

Original Article

Changes in mitochondrial DNA and its encoded products in alcoholic cirrhosis

Chun Tang, Xianchun Liang, Hongming Liu, Liping Guo, Ruxian Pi, Juntao Yang

Department of Hepatobiliary Surgery, Daping Hospital, The Third Military Medical University, Chongqing 400042, China

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Abstract: The purpose of this study was to investigate hepatic mitochondrial DNA (mtDNA) damage and changes in its encoded products in patients with alcoholic cirrhosis (AC) in order to understand disease pathogenesis. We enrolled 23 patients with AC, 26 alcoholics without cirrhosis, and 25 normal subjects in this study. Hepatic mtDNA deletions were positioned using a combination of long and accurate polymerase chain reaction (LA PCR) and gene sequencing. The mtDNA copy number was measured using real-time quantitative PCR. The expression of the mtDNA-encoded cytochrome c oxidase 2 (cox2) was detected by western blotting. A large deletion of bases located at positions 749-15486 was identified in hepatic mtDNA from AC patients. Moreover, the mtDNA copy number was significantly reduced ($P < 0.05$), and its encoded product, cox2, was significantly downregulated ($P < 0.05$). Collectively, our results suggest that specific deletions and reduced copy numbers of hepatic mtDNA in patients with AC is an important pathogenetic factor.

Keywords: Alcoholic cirrhosis, mitochondria, mitochondrial DNA, cytochrome c oxidase 2

Introduction

Alcoholic cirrhosis (AC) is an end-stage alcoholic liver disease (ALD) caused by long-term, excessive drinking. It manifests as chronic inflammation and progressive fibrosis of liver tissue and is the leading cause of death in patients with chronic alcoholism. A 48-month prospective study of 280 patients with severe ALD in the United States found that 30% of patients had alcoholic fatty liver disease, more than half ultimately developed cirrhosis, and two-thirds of cirrhotic patients developed alcoholic hepatitis and ultimately died [1]. An epidemiological survey in China showed that 90-100% of heavy drinkers developed fatty liver, 10-35% of these individuals eventually developed alcoholic hepatitis, and 8-20% progressed to cirrhosis.

AC pathogenesis is complex. Its risk factors include malnutrition caused by long-term alcohol consumption [2, 3], hepatic stellate cell activation [4], fibrosis induced by alcohol and its metabolites [5-10], oxidative stress in liver tissue [11], immune response [12], and genetic poly-

morphisms [13]. Oxidative stress is believed to play a key role in AC development. Mitochondria are particularly vulnerable to the effects of oxidative stress [14]. Excessive oxidative stress injury can induce apoptosis, which is the main mechanism that causes progressive hepatic injury, ultimately leading to cirrhosis [15]. Alcohol has been demonstrated to induce structural and functional damage in hepatic mitochondria [16, 17], but the specific mechanism remains unclear. Mitochondrial DNA (mtDNA) and its encoded products are important regulators of mitochondrial oxidative phosphorylation and respiratory function. A common 4977-base pair (bp) deletion has been identified in the hepatic mtDNA of alcoholic patients with microvesicular steatosis [18]. However, no studies have assessed specific hepatic mtDNA damage in patients with AC.

To this end, tissue specimens were collected from patients with AC in our hospital from June 2007 to June 2011. We investigated mtDNA damage, including changes in copy number and encoded products, in order to explore AC patho-

Table 1. Age and alcohol intake of subjects.

	Group A (Normal controls)	Group B (Alcoholics without cirrhosis)	Group C (Alcoholic cirrhosis)
Number of cases	25	26	23
Gender	Male	Male	Male
Age (years)	52.65±2.53 (36-81)	48.13±2.23(34-71)	53.71±2.82(33-83)
Daily alcohol intake (g)	—	109.2±8.82	117.1±12.29
Years of alcoholism	—	24.83±1.99	26.67±2.79

genesis and stimulate a new way of thinking about its clinical prevention.

Materials and methods

Patients and clinical specimen collection

All experimental procedures were approved by the Medical Ethics Committee of the Third Affiliated Hospital of the Third Military Medical University. Written informed consent was obtained from each subject prior to specimen collection. Collected liver tissue specimens were cryopreserved in liquid nitrogen. AC was diagnosed according to the ALD diagnostic criteria established by the Chinese Medical Association in 2001:1) a long-term history of heavy drinking and alcohol intake ≥ 40 g/d for more than 5 consecutive years; 2) hypohepatia and portal hypertension, cirrhosis confirmed by imaging, and alcoholic liver injury confirmed by a serum enzyme test; 3) negative hepatitis B and C antigen and antibody and DNA tests (to exclude patients with cirrhosis due to other causes). Alcohol intake was calculated as follows: alcohol intake = alcoholic drink intake (ml) \times degree of alcohol (%) $\times 0.8$.

We assessed three groups with the following characteristics: Group A (25 normal subjects): No long-term alcohol consumption; no liver diseases; no significant liver changes found during upper abdominal surgery for other diseases, such as gallbladder stones. Group B (26 chronic alcoholics without cirrhosis): Chronic alcoholism was confirmed according to the above-mentioned diagnostic criteria; normal liver function test results; no significant change in the liver confirmed by imaging; no significant change was found in the liver during upper abdominal surgery for other diseases. Group C (23 patients with AC): The above inclusion criteria were met, and liver scarring was confirmed dur-

ing upper abdominal surgery for AC or other diseases.

All liver tissue specimens were confirmed by pathological examination. There was no significant difference in age between the three groups or in time (years) or daily intake of alcohol (g) between groups B and C (**Table 1**). Because alcoholism is much more common in males than in females in this region, all specimens were collected from male subjects.

Extraction of total hepatic DNA

A tissue DNA extraction kit was purchased from TaKaRa Bio Inc. (Dalian, China). The extraction was conducted according to the manufacturer's instructions. The extracted DNA was stored at -20°C .

Hepatic mtDNA deletion assessment

Long and accurate polymerase chain reaction (LA PCR) and gene sequencing [19] were performed to detect and position specific deletions in hepatic mtDNA. TaKaRa LA Taq (DRR20AM) was used. The primer sequences to amplify full-length mtDNA were as follows: forward (364-391): 5'-AAGAACCCTAACACCAGCCTAACCAGAT - 3', and reverse (336-363): 5'-ATGATGTCTGTG TGGAAAGTGGCTGTGC -3' (Gi: 113 200 490).

A two-tube loading system was used for PCR. Tube A (30- μl total volume) contained 19 μl ddH₂O, 8 μl (2.5 μM each) dNTPs, and 1.5/1.5 μl (10 μM) upstream/downstream primer. Tube B (20- μl total volume) contained 8.5 μl ddH₂O, 3 μl template, 5 μl Mg²⁺-free LA buffer, 3 μl (2.5 μM) Mg²⁺, and 0.5 μl LA Taq. Tube B was incubated in the PCR machine at 75°C for 5 min before it was gently added to tube A. The amplification consisted of initial denaturation for 2 min at 94°C , 30 cycles of 94°C for 15 s, 68°C

for 15 min, primer extension for 15 min at 72°C, and storage at 4°C. After the reaction, 5 µl PCR products was mixed with 2.5 µl of 6X loading buffer. The resulting mixture was analyzed by 0.8% agarose gel electrophoresis (4 V/cm, 60 min).

Gene sequencing was performed to accurately position mtDNA deletions.

Quantitative examination of hepatic mtDNA

For statistical convenience, 20 samples were randomly selected from each group, and the hepatic mtDNA copy number was determined by real-time quantitative PCR using a SYBR® *Premix Ex Taq™* II (Perfect Real Time) kit from TaKaRa.

PCR primers: Because the mtDNA displacement (D)-loop region is highly conserved, the mtDNA copy number was represented by the mtDNA D-loop hypervariable region 1 (HV1), forward: 5'-TTGCACGGTACCATAAATACTTGAC-3', and reverse: 5'-GAGTTGCAGTTGATGTGTGATAGTTG-3', 128 bp. The nuclear β-globin served as an internal control, forward: 5'-CAACTTCATCCACGTTCCACC-3', and reverse: 5'-CAACTTCATCCACGTTCCACC-3', 110 bp.

PCR system and conditions

The PCR system included 12.5 µl SYBR® *Premix Ex Taq™*, 2 µl DNA template (about 100 ng), 1 µl each of upstream and downstream primers (final concentration: 0.4 mmol/L), and 8.5 µl H₂O. The total reaction volume was 25 µl. Quantitative PCR was performed on a Bio-Rad CFX96 real-time PCR system at conditions of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s.

Relative quantitative detection

The relative quantitative value of mtDNA was represented by the ratio of mtDNA (HV1) to β-globin. Each sample was assessed in triplicate.

Determination of mtDNA-encoded products

Cytochrome c oxidase 2 (COX2) was measured using western blotting as a representative mtDNA-encoded protein. Denatured protein samples (50 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred under semi-dry condi-

tions onto nitrocellulose (NC) membranes, and visualized with Ponceau-S staining. Then, the NC membranes were blocked with Tris-buffered saline solution (TBS) containing 5% non-fat dry milk overnight, incubated with COX2 antibody (1:400 dilution) at 4°C overnight, washed in TBS with Tween (TBST) three times (10 min each), incubated with goat anti-rabbit IgG (1:5000 dilution) at room temperature for 1 h, and washed in TBST three times (10 min each). The blots were visualized with enhanced chemiluminescence and X-ray films. Bands were analyzed with optical density, and the values were normalized to β-actin bands.

Statistical analysis

All data are expressed as mean ± SEM. They were statistically assessed with one-way analysis of variance and Student-Newman-Keuls tests. Differences were considered significant if $P < 0.05$.

Results

Positions of hepatic mtDNA deletions in AC

As shown in **Figure 1**, LA PCR did not reveal any mtDNA deletions in long-term alcoholics without cirrhosis or normal controls. In contrast, five deleted mtDNA fragments were detected in AC patients. Their approximate lengths were as follows: A: 1.8 kb; B: 1.0 kb; C: 0.55 kb; D: 0.5 kb; E: 0.4kb. Fragment A was present in all patients. Fragments B, D, and E were only found in single specimen, with an occurrence rate of 4.3% (1/23). Fragment C was present in three specimens, with an occurrence rate of 13.0% (3/23).

We then sequenced fragment A, which had the highest occurrence rate. By comparing the sequencing result with the normal mtDNA sequence, we determined that fragment A was consistent with the mtDNA base sequences 1-748 and 15,487-16,569, with 98.7% consistency. The PCR amplification target bands were confirmed by analyzing the fragment a sequencing result. Moreover, an approximately 14.7-kb specific deletion corresponding to bases 749-15,486 was found in hepatic mtDNA isolated from AC patients.

Quantitative detection of hepatic mtDNA in AC

There was no significant difference in mtDNA

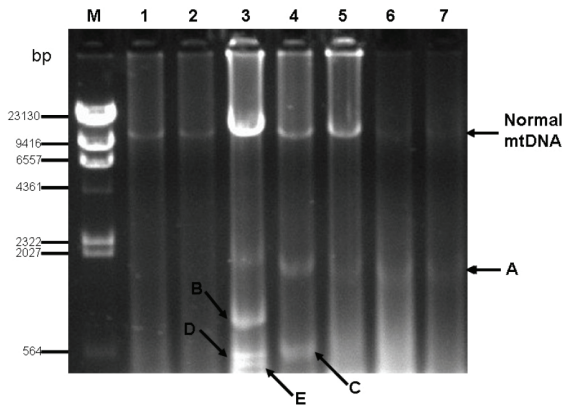
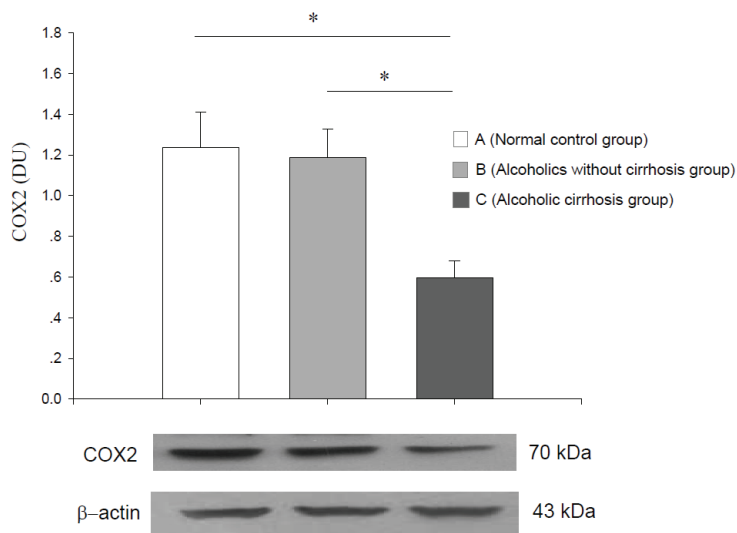


Figure 1. Hepatic mtDNA deletions in AC. Primers were designed to amplify full-length mtDNA (16.5 kb) using long and accurate PCR. M: marker (λ -Hind III digest). Each row contains the following samples: 1, normal control; 2, alcoholics without cirrhosis; 3-7, alcoholic cirrhosis. A 16.5-kb normal mtDNA fragment was observed in all the samples. A-E indicate deleted mtDNA fragments.

Table 2. Quantitative detection of hepatic mtDNA in patients with alcoholic cirrhosis (n=20).

Group	mtDNA (HV1)
Normal controls	4.17±0.47
Alcoholics without cirrhosis	4.09±0.51
Alcoholic cirrhosis	2.64±0.29*#

* P<0.05 vs. Normal controls; # P<0.05 vs. Alcoholics without cirrhosis.



copy number between the normal control group and the alcoholics without cirrhosis group ($P > 0.05$). However, the mtDNA copy number was significantly lower in the AC group compared to both the normal control group and the alcoholics without cirrhosis group ($P < 0.05$) (Table 2).

Western blotting analysis of the hepatic mtDNA-encoded product COX2

Western blotting analysis did not demonstrate a significant difference in COX2 expression between the normal control group and the alcoholics without cirrhosis group ($P > 0.05$). In contrast, its expression was significantly decreased in the AC group ($P < 0.05$). This result indicated that mtDNA damage in AC patients impacted the normal function of mtDNA and resulted in decreased COX2 production (Figure 2).

Discussion

Mitochondria are the cellular centers of energy metabolism. mtDNA is a double-stranded circular DNA molecule that controls organelle function. Its encoded products are related to mitochondrial oxidative phosphorylation and respiratory function [20]. The location of mtDNA makes it particularly vulnerable to oxidative stress, and because it lacks histone protection and a DNA repair system, it is easily damaged [21]. It is believed that increased oxygen free radicals within cells are the major cause of mtDNA damage [22].

The liver is responsible for metabolizing 90% of ethanol. In hepatocytes, ethanol is first oxidized to acetaldehyde via alcohol dehydrogenase, then acetic acid via acetaldehyde dehydrogenase, and it is ultimately metabolized to carbon dioxide and water. After heavy drink-

Figure 2. Changes in the hepatic mtDNA-encoded product COX2 in alcoholic cirrhosis (n=20). A: normal control group; B: alcoholics without cirrhosis group; C: alcoholic cirrhosis group. COX2 expression in the normal control and alcoholics without cirrhosis groups were not significantly different ($P>0.05$), but its expression was significantly lower in the alcoholic cirrhosis group compared with both the normal control and alcoholics without cirrhosis groups ($P<0.05$).

ing, a high plasma ethanol concentration can also activate the microsomal ethanol oxidizing system (MEDS), thus catalyzing acetaldehyde production. However, ethanol-induced MEDS activity not only fails to oxidize ethanol to produce ATP, it also increases oxygen and NADPH consumption. This results in cell hypoxia and increased oxygen free radicals [23]. In addition, the acetaldehyde produced from ethanol metabolism can damage the antioxidant defense system, and it can also directly bind to DNA and inhibit its repair [24]. Chronic heavy drinking can also cause deficiency of mitochondrial glutathione, which plays an important role in mtDNA repair [25]. Collectively, these results may lead to hepatic mtDNA damage. We found an approximately 14.7-kb specific deletion in corresponding to bases 749-15,486 of hepatic mtDNA in 23 AC patients.

Somatic cells contain between 100 and 500 mitochondria, and 1-15 copies of mtDNA are present in each mitochondrion. The mtDNA content (i.e., copy number) varies in different types of cells and tissues. Moreover, cell differentiation, hormone therapy, exercise, and other processes can change the mtDNA copy number [26, 27]. A sufficient copy number of normal mtDNA is required to maintain normal mitochondrial respiratory function [28]. Damaged mtDNA needs to exceed a threshold to cause tissue or organ dysfunction [29-31]. Therefore, we quantified hepatic mtDNA damage in AC patients using real-time quantitative PCR and found that hepatic mtDNA copy number was significantly lower in AC patients than in normal subjects and alcoholics without cirrhosis.

A bioinformatics analysis of the mtDNA deletion at 749-15,486 revealed that this sequence deletion affected virtually all mtDNA-encoded products except the D-loop region. In fact, such a large deletion effectively prevents normal mtDNA encoding functions. The mtDNA D-loop HV1 segment representing the mtDNA copy number is located outside the deleted sequences assessed in this study. Therefore, the mtDNA copy number amplified from this segment included deleted and normal mtDNA, suggesting that the copy number of hepatic mtDNA with normal encoding function in the AC group was less than the actual measured values. This may significantly affect its encoded products and mitochondrial function. Our experiments further demonstrated that the COX2 expression

was significantly decreased in the AC group ($P < 0.05$). COX2 is the terminal enzyme in the electron transfer chain of the eukaryotic inner mitochondrial membrane and the cell membrane of aerobic bacteria. It is responsible for transferring electrons from cytochrome c to oxygen molecules and is encoded by the mtDNA sequence 7,586-8,269, which is located within the deleted sequence detected in this study. Therefore, changes in the amount of COX2, an mtDNA-encoded product, represent to a certain extent the impact of mtDNA deletion on its encoded products.

Based on the above analysis, we conclude that chronic heavy drinking induces MEDS, which leads to an increase in oxygen free radicals in hepatocytes and subsequent hepatic mtDNA damage. This is manifested by a large deletion located at positions 749-15,486 of mtDNA, a reduction in copy number, and a decrease in the expression of mtDNA-encoded products. mtDNA damage can influence cellular energetics, which leads to cell damage and even apoptosis, ultimately resulting in liver cirrhosis. The findings of this study reveal the utility of a new approach to study AC pathogenesis.

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Address correspondence to: Dr. Juntao Yang, Department of Hepatobiliary Surgery, Daping Hospital, The Third Military Medical University, Chongqing 400042, China E-mail: juntaoyang6@gmail.com

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