Changes in Mitochondrial Toxicity in Peripheral Blood Mononuclear Cells During Four-Year Administration of Entecavir Monotherapy in Chinese Patients with Chronic Hepatitis B

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: This study aimed to assess whether long-term entecavir monotherapy induces mitochondrial toxicity in patients with chronic hepatitis B (CHB).

Material/Methods: This was a prospective study in 34 antiviral treatment-naïve patients with CHB who received entecavir monotherapy and were followed up for 4 years. Blood samples were collected after 0, 2, 3, and 4 years of entecavir (ETC) monotherapy (ETC0, ETC2, ETC3, and ETC4, respectively). Mitochondrial DNA (mtDNA) contents were determined using real-time quantitative polymerase chain reaction (qRT-PCR) and mtDNA^{4977} depletions were detected using nested PCR. Levels of hepatitis B virus (HBV) DNA, alanine aminotransferase, alanine aminotransferase, hepatitis B e antigen (HBeAg), creatine kinase, urea nitrogen, and serum creatinine were recorded.

Results: mtDNA contents at ETC0 (9.6±6.3) and ETC4 (10.3±6.2) were markedly higher than at ETC2 (0.8±0.5, P<0.01) and ETC3 (1.3±0.9, P<0.01), but there were no differences between ETC2 and ETC3 or between ETC0 and ETC4. MtDNA^{4977} depletion appeared in 79.4% cases at ETC2 and in 70.6% at ETC3, which were much higher than at ETC0 (32.4%, P<0.01) and ETC4 (8.8%, P<0.01), but there were no differences in mtDNA^{4977} depletion ratio between ETC2 and ETC3, or between ETC0 and ETC4. mtDNA content was negatively correlated to mtDNA^{4977} depletion (partial regression coefficient of –4.555, P<0.001, R^2=0.315). mtDNA content was positively correlated with age (partial regression coefficient of 0.131, P=0.045).

Conclusions: Results suggest that during 4-year entecavir monotherapy for CHB, the mtDNA contents initially decreased and then increased, while the mtDNA^{4977} depletion rates first increased and then decreased.

MeSH Keywords: Hepatitis, Chronic • Mitochondria, Liver • Peripheral Blood Stem Cell Transplantation

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Background

Entecavir (ETC) is a nucleoside reverse transcriptase inhibitor (NRTIs) that revolutionized the treatment of chronic hepatitis B (CHB) by inhibiting hepatitis B virus (HBV) polymerase [1]. The human polymerase gamma is essential for mitochondrial (mtDNA) synthesis, but is also inhibited by entecavir [2]. As a result, mitochondrial function may be disrupted, leading to energy loss, leakage of electrons from the electron transport system, increased reactive oxygen species production, and oxidative damage [3] resulting in peripheral neuropathy, skeletal and cardiac myopathy, nephrotoxicity, pancreatitis, hepatic failure, and lactic acidosis, all of which have been reported in patients with acquired immunodeficiency syndrome (AIDS) under long-term NRTIs treatment [4–8].

An *in vitro* experiment indicated that entecavir concentrations as high as 100 times the maximal clinical exposure did not induce mitochondrial toxicity in HepG2 hepatoma cells after 15 days of culture [9], but these results were obtained after short-term exposure, and it cannot be concluded that entecavir has no mitochondrial toxicity since the human body is more complex than cultured cells and drugs can accumulate in some tissues such as the liver and kidney. Therefore, whether the long-term use of entecavir in humans may cause mtDNA injury needs to be studied.

mtDNA injury may manifest by deletions, point mutations, or quantitative abnormalities of mtDNA copy number per cell [10]. Therefore, we assessed mtDNA contents and the presence of the mtDNA^{4977} mutation to detect mtDNA injury during a 4-year entecavir treatment.

Material and Methods

Patients

Thirty-four treatment-naïve patients with CHB from the Beijing Youan Hospital were enrolled between July 2007 and December 2008. CHB was diagnosed according to the Guidelines on Prevention and Treatment for Chronic Hepatitis B in China (2000). Inclusion criteria were: 1) 18–65 years old; 2) HBsAg-positive with an HBV DNA >10^6 copies/mL within 4 weeks prior to enrolment; and 3) serum alanine aminotransferase (ALT) levels 2-10 times the upper limit of normal within 4 weeks before enrolment. Exclusion criteria were: 1) suspicious hepatic tumors or alpha-Fetoprotein (AFP) >100 ng/mL; 2) cirrhosis; 3) co-infection with hepatitis A, C, D or E virus; 4) co-infection with HIV; 5) other causes of liver disease; 6) serious medical or psychiatric illness; 7) abnormal serum creatinine, thrombocyte count, hemoglobin or serum total bilirubin; or 8) pregnancy. Patients were prospectively followed up once a year for 4 years.

The study protocol was approved by the Ethics Committee of Beijing Youan Hospital (LL-2007-002-S) and written informed consent was obtained from all patients.

Clinical outcomes

Serum ALT, aspartate aminotransferase (AST), total bilirubin (TB), creatine kinase (CK), serum creatinine (SCr), and blood urea nitrogen (BUN) were measured using an Olympus Au5400 automatic biochemistry analyzer (Olympus, Tokyo, Japan). HBV DNA was detected using a COBAS AmpliPrep/COBAS TaqMan 48 analyzer (Roche Diagnostics, Basel, Switzerland).

Isolation of peripheral blood mononuclear cells and DNA

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Hypaque density gradient separation [11] after 0, 2, 3, and 4 years of entecavir (ETC) monotherapy (ETC0, ETC2, ETC3 and ETC4, respectively). Genomic DNA was harvested using a spin-column method (QIAamp DNA Mini Kit; Qiagen, Venlo, Netherlands). About 5×10^6 PBMC in a 1-ml volume were lysed with 20 µl of proteinase K and 200 µl of AL buffer. The solution was incubated at 56°C for 10 min, followed by the addition of 200 µl of 100% ethanol to precipitate DNA. The mixture was then transferred to the QIAamp spin column. After 2 washes with 500 µl of wash buffer, genomic DNA was eluted by the addition of 200 µl of elution buffer. Final DNA concentrations were quantified by SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA, USA) and stored at –80°C until use.

Quantitative real-time PCR for mtDNA

mtDNA copy number was estimated by determining the relative amounts of nuclear DNA (nDNA) and mtDNA by quantitative real-time PCR (ABI Step One Plus Real-time PCR System, Applied Biosystems, Foster City, CA, USA). The highly conserved mitochondrial gene cytochrome C oxidase II (COXII) was measured using: forward 5'-TAT CTT TTG GCG GTA TGC ACT TTT AAC AGT-3', reverse 5'-TGA TGA GAT TAG TAG TAT GGG AGT GG-3' and the probe 5'-FAM-CAC CCC CCA ACT AAC ACA TTA TTT TCC CC-TAMRA-3'. The nDNA gene glyceraldehyde phosphate dehydrogenase (GAPDH) was measured using: forward 5'-GCC ATC CTG CGT CTG GAC CTG GCT-3', reverse 5'-TGA TGA CCT TGG CCA GGG GTG CTA AG-3' and the probe 5'-FAM-GCC GGG ACC TGA CTG ACT ACC TCA TGA-TAMRA-3' [12] The assay was performed in a total reaction volume of 20 µl containing 10 µl of TaqMan Universal 2×PCR Master Mix, 6.5µl of ddH$_2$O, 1 µl of each primer (10 µM), 0.5µl of probe (5 µM) and 1 µl of DNA template. The reaction was performed at 95°C for 10 min, followed by 45 cycles of 10 s at 95°C, and 45 s at 65°C. Each sample was assayed in triplicate and the fluorescence spectra were continuously monitored. To correct the variance

Table 1. Baseline characteristics of patients.

Variables	Pre-treatment n=34	2 years post-treatment n=34	3 years post-treatment n=34	4 years post-treatment n=34	F	χ^2	P
Male, n (%)	26 (76.5%)	–	–	–			
Age	35.59±10.69	–	–	–			
BMI (kg/m²)	22.91±3.77	23.8±3.8	23.8±3.8	23.9±4.0	0.100		0.960
HBV DNA (log IU/mL)	6.8±1.5	0.0±1.7	0.0±1.7	0.0±0.0		81.241	<0.001
HBeAg positive, n, (%)	31 (91.2)	24 (70.6)	20 (58.8)	21 (61.8)		10.483	0.015
ALT (U/L)	86.3±7.5	24.1±1.5	26.0±1.5	26.7±2.0	41.116		<0.001
AST (U/L)	57.4±4.5	25.1±7.9	24.6±7.0	25.7±1.1	27.870		<0.001
TB (μmol/L), median (range)	15.05 (6.50,29.20)	13.60 (6.90,36.30)	13.15 (8.30,32.60)	14.80 (7.40,31.80)		1.134	0.769
PTA (%)	98.51±12.61	95.47±7.94	95.39±5.64	99.13±7.55	1.705		0.169
CK (1/ULN), median (range)	0.63 (0.28,4.06)	0.64 (0.23,5.11)	0.58 (0.18,2.64)	0.63 (0.22,1.69)		1.680	0.641
BUN (1/ULN)	0.7±0.2	0.7±0.1	0.8±0.2	0.8±0.2	0.436		0.728
SCr (1/ULN)	0.68±0.13	0.7±0.1	0.7±0.1	0.7±0.1	0.865		0.462

BMI – body mass index; ALT – alanine aminotransferase; AST – alanine aminotransferase; TB – total bilirubin; PTA – prothrombin activity; CK – creatine kinase; BUN – urea nitrogen; SCr – serum creatinine; ULN – upper limit of normal.

caused by the different DNA content of each sample, the mtDNA/nDNA ratio and RQ value ($2^{-\Delta\Delta CT}$) were used.

Detection of mitochondrial mtDNA[4977] mutation

The common deletion mtDNA[4977] was genotyped at each time point in samples from the 34 patients using a nested PCR protocol [13]. The amplification target was the segment in the 8224-13501 bp region. Primers used for the first-round amplification were: 5'-AAT TCC CCT AAA AAT CTT TGA AAT-3' and 5'-GCG ATG AGA GTA ATA GAT AGG GCT CAG GCG-3' (accession no. NC_012920.1). One µl of the first-round products was used for the second-round amplification using 5'-AAT TCC CCT AAA AAT CTT TGA AAT-3' and 5'-AAC CTG TGA GGA AAG GTA TTC CTG C-3' (accession no. NC_012920.1, product of 301bp) for mtDNA[4977], and 5'-AAT TCC CCT AAA AAT CTT TGA AAT-3' and 5'-AGG CGC TAT CAC CAC TCT GTT TCG-3' (accession no. NC_012920.1, product of 326bp) for wild-type DNA. The nested-PCR products were detected by agarose gel electrophoresis.

Statistical analysis

Repeated measure analysis of variance (ANOVA) was used to analyze normally distributed data, and Kruskal-Wallis *H* tests were used for non-normally distributed data. The chi-square test or Fisher's exact test was used for categorical variables, as appropriate. Multiple linear regression analysis was used to assess the correlations with mtDNA content. Analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). P values <0.05 were considered statistically significant.

Results

Patient characteristics

The 34 patients were followed up once a year for 4 years. No patient was lost to follow-up. Mean patient age was 38.5±10.7 years (range: 18 to 62 years). There were 26 males and 8 females. During the 4-year follow-up, nausea was reported in 1 patient, fatigue in 1, and headaches in 1. CK elevations were reported in 7 patients. BUN elevations were reported in 5 patients. All CK and BUN elevations improved spontaneously. All adverse events were mild, and no patient discontinued treatment due to treatment-related adverse events.

As shown in Table 1, there was no significant difference among successive measurements of BMI, TB, PTA, CK, BUN, and SCr (all P>0.05), while ALT, AST, and HBV DNA amounts improved during the 4 years (all P<0.05).

mtDNA content

The mtDNA contents decreased first and then increased during the 4-year follow-up (*F*=74.910, *P*<0.001). mtDNA levels were 0.7±0.5 at ETC2 and 1.3±1.4 at ETC3, which were much
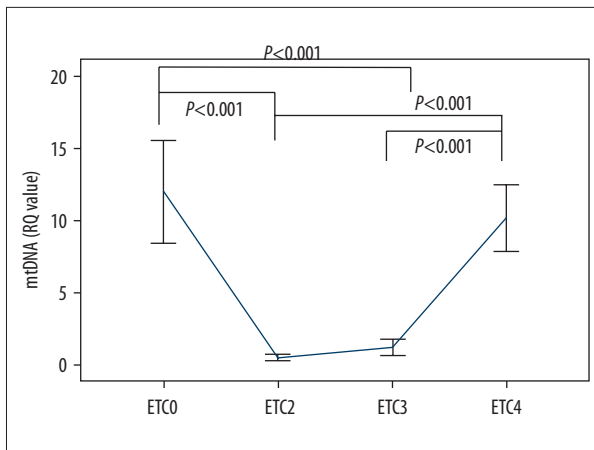
Figure 1. mtDNA contents over time.

lower than at ETC0 (12.1±10.2, P<0.05) and ETC4 (10.1±6.5, P<0.05). No differences were observed between ETC0 and ETC4 (*P*=0.111) or between ETC2 and ETC3 (*P*=0.550) (Figure 1).

MtDNA[4977] depletion

MtDNA[4977] depletion ratio (mtDNA[4977]/wild-type mtDNA) first increased and then decreased during the 4-year follow-up period (χ^2=44.646, P<0.001). MtDNA[4977] depletion was 79.4% at ETC2 and 70.6% at ETC3, which was much higher than at ETC0 (32.4%, χ^2=15.270, 9.950, P<0.001 and P=0.002, respectively) and ETC4 (8.8%, χ^2=34.358, 27.089, both P<0.001). No

significant differences were observed between ETC2 and ETC3 (P=0.401) or between ETC0 and ETC4 (P=0.16) (Figure 2).

Correlation

mtDNA contents were negatively correlated with mtDNA[4977] depletion rates (*R2*=0.315, partial regression coefficient of −4.555, P<0.001). mtDNA contents were positively correlated with age (partial regression coefficient was 0.131, P=0.045).

Discussion

Entecavir toxicity is a controversial topic, although most of the conclusions are that entecavir is relatively safe, is well-tolerated by patients with CHB, and that its adverse effects are usually mild or moderate. Indeed, low rates of serious adverse events were reported even after 168 weeks of entecavir administration [14,15]. However, some serious adverse events may occur [16,17] and can be attributed to mitochondrial toxicity [18,19]. The mitochondrion is an energy-generating organelle that produces reactive oxygen species (ROS) as a byproduct. ROS are known to cause tissue injury and to lead to symptoms such as peripheral neuropathy, skeletal and cardiac myopathy, pancreatitis, hepatic failure, and lactic acidosis [20]. Entecavir is efficiently phosphorylated by human cellular kinase to its active triphosphate form [21,22]. Human DNA polymerases such as DNA polymerase γ (which
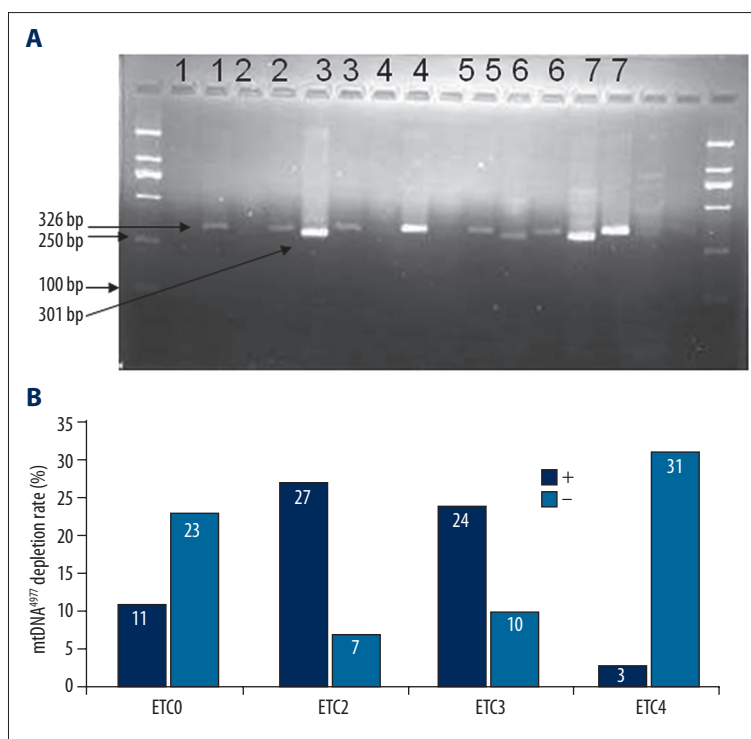

Figure 2. mtDNA[4977] depletion ratios. (**A**) 3% agarose gel electrophoresis of nested PCR products. Lanes 1 and 18 are DL2000 molecular weight ladders. Lanes 1 to 7 are from different patients. The left lanes are mtDNA[4977] (301 bp bands), and the right lanes are wild-type mtDNA (326 bp bands). Therefore, mtDNA[4977] depletion can be seen in lanes 3, 6, and 7, and no mtDNA[4977] depletion can be observed in lanes 1, 2, 4 and 5. (**B**) mtDNA[4977] depletion rates during the 4-year follow-up. "+": mtDNA[4977] depletion; "-": no mtDNA[4977] depletion.

is responsible for the replication and repair of mitochondrial genome) are also inhibited.

In the present study, a repeated-measures approach was used to overcome interindividual variations caused by factors such as the course of disease, age, sex, smoking, alcohol, and eating habit on both mtDNA contents and mtDNA[4977] depletion rates. Previous studies showed that mtDNA content (copies/cell) in PBMC is a reliable and easy method to assess mitochondrial toxicity [23–25]. Results showed that mtDNA copy number first decreased and then increased during the 4-year follow-up. Although this decrease had no relation with changes in HBV DNA, HBx has a potential destructive function on mitochondria. Indeed, Tan et al. [26,27] observed that HBV replication decreased the mitochondrial calcein-AM/CoCl(2) signals by 10% and 14% in HepG2 cells, and by 15% and 10% in Huh7 cells, respectively, suggesting that HBx can induce mitochondrial permeability transition (MPT) and cause calcium effusion into the plasma. Lin et al. [28] showed that HBx can down-regulate COXII expression and inhibit mitochondrial cytochrome C oxidase activity. Koike et al. [29] also showed that protein X stimulates ROS generation in mitochondria.

In the present study, HBV DNA amount decreased from ETC0 to ETC2, but the mitochondrial impairment persisted, resulting in decreased mtDNA content. Montaner et al. [30] found that in symptomatic hyperlactatemia patients treated with NRTIs, the mtDNA/nDNA ratios were on average 69% lower than in HIV-uninfected controls and 45% lower than in HIV-infected asymptomatic/antiretroviral-naïve controls. López et al. [31] reported that HIV-infected infertile women on highly active antiretroviral therapy (HAART) showed significant oocyte mtDNA depletion compared with uninfected controls. Chêne et al. [32] revealed that mtDNA levels significantly decreased with the NRTIs treatment duration. Nevertheless, no clinical adverse events happened in the present study because mtDNA has a threshold effect (i.e., clinical manifestations occur only after a definite threshold) [33,34].

At ETC2 to ETC4, mtDNA contents kept increasing, but did not show significant differences until ETC4. Although entecavir is a polymerase γ inhibitor, its action is too weak to completely stop mtDNA replication, which may explain the slow increase in mtDNA content. However, entecavir may accumulate in some tissues, and it is still uncertain whether entecavir toxicity to mtDNA persists over time. Indeed, most patients will be treated for the rest of their lives, except those experiencing seroconversion. Therefore, longer observation is necessary to correctly assess the long-term effects of entecavir. It is plausible that new agents targeting mitochondrial function could improve mitochondrial biogenesis in humans with degenerative diseases [35], including patients with chronic HBV receiving long-term entecavir treatment.

The mitochondrial respiratory chain complex is an important source of ROS. MtDNA[4977] is a common depletion of mtDNA by ROS [36]. In the present study, mtDNA[4977] depletion ratio first decreased and then increased, and was negatively correlated with mtDNA contents and HBV DNA, which suggest that HBV plays a crucial role in the oxidative damage of mtDNA.

Conclusions

This is the first long-term follow-up study exploring the mitochondrial toxicity of entecavir in patients with HBV. However, it suffers from some limitations. The sample size was small, and there was no control group, which was partly overcome by the use of a repeated measures design. In addition, more than half of the patients did not show seroconversion after 4 years and required longer treatment. Therefore, studies with longer follow-up and larger sample size are necessary.

Results from use of 2 reliable method for assessing mitochondrial damage – mtDNA contents and mtDNA[4977] depletion – suggest that no mitochondrial toxicity was apparent after 4 years of entecavir treatment after the improvement in mtDNA content and oxidative damage.

Conflict of interest

The authors declare that they have no conflict of interest.

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