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Quantification of cell-free circulating mitochondrial DNA copy number variation in hepatocellular carcinoma

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SUMMARY

OBJECTIVE: Hepatocellular carcinoma is the most common primary malignant liver tumor. Mitochondrial DNA copy number has been shown to be associated with various malignancies. However, there has not been any study on the absolute quantification of mtDNA copy number in hepatocellular carcinoma. The aim of this study was to develop a new method for absolute quantification of mtDNA copy number and to relatively quantify the variations in the mtDNA copy number in hepatocellular carcinoma patients in comparison with healthy individuals.

METHODS: Venous blood samples were collected from both hepatocellular carcinoma patients (34) and healthy individuals (34). Circulating cell-free DNAs were isolated and the relative quantification of mtDNA copy number variation was determined using quantitative polymerase chain reaction and digital polymerase chain reaction.

RESULTS: It was found that the relative mtDNA copy number was significantly decreased in hepatocellular carcinoma patients in comparison with the control group ($p<0.05$). The median (range) and average of relative mtDNA/β-actin gene of the patients were determined as 42.8 cp/μL (11.1–88.5) and 45.1 cp/μL, respectively, while the median (range) and average relative mtDNA/β-actin gene of the control group were determined as 102.8 cp/μL (55.1–291.8) and 138.7 cp/μL, respectively ($p<0.05$). When quantitative polymerase chain reaction and digital polymerase chain reaction were compared, mtDNA/β-actin gene copy number ratio of digital polymerase chain reaction results was found to be 1.76-fold more than that of quantitative polymerase chain reaction results.

CONCLUSION: Circulating mtDNA copy number was decreased in hepatocellular carcinoma patients in comparison with healthy individuals, and we suggest that it can be used as a noninvasive biomarker for hepatocellular carcinoma diagnosis in the future.

KEYWORDS: Mitochondria. CfDNA. HCC. dPCR.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor and constitutes 80–90% of all primary malignant liver tumors. It is the fifth most common cancer in adult men and eighth most common cancer in adult women. Also, HCC is ranked the sixth in cancer-related deaths and is the leading cause of liver-related mortality in patients with chronic hepatitis and cirrhosis[1,2].

Mitochondria produce energy through ATP synthesis via the citric acid cycle and oxidative phosphorylation. Since the mtDNA does not have histones and has limited proofreading activity, the mutation rate is about 10 times higher than in the nuclear genome. The noncoded D-loop region of mtDNA region shows a high degree of polymorphism, and mutations in this region lead to changes in the number of mtDNA copies and suppression of mitochondrial gene expression[3,4].

Many clinical, histological, genetic, and biochemical diseases have been associated with mutations in mitochondria[5]. In the literature, the mtDNA copy number alterations have been reported in some malignancies. It has also been published that the number of mtDNA copies were decreased in HCC, but the mechanism has not yet been fully explored[6-9]. There has not been any study on the absolute quantification of mtDNA copy number in HCC, although a few studies were reported on other malignancies.

The aim of this study was to develop a novel method for absolute quantification of mtDNA copy number and to relatively quantify the variations in the mtDNA copy number in HCC patients in comparison with healthy individuals.

METHODS

Human serum samples collection

Serum samples were collected from 34 newly diagnosed HCC patients who were admitted to the Istanbul University Oncology

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The patients who had received radiotherapy and chemotherapy, have confirmed infection and chronic inflammatory diseases, as well as those with tumors in other organs and used antibiotics, anti-inflammatory drugs, or corticosteroids were not included in the study.

As a control group, serum samples were obtained from 34 healthy individuals. Epidemiologic variables and clinical data were collected by physicians. The presence of chronic hepatitis B and C and cirrhosis were recorded according to the laboratory and radiological test results (Table 1). The study was approved by the Ethics Committee of the Istanbul University (Document number 2016/1297).

Cell-free DNAs were isolated using Norgen Plasma/Serum Cell-Free Circulating DNA Purification Kit (Catalog No. 55100) according to the kit's protocol from 500 µL serum samples. The concentration and purity of the isolated DNA was determined using NanoDrop Spectrometer.

Quantitative analysis of cell-free mtDNA

The relative quantification of mtDNA copy number was determined using quantitative real-time PCR (qPCR), and absolute quantification of mtDNA copy number was performed by QuantStudio 3D Digital PCR (dPCR) System using a slightly modified protocol as described previously[10,11]. Briefly, for the amplification of the short segment inside the 7S region of the mtDNA, 2′ Mastermix (SensiFast Probe No-ROX Kit, Bioline) with 900 nM primer each together with 250 nM TaqMan probe and 5 µL DNA were used (Table 2).

As an endogenous gene control, the nuclear low copy gene β-actin (GenBank accession number NM_001101) was used. The qPCR and dPCR conditions of both genes are as follows: an initial denaturation at 95°C for 10 min, denaturation at °C for 15 s, annealing at 64°C for 60 s, and a total of 40 and 39 cycles of PCR, respectively. All samples were measured in duplicate using qPCR and dPCR. Human genomic DNA (sonicated) Hybridime (Cambio Cat No. CA-972-06) was used as a positive PCR control.

For relative quantification of mtDNA by qPCR, ΔC_t method was used as the cycle threshold (C_t) values were obtained from the Light Cycler® 480 Software. After dPCR, mtDNA and β-actin genes of patient and control group samples were analyzed using QuantStudio™ 3D Analysis Suite™ Cloud Software.

Statistical analysis

All data were analyzed using the IBM SPSS statistics v21 package. For the determination of skewness and kurtosis of all data, values were calculated using the Kolmogorov-Smirnov test. Then, all data were analyzed using Mann-Whitney U test as nonparametric statistics. The threshold $p<0.05$ was considered statistically significant.

RESULTS

Epidemiologic data of the patients and healthy individuals are summarized in Table 1. In total, 24 (71%) patients were male with a mean age of 51±9 years. Fourteen had chronic hepatitis B and 11 had chronic hepatitis C. Eight of them were smokers and seven of them were drinkers. As expected,

Table 1. Epidemiologic variation of patients and healthy individuals.

Variables	Patients (n=34, %)	Control (n=34, %)	p-value
Age (years) (mean±SD)	51±9	48±10	>0.05
Male/female	24 (71)/10 (29)	20 (59)/14 (41)	>0.05
Chronic hepatitis B	14 (41)	–	
Chronic hepatitis C	11 (32)	–	
Smoking status			
Never	26 (76)	23 (68)	>0.05
Ever	8 (24)	11 (32)	>0.05
Drinking status			
Never	27 (79)	30 (88)	>0.05
Ever	7 (21)	4 (12)	>0.05
Cirrhosis			
Yes	24 (71)	–	
Family history of cancer			
Yes	11 (32)	4 (12)	<0.05

most of the patients had cirrhosis. In all, 11 patients had a family history of cancer. None of the healthy individuals had cirrhosis. Four of the healthy individuals had a family history of cancer.

There were no significant differences between patients and healthy individuals with respect to gender, age, smoking, and drinking status (all p>0.05). More HCC patients had a family history of cancer than in healthy individuals (p<0.05).

Relative quantification of mtDNA copy number

The mtDNA copy number alteration was determined by qPCR using mtDNA-specific and β-actin gene-specific primers and probes. Then, the ΔC_t analysis of the qPCR data showed that relative mtDNA copy number was significantly decreased in HCC patients in comparison with the control group (p<0.05). The median ratios of mtDNA to β-actin gene in HCC patients and control group individuals were 3.73±0.94 and 4.53±0.54, respectively (p<0.05).

Absolute quantification of mtDNA copy number

For the successful determination of the alterations in the mtDNA copy number, the ratio of mtDNA to β-actin gene was measured using dPCR. The ratio of mtDNA to β-actin gene was decreased in HCC patients in comparison with that of control group (p<0.05). The median (range) and average of the patient relative mtDNA/β-actin gene were 42.8 cp/μL (11.1–88.5) and 45.1 cp/μL, while the median (range) and average of the control group relative mtDNA/β-actin gene were 102.8 cp/μL (55.1–291.8) and 138.7 cp/μL, respectively (p<0.05).

When dPCR results were compared, the mtDNA/β-actin gene ratio of some HCC patients was much less than that of other status of patients (Table 3). Also, when chronic hepatitis B was evaluated with drinkers and smokers, drinkers and smokers had much less cell-free mtDNA copy numbers than in other HCC patients.

Quantitative polymerase chain reaction (qPCR) and dPCR results were also compared and it was shown that mtDNA/β-actin gene copy number ratio of dPCR results was 1.8-fold more than that of qPCR results.

DISCUSSION

In recent years, tumor markers were discovered and utilized from the blood (liquid biopsy specimens) of the patients. The biggest advantage of these markers is that they contain genetic information that may represent tumor tissue in a liquid biopsy specimen taken without the need for surgery[12,13]. In this study, we investigated the mtDNA copy number alterations in liquid biopsy samples of HCC patients. In the literature, it has been reported that the number of mtDNA copies has been increased in some malignancies such as breast cancer, lung cancer, endometrial adenocarcinoma, and colorectal cancer[14-16]. In contrast, the number of mtDNA copies has decreased in some malignancies such as ovarian cancer, gastric cancer, Ewing sarcoma, and HCC[6-9]. It is believed that this decrease is due to point mutations that may occur in the noncoding D-loop region that regulates mitochondrial genome replication. mtDNA copy number has also decreased in HBV-related HCC patients[17].

For clinical and diagnostic approach, precise and accurate nucleic-acid quantification is required to prevent incorrect diagnosis. Digital PCR is considered the reference method for absolute nucleic-acid copy-number detection[18,19].

In this study, mtDNA was quantified for the first time by dPCR in HCC patients. Using qPCR, it was found that mtDNA copy number decreased 1.7-fold in newly diagnosed HCC patients (p<0.05). The mtDNA/β-actin gene ratio was found to be decreased 1.8-fold using dPCR measurements. Since dPCR is considered the reference measurement system, we consider that dPCR gives higher accuracy in results.

A survey including smoking status, drinking status, and family history of cancer was filled by patients during serum collection from the patients and healthy individuals. The dPCR data showed that the mtDNA copy number was significantly lower in both smokers and drinkers than in other patients. It was reported that consuming more than two portions of alcoholic beverages per day was significantly associated with 2-fold higher risk of developing HCC[20,21]. Also, currents or past smokers have been significantly associated with HCC[22]. It has been reported that smoking decreases mtDNA copy number, proving that it may also be associated with mitochondrial dysfunction[23].

Table 2. Primer and probe sequences of β-actin and mtDNA gene for quantitative polymerase chain reaction and digital polymerase chain reaction.

	Primer/probe	Sequence	PCR product size (bp)
β-actin	Forward	5′-GGCACCACACCTTCTACAATGAG-3′	104
	Reverse	5′-GGTCATCTTCTCGCGGTTGG-3′	
	Probe	HEX 5′-TGCTGCTGACCGAGGCCCCC-3′ BHQ1	
mtDNA	Forward	5′-CATAAAGCCTAAATAGCCCACACG-3′	85
	Reverse	5′-CCGTGAGTGGTTAATAGGGTGATA-3′	
	Probe	FAM 5′-AGACATCACGATGGATCACAGGTCT-3′ BHQ1	

Table 3. mtDNA/β-actin gene ratio in hepatocellular carcinoma patients.

	Female	Male	Hepatitis B	Hepatitis C	Smoking	Alcohol	Cirrhosis	Family hist. of cancer
	Average (n) Median Range	Average (n) Median Range	Average (n) Median Range	Average (n) Median Range	Average (n) Median Range	Average (n) Median Range	Average (n) Median Range	Average (n) Median Range
Gender	53.0 10 55.6 16.6–82.1	41.6 24 41.4 11.2–88.5						
Hepatitis B	73.1 3 74.9 62.4–82.1	39.6 11 39.3 16.2–73.8	47.3 14 44.1 16.2–82.1					
Hepatitis C	52.5 5 50.9 26.9–74.9	32.6 6 36.3 11.7–44.5	47.2 5 44.1 30.9–74.9	41 11 41.8 11.7–74.9				
Smoking	54.5 2 54.5 40.7–68.3	28.3 6 23.3 11.2–63.3	27.8 3 30.4 16.2–36.8	40.3 4 40.7 11.7–68.3	34.8 8 33.6 11.2–68.3			
Alcohol	–	32.7 7 30.4 11.7–69.6	32 4 33.6 16.2–44.5	28.1 2 28.1 44.5–18.7	23.8 4 23.3 11.7–36.8	32.7 7 30.4 11.7–69.6		
Cirrhosis	71.6 5 71.6 68.3–71.9	39.7 19 42.9 16.7–73.8	50.9 17 44.5 24.3–74.9	48.4 15 44.3 16.7–74.9	–	32.2 6 32.2 19.9–44.5	46.1 24 44.3 16.7–74.9	
Family hist. of cancer	52.7 4 54.5 26.9–74.9	40 7 30.9 19.9–69.6	40.4 5 30.9 24.3–74.9	47.3 6 41.8 26.9–74.9	50.7 4 52 30.4–.68.3	39.9 3 30.4 19.9–69.6	45.8 5 41.8 19.9–74.9	44.6 11 40.7 19.9–74.9

The decreased mtDNA copy numbers in HCC patients who were smokers and drinkers may be associated with the chemicals that target the mtDNA.

This study has some limitations. First of all, the study population was relatively small. Moreover, this study involved patients only from two medical centers from Istanbul, Turkey. However, our new and successful technique renders our study interesting and favorable.

CONCLUSIONS

This is the first study that performs absolute quantification of cell-free mtDNA to β-actin gene ratio in HCC patients using qPCR in addition to dPCR. Although mtDNA/β-actin gene ratio was found to be decreased in both platforms, there was about 1.8-fold difference in them. Since dPCR is considered the reference measurement system, we have higher confidence in the results of dPCR than in qPCR. It was shown that circulating mtDNA copy number was decreased in HCC patients using both platforms. Thus, dPCR has the potential to be used as a noninvasive biomarker for HCC diagnosis in the future.

AUTHORS' CONTRIBUTIONS

BY: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **DT:** Formal Analysis, Investigation, Resources. **FG:** Formal Analysis, Investigation, Resources, Writing – original draft. **MA:** Project administration, Supervision. **SP:** Project administration, Supervision.

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