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Research Article

Short Telomere Length in Plasma of Sudanese Patients with Hepatocellular Carcinoma and Chronic Liver Diseases

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Abstract

Background: Telomeres, a protective sequence of DNA at the end of chromosomes, are essential for the maintenance of chromosomal integrity and stability. With each cell division, telomeres are shortened until a critical length is reached. Several cancers have been linked to shortened telomere length (TL). The current cross-sectional study aims to investigate the TL in the plasma of Sudanese patients with hepatocellular carcinoma (HCC) and chronic liver diseases (CLD).

Methods: Blood samples were obtained from 113 patients with HCC and CLD as well as from 50 healthy controls. The assessment of TL in blood samples was carried out using the relative quantitative PCR method.

Results: Patients with HCC had significantly shorter TL than healthy controls (0.66 vs 0.89; $P < 0.01$), whereas insignificant shorter TL was detected in HCC patients as compared to those with CLD. A significant decrease in telomeres copy numbers was observed in HCC patients when compared to those with CLD ($P < 0.01$).

Conclusion: The results of the present study demonstrate that TL is shorter in patients with HCC and CLD compared to healthy controls.

Keywords: telomere's length, telomeres copy numbers, hepatocellular carcinoma, CLD, Sudan

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1. Introduction

The ends of eukaryotic chromosomes are protected by special structures called telomeres. The highly conserved telomeric DNA sequences consist of 4–12 kb of short tandem $(TTAGGG)_n$ repeats oriented 5′–3′ toward the end of the chromosome [1]. Human telomeres act as a shield structure protecting and stabilizing both ends of the chromosome, regulate cell life span, and maintain genomic stability [2]. DNA polymerase's end replication incompetency results in telomeres becoming shorter by 50 to 200 base pairs within each cell division cycle in normal somatic cells [3–5]. The critical shortening of the telomere leads to genomic instability that triggers tumor growth, which otherwise, prevented by replicative senescence and apoptosis [6, 7]. Preserving telomere to a specific length is essential for cellular immortality. Telomere maintenance can be achieved by using one of two known methods to overcome the end-replication issue and attain genomic stability: alternative lengthening of telomeres (ALT) or telomerase activation [8]. Telomerase is an enzymatic complex of ribonucleoprotein which extends the telomeric repeats at the chromosomal ends and maintains the telomeres. Thus, it is the main positive regulator of telomere length (TL). Telomerase activation can induce cellular immortality by maintaining the length of its telomeres [9]. TL can be significantly shortened in the absence of adequate telomerase activity and under unmanageable cells division, resulting in dysfunctional telomeres and ultimately triggering either cell aging or programmed cell death [10]. Additionally, environmental factors such as chemical toxins, radiation, and infection can affect TL regulation during growth and development [11–15].

During the early stages of carcinogenesis, cells experience broad proliferation which results in a significant and critical shortening in TL. Shortened telomeres have been accounted in chromosomal instability in hepatocytes, particularly in viral-related hepatocarcinogenesis [16]. The relationship between cirrhosis and telomere shortening have been investigated by several studies which demonstrated that telomere shortening may be a marker of cirrhosis formation correlating with the accumulation of senescent hepatocytes [17]. Several studies reported shorter telomeres in hepatocellular carcinoma (HCC) and chronic liver diseases (CLD) as compared to non-carcinoma tissue [18–20].

TL can be determined in solid tumor tissues, peripheral blood, and cell-free DNA (cfDNA) which can be obtained from plasma, serum, and other body fluids. Plasma (cfDNA) concentration increases in pregnancy, cancer, and some other diseases, so that (cfDNA) can be used as biomarker for early diagnosis [21]. In patients with cancer, plasma cfDNA TL was significantly shorter compared to the whole blood and tumor tissue samples, indicating that plasma cfDNA could be used as a cancer biomarker, and cfDNA analysis was more sensitive than tumor tissue analysis [22].

Our study examined the TL in the plasma of patients with HCC, CLD, and control group using RT-PCR to provide more information for accurate diagnosis of HCC.

2. Materials and Methods

2.1. Study design and population

In this cross-sectional study, 113 participants (86 males and 27 females, aged 19–80) and 50 controls (34 males and 16 females, aged 21–80) were recruited from Ibn Sina Provisional Hospital for Gastrointestinal Diseases in Khartoum State, Sudan. The 113 participants were composed of three distinct groups: patients with HCC (n = 33), patients with cirrhosis (n = 33), and patients

with hepatitis (n = 47). Patients (aged >18 years) with confirmed HCC as well as those with CLD were included in this study. Patients, who had malignancies other than HCC, severe heart and brain disease, ulcerative colitis, and atherosclerosis were excluded from the study. Healthy controls with no history of related conditions were included in this study.

The diagnosis of HCC was based on either cytology confirmation or according to the guidelines of American Association for the Study of the Liver Disease (AASLD). The diagnosis of cirrhosis was based on clinical picture ultrasonography (US) combined with clinical signs and symptoms. Diagnosis of hepatitis was determined by serological test. In controls, HCC, liver cirrhosis, and hepatitis were ruled out by abdominal US examination, AFP level, and serological test.

2.2. DNA extraction

Plasma samples were obtained from all 113 patients with confirmed HCC (29%), cirrhotic (29%), and with hepatitis (42%), and 50 healthy controls. DNA was extracted from plasma samples using QIAGEN kit (FSC, Germany) as per the manufacturer's protocol. DNA was then stored at –20°C. DNA concentration and purity were determined for each extract using the Gene Guant spectrophotometer (Amersham Biosciences, America Serial No. 92375). The instrument was set to measure the absorbance at 260 and 280 nm to determine the DNA concentration.

2.3. Telomere length (TL) measurements

Quantitative polymerase chain reaction (qPCR) was conducted to measure the relative average TLs as described by Rahamtalla *et al.* [23]. In this technique, the quantities of telomere as well as albumin were compared in a PCR reaction with the assumption that both amplified products demonstrate similar efficiency (the selection of the albumin primer pair as the single copy reference gene was made because it lacks single nucleotide polymorphisms [SNPs] in the target sequence and did not generate a product in the negative control) [24]. The oligonucleotide primer sequences used for TL measurement and albumin were described in Rahamtalla *et al.* and Cawthon's studies [23, 24].

Reaction was performed within a total volume of 20 μL containing 1.5 μl genomic DNA, 0.6 μl of primer Mix, and 4 μl of EvaGreen qPCR Supermix (Solis BioDyne, European Union) which composed of DNA polymerase, buffer, $Mmcl_{2,}$ dNTPs, dye, internal reference based on ROX dye, GC-enhancer and blue visualization dye, with 13.9 μl of H_2O added to get the final volume of 20 μl. The qPCR conditions for amplification for telomere were carried on the thermal cycler – an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 59°C for 15 sec, extension at 72°C for 20 sec, and the last 1 min at 95°C, 10 sec at 60°C, and 15 sec at 72°C. Whereas, the qPCR conditions for amplification for albumin included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 48.4°C for 45 sec, extension at 72°C for 30 sec, and the last 1 min at 95 °C, 30 sec at 48.4°C, and 30 sec at 95°C.

The PCR products and 5 μl of 50 bp DNA ladder RTU (GeneDire, Cat. No.DM012-R500S) were subjected to electrophoresis on a 3% agarose gel which was stained by Ethidium bromide dye and then visualized using specialized gel documentation systems (Syngene, Bio Imaging).

Furthermore, real-time PCR products were validated using melting curves generated from amplified genomic DNA with both telomere and albumin primers. Every sample was executed in

duplicate. For each sample the Ct values for both telomere and albumin were obtained (Ct: cycle thresholds indicate the number of fractional cycles at which the amount of amplified target reaches a fixed threshold). Relative quantity of telomere versus albumin (T/S) was calculated using the following equation:

Number of telomeric DNA copies = $2^{--(\Delta\Delta Ct)}$,

where, ΔCt = $Ct_{telomere} - Ct_{albumin}$, Δ ΔCt = $\Delta Ct_{patients} - \Delta Ct_{healthy\ controls}$ [25].

A direct correlation between the average T/S and the length of the telomere per cell is anticipated as compared to the standard DNA samples. The average TL of samples with a T/S >1.0 was longer than that of standard DNA samples, while the average TL with a T/S <1.0 was shorter than the standard DNA samples, but T/S = 1 when both telomere repeats copy number and reference single copy DNA number are equal [24].

2.4. Statistical analysis

The numerical statistical tests comprised the Student's *t*-test and ANOVA test, mean separation was obtained by using Duncan's New Multiple Range Test (DNMRT). Results were expressed as mean ± SD. Correlation between different parameters was performed using Chi-square and Pearson's techniques. Statistical analyses were all considered significant at *P*-value ≤ 0.05. The significance of a *P*-value ≤ 0.05 was observed in all statistical analyses. All statistical measure was performed using Windows version 16 SPSS software.

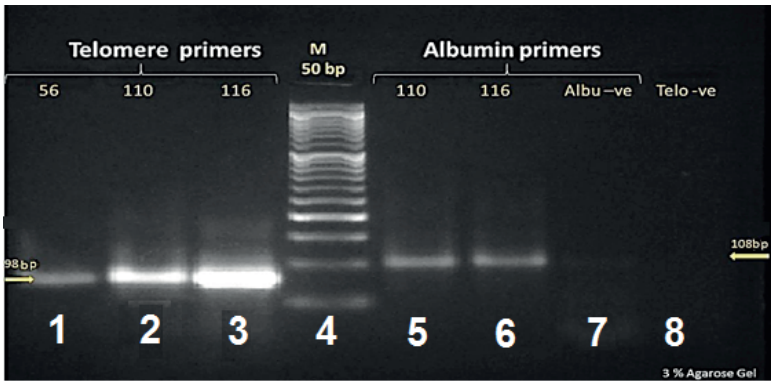

Figure 1: The PCR product electrophoresed in (3%) agarose gel amplified by real-time PCR. Left region amplified with telomere primer (98 bp). Lane 4: 50 bp DNA Ladder; Lanes 1–3: amplified samples. Right region amplified with albumin primer (108 bp). Lane 5, 6: amplified samples. Lanes 7, 8: albumin –ve and telomere –ve controls.

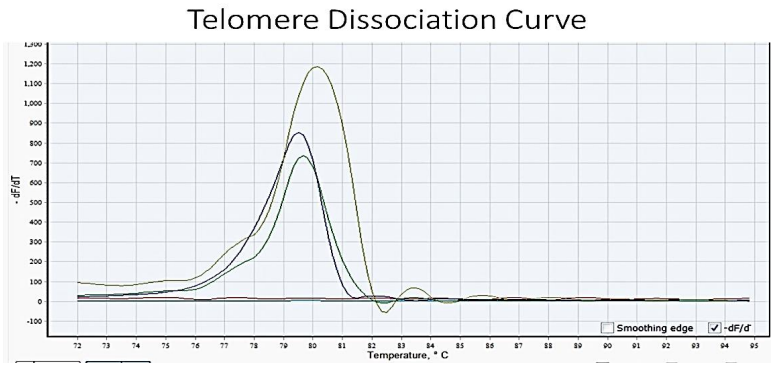

Figure 2: Melting curves for telomere. Three template genomic DNA melting curves are shown in brown, blue, and green colors. Template control melting curve is in red color. Telomere primer was fully melted in 88°C.

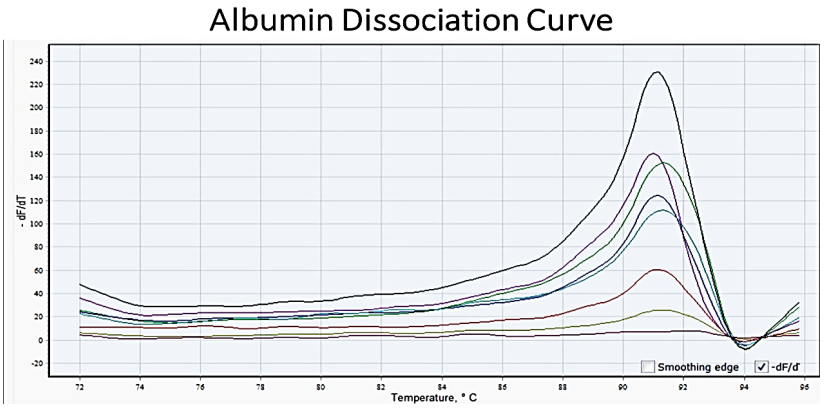

Figure 3: Melting curves for albumin. Seven template genomic DNA melting curves are in black, violet, blue, Indigo, green, deep green, and red colors. Template control melting curve is in deep violet color. Albumin primer was fully melted in 94ºC.

Table 1: The ultrasonography features of HCC.

Features	HCC (n = 33)
Site of focal liver lesion	
Right lobe	18 (55%)
Left lobe	8 (24%)
Both lobes	3 (9%)
Infiltrate	4 (12%)
Size of focal liver lesion (cm)	
2–5	16 (48.5%)
>5	15 (45.5%)
Unknown	2 (6%)
Number of focal lesion	
Multiple	20 (60.6%)
Single	13 (39.4%)
Tumor grade	
Grade 1	13 (39.4%)
Grade 2	15 (45.5%)
Grade 3	5 (15.1)

cm, centimeter; HCC, hepatocellular carcinoma

Table 2: Telomere length (TL) and number of telomeric DNA copies.

Parameters	Control (n = 50)	HCC (n = 33)	Cirrhosis (n = 33)	Hepatitis (n = 47)	All patients (n = 113)
TL Mean ± SD	0.89^{a} ± 0.28	0.66^{b} ± 0.14	0.65^{b} ± 0.09	0.57^{b} ± 0.06	0.673^{**} ± 0.13
Telomere DNA copies	–	90.51	121.10	2546.8.	2757.2

**Significant correlation at < 0.01, according to Duncan's New Multiple Range Test (DNMRT): similar letters within rows are insignificant at *P*-value ≤ 0.05
[a]Significant difference from patients; [b]significant difference from control
HCC, hepatocellular carcinoma; SD, standard deviation

Table 3: Telomere length (TL) affected by age group.

Parameters	Age (yrs)			
	19-34	35-50	51-66	67-82
TL:	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control	1.03 ± 0.22	0.76 ± 0.29	0.88 ± 0.28	1.03 ± 0.29
HCC patients	0.57 ± 0.00	0.64 ± 0.14	0.65 ± 0.12	0.68 ± 0.20
Cirrhotic patients	0.74 ± 0.12	0.62 ± 0.09	0.63 ± 0.06	0.67 ± 0.11
Patients with hepatitis	0.56 ± 0.04	0.57 ± 0.06	0.61 ± 0.06	0.53 ± 0.02

Table 4: Telomere length (TL) affected by HCC causes.

HCC cause	Mean ± SD
Unknown	0.15±0.69
Cirrhosis	0.23±0.68
Hepatitis	0.10±0.63
Cirrhosis + hepatitis	0.63 ± 0.06

Table 5: Correlation between telomere length (TL), age, and gender in patients and controls.

	Coefficient	
Variables	r	P-value
Patients		
Age	–0.146	0.131
Gender	0.078	0.423
Control		
Age	–	0.04*
Gender	0.514	0.00**

**Significant correlation at 0.01; *significant correlation at 0.05

3. Results

3.1. Characteristics of the study population

The current study shows that the distribution of mean age for all patients was 49.03 ± 14.57 years, with the mean age of HCC patients being 48.39 ± 13.28, of cirrhotic patients 51.21 ± 14.93, of patients with hepatitis 44.34 ± 14.30, and that of controls 35.94 ± 12.58 years. HCC caused by hepatitis was 30.3%, cirrhosis 15.2%, both cirrhosis and hepatitis 21.2%, and unknown causes 33.3%. Among HCC patients, 13 (39.4%) were in grade 1, 15 (45.5%) in grade 2, whereas the remaining 5 (15.1%) were in grade 3. The US features of focal liver lesions revealed that 55% of HCC patients had right lobe lesion, 24% had lesion in left lobe, while only 9% had lesions in both lobes. Infiltrate lesion was reported in 12% of HCC patients. The size of focal liver lesion was 2–5 cm in 48.5%, >5 cm in 45.5%, and size was unknown in the remaining (6%). Moreover, most HCC patients (20 [60.6%]) had multiple focal liver lesions, while few (13 [39.4%]) had a single focal liver lesion, as shown in Table 1. Furthermore, there was no evidence of extra-hepatic spread or metastasis or portal vein thrombosis.

3.2. Telomere length (TL) and number of telomeric DNA copies

Differences in TL were assessed across patients and control groups, with significant findings discussed in Table 2. TL was significantly ($P < 0.001$) shorter in patients (0.673 ± 0.13) as compared to controls (0.89 ± 0.29). However, TL in the same HCC group of patients (0.66 ± 0.14), cirrhosis (0.65 ± 0.09), or hepatitis (0.57 ± 0.06) was insignificantly different. The mean TL affected by age group are provided in Table 3. The TL was shorter in those aged 35–50 (control: 0.76 ± 0.29; cirrhotic patients: 0.62 ± 0.09; and HCC patients: 0.64 ± 0.14) as well and 51–66 (control: 0.88 ± 0.28; cirrhotic patients: 0.63 ± 0.06; and HCC patients: 0.65 ± 0.12) than in those aged 67–82 (control: 1.03 ± 0.29; cirrhotic patients: 0.67 ± 0.11; and HCC patients: 0.68 ± 0.20).

Moreover, Table 4 presents data on how TL is affected by various causes of HCC. As illustrated in the table, TL was shorter in HCC caused by hepatitis (0.63 ± 0.10), cirrhosis (0.68 ± 0.23), and the combination of cirrhosis and hepatitis (0.63 ± 0.06) compared to that caused by unknown causes (0.69 ± 0.15).

On the other hand, the telomeric DNA copies number was significantly ($P < 0.001$) decreased in HCC (90.51) as compared to cirrhosis (121.10) and hepatitis (2546.8; Table 2).

3.3. Correlation between telomere length (TL), age, and gender

Our analysis revealed no significant correlations between age, gender, and TL in the patient groups. In contrast, a significant correlation was observed between age, gender, and TL in the healthy control group, as detailed in Table 5.

3.4. Melting curve and agarose gel electrophoresis

The agarose gel electrophoresis revealed only the expected (98 bp) product for telomere and (108 bp) product for albumin (Figure **1**). The melting curves analysis validates the result and showed that the cycle thresholds for the telomere signals could be collected at 74–88°C. The sharp melting curve for PCR product was consistent with specific, fixed-length product formation. No template control melting curves was present (Figures 2 & 3).

4. Discussion

Identification of valuable biomarkers for HCC is needed for disease management. In this study, relative TL was measured and found to be significantly shorter in patients with HCC as compared to healthy controls. This finding coincided with several studies reporting a shorter telomer length of cancerous liver tissues compared to normal tissues [11, 26, 27]. Telomere shortening may contribute to the genetic aberrations that drive genome instability and hence stimulate malignant growth. Many studies have associated shortened telomeres to the progression of the earliest stages of certain cancers and confirmed that a shortened TL was correlated with the degree of fibrosis, suggesting that telomere shortening may contribute to and be a marker of cirrhosis [28–31].

On the other hand, insignificant difference in TL was reported in this study between HCC patients as compared to CLD patients. Our findings are in agreements with other studies that reported insignificant difference between mean TL in HCC and chronic hepatitis [31]. The findings, on the other hand, are contradictory to those done in China and Korea where HCC patients were reported to have significantly longer telomere, compared to patients with CLD and health control [32, 33]. However,

this may be due to increased telomerase activity, more advanced tumor stage, poor prognosis [33], or significant racial differences in the distribution of TL [6]. Researchers hypothesized that Blacks would have shorter TL than Whites [34]. Nevertheless several studies have shown that the length of the telomere is partially genetically determined and that the heritability ranges from 34% to 82% in humans [35, 36]. This could also be due to other biological mechanism such as oxidative stress [37, 38].

On the other hand, TL was shorter in those aged 50–65 in both the patient and control groups with the length increasing gradually in older ages. Many studies have found that TL shortens most rapidly in the first few years of life, with the shortening rate slowing in young adulthood and possibly speeding up or slowing down again at older ages [39].

Furthermore, in this study TL was found to be shorter in HCC caused by combination of cirrhosis and hepatitis compared to unknown causes. This results in agreement with several studies that have investigated the relationship between cirrhosis and telomere shortening and showed that shortened TL in hepatocytes is correlated with degree of fibrosis and it may be considered as a marker of cirrhosis formation [40]. Other studies have found the relationship between telomere shortening and liver cirrhosis, and that TL is increasingly shorter in cirrhotic and chronic hepatitis livers [41].

In the present study, the number of telomere copies was calculated, and the results revealed that the numbers were significantly reduced in patients with HCC as compared to patients with cirrhosis, HBV, HCV, and with both BCV and HBV. Several previous studies have reported reduction of mtDNA copy number associated with HCC [42], renal cancer [43], breast cancer [44], and bladder cancer, while it increased in other types such as thyroid, colorectal, and endometrial cancers [45]. A Sudanese study conducted in 2016 to explain the role of variation in the number of mitochondrial copies in breast cancer reported a decrease in mtDNA copies in tumor tissue compared to normal tissue [46]. In addition, no significant correlation was found between age, gender, and TL of all patients. However significant correlation was found between age, gender, and TL of controls with longer telomere in females than males. Our result disagrees with that of other studies that have found correlation between shorter telomeres, with higher abrasion rates, and age of males [47–49].

The rate of telomere shortening with age [48] is different between men and women and can be influenced by several factors that accelerate aging and the risk of premature death by negatively affecting TL. Possible explanation of females having longer telomere is that estrogen directly activates the promoter of telomerase and indirectly affects DNA repair through the p53 pathway and telomerase activation and nitric oxide pathways [50]. Moreover, a systematic review also concluded that the length of telomere in females is longer than in males [49], but the strength of the association varied according to measuring methods [47].

The new scientific contribution of the present study focused on the measurement of relative TL and telomere copy numbers. A significant shorter telomere was found in HCC as an independent diagnostic indicator for HCC. Telomere copy numbers in HCC patients were found to be significantly reduced as compared to those with CLD ($P < 0.01$). The current study demonstrates the association of short telomeres length with increased risk of HCC.

5. Limitations

A notable limitation of our study is the gender imbalance, with a higher number of male participants compared to females. Future research

should consider a more balanced gender distribution to provide a clearer understanding of how gender impacts TL.

6. Conclusion

The results of the present study demonstrates that TL is shorter in HCC and CLD patients compared to healthy controls. This finding suggests a potential association between shorter TL and increased risk of HCC. However, longitudinal studies are necessary to validate these findings and assess the utility of TL as a diagnostic biomarker for HCC.

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Ethical Considerations

The study was approved by the Ethics and Research Committee of Faculty of Medical Laboratory Science, Omdurman Islamic University and was conducted in accordance with international ethical guidelines. Informed consent was obtained from all participants.

Availability of Data and Material

Data used to support the findings of this study are included within the article.

Competing Interests

None.

Funding

None.

Abbreviations and Symbols

AASLD: American Association for the Study of Liver Disease
ALT: Alternative lengthening of telomeres
ANOVA: Analysis of variance
bp: Base pairs
°C: Centigrade
cfDNA: Cell free DNA
CLD: Chronic liver disease
Cm: Centimeter
Ct: Cycle thresholds
DNA: De-oxy nucleic acid
DNMRT: Duncan's new multiple range test
FSC: Forest stewardship council
HBV: Hepatitis virus B
HCC: Hepatocellular carcinoma
HCV: Hepatitis virus C
kb: Kilobyte
mins: Minutes
μl: Microliter
mtDNA: Mitochondrial DNA
nm: Nano meter
dNTP: Deoxynucleoside triphosphate
PCR: Polymerase chain reaction
qPCR: Quantitative polymerase chain reaction

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