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Antiretroviral Therapy-Induced Toxicity is Associated with Increased mRNA Expression of Telomerase

Min Li, PhD¹, Yram Foli, MS¹, Nana Y Amakye², Taline Klein, BS³, Shanmugapriya Selvaraj, MS¹, Lingeng Lu, MD, PhD⁴, and Elijah Paintsil, MD^{1,4,5,#}

¹Department of Pediatrics, Yale School of Medicine, New Haven, Connecticut, USA

²Department of Cell Physiology and Pharmacology, School of Biological Sciences, University of Leicester, Leicester, UK

³Department of Internal Medicine, University of São Paulo Medical School at Ribeirão Preto, São Paulo, Brazil

⁴Department of Epidemiology & Public Health, Yale School of Medicine, New Haven, Connecticut, USA

⁵Department of Pharmacology, New Haven, Connecticut, USA

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Antiretroviral therapy; mitochondrial toxicity; telomere length; telomerase; immune activation

Introduction

The widespread use of combination antiretroviral therapy (cART) has dramatically decreased AIDS-associated morbidity and mortality in HIV-infected individuals ¹. However, HIV treatment-experienced patients have a high prevalence of aging-associated diseases such as malignancies and cardiovascular diseases ². Given the canonical role of the telomerase system in cellular aging, it has been implicated in the pathogenesis of these non-AIDS defining illnesses in HIV-infected individuals ³. Telomerase is a ribonucleoprotein comprised of two subunits—an RNA template (TERC) and a human reverse transcriptase (hTERT). hTERT synthesizes tandem repeats of a “TTAGGG” sequence (telomere) at the ends of eukaryotic chromosomes ⁴. In somatic cells, progressive shortening of telomeres during cell division results in replicative senescence when a critical telomere length is reached ⁴.

Although many pathways undoubtedly operate in concert to induce aging, the mitochondrion is believed to be the master orchestrator ⁵. Antiretroviral therapy (ART)

[#]Correspondence to: Elijah Paintsil, MD., Departments of Pediatrics and Pharmacology, Yale University School of Medicine, 464 Congress Avenue, New Haven, Connecticut 06520, USA. Phone: 203-785-6101 Fax: 203-785-6961; elijah.paintsil@yale.edu.

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causes mitochondrial dysfunction, oxidative stress, and mitochondrial DNA (mtDNA) defects; aging is associated with mitochondrial dysfunction and oxidative stress⁶. Mitochondrial dysfunction is considered a pre-requisite for the onset of aging process⁷. Mitochondrial DNA (mtDNA) polymerase- γ is inhibited by nucleoside reverse transcriptase inhibitors (NRTIs) leading to mtDNA depletion and subsequent mitochondrial dysfunction⁸. *In vitro* studies have shown that NRTIs can also inhibit hTERT activity leading to progressive shortening of telomere length, although *in vivo* studies have not corroborated this finding^{9,10}. It has been hypothesized that inhibition of hTERT by ART could contribute to the premature aging in HIV-infected individuals⁶. We assessed whether there is an association between ART-induced mitochondrial toxicity and the telomere-telomerase system among HIV treatment-experienced patients.

Materials and Methods

Study population and design

The study protocol was approved by the Institutional Review Board of Yale School of Medicine. All participants gave their written informed consent before participation in the study. The details of the study design have been previously published¹¹. In brief, this was a case-control study. The cases comprised HIV-infected individuals on a stable NRTI-based ART regimen for at least 12 months at the time of study enrollment with one or more clinical or laboratory toxicities that have been associated with ART-induced mitochondria toxicity¹². For each case, two controls were enrolled: (1) an HIV-infected individual on a stable NRTI-based ART for at least 12 months at the time of study enrollment without any of the observed clinical or laboratory toxicities (positive controls) and (2) an HIV-uninfected volunteer (negative controls). The controls were matched to the cases by age, sex, and race/ethnicity.

Patient procedures

At enrollment, participants answered a brief survey comprised of questions regarding past medical history and demographic characteristics. Medical records of HIV-infected participants were reviewed. Approximately 20 ml of venous blood was collected from each participant. Plasma was separated from whole blood by centrifuging at 1000×g for 15 min. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient (Ficoll-Hypaque; ICN) according to manufacturer's instructions. Aliquots of plasma and PBMCs were immediately stored at -80°C.

Measurement of telomere length

Genomic DNA was extracted from PBMCs using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. We used a previously published quantitative PCR (qPCR) method that compares the copy number of telomeres to a single copy gene (36B4)⁹. The primers for telomere and 36B4 are listed in supplementary Table 1. The telomere length (TL) was determined by the relative quantity of telomere versus 36B4 (T/S ratio) using the formula 2^{-Ct} , where $Ct = Ct_{\text{telomere}} - Ct_{36B4}$ as previously described¹³.

Quantitative RT-PCR for expression of telomerase (hTERT) and human leukocyte antigen-DRA (HLA-DRA)

Total RNA was extracted from PBMCs using TRIzol[®] Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The quantitative real-time PCR protocols used for hTERT, HLA-DRA and GAPDH have been described previously^{14,15}. The primers for hTERT, HLA-DRA, and GAPDH (GAPDH1) are listed in supplementary Table 1. The threshold cycle (Ct) value of the mRNA expression of gene of interest (e.g., hTERT or HLA-DRA) for each participant was determined. The expression index (EI) was derived from a formula previously described¹⁶; $EI = 1000 \times 2^{-Ct}$, where $Ct = C_{t_{telomerase\ (HLA-DRA)}} - C_{t_{GAPDH}}$. The mRNA expression of hTERT was used as a measure of telomerase activity.

Detection of hTERT splice variants

The protocol for the amplification of hTERT alternative splice variants has been published previously¹⁷. The hTERT primer set (hTERT 2162/2580) listed in supplementary Table 1 allowed for the detection of full-length hTERT transcript, α -deletion (hTERT- α), β -deletion (hTERT- β), and α - β -deletion (hTERT α/β -). The PCR products were subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized under UV light. The intensity of the bands was quantified using ImageJ 1.48v software (<http://rsbweb.nih.gov/ij/index.html>).

Data analysis and statistics

The data are presented as medians with interquartile ranges (IQRs) and as frequencies with percentages for continuous and categorical variables, respectively. Spearman's rank correlations were used to examine bivariate associations between telomere length and hTERT mRNA expression, or participant characteristics, e.g., age, sex, ethnicity, CD4+ T cell count, viral load, duration of HIV infection, and duration of ART treatment. Wilcoxon rank sum and Fisher's exact tests were used to compare continuous and categorical variables among the study groups, respectively. P-values are two sided and considered significant if <0.05 .

Results

Characteristics of study participants

We enrolled 21 cases, 21 positive controls, and 21 negative controls from April 2011 to March 2013. The demographic and clinical characteristics of participants are illustrated in Table 1. Among cases, 48% and 52% had one and multiple manifestations of toxicity, respectively. Of note, cases compared with HIV-positive controls had higher viral loads ($p=0.02$), shorter duration of HIV diagnosis ($p=0.001$) and shorter NRTIs treatment ($p=0.03$).

Differences in telomere length among study groups

We determined telomere length as the relative quantity of telomere copy number to 36B4 (single copy gene) number, i.e., T/S ratio. The median (IQR) T/S ratio in negative controls,

positive controls, and cases, was 97.4 (63 – 118), 73.0 (54 – 119), and 107.4 (63 – 135), respectively (Figure 1A). There was no statistically significant difference in telomere length among the groups.

Differences in and determinants of telomerase mRNA expression among study groups

We next investigated the mRNA expression of hTERT among the study groups. The median (IQR) expression index of hTERT mRNA in the negative controls, positive controls, and cases, was 1.7 (1 – 6), 2.0 (0.5 – 17), and 54.0 (4 – 115), respectively (Figure 1B). Cases had significantly higher mRNA expression of hTERT compared to positive controls ($p<0.01$) and negative controls ($p<0.0001$). There was no statistically significant difference in the expression of hTERT mRNA between positive controls and negative controls ($p=0.5$).

We investigated whether the higher expression of hTERT mRNA observed in cases was associated with the activation status of their PBMCs at enrollment. The level of expression of HLA-DRA on cell surfaces has been used as marker of immune activation¹⁸. The median (IQR) expression index of HLA-DRA in negative controls, positive controls, and cases was 987.3 (529.0–1392.0), 1019.7 (541.6–1417.5) and 468.1 (139.9–1105.7), respectively. As illustrated in Figure 1C, cases had a lower HLA-DRA expression when compared to positive controls ($p=0.056$) and negative controls ($p=0.03$). Thus, the high expression of hTERT mRNA in cases cannot be explained by the state of activation of their PBMCs.

The hTERT gene is known to generate at least six alternative splice forms and multiple transcripts, which play important roles in regulation of telomerase enzyme activity¹⁹. We focused on the most common variants: hTERT full length (418 bp), α -deletion (382 bp), and β -deletion (236 bp). Although, we observed variability in both the intensity and the number of hTERT splice variants, there was no statistically significant difference in hTERT full length (Figure 1D), hTERT- α (Figure 1E), and hTERT- β variant (Figure 1F) among study participants.

Discussion

In our cross-sectional, case-control study, HIV treatment-experienced patients diagnosed with mitochondrial toxicity had high expression of hTERT mRNA compared with age, sex, and ethnicity matched HIV-infected patients without toxicity and HIV-uninfected controls. Surprisingly, there was no statistically significant difference in the telomere length among the groups. Increased telomerase mRNA expression in cases without a significant change in telomere length may be a compensation signaling replicative stress in HIV-infected individuals with ART-induced mitochondrial toxicity¹¹. Thus to maintain the length of the telomere in patients with ART-induced mitochondrial toxicity there is a compensatory increase in hTERT mRNA expression.

The HIV-1 reverse transcriptase (RT) enzyme is structurally and mechanistically similar to the hTERT enzyme²⁰. HIV NRTIs have been reported to inhibit the hTERT enzyme, leading to progressive shortening of telomere length during *in vitro* studies⁹. We observed no significant difference in telomere length in cases compared to HIV treatment-experienced positive controls and HIV-uninfected negative controls. Our finding is consistent with a

recent study that found no statistically significant difference in leukocyte telomere length among HIV treatment-experienced mothers, HIV treatment-naïve mothers, and HIV-uninfected mothers ²¹. Interestingly, Kaushal et al observed an increase in T cell telomere length in HIV-infected individuals after initiating ART ²². Other recent studies have reported significantly shorter telomere length in PBMCs of HIV-infected individuals, regardless of treatment status, compared to matched HIV-uninfected individuals ^{23,24}. Srinivasa et al found a significant inverse relationship between soluble CD163 (sCD163), a marker of monocyte and macrophage activation, and telomere length in a cohort of HIV treatment-experienced individuals with undetectable viral load ²⁴. In our study, cases had a significantly lower expression of HLA-DRA than positive and negative controls (Figure 1C).

At least six alternative splice hTERT variants have been identified ²⁵. The splice variants usually either lack a critical reverse transcriptase motif or produce a non-functional reverse transcriptase. We observed variability in both the intensity and number of hTERT splice variants among study participants. There was no statistically significant difference in hTERT full length, hTERT- α , or hTERT- β variant among study participants. The small sample size did not allow for further correlation analyses of splice variants and telomere length, or hTERT mRNA expression.

Telomerase also has extra-telomeric functions. hTERT has been shown to have a mitochondrial leader sequence which targets hTERT protein to the mitochondria and improves respiratory chain function and provides protection from oxidative stress ⁷. Moreover, hTERT has been reported to have anti-apoptotic function ²⁶. Most of these extra-telomeric functions of hTERT do not require binding to the RNA template (TERC) or a functional hTERT catalytic domain. The increased hTERT mRNA expression observed in our cases might be beneficial if it resides in the causal pathway of any of the extra-telomeric functions enumerated. It is also plausible that the increased mRNA expression of hTERT in cases signals replicative stress and it may be a transient phenomenon. If mitochondrial dysfunction continues, telomerase activity may decrease leading to shortening of telomere length. This could explain the high prevalence of premature and accelerated aging in HIV-infected individuals ⁶.

Our study has several limitations. First, like all cross-sectional studies we cannot prove that mitochondrial toxicity in HIV-infected individuals causes increased telomerase mRNA expression. Second, the diagnosis of mitochondrial toxicity was not confirmed with tissue biopsy. Third, we did not use the gold standard for assessing telomerase activity (i.e., the telomerase repeats amplification protocol assay TRAP assay ⁴) since we used stored PBMCs. However, a positive correlation between hTERT mRNA expression, hTERT protein expression, and telomerase activity have been reported previously ^{19,25}. Therefore, hTERT mRNA expression is a good surrogate for telomerase activity. Fourth, the small sample size did not allow us to take into account other factors that could potentially confound our findings, such as HIV viral load, past and present ART regimens, levels of physical activity, and co-infections with other viruses. The strengths of our study are that cases were age, sex, and race matched to positive and negative controls, and it is the first

study to associate ART-induced toxicity with telomere length and hTERT mRNA expression.

In conclusion, based on our findings and previously published data, we hypothesize that ART-induced mitochondrial toxicity resulting in mtDNA damage might induce transient expression of hTERT mRNA. The induced hTERT mRNA may be responsible for maintaining telomere length, thereby preventing replicative senescence and protecting mtDNA from further damage. Furthermore, telomerase mRNA expression might be a surrogate biomarker of ART-induced mitochondrial toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Telomere length and telomerase mRNA expression among study participants. A. Telomere length. The telomere length was determined using quantitative PCR (qPCR) as detailed in the “Methods”. The relative quantity of telomere copy number to 36B4 single copy gene number (i.e., T/S ratio) was determined based on the formula 2^{-Ct} , where $Ct = Ct_{\text{telomere}} - Ct_{36B4}$. B. Telomerase mRNA expression. qPCR was performed using RNA extracted from peripheral mononuclear cells (PBMCs) to determine the mRNA expression of telomerase (hTERT) compared with GAPDH. The mRNA expression of hTERT was calculated as an expression index (EI), where $EI = 1000 \times 2^{-Ct}$, where $Ct = Ct_{\text{hTERT}} - Ct_{\text{GAPDH}}$. C: HLA-DRA expression, QPCR was performed using RNA extracted from PBMCs to determine the mRNA expression of HLA-DRA and compared with GAPDH. The mRNA expression of HLA-DRA was calculated as an expression index (EI), where $EI = 1000 \times 2^{-Ct}$, where $Ct = Ct_{\text{HLA-DRA}} - Ct_{\text{GAPDH}}$. D. Band intensity of hTERT full length normalized with that of GAPDH band. E. Band intensity of hTERT- α splice variant normalized with that of GAPDH band. F. Band intensity of hTERT- β splice variant normalized with that of GAPDH band.

Data represent values obtained from at least 2 independent experiments done in duplicates. Data are represented as box-and-stem plots, in which boxes represent the median values and the second and third quartiles, and stems extend to the 10th and 90th percentiles. The differences between groups were analyzed with Wilcoxon test.

Table 1

Demographic and clinical characteristics of study participants

	HIV-uninfected (n=21)	HIV-infected without toxicity (n=21)	HIV-infected with Toxicity (n=21)	P Values
Sex				
Female	33.4%	33.4%	33.4%	1.00
Male	66.6%	66.6%	66.6%	1.00
Age (Years)	52 (49–72)	53(50–68)	53(50–72)	1.00
Race				
White non-Hispanic	28.6%	28.6%	28.6%	1.00
White Hispanic	4.8%	4.8%	4.8%	1.00
African American	66.6%	66.6%	66.6%	1.00
CD4 Count (count/uL)	NA	690 (306–876)	545 (588–929)	0.23 [*]
Viral Load [†] (copies/mL)	NA	20 (20)	20 (20–131.5)	0.02 [*]
Duration of HIV infection (years)	NA	16 (7.5–19.5)	9 (6–10)	0.001 [*]
Duration of current Nucleoside exposure (years)	NA	5 (3.8–6.3)	3 (1.8–5.5)	0.03 [*]
Current Nucleoside Analog				
TDF	NA	76.2%	71.4%	0.73 [*]
FTC	NA	76.2%	76.2%	1 [*]
3TC	NA	23.8%	28.6%	0.73 [*]
EFV	NA	42.9%	14.3%	0.04 [*]
ABC	NA	4.8%	0	0.31 [*]
AZT	NA	4.8%	0	0.31 [*]
Toxicity				
Single toxicity	NA	NA	48%	-
Multiple toxicity	NA	NA	52%	-

Data were shown as percentage or median (25%–75%); P values were calculated using paired t-test for continuous variables and Chi-square test for categorical variables;

^{*} P value between HIV-infected without and with toxicity only; P-values are two sided and considered significant if <0.05.

[†] Limit of detection of the viral load assay was 20 copies/mL; patients with undetectable viral load were assigned a value of 20 copies/mL;

NA, not applicable; TDF, Tenofovir; FTC, Emtricitabine; AZT, Zidovudine; 3TC, Lamivudine; ddI, Didanosine; D4T, stavudine; ABC, Abacavir; EFV, Efavirenz.