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Mitochondrial DNA mutations in blood samples from HIV-1-infected children undergoing long-term antiretroviral therapy

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ABSTRACT

We have analyzed mutations in whole mitochondrial (mt) genomes of blood samples from HIV-1-infected children treated with long-term antiretroviral therapy (ART), who had an excellent virological response. HIV-1-infected children who have undergone ART for 4 y with an excellent virological response (group A; 15 children) and ten healthy children (controls) without HIV-1 infection were enrolled retrospectively. Peripheral blood mononuclear cells (PBMCs) were obtained and mt DNA mutations were studied. The total number of mtDNA mutations in group A was 3 H more than in the controls (59 vs. 19, $P < 0.001$) and the same trend was seen in all mtDNA regions. Among these mtDNA mutations, 140 and 28 mutations were detected in group A and the controls, respectively. The *D-loop*, *CYTB* and *12s rRNA* were the three most common mutation regions in both groups, with significant differences between the groups observed at nucleotide positions C309CC, T489C CA514deletion, T16249C and G16474GG (D-loop); T14783C, G15043A, G15301A, and A15662G (*CYTB*); and G709A (*12s rRNA*). G15043A and A15662G had been associated with mitochondrial diseases. Our findings suggest that mtDNA mutations occur frequently in long-term ART-treated, HIV-1-infected children who have an excellent virological response, although they did not have obvious current symptoms. The *CYTB* region may play an important role in mtDNA mutation during ART, which might contribute to the development of subsequent mitochondrial diseases.

1. Introduction

Since highly active antiretroviral therapy (HAART) was introduced to the clinic, morbidity and mortality of children infected with human immunodeficiency virus (HIV) have decreased significantly [1,2]. However, long-term treatment has been associated with potentially severe adverse effects, including lactic acidosis, hepatic steatosis, peripheral neuropathy, myopathy, cardiomyopathy, pancreatitis, lipodystrophy, etc. Nucleoside reverse transcriptase inhibitors (NRTIs), as the backbone of HAART, are responsible for significant adverse effects resulting in mitochondrial (mt) toxicity [1,2].

Mammalian mitochondria contain circular double-stranded genomes (16,569 bp) encoding 22 tRNAs, 2 rRNAs, and 13 polypeptides. DNA polymerase γ is the key enzyme for mitochondrial DNA replication and base excision repair. Mitochondrial DNA is critical for normal mt function, and mutations in this genome can cause a wide range of human diseases [3]. The toxicities noted above are consequences of mt dysfunction resulting from the depletion or deletion of mt DNA caused by NRTI-induced inhibition of DNA polymerase γ [4,5].

Research has focused on mtDNA copy number and has attempted to find clinical correlations between mtDNA levels and mt toxicities, even though the assay for mtDNA levels remains controversial [6–9]. Due to the lack of protective histones and effective repair ability, mtDNA is highly susceptible to oxidative damage.

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Table 1
Clinical characteristics of group A and controls.

	Group A (n = 15)	Controls (n = 10)	P value
Age, median years (Range)[a]	9 (6–14)	9 (5–13)	0.950
Male, no. (%)[b]	5 (33.33)	7 (70.00)	0.456
Treatment Regimen, no. (%)[b]			NA
AZT + 3TC + NVP	6 (40)	0 (0)	
d4T + 3TC + NVP	9 (60)	0 (0)	
CD4T cell counts, median cells/μl (Range)[a]	787 (438–1635)	947 (784–1395)	0.065

Lewis et al. suggested that NRTIs may cause an increased risk of mtDNA mutation [4,5]. Martin et al. investigated mtDNA changes in HIV-infected adults and found that NRTIs provided conditions favorable for mtDNA mutations [10]. However, little information on the complete set of mtDNA mutations in HIV-1-infected children is available. Our group previously reported mt toxicity in peripheral blood mononuclear cells (PBMCs) of children from the Chinese national pediatric HAART cohort. That study indicated that the NRTI treatment group exhibited significant mtDNA loss, and HIV-1-infected children in this cohort on long-term HAART could suffer severe mt injury [11]. Here, we sequenced and analyzed the complete mt genome from PBMCs to investigate whether long-term ART-treated, HIV-infected children achieving virologic suppression have different mtDNA mutation characteristics from age-matched healthy children.

2. Materials and methods

2.1. Study population

Individuals were retrospectively included from our previous study [11]. Specifically, a subset of the Pediatric AIDS Clinical Trial Group (PACTG) from China of HIV-1-infected children was chosen as the ART-treated HIV-1-infected group (group A). These children: (a) had started a stavudine- (d4T) or zidovudine- (AZT) containing combination ART regimen for 4 years and never changed their regimen; (b) had an excellent virological response to ART (namely virologic suppression, defined as a viral load (VL) <50 copies/mL at each follow-up point after initiation of ART); and (c) did not have obvious symptoms. Healthy children without HIV-1 infection were included as controls. The study was approved by the ethical review committee of Beijing You An Hospital, the parents of HIV-infected children signed written consent forms to participate in the research study prior to blood and clinical data collection.

2.2. DNA isolation, mtDNA DNA amplification, cloning, and sequencing

PBMCs were isolated from fresh whole blood and stored at −135 °Cin cellular freezing solution until analysis. Total DNA was extracted from PBMCs using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The patient's entire mt genome DNA was amplified by a nested polymerase chain reaction (detailed in the Supplemental Materials). The amplified fragments were gel-purified by using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and cloned into the plasmid vector pMD-18 T at the EcoRV site. Recombinant plasmids were sequenced with M13 universal primers on an ABI 3730XL sequencer (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Sequence analysis

Multiple sequence alignments from each subject were performed and manually adjusted using NCBI BLAST and BioEdit software (version 7.1.3). MtDNA mutations were then checked against the revised Cambridge Reference Sequence (rCRS, GenBank NC_012920) using the MITOMASTER web tool in the Mitomap database (http://www.mitomap.org/). Shannon Entropy as a measure of variation in mtDNA sequence alignments was applied to determine if there was greater variability in group A relative to controls (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_two.html).

2.4. Statistical analysis

Comparisons of the differences between group A and controls were calculated using Mann-Whitney or Chi-square tests. Statistical significance was set at a P value < 0.05 and for two-sided tests. All statistical analyses were performed using SPSS software package (version 17.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Patient and control group characteristics

Fifteen HIV-infected children (median age, 9 y; range, 6–14 y) and ten healthy children (median age, 9 y; range, 5–13 years) as controls were included in the study. The entire group A started a d4T- or AZT-containing combination ART regimen. The clinical characteristics of the patients are summarized in Table 1. The age, gender, and CD4+ T cell count did not differ significantly between group A and controls. A total of 25 whole mtDNA sequences from group A and the control group were generated and analyzed to determine the genetic characteristics of mtDNA.

3.2. The whole mitochondrial genomic-mtDNA mutation ratio in patient group was significantly higher than in the control group

To analyze the whole mt genomic information from group A and normal controls, two 8.5-kb amplicons of mtDNA PCR products, which covered the whole mt genome from the PBMCs, were amplified. More mtDNA mutations occurred in the PBMCs of the ART-treated children. There were significant differences in the numbers of mtDNA mutations between the two groups when compared with the reference mtDNA. The median numbers of total nucleotide variation sites from the whole mt genome were 59 (45–72) in group A vs. 19 (15–20) in controls (P < 0.001), respectively. Next, we individually analyzed the distinct regions of mtDNA that showed trends toward higher mtDNA mutation numbers in group A, including the mtDNA mutation ratio from the mtDNA D-loop, tRNA/ribosomal RNA, and mtDNA-encoded polypeptides (Fig. 1a). The D-loop, CYTB and 12 s rRNA were the top three mutation regions in both groups. Among the above mtDNA mutations, 140 and 28 mutations were detected in group A and control, respectively (Fig. 1b).

3.3. High level of D-loop mutations in PBMCs of patient group

The ratio of mtDNA D-loop mutations/polymorphisms was 1–15 sites from group A vs. 0–10 sites in control children (Table 2). In ART-treated children, 5/15 cases had a 309C insert, 4/15 had