Telomere length in patients with alcohol-associated liver disease: a brief report

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ABSTRACT
The intact telomere structure is essential for the prevention of the chromosome end-to-end fusions and maintaining genomic integrity. The maintenance of telomere length is critical for cellular homeostasis. The shortening of telomeres has been reported in patients with chronic liver diseases. The telomere length has not been systemically studied in patients with alcohol-associated liver disease (ALD) at different stages, such as alcoholic hepatitis and alcoholic cirrhosis. In this brief report, we observed evidence of telomere shortening without changes in the telomerase activity in the liver of patients with alcoholic hepatitis and alcoholic cirrhosis when compared with controls. The alterations in the genes associated with telomere binding proteins were only observed in patients with alcoholic cirrhosis. Future studies are required to determine the mechanism of how alcohol affects the length of the telomere and if the shortening impacts the disease progression in ALD.

INTRODUCTION
Telomeres are the specialized nucleoprotein complex structures at the end of each linear chromosome.[1] The intact telomere structure, a functional telomere cap, is essential for the prevention of the chromosome end-to-end fusions and maintaining the genomic integrity.[2] The loss in the integrity of the telomere cap causes dysfunctional telomeres; the condition has been found in several disease conditions.[2–4] Human telomeric DNA consists of conserved tandem repeats; the sequence is synthesized de novo and added to the telomere end on the chromosome by enzyme telomerase.[5] During the DNA replication in the S-phase of the cell cycle, the terminal portion of the DNA at the end of the chromosome cannot be fully replicated by DNA polymerase, resulting in the shortening of telomeres during each cell division.[5 6] In cell types with rapid divisions such as germ cells, the enzyme telomerase, therefore, is active.[7] Dysregulation in this process may lead to telomere shortening which has downstream effects, causing cellular senescence, chromosomal instability, and impaired cellular homeostasis.[8]

Telomere shortening has been reported in patients with chronic hepatitis, notably hepatitis C, non-alcoholic fatty liver disease, cirrhosis, and hepatocellular carcinoma.[9–11] The telomere length has not been systemically studied in patients with alcohol-associated liver disease (ALD), the disease with a spectrum of histopathological changes from steatosis, alcohol-associated hepatitis (AH), and alcohol-associated cirrhosis (AC).[12 13] In this brief report, we analyzed the telomere length in the liver of patients with AH and AC compared with normal liver.

MATERIALS AND METHODS
Human liver specimens
The deidentified human liver specimens for healthy controls and patients with AC who underwent liver transplantation were obtained under the Institutional Review Board-approved protocol (number 1011004278) at the Indiana University Purdue University Indianapolis. AC was diagnosed, as previously described.[14 15] In brief, patients reported a history of alcohol consumption averaging at least 80 g/day (for men) or 50 g/day (for women). All had a history of portal hypertension complications and evidence of cirrhosis in the explants, with the exclusion of other known causes of liver diseases such as viral hepatitis B or C, autoimmune liver disease, hemochromatosis, or Wilson's disease. Human liver tissues from patients with AH who underwent liver transplantation were provided by the Department of Surgery at John Hopkins Hospital under funding from the NIAAA (R24AA025017, Clinical resources for alcoholic hepatitis investigators) under the IRB-approved protocol (number 00107893). The diagnosis of AH was made based on the standard definitions and recommendations from the National Institute of Health/National Institute on Alcohol Abuse and Alcoholism AH Consortia. All subjects provided informed consent. The baseline demographic and laboratory data at the time of liver collection are shown in table 1.

Telomere length measurements
We isolated the genomic DNAs from human liver tissues using Monarch genomic DNA isolation kit (New England Biolabs, Catalog # T3010L). Once isolated, they were digested with *Hinf*I and *Rsa*I. The DNA fragments were separated by electrophoresis on 0.8% MP agarose gels (Roche, Catalog # 11388983001)

Table 1 Baseline demographics and clinical characteristics of the study cohort

Variables	Alcohol-associated hepatitis (n=5)	Alcohol-associated cirrhosis (n=5)
Age (years)	40.8±7.7	58.2±6.3
Sex	3M/2F	3M/2F
White cell count (×10^9/L)	13.8±7.05	4.5±1.1
Hemoglobin (g/dL)	7.7±0.4	10.2±1.4
Platelet count (×10^9/L)	83.2±48.7	90±65.7
Total bilirubin (mg/dL)	32.4±12.6	2.6±0.8
AST (U/L)	125.6±19.5	52.2±25.3
ALT(U/L)	52.4±13.8	27.6±16.8
Alkaline phosphatase (U/L)	137.8±42.8	133.4±30.5
Albumin (g/dL)	3.34±0.49	3.0±0.3
Creatinine (mg/dL)	1.64±0.76	1.1±0.4
MELD score	37.4±4.6	15.2±3.1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; M, male; MELD, model for end stage liver disease.

and hybridized with DIG-telomere probe (Roche Diagnostics, Catalog # 12209136001). Southern hybridization and non-radioactive detection of telomere signals were performed following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The mean telomere restriction fragment (TRF) lengths were determined following the standard protocol.[16 17] The qPCR for telomere length measurements were performed in triplicate in 20 µL reaction volumes with reaction conditions of 10 min at 94°C, two cycles for 10 s each at 94°C and 15 s at 49°C, followed by 35 cycles at 94°C for 10 s each, 62°C for 15 s, and 74°C for 30 s with fluorescence signal acquisition.[18] The amount of the telomeric DNA (T) in the experimental samples was normalized with the amount of standard telomeric DNA. Likewise, the amount of a single copy gene (S) (*Albumin*) in the experimental sample was normalized with the single-copy gene in a standard DNA sample. T/S ratio was calculated following the method described elsewhere.[18] The average T/S ratio is proportional to the average telomere length per cell. The reference genomic DNA sample with known telomere length was used as a reference for calculating the telomere length of target samples.

Telomerase repeated amplification protocol (TRAP) assay

TRAP assays to measure telomerase activity were performed on 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) extracts containing 1.0 µg of total protein using the TRAPeze telomerase detection kit (Millipore, Catalog # S7700FR), and PCR products were resolved by electrophoresis in 12.0% polyacrylamide gels and visualized by SYBR Safe using BioRad Chemidoc Imaging System.[19]

Total RNA extraction and quantitative real-time PCR analysis

Trizol reagent (Invitrogen, Catalog # 15596018) was used to extract total RNA from human liver tissue. cDNA was synthesized from 1 µg of total RNA using high-fidelity cDNA synthesis kit (Thermo Fisher Scientific, Catalog # 4374966) following manufacture's instruction, and 1 µL

of cDNA was used for quantitative real-time PCR analysis kits (iTaq Universal SYBR green mix, Bio-Rad, Catalog # 1725124). The PCR and the mRNA expression levels were analyzed following the standard protocol published elsewhere.[20]

Statistical analysis

The data were presented as the mean±SE of the mean (SEM). Statistical analysis was carried out using one-way analysis of variance in GraphPad Prism V.9.2. $p<0.05$ was considered statistically significant.

RESULTS

Telomere length in patients with AH and AC

We first determined the mean TRF length by the Southern blot hybridization method.[19 21 22] We found a progressive decrease in the mean telomere length in patients with AH (7.39±0.73 kb) and AC (6.22±0.75 kb) when compared with that of controls (9.66±0.65 kb, figure 1A,B). To confirm our observation, we next determined the telomere lengths, by calculating the T/S ratio, using the quantitative PCR method. Consistent with our TRF data, we found a significant decrease in the T/S ratio in the liver of patients with AH (0.75±0.14) and AC (0.64±16) when compared with that of controls (1.13±0.2, figure 1C). There was also a strong correlation in the measurement of telomere length using both methods (r^2=0.70, figure 1D).

Altered expression of genes associated with telomere binding proteins and telomerase in patients with AH and AC

The telomeres are associated with several telomere binding proteins which are encoded by *TERF1* (*TRF1*) (*telomeric* repeat factor 1), *TERF2* (*TRF2*) (*telomeric* repeat-binding factor 2), *POT1* (protection of telomeres 1), *ACD* (*TPP1*) (adrenocortical dysplasia protein homolog/tripeptidyl peptidase 1), *TERF2IP* (*RAP1*) (telomeric repeat-binding factor 2-interacting protein 1/repressor/activator protein 1), and *TINF2* (TERF1 interacting nuclear factor 2). The expression of these genes was significantly higher in the liver of patients with AC, but not AH when compared with healthy control (HC) (figure 2A). We next tested if the shortening of telomere in patients with AH and AC affected the activity of telomerase enzyme using the following approaches. First, we determined the gene expression of the telomerase template RNA component (*TERC*) and its catalytic subunit, telomerase reverse transcriptase (*TERT*). We observed an increase in the expression of both *TERC* and *TERT* in the liver of patients with AH and AC compared with HC but the differences were not statistically significant (figure 2B,C). Second, we performed the TRAP assay and found no detectable telomerase activity in both AH and AC samples (figure 2D). Taken together, we observed evidence of telomere shortening without changes in telomerase activity in the liver of patients with AH and AC when compared with controls. The significant changes in the gene associated with telomere binding proteins were only observed in the samples from patients with AC.
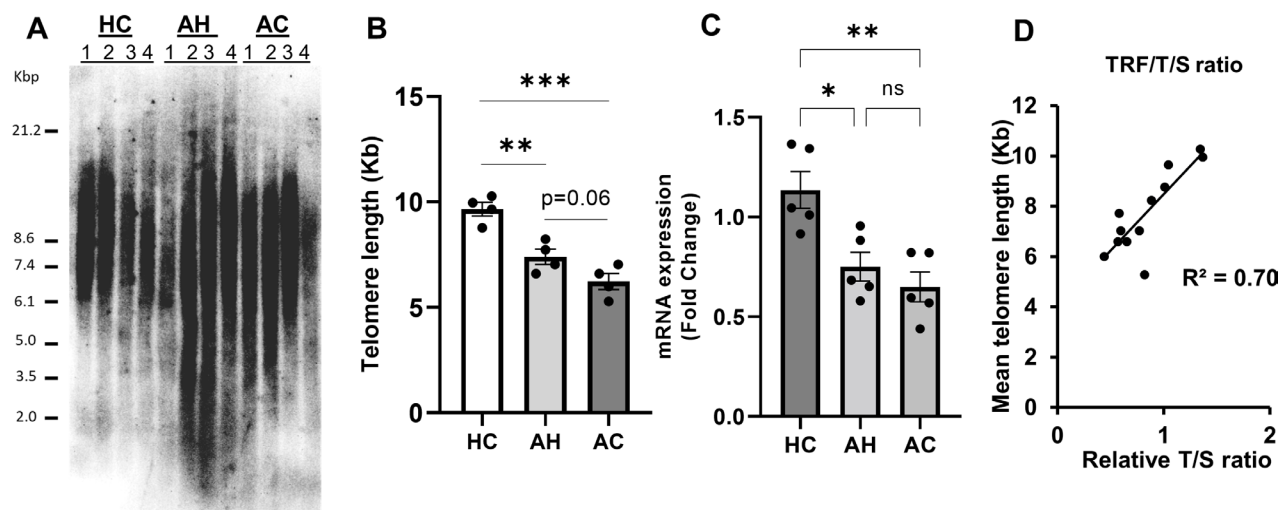
Figure 1 Telomere length measurement using telomere restriction fragment (TRF) by Southern blot hybridization (A and B) and qPCR (C) in the liver of healthy controls (HC), alcoholic hepatitis (AH), and alcoholic cirrhosis (AC). (D) Correlation between the TRF and T/S ratio. The data were presented as the mean±SE of the mean (SEM). **$p < 0.01$, ***$p < 0.001$.

DISCUSSION

In this brief report, we found a significant shortening of telomere length in the liver of patients with AH and AC. Telomere length homeostasis is important for cell survival as short telomeres initiate DNA damage, induce senescence and apoptosis,[8] a possible mechanism underlying alcohol-induced liver injury. An increase in the expression of genes regulating telomere binding protein is likely in response to telomere shortening, especially in patients with AC; a previous study has shown that TRF1 is involved in the negative feedback mechanism to stabilize the length of telomeres.[23] The expression of these
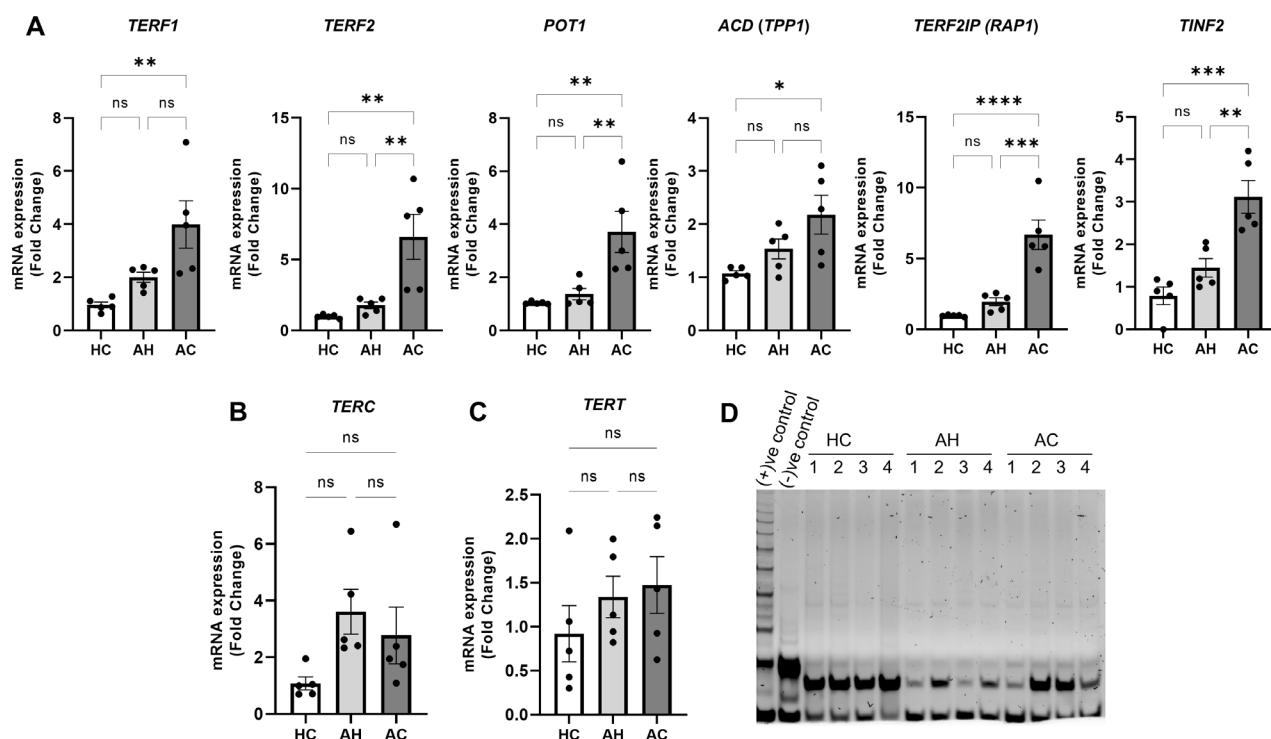

Figure 2 Expression of genes associated with telomere binding proteins and telomerase in alcohol-associated liver disease (ALD). (A) The mRNA expression of telomere-associated genes, *TERF1, TERF2, POT1, ACD,* TERF2IP, and *TIN2,* determined by qPCR. (B, C) TERC and TERT mRNA expression in healthy control (HC), alcoholic hepatitis (AH), and alcoholic cirrhosis (AC) livers. (D) Telomerase activity (telomerase repeated amplification protocol) in 0.1 µg protein-containing lysates of HC, AH, and AC livers. All the experiments described here were performed in triplicates. The data were presented as the mean±SE of the mean (SEM). Statistical analysis was carried out using one-way analysis of variance in Prism V.9.0. $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

genes may be influenced or inhibited by an inflammatory process as we failed to observe an induction in the expression of these genes in patients with AH, a hypothesis deserved further testing. The shortening of telomere length is normally compensated by addition of telomere sequences by telomerase.[7] This is not the case for patients with AH and AC, suggesting the dysregulation in this compensatory process. A disproportionate increase in telomerase activity may perpetuate further shortening of telomere and liver injury in patients with ALD.

While our data are interesting, we acknowledge a few shortcomings. First, our study is based on a limited sample size of patients in each group. Second, we did not capture a detailed alcohol consumption history at the time of liver tissue collection in controls and patients with AH and AC. Alcohol itself may affect telomere structure and function, independent of underlying liver disease.[24]

In conclusion, our results from patients with ALD augmented the existing evidence of telomere shortening in patients with chronic liver disease.[9–11] In our opinion, future studies are required to determine the mechanism of how alcohol affects the length of the telomere and if the shortening impacts the disease progression in ALD. In the same context, the strategy to exploit the telomere length as a potential therapeutic intervention for ALD should be explored.

Competing interests SL is a *Journal of Investigative Medicine* Editorial Board member. All other authors declare no competing interests.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Indiana University Purdue University Indianapolis IRB # 1011004278. Participants gave informed consent to participate in the study before taking part.

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