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Original Article

Real-time PCR for quantitative detection of mitochondrial DNA from peripheral blood mononuclear cell in patients with HBV-related hepatocellular carcinoma

Tian-Bin Chen[1,2*], Zhen Xun[1,2*], Jin-Piao Lin[1,2], Can Liu[1,2], Ya Fu[1,2], Wen-Nan Wu[1,2], Xiao-Chun Fu[1,2], Yu-Hai Hu[3], Qi-Shui Ou[1,2]

[1]First Clinical College, Fujian Medical University, Fuzhou, China; Departments of [2]Laboratory Medicine, [3]Hepatopancreatobiliary Surgery, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China. [*]Equal contributors.

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Abstract: The alteration of mitochondrial DNA (mtDNA) content could affect the expression of genes which causes many tumor diseases. However, the association between mtDNA content in peripheral blood mononuclear cell (PBMC) and hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) remains undetermined. First of all, establishing a reliable assay to detect mtDNA content is of great clinical significance. In this study, the method of real-time quantitative polymerase chain reaction (RT-qPCR) with SYBR Green I was established to evaluate mtDNA content in PBMC of healthy controls (n=23) and non-surgical HBV-related HCC cases (n=46). Receiver operating characteristic (ROC) curve analysis was carried out to assess the clinical significance of mtDNA content for diagnosing HCC. Consequently, linear range of the assay was between 1×10^{10} copies/μl and 1×10^{3} copies/μl. Sensitivity was 800 copies/μl. Besides, HCC cases had a significantly lower mtDNA content than healthy controls (378.55 [58.20-784.85] vs 715.48 [292.00-1280.00]; P<0.001). When 489.90 copies/μl was set as the cut-off point, the sensitivity and specificity of mtDNA content to diagnose HCC were 82.6% and 71.7%, respectively. In conclusion, a simple, cost-effective, highly sensitive and specific method to detect mtDNA content is established. This method can be applied to clinical laboratory for detecting mtDNA content. Moreover, our study provides the first epidemiological evidence that mtDNA content in PBMC is significantly associated with HCC. mtDNA content in PBMC could serve as a novel clinical diagnostic indicator for HCC which may need more researches to validate.

Keywords: Mitochondrial DNA, peripheral blood mononuclear cell, hepatocellular carcinoma, real-time PCR, ROC curve, clinical diagnostic indicator

Introduction

Mitochondria are dynamic double-membrane-bound organelles which participate in a wide range of cellular processes, including ATP production, programmed cell death, calcium homeostasis and signaling transduction [1]. Human mitochondria also possess their own genomes (mtDNA) which are circular double-stranded, maternally inherited DNA [2]. Additionally, human mtDNA exists at extremely high levels with up to 10^{3}~10^{4} copies in each cell [3]. However, because of the different requirement of ATP, mtDNA content varies in different cell types or tissue origins [4]. As well, the amount of mtDNA varies on different age stage of the same individual [5].

Due to the lack of protection from histones and inefficient damage repair mechanisms, mtDNA is more susceptible to reactive oxygen species (ROS) or other genotoxic damages which lead to the copy number alterations [6]. Numerous reports have shown that the change of mtDNA content is associated with many malignancies, including an increase of mtDNA content in prostate cancer [7], colorectal cancer [8], esophageal squamous cell carcinomas [9] and a decrease in renal cell carcinoma (RCC) [10], gastric cancer [11], breast cancer [12]. Therefore, it

Table 1. Primers for RT-qPCR

Gene	Primer names	Primer sequences (5' to 3')	Amplicon size (bp)
ND1	ND1 F	5'-CCCTAAAACCCGCCACATCT-3'	20
	ND1 R	5'-GAGCGATGGTGAGAGCTAAGGT-3'	22
β-globin	β-globin F	5'-CTATGGGACGCTTGATGT-3'	18
	β-globin R	5'-GCAATCATTCGTCTGTTT-3'	18

is suggested that the alteration of mtDNA content might play a vital role in tumorigenesis and progression. Nevertheless, there are few commercial kits for detection of mtDNA content. Although several approaches for detecting mtDNA content have became available in recent years, some limitations have also been found, such as high testing costs [13] or narrow linear range [14]. Therefore, establishing a simple, rapid, sensitive and specific assay to detect mtDNA content is of great clinical significance.

HCC has been qualified as one of the most common malignant tumors in the world and 50% of HCC cases were occurred in China [15]. Furthermore, HBV infection is the most predominant risk factor of HCC. Numerous studies have proved that mtDNA content variation in PBL or serum samples was a predicated factor for HCC. However, the association between mtDNA content in PBMC samples and non-surgical HBV-related HCC remains undetermined. In the present study, we firstly establish a simple, cost-effective, highly sensitive and specific method which is applied to clinical laboratory to detect mtDNA content. After that, we use this method to detect mtDNA content in PBMC of patients with non-surgical HBV-related HCC. At last, the clinical significance of mtDNA content for diagnosing HCC is explored.

Materials and methods

Subjects

We enrolled consecutive patients from Department of Hepatopancreatobiliary Surgery and Center of Physical Examination in The First Affiliated Hospital of Fujian Medical University. A total of 46 whole blood samples were obtained from patients with non-surgical HBV-related HCC. Of these patients without HCV or HIV infection, 41 (89.1%) were males and 5 (10.9%) were females with an average age of 55.96 (55.96±14.96) years old. None of the cases had previous history of other cancers. Additional 23 whole blood samples were recruited from healthy controls. No statistical difference was found in age and gender between HCC cases and healthy controls. The study was in accordance with the approval of the ethics committee of The First Affiliated Hospital of Fujian Medical University and the ethical principles of the 1975 Declaration of Helsinki. Written informed consent was also obtained before collecting blood samples and studying the clinical features of each patient.

Genomic DNA extraction from PBMCs

Peripheral blood mononuclear cells were extracted from all whole blood samples using Lymphocyte Separation Medium (TBDscience, Tianjin, China) and then genomic DNA was extracted from PBMCs using the TIANamp Blood DNA Kit (Tiangen Biotech Company, Beijing, China) according to the manufacturer's instruction.

Primers for RT-qPCR

Primers for RT-qPCR were designed using Primer Premier 5.0 software (Premier Biosoft International, USA). In brief, the copy number of mtDNA was determined using forward primer (ND1 F) and reverse primer (ND1 R) for amplifying mtDNA gene ND1. The human single-copy nuclear gene β-globin was amplified as a control gene (**Table 1**). Blast analysis was performed to demonstrate the specificity of the primers.

Preparation of ND1 and β-globin plasmids for standard curves

Genomic DNA of two clinical healthy controls was used for plasmids construction. Target DNA was amplified with primers (ND1and β-globin) listed in **Table 1**. PCR was performed in a 25 μl reaction mixture containing 12.5 μl of 2× Premix Taq™ (Takara, Japan), 1.0 μl of each primer (10 μM), 8.5 μl of ddH_2O and 2.0 μl of genomic DNA. Thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, then 35 cycles with denaturation at 95°C for 30 s, annealing at 58°C for 35 s and extension at 72°C for 30 s, and a final elongation step at 72°C for 15 min. PCR products were electrophoresed on a 2.0% agarose gel and purified via Universal DNA Purification kit (Tiangen Biotech Company, Beijing, China).

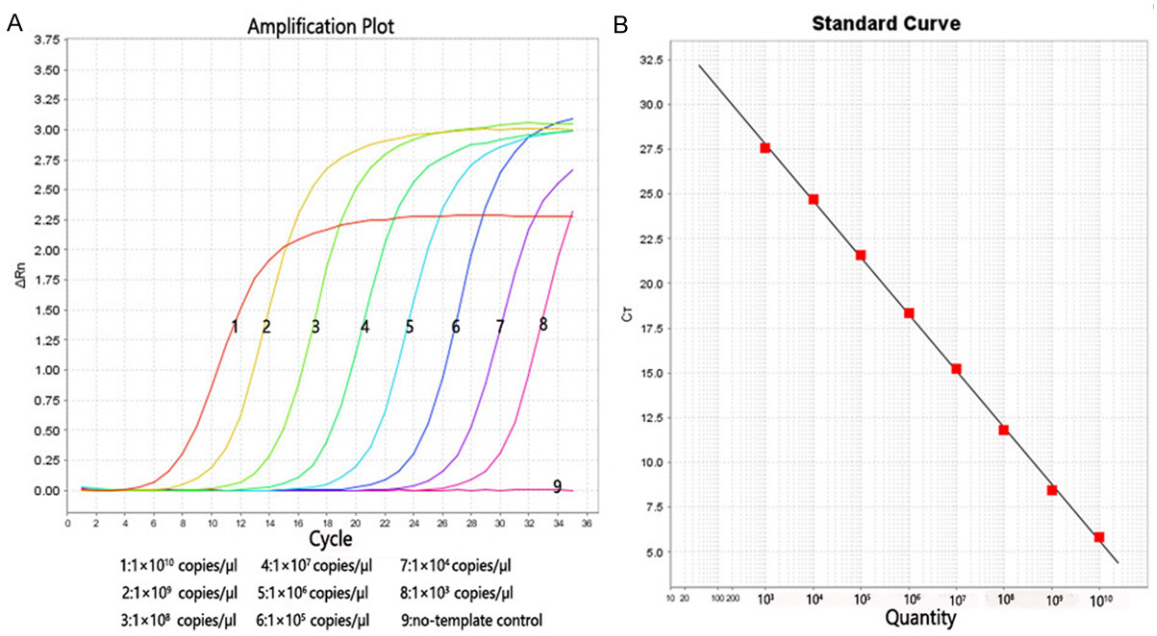

Figure 1. Amplifications plot and standard curve of ND1: A. Amplification plots with different colors represented different concentrations of ND1 recombinant plasmids which were illustrated in the figure. B. Standard curve of ND1: Y=-3.166X+37.268 (R^2=0.999).

Then PCR products were cloned into pEASY-T3 vector (Transgen Biotech Company, Beijing, China) and transformed into Trans1-T1 Phage Resistant Chemically Competent Cell (Transgen Biotech Company, Beijing, China). The positive plasmids were sequenced and then isolated using TIANprep Mini Plasmid kit (Tiangen Biotech Company, Beijing, China). Plasmids were quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The corresponding copy number was calculated and 10-fold serially dilution from 1×10^{10} copies/μl to 1×10^{1} copies/μl using Easy Dilution (Takara, Japan) was to generate standard concentrations.

RT-qPCR for detection of mtDNA copy numbers

RT-qPCR assay was performed on ABI 7500 Real-Time PCR system (Life Technologies, USA). The 25 μl PCR amplification reaction mixtures contained 12.5 μl of 2× SYBR® Premix Ex Taq™ (Takara, Japan), 1.0 μl of each primer (10 μM), 0.25 μl of 50× ROX II reference dye (Takara, Japan), 9.25 μl of ddH_2O and 1.0 μl of genomic DNA. Real-time PCR conditions were: initial denaturation at 95°C for 20 s, followed by 35 cycles of denaturation at 95°C for 3 s, and annealing/extension at 58°C for 30 s. The post-amplification melting curve analysis was performed to confirm whether the nonspecific amplification was generated. All samples were amplified in duplicate. In each run, negative and positive controls and a standard curve were included. The R^2 for each standard curve was ≥0.99, if not, the test was repeated. The ratio of mtDNA copy number to β-globin copy number (also referred as relative mtDNA content) for each object from standard curves was calculated to further analysis.

Statistical analysis

Data were analyzed with SPSS version 20.0 (SPSS Inc, USA), and the significance level was set at P<0.05. Normality test was performed to determine whether Student's t-test or Wilcoxon rank sum test should be chosen to analyze mtDNA content. Finally, the results of mtDNA content were compared between HCC cases and healthy controls with Student's t-test. In addition, receiver operating characteristic (ROC) curve analysis was carried out to assess the clinical significance of mtDNA content for diagnosing HCC.

Results

Identification of recombinant plasmids by sequencing

Gel image showed no second bands for the products of ND1 and β-globin recombinant

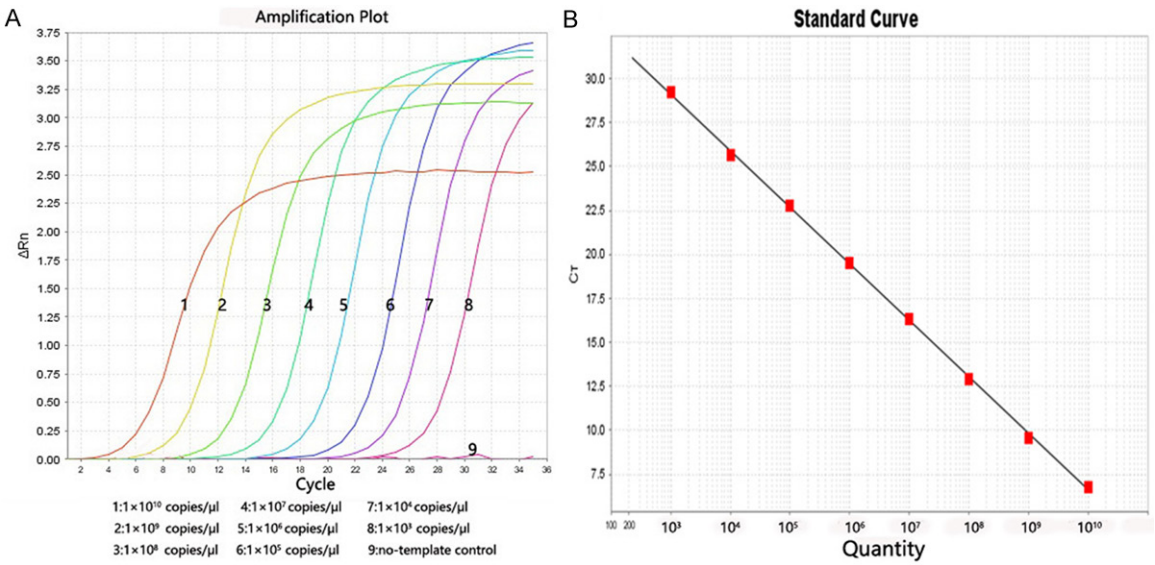

Figure 2. Amplifications plot and standard curve of β-globin: A. Amplification plot with different colors represented different concentrations of β-globin recombinant plasmids which were illustrated in the figure. B. Standard curve of β-globin: Y=-3.216X+38.752 (R^2=1).

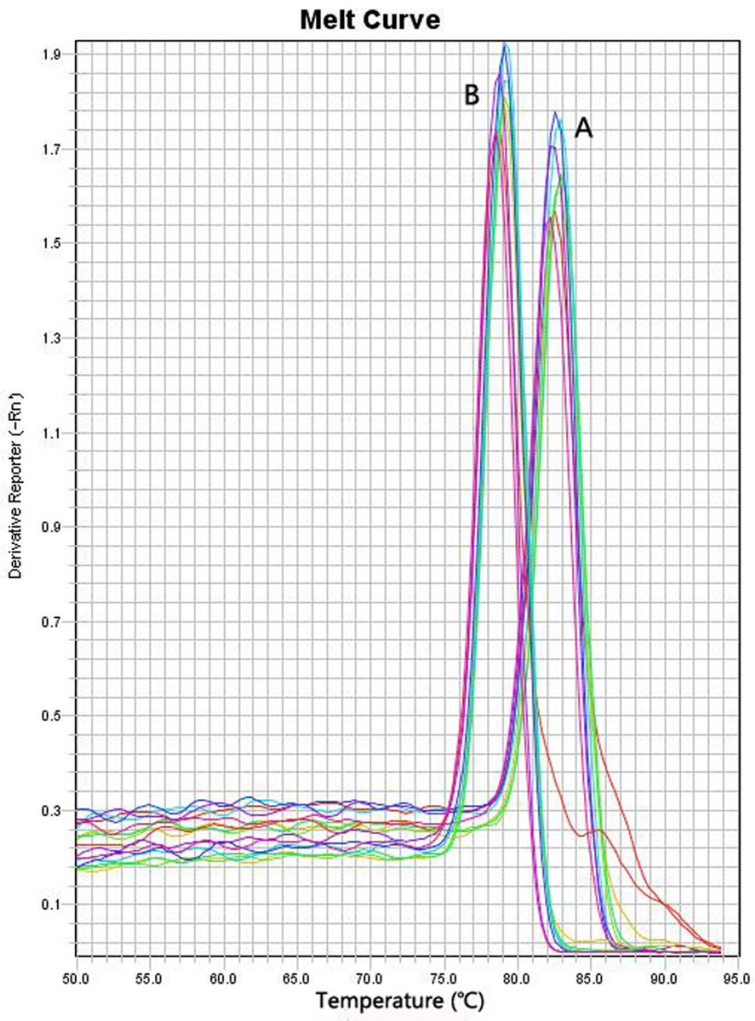

Figure 3. Specificity test: melt curves for different concentrations of ND1 (A) and β-globin (B) recombinant plasmids.

plasmids. The ND1 and β-globin recombinant clones were picked out and sequenced. Then, the obtained DNA sequences were subjected to BLAST alignment against mitochondrial and nuclear genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Blast results indicated the sequences were completely consistent with the mitochondrial and nuclear reference genome (GeneBank accession number: NC_012920 and NC_000011, respectively), which meant that the ND1 and β-globin recombinant plasmids were successfully constructed.

Linear range

The 1×10^{10} copies/μl~1×10^{1} copies/μl recombinant plasmids were used to test the linear range of RT-qPCR (**Figures**

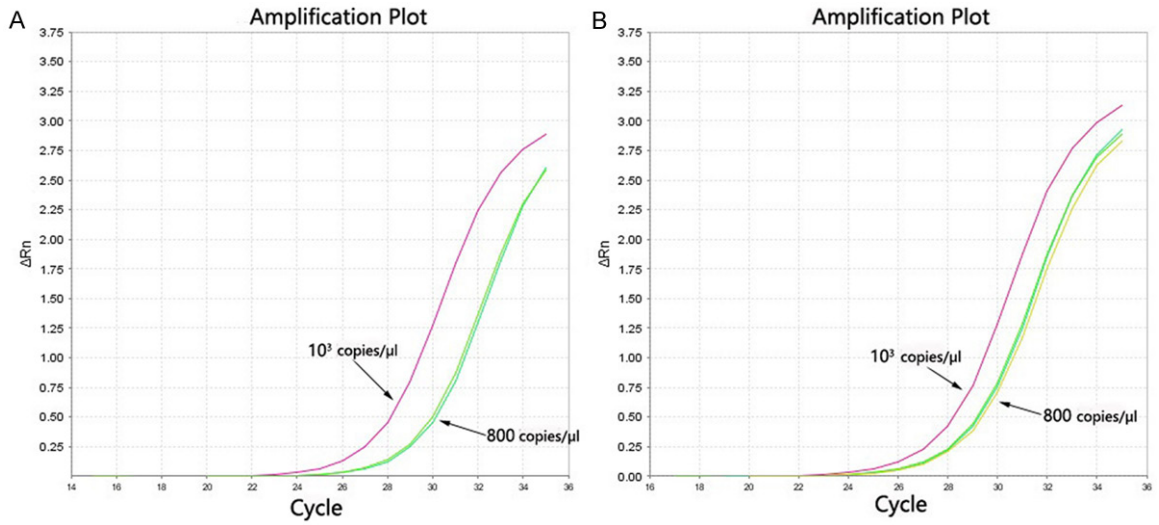

Figure 4. Sensitivity test: amplification plot with different colors represented different concentrations of ND1 (A) and β-globin (B) recombinant plasmids which were illustrated in the figure.

1 and **2**). There was an excellent linear correlation between cycle number and mtDNA copy number from the concentration of 1×10^{10} copies/μl to 1×10^{3} copies/μl with correlation coefficients of 0.999 and 1 for ND1 and β-globin, respectively. The amplification efficiency of ND1 and β-globin standard curves was 106.922% and 104.623%, respectively.

Specificity test

The melt curve image showed 2 clear peaks for ND1 and β-globin recombinant plasmids (**Figure 3**) which indicated that the primers were high specificity.

Reproducibility test

The high (1×10^{7} copies/μl), medium (1×10^{5} copies/μl) and low (1×10^{3} copies/μl) concentrations of ND1 and β-globin recombinant plasmids were used as templates for 20 separate and simultaneous measurements by RT-qPCR. The intra-assay coefficient of variation (CV) was 0.50%, 0.32%, 1.34% for ND1 recombinant plasmids and 0.75%, 0.90%, 1.28% for β-globin recombinant plasmids. The quantitative assay was performed for 20 days consecutively. The inter-assay CV was 2.78%, 1.42%, 2.35% for ND1 recombinant plasmids and 4.14%, 1.26%, 1.37% for β-globin recombinant plasmids.

Sensitivity test

The limit concentration for both ND1 and β-globin recombinant plasmids was 800 copies/μl (**Figure 4**) which can be accurately and steadily detected by the method.

Clinical significance of mtDNA content in PBMC for non-surgical HBV-related HCC

Two-sample t-test revealed that relative mtDNA content in PBMC of non-surgical HBV-related HCC cases was significantly lower than that of healthy controls (mean [range]: 378.55 [58.20-784.85] vs 715.48 [292.00-1280.00]; P<0.001) (**Figure 5A**). ROC curve analysis showed that the area under the curve was 0.845 and the standard error was 0.047. Moreover, the asymptotic 95% confidence interval was from 0.752 to 0.938. When 489.90 copies/μl was set as the cut-off point, the sensitivity and specificity of mtDNA content to diagnose HCC were 82.6% and 71.7%, respectively. These results suggested that mtDNA content in PBMC was significantly associated with HCC and could serve as a novel clinical diagnostic indicator for HCC (P<0.001) (**Figure 5B**).

Discussion

The alteration of mtDNA copy number could affect the expression of mitochondrial genes as well as a wide range of mitochondrial functions such as energy production, signaling transduction, cell cycle regulation, cellular growth, differentiation and apoptosis [16]. Therefore, establishing a simple, rapid, cost-effective, highly sensitive and specific assay to detect mtDNA is of great clinical significance. Compared with

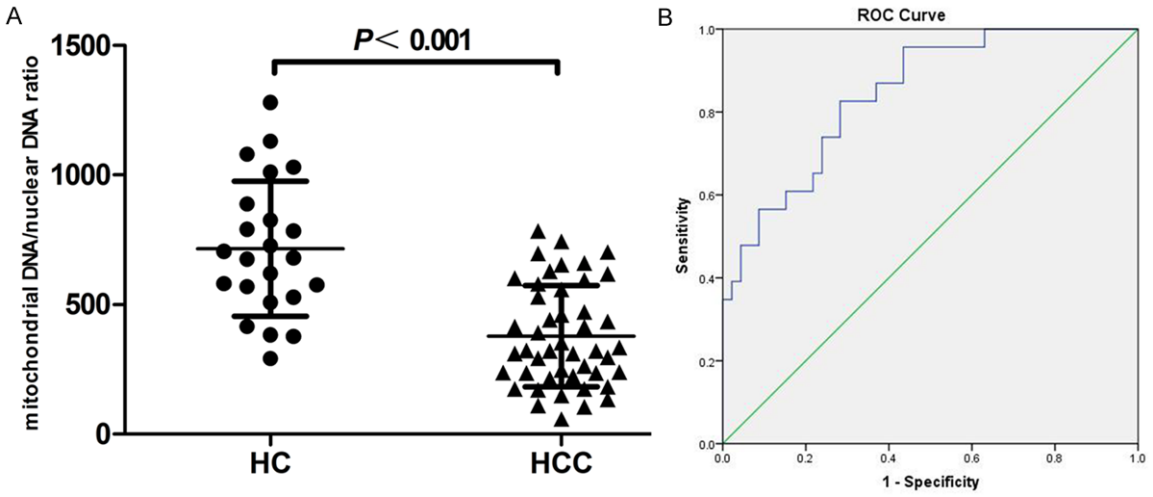

Figure 5. A. Relative mtDNA content in healthy controls (n=23) and patients with non-surgical HBV-related HCC (n=46). B. ROC curve between healthy controls and patients with HCC. HC: healthy controls. HCC: hepatocellular carcinoma.

other methods [13, 14, 17, 18], our experiment had a much wider linear range. Moreover, the assay is SYBR Green I-based real-time quantitative PCR and there is no necessary to design target-specific probes, which is expensive and complicated. In addition, unlike ΔΔCt calculation method, this method uses standard curves in each run to absolutely quantify mtDNA copy number. Therefore, the accuracy of our assay is much more trustworthy. However, there is a limitation with detecting the lowest concentration of 800 copies/μl. As our data suggests that mtDNA content in PBMC samples of patients with HCC is relatively high (above 1000 copies/μl), the limitation can be ignored to some extent. Therefore, the method established in this study is reliable and applicable for clinical laboratory to measure mtDNA content.

Recently, numerous studies have shown that lower mtDNA content in PBL and serum samples can predict the increased risk of HCC [19, 20]. Another research elucidates that decreased mtDNA content in serum can increase cirrhosis risk in HBV infected patients [21]. However, whether mtDNA content in PBMC samples can also be regarded as a predictor for HCC remains to be determined. In this study, we conclude that mtDNA content in PBMC was significantly associated with HCC and when 489.90 copies/μl was set as the cut-off point, the sensitivity and specificity of mtDNA content to diagnose HCC were 82.6% and 71.7%, respectively. So mtDNA content may serve as a novel clinical diagnostic indicator for HCC. Besides, all of these researches demonstrate that mtDNA content is obviously influenced by HBV. While the exact mechanism behind the decrease of mtDNA content in HBV-related diseases is not clear.

After HBV invading liver cells, HBx protein would locate in the voltage dependent anion channel (VDAC) which exists at mitochondrial outer membrane. This process causes excessive ROS and oxidative stress inducing the damage of mitochondrial membrane, protein and mtDNA [22]. The damage of mtDNA could affect the transcription and translation of mitochondrial genes, which may lead to the reduction of mtDNA content. In this circumstance, decreased mtDNA content in patients with HBV-related HCC is bound up with HBV infection. Chronic HBV infection causes oxidative damage of cells which couldn't restore to normal condition temporarily. So we will continue to track on mtDNA content, biological characteristics and survival condition of each patient in subsequent studies to elucidate the roles played by mtDNA in the development of HCC. Meanwhile, further elucidation of the mechanisms underlying the association of mtDNA content with HBV-related diseases will lead to a better understanding of the roles played by mitochondria in the development of liver diseases.

In conclusion, a simple, rapid, cost-effective, highly sensitive and specific method for detection of mtDNA content is established. This method can be applied to clinical laboratory. Meanwhile, our study provides the first epidemiological evidence that mtDNA content in PBMC is significantly associated with HCC. When 489.90 copies/μl was set as the cut-off point, the sensitivity and specificity of mtDNA content to diagnose HCC were 82.6% and 71.7%, respectively. These results suggest that mtDNA content could serve as a novel clinical diagnostic indicator for HCC. However, more patients are warranted to be collected and more investigation to the mechanism of the decrease of mtDNA content should be further executed to validate the role of mtDNA content in the HBV-related HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qi-Shui Ou, First Clinical College, Fujian Medical University, Fuzhou, China; Department of Laboratory Medicine, The First Affiliated Hospital of Fujian Medical University, 20 Chazhong Road, Fuzhou, China. Tel: 86-591-83340702; Fax: 86-591-83340702; E-mail: ouqishui@fjmu.edu.cn

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