cancer progress and poor prognosis[19]. These studies suggested that ccf-mtDNA is a potential tumor molecular marker, and further studies are needed to reveal the mechanism and clinical value of changes in ccf-mtDNA content.

Transarterial chemoembolization (TACE) is the firstline treatment for intermediate stage disease, which includes asymptomatic patients with limited unresectable multinodular lesions, without vascular invasion or extrahepatic spread and who have well-preserved liver function[20], although its clinical effect is still far

from satisfactory. Meta-analysis showed Traditional Chinese Medicine (TCM) improves immune response for unresectable hepatocellular carcinoma (UHCC) after transcatheter arterial chemoembolization (TACE)[21]. TCM is gathering increasing interest due to the immunoregulatory properties of certain compounds, it can restore immunosurveillance in HCC to promote anti-tumor effects in several ways, including the upregulation of immunostimulatory factors and the downregulation of immunosuppressive factors[22]. And also clinical studies have demonstrated the utility of TCM in relieving adverse events after TACE, Tang et al. found the chinese medicine Jianpi Ligan decoction (JLD) was effective for reduction of side effects and improvement of long-term survival for patients with unresectable HCC treated by TACE[23]. However, the cellular and molecular mechanisms of TCM-mediated anti-tumor effect is still not clear, and effective predictive biomarkers are needed to increase efficacy and survival for HCC patients receiving TACE combine with TCM treatment.

Due to the limitation of sample size in this study, some results can't fully reflect the role of ccf-mtDNA content in the prognosis of HBV-HCC patients with TACE combined with TCM treatment. Therefore, further researches are needed to verify these finding with large number of samples. In summary, this study showed that the ccf-mtDNA content in serum has a certain value for the prognosis assessment of HBV-HCC patients receiving TACE combined with TCM treatment, and it may be used as a potential molecular marker for the prognosis of HCC patients.

Abbreviations

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; TACE: trans-hepatic arterial chemoembolization; TCM: traditional Chinese medicine; BCLC: Barcelona clinic liver cancer; mtDNA: The circular genome of mitochondrial; ccf-mtDNA: circulating cell-free mtDNA; JLD: Jianpi Ligan decoction;

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SW and GZ conceived and designed the experiment, GZ and JX wrote the manuscript, YL, SL and HL performed the experiments, FX, XL and QZ collected the samples and clinical data, SW and GZ analyzed the data. All authors are in agreement with the content of the manuscript and this submission.

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Tables

Table 1. The characteristics of study population

Characteristics		Number（n=141）（%）
Age		
	≤57	71（50.4）
	（57	70（49.6）
Gender		
	Male	118（83.7）
	Female	23（16.3）
Smoking		
	No	114（81.9）
	Yes	27（19.1）
Drinking		
	No	114（80.9）
	Yes	27（19.1）
Family history of cancer		
	No	103（73.0）
	Yes	38（27.0）
Liver cirrhosis		
	No	96（68.1）
	Yes	45（31.9）
Child-Pugh class		
	A	24（17.0）
	B	69（48.9）
	C	48（34.1）
TACE treatment		
	No	71（50.4）
	Yes	70（49.6）
TCM adjuvant treatment		
	No	82 (58.2)
	Yes	59 (41.8)

Vital status	
Dead	88 (62.4)
Live	53 (37.6)
Survival time (Month) (median, quartile range)	11.0 (7.5-22.0)
ccf-mtDNA content (median, quartile range)	1.98 (1.17-3.68)

Table 2. The association between ccf-mtDNA and overall survival of HBV-HCC patients

ccf-mtDNA	Number of patient	Number of death	Univariate		Multivariate†		Multivariate‡	
			HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
By median								
Lower	71	45	1.00		1.00		1.00	
Higher	71	43	0.93 (0.86-4.74)	0.74	1.49 (0.94-2.34)	0.09	1.44 (0.91-2.29)	0.12
By tertile								
1st tertile	47	28	1.00		1.00		1.00	
2nd tertile	47	32	1.45 (0.87-2.41)	0.16	1.58 (0.89-2.80)	0.12	1.60 (0.90-2.84)	0.11
3rd tertile	47	28	1.18 (0.70-1.99)	0.55	1.76 (1.00-3.11)	0.05	1.66 (0.93-2.94)	0.08
P for trend				0.36		0.13		0.17

Notes: †Multivariate analysis adjusted for age, gender, drinking and smoking status, history of family cancer, liver cirrhosis, and TACE treatment. ‡Multivariate analysis adjusted for age, gender, drinking and smoking status, history of family cancer, liver cirrhosis, TACE treatment, and adjuvant treatment with Chinese medicine.

Table 3. The characteristics of subpopulation with TACE and TCM treatments

Characteristics	Number（n=50, %）
Age	
≤55	25（50.0）
（55	25（50.0）
Gender	
Male	43（86.0）
Female	7（14.0）
Smoking status	
No	35（70.0）
Yes	15（30.0）
Drinking status	
No	35（70.0）
Yes	15（30.0）
Family history of cancer	
No	33（66.0）
Yes	17（34.0）
Liver cirrhosis	
No	34（68.0）
Yes	16（32.0）
Child-pugh classification	
A	14（28.0）
B	26（52.0）
C	10（20.0）
Tumor number	
Single	32（64.0）
Multiple	18（36.0）
Tumor Size	
≤5	27（54.0）
>5	23（46.0）

Vital status	
Death	23（46.0）
Live	27（54.0）
Survival time (Month) (median, quartile range)	20.0 (12.0-29.0)
ccf-mtDNA content (median, quartile range)	1.58 (0.77- 3.27)

Table 4. The association between ccf-mtDNA and prognosis of HBV-HCC patients with TACE and TCM treatments

ccf-mtDNA	Number of patient	Number of death	Univariate		Multivariate †	
			HR（95%CI）	P value	HR（95%CI）	P value
By median						
Lower	25	9	1.00		1.00	
Higher	25	14	2.02（0.86-4.74）	0.11	**4.01（1.25-12.84）**	**0.02**
By tertile						
1st tertile	17	6	1.00		1.00	
2nd tertile	17	10	2.66（0.94-7.52）	0.07	**4.55（1.16-17.81）**	**0.03**
3rd tertile	16	7	1.60（0.53-4.85）	0.40	**5.32（1.05-26.91）**	**0.04**
P for trend				0.17		0.07

Notes: †Multivariate analysis adjusted for age, gender, drinking and smoking status, history of family cancer, liver cirrhosis, tumor number and tumor size.

Figures

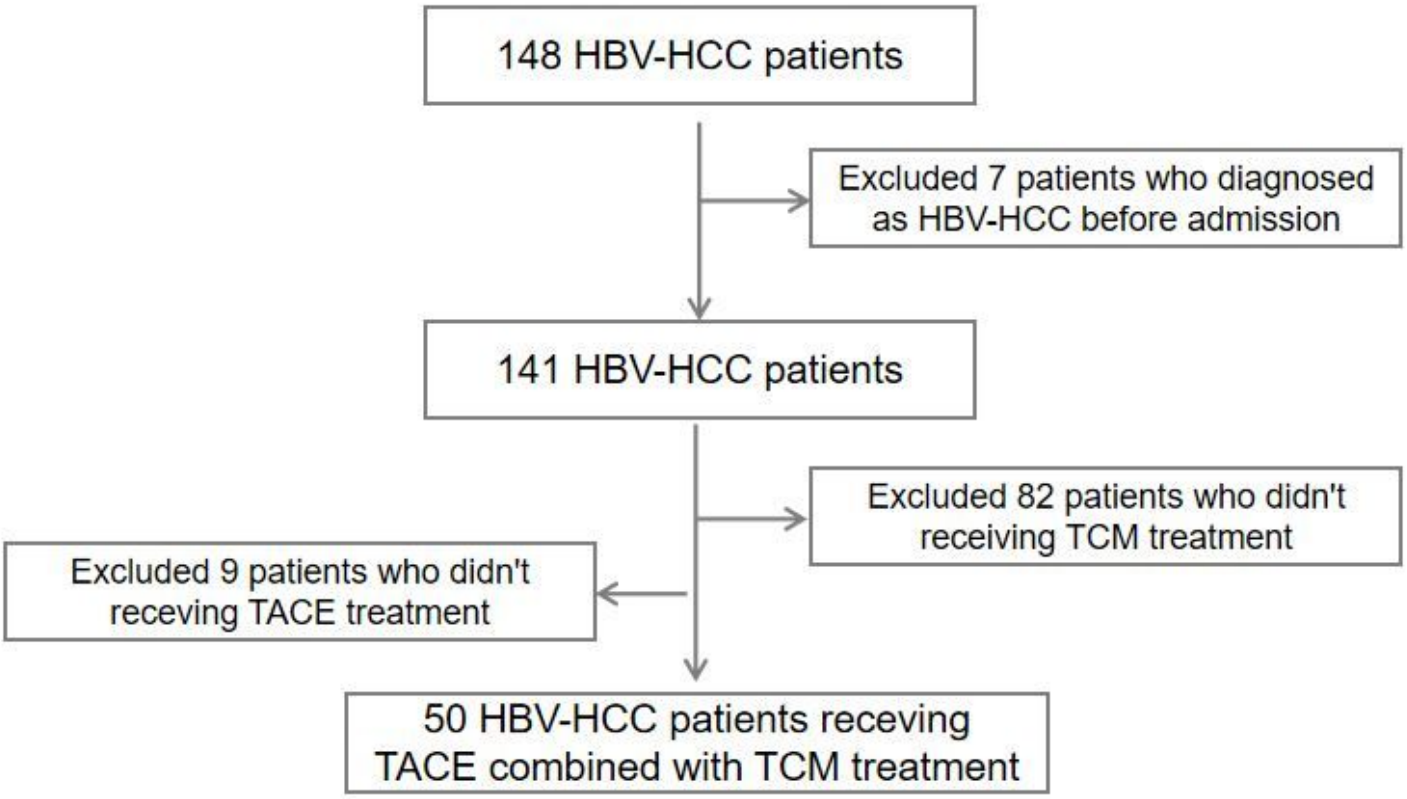

Figure 1

The flowchart of patient enrollment.

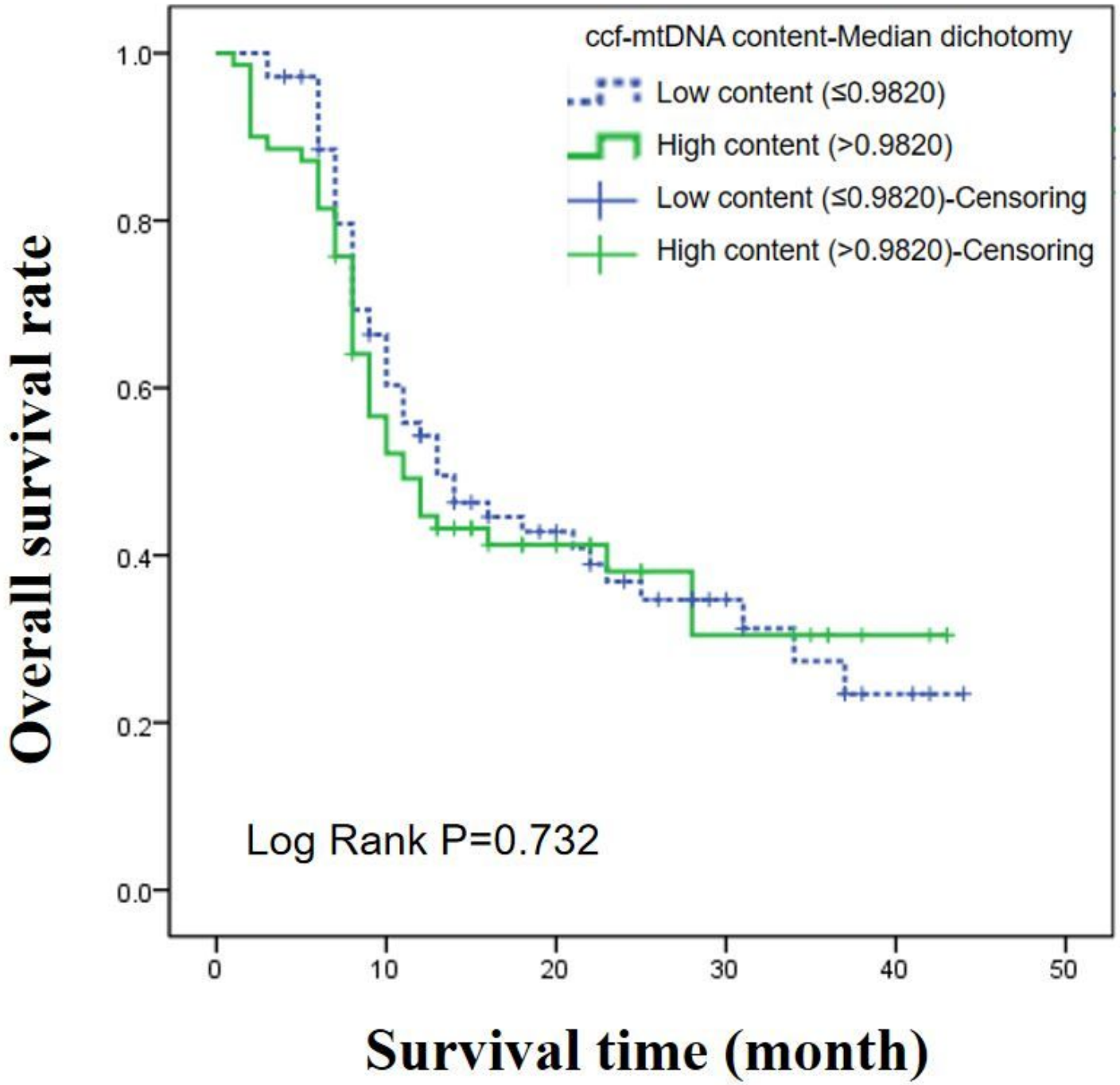

Figure 2

Kaplan-Meier curve analysis of ccf-mtDNA content and HBV-HCC patients

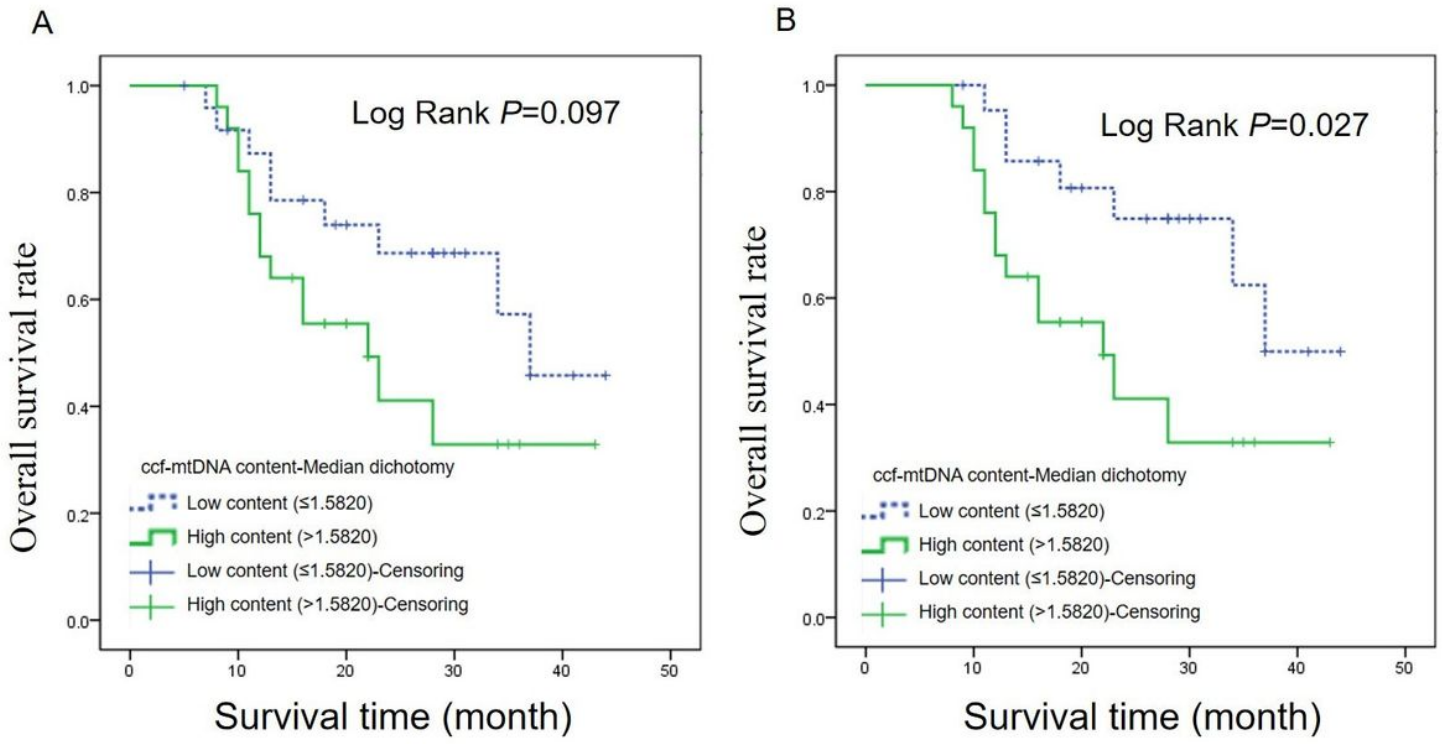

Figure 3

Kaplan-Meier curve analysis of ccf-mtDNA content and HBV-HCC patients receiving TACE combined with TCM treatment. Panel A included all HBV-HCC patients (n=50) treated with TACE and TCM, panel B included patients (n=47) with excluding 3 patients died less than six months after admission.