Title

Telomerase: a key player in the pathogenesis of non-alcoholic fatty liver disease?

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Declaration of interest

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Abstract

Introduction: Telomerase is a basic nuclear protein reverse transcriptase, which plays a key role in maintaining telomere stability, genome integrity, long-term cell activity and potential continued proliferation.

Area covered: This narrative review discusses key research advances involving telomerase in the development and progression of non-alcoholic fatty liver disease (NAFLD). The review evaluates: a) whether the assessment of telomerase can be used as a non-invasive diagnostic tool; and b) whether modification of telomerase function might be a useful potential therapeutic target for treatment of NAFLD. Furthermore, the relationship between telomerase and other chronic metabolic diseases is evaluated.

Expert opinion: Several experimental and preclinical studies have suggested that telomerase plays an important role in the development of NAFLD. However, further mechanistic studies are needed to prove a causal relationship and to better elucidate whether the measurement of telomerase has utility as a diagnostic tool or whether pharmacological manipulation of telomerase has therapeutic potential in NAFLD treatment.

Keywords: telomerase, nonalcoholic fatty liver disease, liver fibrosis, lipid metabolism, hepatocellular carcinoma, metabolic disorders, therapeutic targets.

Highlights

- The specific role of telomerase function in hepatocytes is discussed.

- Fatty degeneration, cellular inflammation, oxidative stress, mitochondrial dysfunction, as well as fibrosis and increased susceptibility to cancer in the liver are considered to be pathological changes potentially induced by telomerase dysfunction.
- A novel therapeutic strategy to regulate telomere length by improving telomerase function in NAFLD is also considered.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is now one of the most common liver diseases due to the increasing prevalence of metabolic diseases, such as obesity and type 2 diabetes [1]. Because more than a quarter of people suffer from NAFLD, this liver disease now represents an increasing global health problem and economic burden [2,3]. In addition to multiple environmental risk factors, there is also a strong association between NAFLD and genetic factors [4]. Indeed, somatic genetic mutations occurring in multiple *loci* may affect hepatic lipid metabolism, leading to the development of NAFLD and accelerating its progression to non-alcoholic steatohepatitis (NASH) and cirrhosis [5]. Among these genetic factors, telomere and telomerase play important roles in the development of NAFLD [6]. In particular, telomerase dysfunction may lead to telomere shortening that affects the normal function of liver cells, lipid metabolism, and hepatic fibrogenesis and may promote progression of NAFLD to NASH [7], cirrhosis and hepatocellular carcinoma (HCC) [8]. This narrative review discusses key research advances regarding the link between telomerase and NAFLD development and progression. Whether telomerase could be used as a non-invasive diagnostic tool and potential therapeutic target for patients with NAFLD is also discussed, together with the relationship between telomerase and other related chronic metabolic diseases.

2. Telomeres and telomerase

In the human genome, telomeres are a small DNA-protein complex located at the end of the chromosome. The length of telomeres' DNA varies from 5kb to 15kb [9].

Telomeres are composed of hexanucleotide repeat segments (TTAGGG) [10] (**Figure 1**), which improve the structural stability of chromosomes and protect the end portion of chromosomes from end-to-end fusion, rearrangement, and translocation during the processes of cell proliferation. However, telomeres lose about 50-100 base pairs in each mitotic cycle, since DNA polymerases cannot fully replicate the 3' end of the lagging chain (due to a process called "telomere attrition"), and therefore the telomere length gradually shortens during cell proliferation [11,12]. Human cells can only replicate a certain number of times before they undergo programmed cell death, according to the Hayflick limit concept on cell aging. When the telomere is shortened to the Hayflick limit [13], the cell stops mitosis, activates the DNA apoptosis program, and enters into the cellular aging process. Therefore, telomere length can be used as a "life clock" to describe the aging of cells [14].

Telomerases are ribonuclease complexes, closely related to the telomeres, participating in the mitotic phases of the cells, which are involved in the process of DNA replication, as well as in the synthesis of new DNA sequences. Adding DNA to the ends of chromosomes allows them to stabilize the telomere length and reduce the speed of telomere shortening. Telomerase consists of two main parts: the telomerase RNA component (hTERC) and the telomerase reverse transcriptase (including its RNA template) (hTERT) [15]. hTERC combines with the Dyskerin complex, which is composed of four nucleolar proteins: Dyskerin (DKC1) and members 1, 2, and 3 of nucleolar protein family A (i.e., NOLA1, NOLA2, and NOLA3). The function of the

Dyskerin complex is to stabilize and protect the structure and function of hTERC [16-18].

The human Shelterin complex consists of six core proteins: telomeric repeat-binding factor 1 and 2 (TRF1, TRF2), TRF1-interacting protein 2 (TIN2), TRF2-interacting protein 1 (RAP1), protection of telomeres 1 (POT1), and telomere-binding protein POT1-interacting protein 1 (TPP1) [19]. TRF1 and TRF2 bind to telomere double-stranded DNA. TPP1 combines with POT1, to recruit POT1 to the telomere region. POT1 can, specifically, recognize telomere single-stranded DNA. RAP1 binds with TRF2, to access the telomerase. Finally, TIN2 acts as a bridge, connecting POT1-TPP1 to TRF1 and TRF2, thus binding the whole Shelterin complex together. The Shelterin complex, by binding to telomeres specifically, helps to cap telomeres. Additionally, as shown in **Figure 1,** the Shelterin complex also plays an important role in regulating telomere length, in maintaining telomere structure, and in adding functional integrity [20].

3. Telomerase in NAFLD

Previous studies have shown that the dysfunction and somatic genetic mutations of both hTERT and hTERC are relevant to several chronic liver diseases [21]. Dysfunction or gene mutations of both hTERT and hTERC affect telomere shortening that will consequentially lead to a decline of hepatocyte regenerative ability, and metabolic dysfunction [22], making the liver prone to fatty degeneration,

inflammation and fibrosis. Furthermore, after liver damage occurs, with increasing levels of fibrosis, the liver is increasingly susceptible to the development of HCC. Therefore, telomere length may be a reliable genetic marker to monitor the progress of pathologic liver conditions, especially, the development and progression of chronic liver diseases [23-25].

3.1 Telomerase in fatty liver degeneration

Recent findings suggest that impaired lipid handling by hepatocytes plays a key role in the pathogenesis of NAFLD by triggering inflammation, fibrogenesis, and carcinogenesis [26]. NAFLD can be observed in individuals with telomerase dysfunction, suggesting that telomerase may be involved in hepatic lipid metabolism. It is currently uncertain whether telomerase function may be regulated to improve the liver disease, or whether telomerase function could be used to predict the progression and evaluate the prognosis of NAFLD. Additionally, telomerase is closely related to cellular aging, and it is plausible that hepatocytes are more susceptible to steatosis with aging [27].

Studies have shown that there are patients with severe hepatic steatosis who have impaired mitochondrial telomerase function (since hTERT protects mitochondrial DNA from damage [28]), regardless of the presence of NASH [29,30]. It is plausible that increased uptake of free fatty acids (FFA) into hepatocytes amongst patients with NAFLD increases intracellular amounts of reactive oxygen species (ROS), due to the

increase of oxidative stress *in vivo* (oxidation of base G in the telomere sequence) [31]. This effect would delay telomerase activation and decrease telomerase activity, resulting in the breaking and shortening of the telomere, under non-replicative conditions [32]. Thus, we surmise that in patients with NAFLD, the oxidative damage caused by high levels of ROS in hepatocytes may be the main cause of telomere shortening and that the degree of hepatic steatosis, rather than that of fibrosis, might play a leading role in telomere shortening [33] (see **Figure 2**).

In addition, the Shelterin complex binding to telomerase plays a role in protecting the length of the telomere at the end of the chromosome [34]. RAP1 is one of the components involved in the process of lipid metabolism [35]. It has been experimentally demonstrated that if RAP1 is absent (resulting in telomerase dysfunction), fatty liver degeneration, impaired glucose tolerance, and increased abdominal fat accumulation may occur in mice [36].

Furthermore, hTERT mutation may induce telomerase dysfunction, leading to a series of familial liver diseases. These familial liver diseases are usually characterized by hepatic steatosis and iron overload of hepatocytes, which may be caused by erythropoiesis-related disorders [37]. In summary, the dysfunction of telomeres and telomerase may lead to fat accumulation in hepatocytes and the occurrence of NAFLD.

3.2 Telomerase in liver fibrosis

Somatic genetic mutations of the telomerase gene (especially hTERT) may accelerate the process of telomere shortening and affect the regenerative ability of hepatocytes, thereby promoting hepatic fibrogenesis [38,39]. The causal effect of this gene mutation has been confirmed in mouse models with telomerase deficiency. After three generations, the telomeres of these mice became shorter, and the development of fibrosis accelerated after liver injury [40,41]. Conversely, over-expression of hTERT activity improved the stability of telomerase and length of telomere, delayed the progress of liver fibrosis in these mice.

In chronic liver diseases (such as cirrhosis), tissue regeneration is increased, cell division is expedited, and telomere shortening is accelerated. When telomeres become extremely short, cell apoptosis occurs, leading to further reduction of the number of hepatocytes [42]. Therefore, telomere shortening is also considered a marker of cirrhosis [43]. In cirrhotic patients, telomere shortening is associated with the expression of some cellular senescence markers, such as β-galactosidase, p16, p21, and p53 proteins [44,45]. P53 protein is the key regulatory point within the cell apoptosis signal pathway. P53 phosphorylation induces p21 expression and p21 binds to cyclin-dependent kinase (Cdk) complex. Binding to the cyclin-dependent kinase (Cdk) complex inhibits its activity, resulting in the accumulation of nuclear protein pRB in cells, inhibiting the transcription of E2F target gene required for cell cycle progression, and causing G1 phase arrest of cell division. Nuclear protein pRB can

also be activated by p16 protein, which is also an inhibitor of Cdk activity, usually found in aging cells [45].

It has been reported that telomere shortening in peripheral blood leukocytes is independently associated with an increased risk of advanced liver fibrosis. In fact, Kim et al. reported that the association between telomere length in blood leukocytes and advanced liver fibrosis remained statistically significant even after adjusting for obesity and other risk factors, suggesting that the telomere length of these cells may play a role in mediating the link between NAFLD and advanced fibrosis, especially in the elderly [46].

3.3 Telomerase in NAFLD-related HCC

In immortalized cells (such as cancer cells, stem cells and germ cells), telomerase activity increases, delaying telomere shortening, and even extending telomere length [25]. Some studies have found that the progress of cancer is related to the reactivation of telomerase, which is a necessary condition for the immortalization of tumor cell clones [47,48]. hTERT was up-regulated in dysplastic liver tissues, and hTERT activity in dominant liver cancer tissues was more than 10 times greater than that in surrounding non-tumor tissues [49]. Furthermore, some studies suggest that telomere length may be able to predict the survival time of humans and propose tumor inhibition mechanisms that limit cell proliferation [50].

In contrast, telomere length in peripheral blood leukocytes of patients with NAFLD-related HCC has been shown to be shorter than that in healthy controls [51]. Telomere shortening plays a key role in inducing genomic changes. Shorter telomeres are likely to lead to exhaustion, aging, and increased fibrogenesis within various tissue/organ compartments (including the lung, liver and hemopoietic systems); and increase the development of various types of cancers by promoting genomic instability (such as chromosome separation defects) [52]. In fact, telomere shortening is associated with the typical karyotype changes of HCC (i.e., alterations in chromosome 8), especially in the case of concomitant TP53 mutation [53,54].

Similarly, the development of NAFLD-related HCC is also affected by genetic factors, but the gene determinants influencing the progression of NAFLD-related HCC remain mostly unknown, because rare gene mutations may promote the progression of this liver disease [55-57]. hTERT mutation, which is a key factor in the progression of liver fibrosis and carcinogenesis [40,58-60], is associated with the development of NAFLD-related HCC [61], and in this work, an association between shorter peripheral blood telomeres and NAFLD-HCC development was also demonstrated, suggesting that rare germline mutations in hTERT may predispose to HCC in NAFLD. In patients with NAFLD-related HCC, rare coding variants of hTERT were found to be mostly located in the N-terminal (template-binding domain), such as Ala67Val mutation, while another rare Glu668Asp mutation was located in the catalytic domain of hTERT. Both of these mutations may lead to the decrease of

intracellular protein and interfere with the normal functioning of telomerase, resulting in the telomere shortening [62], while also affecting the ability of DNA repair that maintains the stability of the chromosome structure [63]. Meanwhile, other rare 'driver gene' mutations, such as FGA and SYNE1, were also found in patients with NAFLD-related HCC, which were considered as new recurrent mutations in NAFLD-related HCC [64]. In addition, in HCC, hTERT promoter mutations were identified with an overall prevalence of between ~20% and 80%, as the most frequent somatic genetic alterations [65]. Both hTERT promoter mutations and CTNNB1 activation mutations may be involved in the liver malignant transformation [66,67], by co-regulating gene expression. Soo Ki Kim et al. investigated genetic alterations in NAFLD-HCC and performed targeted sequencing of eleven NAFLD-HCC samples and 10 matched non-tumorous liver tissues. CTNNB1 mutations were observed in 5 of 11 HCC cases (45%), of which 3 mutations were located in the mutation hotspot of CTNNB1. Additionally, hTERT promoter mutations were detected in all 5 NAFLD-related HCC patients with CTNNB1 activation mutations, indicating that hTERT is closely related to CTNNB1 [64]. Therefore, hTERT mutation may accelerate the progression of NAFLD to HCC.

HCC cases with ''stemness''-related marker expression were characterized by longer telomeres and a greater amount of alterations in the process of DNA compared to traditional HCC cases [68]. The increased expression of TRF2, POT1, RAP1, TPP1, and hTERT in these HCC cases suggests a role of these genes in maintaining telomere

length in these tumors, which is associated with poor prognosis and lower survival rates [68]. Kim et al. confirmed that the expression of TPP1 is related to the expression of hTERT, suggesting that TPP1 is not only a positive regulator of telomere length maintenance [69] but is also a potential target for cancer treatment since it plays a leading role in hTERT recruitment to telomeres. Therefore, hTERT, which is a stimulator gene related to NAFLD-related HCC [66], capable of regulating its activity and delaying the shortening of telomere length, could also be a promising drug molecular therapeutic target for patients with NAFLD-related HCC.

4. Telomerase in metabolic diseases

It is well known that obesity and type 2 diabetes are strongly associated with the development of NAFLD. It has been reported that accelerated telomere attrition in peripheral blood leukocytes is closely associated with weight gain and insulin resistance in young adults from the Bogalusa Heart study, who were followed for a period of nearly 10 years [70]. This finding suggests a nexus of telomere biology with metabolic disorders (adiposity and insulin resistance) that adversely impacts the early development of NAFLD in humans.

It has been reported that there is a significant association between telomere length and type 2 diabetes [71]. Moreover, in hTERC-deficient mice, insulin-producing pancreatic beta cells were found to be smaller and more likely to have impaired glucose tolerance, thus suggesting that telomerase dysfunction can lead to impaired

regeneration of pancreatic beta cells and affect normal glucose metabolism [72,73]. Under the condition of a high-fat diet, hTERC gene knockout mice also showed impaired glucose tolerance and overexpression of p53 (while overexpressing of p53 in adipose tissue, causing low-grade inflammation and insulin resistance) [74]. Concurrently, insulin resistance may increase the oxidative stress response in cells, which aggravates the degree of telomere shortening, thus increasing the risk of developing obesity, and other metabolic diseases [75]. Similarly, hTERT deficiency may cause metabolic dysfunction and cell damage in hepatocytes, resulting in fat accumulation and mitochondrial dysfunction in the liver [76]. As shown in **Figure 3**, telomerase dysfunction has also been shown to alter metabolic function, by inhibiting the PGC-1α dependent process [77].

5. Therapeutic targeting of early disease severity

Early diagnosis and intervention play an important role in the treatment of NAFLD and other chronic liver diseases. If the early stages of liver disease in NAFLD can be identified, and the patient can make appropriate lifestyle modifications that improve the dysregulation of lipid metabolism, then the progression of NAFLD to NASH and cirrhosis can be ameliorated and perhaps prevented. Liver biopsy has unique advantages in the assessment of the severity of NAFLD and, specifically, in the quantification of hepatic steatosis, inflammation and ballooning, as well as in the assessment of the stage of liver fibrosis. Liver biopsy is still the ‘gold standard’ for the diagnosis and staging of NAFLD [78-80]. However, this method is invasive and is

associated with significant morbidity (e.g. hemorrhage), so it is important to find new non-invasive, and alternative diagnostic tests for diagnosing and staging NAFLD.

We speculate that hTERT promoter mutations could be reliable markers of increased risk of malignant transformation in HCC and potential biomarkers for the diagnosis and/or follow-up of these patients using circulating tumor DNA. We suggest using venous blood sampling to detect telomere shortening in peripheral blood leukocytes as a much simpler investigation method than performing a liver biopsy. Patient identification for further investigation may be possible, based on the presence of promoter mutations, or the amount of hTERT overexpression. That said, validation of such an approach is required.

As discussed above, the shortening of telomere length may lead to impairment of hepatocyte regenerative ability and promotion of disease progression in NAFLD. It has been suggested that the association between NAFLD and telomere length may be useful in early diagnosis, disease staging and prediction of this liver disease. Leucocyte telomere length has been shown to be significantly associated with advanced liver fibrosis in patients with T2DM [71]. Moreover, telomerase could also be used as a new therapeutic target to treat NAFLD. QPCR analysis of hTERT, hTERC, and TERF1 also showed that liver telomerase activity was decreased after hepatic ischemia-reperfusion and irisin increased telomerase activity, via inhibition of phosphorylation of JNK during hepatic ischemia-reperfusion [81]. Experimentally, it

has been shown that in high-fat diet-fed mice, high doses of activated carbon N-acetylcysteine (ACNAC) significantly increased the levels of hTERC and telomerase activity, while prolonging telomere length to improve the regenerative ability of hepatocytes [82].

Therefore, we suggest that a therapeutic approach could be to increase the biological function of telomerase, improve the stability of telomere, delay the shortening of telomere length, and thereby potentially reduce the progress of hepatic steatosis and fibrosis. We also reason that it is important to evaluate the validity of telomere length measurement as a prognostic marker in NAFLD, and to determine whether it could be a new non-invasive test for monitoring NAFLD.

6. Expert opinion

The main risk factors for NAFLD are obesity and type 2 diabetes, which are also closely associated with telomere shortening and telomerase dysfunction [83-85]. In NAFLD, telomere shortening has been described and telomere shortening is associated with increased hepatic fat accumulation and fibrosis [86]. **Figure 4** shows the main processes that are involved in linking telomere shortening to the development of hepatic fat accumulation, fibrosis and HCC in NAFLD. We suggest that a better understanding of how telomere shortening is involved in the pathogenesis of NAFLD could ultimately lead to future randomized controlled trials, to test whether strategies to delay telomere shortening are effective in the prevention and

treatment of NAFLD.

The occurrence and development of cancer is closely related to telomerase and telomere length. In most cases, the reactivation of telomerase is considered to be one of the necessary conditions for the unrestricted growth of cancer cells. In the early stages of cancer occurrence, there are studies that have found that the shortening of telomere length will promote further deterioration of cancer. Therefore, the specific mechanism of telomerase function and the effect of telomere length on cancer needs to be further studied and determined. In a state of imbalance, what is the relationship between the inhibition or activation of telomerase function and disease development? What regulatory mechanism does the upstream promoter gene use to achieve the conversion between inhibition and activation of telomerase function? These issues need further exploration and more attention.

Due to the important role of telomerase in the metabolic process of the body, the detection of telomere length may be one of the methods for prognostic evaluation and disease monitoring of NAFLD patients. Those patients with short length of telomeres and no significant improvement in liver condition may consider telomerase as a target to intervene. The method of detecting telomere length more quickly and accurately can be one of the goals of clinical testing methods in the future. Moreover, mutations in the promoter gene of telomerase, hTERT, may be suggested as markers for the risk of malignant transformation, as well as a potential biomarker for diagnosis and

follow-up using circulating tumor DNA. Therefore, usage of peripheral blood of follow-up patients for hTERT gene detection, to stratify patients according to the hTERT promoter mutation status or the level of hTERT overexpression, needs to be verified clinically prior to implementation as predictive biomarkers.

At the same time, over inhibition or activation of telomerase function may potential result in adverse effects in human, more meticulous attention is suggested for considering telomerases for possible therapeutic target. Once the telomerase length is too long or too short, it will cause adverse effects. What is the proper range of the length of the telomere to maintain the body in a healthy status? What kind of target therapeutic drugs or methods can activate telomerase properly, thereby stabilizing the length of telomeres within a moderate range and maintaining the normal physiological functions of cells? These clinical problems need to be further verified by randomized placebo control experiments.

7. Concluding remarks

We hypothesize that there is a key role for telomere shortening in NAFLD. When the telomere length is shortened to such a degree that the cell enters the apoptosis process, the cell sends out stop signals. However, if the protection mechanism against this "stop signal" (such as the p53 tumor suppressor gene) fails, and the cell proliferation process does not stop, the telomere becomes extremely short, producing chromosomal instability affecting the regenerative ability of liver cells. Some stem cells (such as

hematopoietic stem cells) are also activated in the damaged area of the liver and promote fibrogenesis. In such cases, if hTERT gene mutation and other factors leading to loss of telomerase function further accelerate telomere shortening and the progression of fibrosis [87], it can cause liver injury and create a favorable microenvironment for the development of cirrhosis and HCC [88] (**Figure 4**).

The specific mechanisms of telomere length that change during the normal functioning of hepatocytes remains poorly understood. We suggest further study of the molecular mechanisms of telomere shortening in the pathogenesis of NAFLD is urgently needed, as are cohort studies testing whether telomere shortening is associated with, and predicts the severity of NAFLD.

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Figure Legends

Figure 1. Location and composition of telomeres and the structure of telomerase. In the human genome, telomeres are a small DNA-protein complex located at the end of chromosome and composed of hexanucleotide repeat segments (TTAGGG) in 3' end of the lagging chain. Telomerase binds to telomere and is mainly composed of hTERC and hTERT. Among them, Dyskerin complex adheres to hTERC to stabilize and protect it; Shelterin complex interacts with telomere to regulate telomere length.

Figure 2. Telomerase is related to fat metabolism *in vivo*. The increase of free fatty acid (FFA) uptake in cells leads to the increased production of reactive oxygen species (ROS). Oxidative stress causes the oxidation of base G in telomere sequence, resulting in delayed telomerase activation and decreased telomerase activity, resulting in telomere breakage and shortening under non-replicative conditions; Rap1 is involved in the lipid metabolism process *in vivo*. If the telomerase dysfunction occurs due to the lack of Rap1, it will lead to hepatic steatosis, impaired glucose tolerance and increased abdominal fat accumulation in mice; the disorder of red blood cell formation will lead to steatosis and iron overload in liver cells, which will eventually lead to lipid metabolism disorder and increase the susceptibility to NAFLD.

Figure 3. Telomerase is associated with metabolic diseases. With a high-fat diet, hTERC knockout mice showed impaired glucose tolerance and over-expression of p53, which led to inflammation and insulin resistance in pancreatic beta cells. Insulin

resistance may increase the intracellular oxidative stress response, further aggravate telomere shortening, increase body weight, thus making the body more prone to the development of NAFLD and metabolic diseases.

Figure 4. Telomere shortening in NAFLD. When the telomere length is shortened sufficiently to promote apoptosis, and the stop signal (e.g. the p53 tumor suppressor gene) fails, the telomere becomes extremely short promoting chromosomal instability. If hTERT gene mutation or other factors leading to a loss of telomerase function occurs there is increased risk of liver, steatosis, fibrosis and HCC.