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Original Article

Shortened leucocyte telomere length is associated independently with fibrosis stage in non-alcoholic fatty liver disease

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Abstract: This study aimed to assess the difference in leucocyte telomere length (LTL) between non-alcoholic fatty liver disease (NAFLD) patients and healthy controls. We measured LTL using a real-time PCR based method in 100 NAFLD patients and 100 healthy controls, and the relationship between short LTL and the risk and severity of the disease was examined using multivariate logistic regression analyses. We found mean LTL was significantly shorter in NAFLD patients than in controls ($P < 0.001$). Subjects with short LTL had a significantly elevated risk of NAFLD (OR: 3.095, 95% CI: 1.594-6.010; $P = 0.001$). Moreover, LTL was observed markedly shorter in NAFLD patients with fibrosis compared to patients with non-fibrosis ($P = 0.010$), multivariate logistic regression analysis indicated that short LTL was independently associated with a higher risk of severe fibrosis stage in NAFLD (OR: 10.799, 95% CI: 1.168-99.832, $P = 0.036$). Further analysis showed a linear correlation between LTL and the expression of hepatocyte senescent marker p21 ($r = -0.413$, $P = 0.011$). In conclusion, these results suggest that short LTL is closely associated with NAFLD and the severity of NAFLD fibrosis, and LTL has potential as a possible non-invasive prognosis biomarker for monitoring the severity of NAFLD fibrosis.

Keywords: Ageing, fibrosis, leucocyte telomere shortening, non-alcoholic fatty liver disease

Introduction

Telomeres are noncoding regions of repetitive nucleotide sequences (5'-TTAGGG-3'; telomeric repeats) located at the ends of chromosomes where they participate in maintaining the integrity and stability of the genome during replication [1]. Telomere length is an indicator of the biological age of cells and telomere attrition is closely connected with replicative senescence. The length of telomere shortens each time cells divide, when it reaches a critical point, DNA repair pathways are recruited and cells enter a state of inactivity such as cell cycle arrest or even apoptosis [2].

Human telomere length is determined by multiple inputs, not only influenced by genetic factors but also by external environmental and lifestyle factors [3, 4], with the most significant of these being oxidative stress. Oxidative stress is implicated in various pathologies, including metabolic disorders and chronic inflammatory conditions. There are also emerging evidences that describe associations between shortened LTL and metabolic and inflammatory diseases, such as cardiovascular dysfunction [5, 6], diabetes type 2 [7], obesity [8], insulin resistance [9] and infections [10]. Thus, shortened LTL reflects the cumulative load of the oxidative, metabolic and inflammatory processes and may serve as a feasible and reliable non-invasive indicator for determining the risk and prognosis of these diseases.

Previous studies have suggested that hepatocellular senescence might be involved in the progression of the NAFLD and serve as a mark-

er to predict patient clinical outcomes [11-13]. These studies have been focused on hepatic tissues rather than the easily accessible blood sample, thus to develop novel and non-invasive biomarkers is necessary. NAFLD is recognized as the hepatic manifestation of metabolic syndrome and shortened LTL is associated with metabolic diseases, as mentioned above. However, whether there is a link between shortened LTL and NAFLD has not yet been reported until now. In our present study, we aimed to assess the difference in LTL between NAFLD patients and healthy controls, and to investigate the association of leucocyte telomere attrition with the disease severity.

Materials and methods

Patients and specimens

A total of 100 patients with NAFLD were selected from the Diagnosis and Treatment Center of Hangzhou Normal University Affiliated Hospital (Hangzhou, China) during the period between December 2014 and September 2016. Tissue specimens were obtained from 37 NAFLD patients of them who were performed liver biopsy operation. All patients we studied fulfilled the Chinese diagnostic criteria for NAFLD [14]. In addition, no patients had any clinical evidence of cancer, cardiovascular disease, diabetes and other diseases. At the same time, we selected 100 healthy individuals in Physical examination center of Hangzhou Normal University Affiliated Hospital as normal controls. The study protocol has been approved by the Ethics Committee of Hangzhou Normal University Affiliated hospital. A written informed consent was signed by all individuals participating in the study.

Blood samples collection and biochemical analyses

Blood samples were obtained from the cubital vein in 3 ml sterile vacutainers coated with EDTA (ethylenediaminetetraacetic acid). The biochemical indexes which included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT) Albumin (ALB), Total bilirubin (TB), total cholesterol (TC), triglyceride (TG) and fasting plasma glucose (FPG) were measured by an automatic biochemical analyser (model 7180; Hitachi, Japan) using standard methods.

DNA extraction

Genomic DNA was extracted from the blood using a QIAmp DNA Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. All DNA samples were tested for concentration and quality using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific, USA) and a 1% agarose gel electrophoresis (Bio Rad Power Pac 300, USA). Then DNA samples were stored in TB at -80°C temperature until the time of assay.

Telomere length measurement

Telomere length was determined using a quantitative real time polymerase chain reaction (q-PCR) based method. This method provides a relative measure of average LTL by calculating the ratio of telomere repeats copy number to a single housekeeping gene (36B4) copy number across all chromosomes for all cells [15, 16]. The primers used for the telomere and the 36B4 amplification were synthesized by Sangon Biotech Co., Ltd (shanghai). The primer sequences were as follows: telomere forward: 5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3'; telomere reverse: 5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3'; 36B4 forward: 5'-CAG CAA GTG GGA AGG TGT AAT CC-3'; 36B4 reverse: 5'-CCC ATT CTA TCA TCA ACG GGT ACAA-3'. DNA samples were diluted to a concentration of 5.0 ng/μl prior to performing q-PCR. To each well, 10 μL 1× real-time quantitative PCR SYBR Green Master (Applied Biosystem, USA), 7 ul water, 2 ul the respective primers of working concentration (4 uM) and 1 μl of diluted DNA sample were added. T*elomere* and 36B4 were run in duplicate within the same plate. The same reference sample was included in all plates as internal control to allow the comparisons of results between different runs. A no-template control was also included for quality control. Q-PCR was performed on the Applied Biosystems 7900HT Sequence Detection System (Foster City, CA, USA) with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and an annealing temperature 60°C for 1 min. The relative T/S values were calculated according to the $2^{-\Delta\Delta CT}$ method.

Table 1. Characteristics of NAFLD patients and healthy controls

	NAFLD patients (N = 100)	Controls (N = 100)	*P*-value
Age (years)	41.88 ± 11.83	39.84 ± 10.43	0.171
Male (percentage)	73 (73%)	65 (65%)	0.221
BMI (kg/m^2)	27.42 ± 3.87	22.4 ± 2.5	< 0.001
FPG (mmol/L)	6.09 ± 1.83	5.42 ± 0.43	0.001
SBP (mmHg)	128.56 ± 18.93	121.43 ± 17.15	0.001
DBP (mmHg)	84.61 ± 8.57	75.94 ± 9.87	< 0.001
ALB (g/L)	47.16 ± 2.68	45.75 ± 1.89	0.006
ALT (IU/L)	48.00 (30.00-72.00)	18.00 (15.00-24.00)	< 0.001
AST (IU/L)	28.00 (24.00-44.50)	22.00 (16.00-24.00)	< 0.001
ALP (IU/L)	134.00 (117.00-169.00)	87.00 (72.00-117.00)	< 0.001
GGT (IU/L)	47.00 (27.00-94.00)	17.00 (14.00-30.00)	< 0.001
TB (umol/L)	16.89 ± 5.51	16.27 ± 4.58	0.582
TC (mmol/L)	5.06 (4.51-5.94)	4.76 (4.32-5.62)	0.145
TG (mmol/L)	2.14 (1.40-3.30)	1.10 (0.65-1.33)	< 0.001
SOD (U/mL)	89.48 ± 19.61	112.39 ± 22.09	< 0.001
MDA (nM/mL)	8.54 ± 2.53	3.96 ± 1.01	0.012

NAFLD, non-alcoholic fatty liver disease; BMI, Body mass index; FPG, fasting plasm glucose; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; ALB, Albmin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosph tase; GGT, gamma-glutamyl transpeptidase; TB, Total bilirubin; TC, Total cholesterol; TG, Triglyceride; SOD, superoxide dismutase; MDA, malonyldialdehyde; LTL, Leucocyte telomere length.

Assessment of liver histology

Hematoxylin and Eosin (HE) and Masson's Trichrome stainings were performed to evaluate steatosis, inflammation and fibrosis, respectively. Slides were read by a single experienced hepatopathologist, who was blinded to subject' details. A histological scoring system (non-alcoholic fatty liver activity score, NAS) [17] was used to grade the degree of liver steatosis, lobular inflammation and fibrosis. The degree of steatosis and inflammatory were scored using an 8-point scale (steatosis 0-3; lobular inflammation 0-3; ballooning hepatocyte degeneration 0-2). A NAS of five or higher were defined as NASH, while a NAS of two or lower were defined as simple fatty liver. The stage of fibrosis was scored using a 6-point scale (1a, b = mild (1a)/moderate (1b) zone 3 perisinusoidal fibrosis; 1c = portal fibrosis only; 2 = zone 3 and portal/periportal fibrosis; 3 = bridging fibrosis; 4 = cirrhosis).

Tissue processing and immunostaining

Tissue processing and immunostaining was performed on liver tissue sections using the method developed in our laboratory [18]. The liver biopsy specimens were fixed in 10% formalin, embedded in paraffin and cut into 5 μm slides, and then the slides were dewaxed in xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed using a high-temperature and high-pressure antigen repairing method [2 min 40 sec in sodium citrate buffer (pH = 6)], and endogenous peoxidase activity was quenched with 3% hydrogen peroxide at room temperature for 10 min. 0.5% Triton X-100 was applied for 20 minto permeate the membranes. The slides were then incubated with anti-p21 primary antibody (ZSGB-BIO, china) overnight at 4°C. After washed with PBS, the slides were subsequently incubated in the horseradish peroxidase conjugated goat anti-mouse IgG antibody (Maixin Bio, china) for 15 min at room temperature. The visualization of the immunoreaction was performed with diaminobenzidine (Maixin Bio, china). The slides were then counterstained with Meyer hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted. Human cervical carcinoma tissue was used as positive control, and negative control was obtained by omitting the primary antibody.

Immunohistochemistry was evaluated in a quantitative way to ensure objectivity. Brown nucleus represents a p21-positive cell. Three non-overlapping regions fields were selected from each slice. The result was expressed as a p21-positive index which was the mean percentage of the number of positive hepatocytes relative to the total number of hepatocytes [13, 19]. The expressions were analyzed by two individual researchers on a blinded basis under a Nikon (Tokyo, Japan) 80i microscopy.

SOD activity and MDA level

Antioxidant index (SOD activities) or oxidant index (MDA levels) in the plasma were separately determined by the method of xanthine

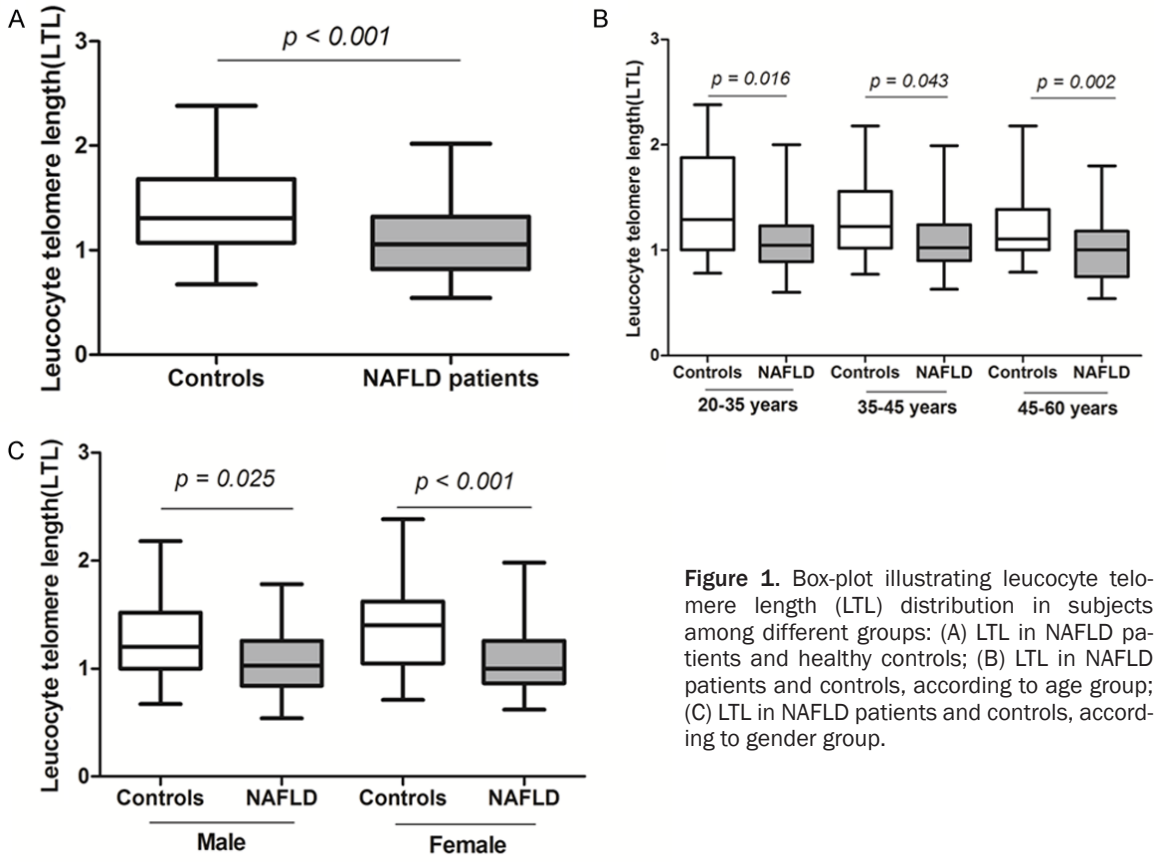

Figure 1. Box-plot illustrating leucocyte telomere length (LTL) distribution in subjects among different groups: (A) LTL in NAFLD patients and healthy controls; (B) LTL in NAFLD patients and controls, according to age group; (C) LTL in NAFLD patients and controls, according to gender group.

oxidase or thiobarbituric acid using the SOD and MDA kits, according to the manufacturer's protocols (Nanjing Jiancheng Biological Engineering Research Institute, China). All samples were assayed in triplicate.

Statistical analysis

Mean ± SD was used to describe continuous normally distributed variables, while median and interquartile range (IQR) were used for variables without a normal distribution, and percentage (%) was used for categorical variables. The comparisons between two groups were analyzed using the Student's t-test, non-parametric test, Chi-squared test, or analysis of covariance (ANCOVA) where appropriate. Pearson's correlation coefficient, Spearman's rank correlation coefficient, univariate and multivariate logistic regression analyses were used to define the relationship between variables where appropriate. All tests in this study were two sided, and $P < 0.05$ was considered statistically significant. All analyses were performed using the SPSS software (version 17, SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the participants

The characteristics of the 100 patients with NAFLD and 100 healthy controls are listed in **Table 1**. As expected, NAFLD patients had higher BMI, FPG, SBP, DBP and serum ALB, TG, ALT, AST, ALP, GGT levels than the control subjects (all $P < 0.05$). And compared with the controls, MDA levels were higher while SOD activities were lower in the NAFLD patients (all $P < 0.05$).

LTL level in NAFLD and control groups and correlation of LTL with NAFLD clinical characteristics

Since telomere length might also be influenced by age, gender, blood glucose, and blood pressure, the comparison of LTL between NAFLD patients and the healthy controls was analyzed

Table 2. Association between LTL and NAFLD clinical characteristics

Variables	r	*P*-values
Age (years)	-0.235	0.018
Genders	-	0.036
BMI (kg/m^2)	-0.392	< 0.001
FPG (mmol/L)	-0.063	0.389
SBP (mmHg)	-0.030	0.686
DBP (mmHg)	-0.045	0.330
ALB (g/L)	0.039	0.792
ALT (IU/L)	-0.209	0.038
AST (IU/L)	-0.147	0.152
ALP (IU/L)	-0.039	0.704
GGT (IU/L)	-0.222	0.029
TB (umol/L)	-0.186	0.211
TC (mmol/L)	-0.011	0.919
TG (mmol/L)	-0.352	< 0.001
SOD (U/mL)	0.210	0.024
MDA (nM/mL)	-0.099	0.385

LTL, Leucocyte telomere length; NAFLD, non-alcoholic fatty liver disease; r: correlation coefficient; BMI, Body mass index; FPG, fasting plasma glucose; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; ALB, Albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; TB, Total bilirubin; TC, Total cholesterol; TG, Triglyceride; SOD, superoxide dismutase; MDA, malonyldialdehyde.

by ANCOVA to control the role of these variables. Overall, LTL was markedly shorter in NAFLD patients than in controls ($P < 0.001$), as presented in **Figure 1A**.

Then we classified the participants of both groups into 3 age categories (20 to 35 years; 35 to 45 years; and 45 to 60 years). A significantly shortened LTL was observed in NAFLD patients contrast to the controls within each of the 3 age categories (all $P < 0.05$), as shown in **Figure 1B**. We further explored LTL from subgroups according to gender, and both in male and female groups, NAFLD patients had significantly shorter LTL than healthy controls (all $P < 0.05$), as shown in **Figure 1C**.

The correlation between LTL and NAFLD clinical characteristics are shown in **Table 2**. As expected, there was a significant relationship between LTL and age, with LTL being shorter in older patients ($P < 0.05$). And LTL was found to be inversely correlated with BMI, ALT, GGT, TG and positively correlated with SOD activities (all $P < 0.05$).

Association of short LTL with the risk of NAFLD

Logistic regression analysis was employed to investigate the association of LTL changes with the risk of NAFLD. We divided participants into two groups based on the median value (1.31) of LTL in healthy controls. Both univariate and multivariate analyses indicated that subjects with shorter LTL had a significantly elevated risk of NAFLD as compared to subjects with longer LTL, with an hazard ratio of 3.167 (unadjusted, 95% CI, 1.732-5.791; P < 0.001) and 3.095 (adjusted for age and gender, 95% CI, 1.594-6.010; P = 0.001), respectively.

Comparison of LTL between different subgroup of patients stratified by histopathological characteristics

The histopathological characteristics of 37 NAFLD patients are shown in **Table 3.** We compared LTL among subgroups of NAFLD patients according to the severity of steatosis, lobular inflammation and fibrosis. As shown in **Table 4**, a significantly shortened LTL was observed in patients with fibrosis 2-4 contrast to the patients with fibrosis 0-1 ($P = 0.010$), and advanced fibrosis patients had shorter LTL than non-advanced fibrosis patients ($P = 0.043$). But, there were no significant differences in LTL among NAFLD patients with less or more than 33% steatosis, lobular inflammation of grade one or more, mild and more severe ballooning degeneration.

Association of short LTL with the risk of NAFLD fibrosis

We further investigated the association of short LTL with the severity of liver fibrosis using logistic regression analysis. The results showed patients with shorter LTL were more likely to have increased risk of fibrosis stage than those with longer LTL, both in univariate (unadjusted, OR: 12, 95% CI: 1.341-107.363, $P = 0.026$) and multivariate analysis (adjusted for age and gender, OR: 10.799, 95% CI: 1.168-99.832, $P = 0.036$).

Correlation analysis between LTL and the hepatocyte p21 expression in NAFLD

We measured the expression of hepatocyte p21 in 37 NAFLD patients. The expression of hepatocyte p21 was found positively correlated

Table 3. The histopathological grades of 37 NAFLD patients with liver biopsy

Histopathological featur	Number
Steatosis (grade: 0/1/2/3)	1/15/7/14
Ballooning degeneration (grade: 0/1/2)	2/17/18
Lobular inflammation (grade: 0/1/2/3)	0/15/17/5
Fibrosis (stage: 0/1/2/3/4)	0/22/9/5/1
NASH/non-NASH	26/11
Non-advanced fibrosis/Advanced fibrosis	6/31

Non-alcoholic fatty liver activity score (NAS) was used to grade the degrees of liver steatosis, lobular inflammation and fibrosis. NASH (NAS ≥ 5); non-NASH (NAS < 5); non-advanced fibrosis (fibrosis stage 0-2); Advanced fibrosis (fibrosis stage 3-4).

Table 4. Comparison of LTL among different subgroups of patients stratified by histopathological characteristics

Variables	LTL	*P*-values
Steatosis		
Grade (0-1)	1.05 (0.76-1.17)	0.114
Grade (2-3)	1.06 (0.92-1.38)	
Ballooning degeneration		
Grade (0-1)	1.06 (0.94-1.18)	0.589
Grade (2)	1.05 (0.76-1.43)	
Lobular inflammation		
Grade (0-1)	1.06 (0.91-1.25)	0.843
Grade (2-3)	1.06 (0.86-1.26)	
Fibrosis		
Stage (0-1)	1.12 (0.93-1.40)	0.010
Stage (2-4)	1.04 (0.74-1.06)	
NASH (NAS ≥ 5)	1.06 (1.01-1.18)	0.940
Non-NASH (NAS < 5)	1.05 (0.80-1.31)	
Non-advanced fibrosis (stage 0-2)	1.06 (0.93-1.28)	0.043
Advanced fibrosis (stage 3-4)	0.86 (0.67-1.03)	

with the severity of liver fibrosis (r = 0.518, P = 0.001), and LTL was inversely correlated with the hepatocyte p21 expression in NAFLD (r = -0.413, P = 0.011).

Discussion

Several studies have shown that there were shortened telomere length, high-level expression of p21, increased r-H(2)AX expression, nuclear enlargement in hepatocyte of NAFLD patients [11, 13]. P21 plays a vital role in the induction and maintenance of cellular senescence and increased hepatocyte p21 expression is associated independently with fibrosis stage and an adverse liver-related outcome in NAFLD [11, 13, 20]. Many researchers also demonstrated the relationship between telomere length shortening in liver tissue and cirrhosis, they observed that a shortened hepatic telomere length was correlated with the degree of fibrosis [21-24]. The senescence appears to be profibrogenic by as-yet undefined mechanisms and may be a marker of cirrhosis [25]. However, these studies have been focused on hepatic tissues rather than the easily accessible blood sample, thus to develop novel and non-invasive biomarkers is necessary.

To our best knowledge, this is the first study demonstrating an association between short LTL and NAFLD in human subjects. We found NAFLD patients had substantially shorter LTL than healthy controls (P < 0.05), and short LTL was independently associated with a higher risk of NAFLD (P < 0.05). Patients with severe fibrosis had increased telomere erosion as compared to mild fibrosis patients (P < 0.05) and shorter LTL patients were more likely to have increased risk of fibrosis stage (P < 0.05). These findings confirm our hypothesis that a decrease in LTL is correlated with the risk and severity of NAFLD. LTL has potential as a possible non-invasive prognosis biomarker for monitoring the severity of NAFLD fibrosis. Moreover, the present study found LTL shortening was correlated with the up-regulation of hepatocyte p21 expression (r = -0.413, P = 0.011), which suggests that leucocyte telomere erosion might be consistent with the senescence of liver, raising the possibility that LTL might be used as an easily accessible non-invasive biomarker to predict NAFLD fibrosis outcomes.

The mechanism of LTL shortening is not readily available. In NAFLD, telomere attrition might be accelerated by dysregulated metabolic factors which could induce oxidative stress and chronic inflammation [26, 27]. Oxidative stress could promote the double-strand breaks of telomeric DNA, leading to the disruption of telomere length [28]. Inflammation triggers proliferation and repair processes, which accelerates cell

turnover and ultimately results in excessive telomere shortening [29]. The results in the present study showed that LTL was inversely correlated with ALT, GGT and positively correlated with SOD activities (all $P < 0.05$), suggesting LTL might be influenced by chronic inflammation and oxidative stress in NAFLD, which are in agreement with the findings reported by Wolkowitz and coworkers [30]. To the extent, oxidative and inflammatory stresses are chronically increased in NAFLD patients resulting in increased leucocyte turnover and telomere shortening. The exact mechanism underlying the association of shortened telomere length in leucocytes and NAFLD remains a mystery and requires further studies.

However, several limitations should be considered in our study. First, this study was cross-sectional in which cause-and-effect associations could not be determined, and prospective longitudinal studies are needed to confirm the prognostic value of LTL as a predictive marker for NAFLD progression. Second, we measured LTL using a PCR based method that only could quantify a relative density of telomeres and the absolute telomere length cloud not be examined. Thus random measurement error still exists. Third, the association reported in this study was in a very small number of subjects. Larger study samples are required to throw more insight into the association of short LTL with NAFLD risk and prognostic prediction of NAFLD fibrosis. In view of the limitations discussed above, the results should be regarded as preliminary and further studies with larger samples and prospective design are needed to confirm or refute our findings.

In conclusion, our study demonstrated the association of short LTL with NAFLD risk and the severity of NAFLD fibrosis. These findings provide valuable information towards accelerated biological aging in NAFLD pathology and open a way for future work aimed at identifying leucocyte telomere biomarkers for cirrhosis outcome prediction. However, leucocyte telomere length shortening is not specific for NAFLD, and our study also has some limitations, therefore, in future, further and better designed studies are still needed.

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Disclosure of conflict of interest

None.

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