Egyptian Journal of Medical Human Genetics

REVIEW

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Pathogenic effects of telomerase reverse transcriptase (TERT) promoter mutations in nonalcoholic steatohepatitis (NASH) related hepatocellular carcinoma (HCC) and its potentials as a diagnostic biomarker

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Abstract

Background Hepatocellular carcinoma (HCC) is the most common type of liver cancer globally due to its diverse aetiologies, poor diagnosis and prognosis as well as low survival rate. Nonalcoholic steatohepatitis-related HCC (NASH-HCC), the progressive form of nonalcoholic fatty liver, is the most prevalent subtype of HCC in this century, and genetic predisposition significantly influences its pathogenesis. Several genes associated with NASH–HCC development have been recently studied. One key regulatory gene is the *TERT* gene encoding for the *TERT* protein.

Main body of the abstract Hence, the goal of this mini-review is to present the most recent findings about *TERT* promoter mutations, the mechanism of upregulation of *TERT* gene expression, the downstream mechanism of promoting NASH-HCC, and its potential as a NASH-HCC diagnostic biomarker.

Conclusion Relevant and up-to-date findings were presented in this review, but more thorough researches in multiethnic and diver population are needed to determine the prevalence of *TERT* promoter mutations, its gene expression levels and their potentiality as early diagnostic molecular biomarkers with application in clinical settings.

Keywords Hepatocellular carcinoma (HCC), NAFLD, NASH-HCC, Telomeres, *TERT* gene, *TERT* promoter mutations, *TERT* mRNA, Molecular biomarker, Diagnosis

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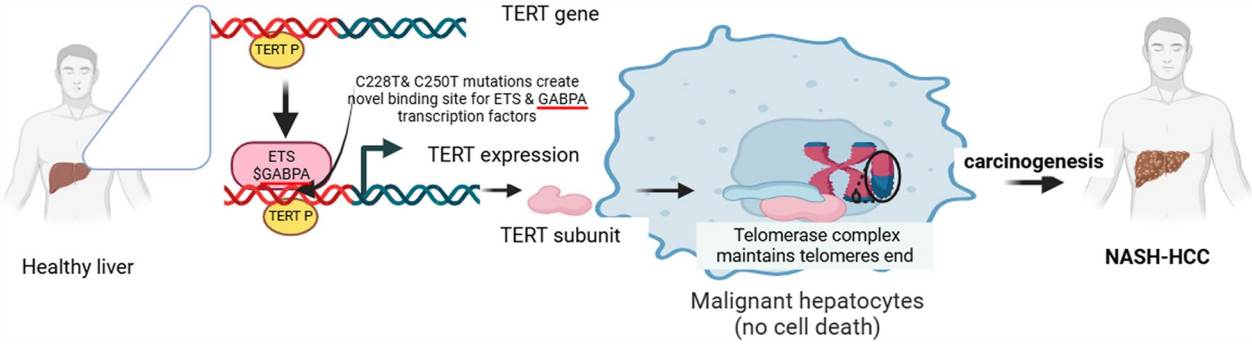
Graphical Abstract



Background

Liver cancer is ranked sixth in incidence and third most prevalent cause of cancer-related deaths globally [1, 2]. Liver cancer remains a spectrum disease with heterogeneity that encompasses hepatocellular carcinoma (HCC), intrahepatic and extrahepatic cholangiocarcinoma (iCCA and eCCA), and ampullary carcinoma (AC) among others [3, 4]. HCC is the most prevalent and aggressive subtype of liver cancer and remains the major cause of liver cancer-related mortality over decades [5–7]. About 80% of HCC cases are reported in Eastern Asia and sub-Saharan Africa [8]. Chronic hepatitis B and C virus infections, alcohol abuse, aflatoxin B1 exposure, and nonalcoholic fatty liver disease (NAFLD) are the major risk factors for the development of HCC [9]. The impact of conventional risk factors of HCC, like hepatitis B and C viral infections, high alcohol intake, and aflatoxin in HCC developments, are significantly declining, while the effect of NAFLD is emerging [10]. Risk factors for NASH-HCC development can be broadly classified as non-genetic and genetic. There are several non-genetic factors, including obesity, type 2 diabetes, dyslipidemia, sedentary lifestyle, and poor dietery habits, reported to impact NASH–HCC development. The genetic predispositions to NAFL and NASH-HCC have been analysed in recent decades, especially single nucleotide polymorphisms (SNPs) in genes related to cellular proliferation, which includes Telomerase Reverse Transcriptase (*TERT*) promoter mutations, Catenin Beta 1 (*CTNNB1*), Tumour Protein 53 (*TP53*), Activin A Receptor Type 2A (*ACVR2A*). Among these genes, the *TERT* promoter region is reported as the most prevalent in NAFL and NASH-HCC patients. Two common SNPs termed C228T and C250T, are located at − 124 bp and − 146 bp upstream of the *TERT* transcription site.

The most reported consequence of C228T and C250T SNPs is upregulation of *TERT* gene expression, which stabilises and maintains the length of the telomere's ends, which in turn prevents cancerous hepatocytes from undergoing conventional apoptosis, hence, constitutively maintains their survival. However, little is known about the actual mechanism (s) through which it upregulates hepatocarcinogenesis in NASH-HCC. Considering the global increase in the prevalence of NASH-HCC, this review presents the current studies on *TERT* promoter mutations and their impact on NASH-HCC progression in some part of the wold. This review also highlights the *TERT* promoter mutations correlating with *TERT* gene expression levels using TERT mRNA to assess their potential roles as early diagnostic molecular biomarkers for NASH-HCC since TERT activity is upregulated in cancerous cells but stable in normal cells. High expression levels of TERT subunit may represent its constitutive expression due to C228T and C250T SNPs that recruits unique transcription factors that upregulate its expression.

Nonalcoholic fatty liver disease (NAFLD)

NAFLD, or the recently renamed "metabolic dysfunction-associated steatotic liver disease (MASLD)" encompasses a range of liver diseases from nonalcoholic and non-viral -based risk factors that stimulate the initiation of benign steatosis to NASH-HCC [11–15]. NAFLD patients sometimes have steatosis without fibrosis or inflammation and are asymptomatic during HCC development [16]. The onset of NAFLD is due to hepatocyte lipid accumulation, insulin resistance, oxidative stress, dyslipidemia, inflammatory changes, dysmicrobiota, thyroid dysfunction, vitamin D deficiency, genetic predisposition, and decreased lysosomal acid lipase (LAL) activity [17, 18]. Apart from the

recognised metabolic syndromes, NAFLD is also influenced by several pathophysiological processes that further complicate its intricate genesis. This unique feature sets NAFLD apart from other HCC aetiologies, including viral infection and alcoholism.

Nonalcoholic steatohepatitis (NASH) related hepatocellular carcinoma (HCC)

Nonalcoholic steatohepatitis (NASH-HCC) is a progressive stage of NAFL described in 1980 by Ludwig and associates which is recently renamed as metabolic dysfunction-associated steatohepatitis (MASH-HCC) [15, 19]. The transformation of NAFL into NASH-HCC is impacted by associated risk factors which include a sedentary lifestyle, poor eating habits, metabolic syndromes, and genetic predispositions. NASH-HCC was first explained by the "two-hit" theory, which states that lipid accumulation is the first hit while hepatocyte inflammation is the second hit. The fundamental mechanism of the "two-hit" hypothesis of NASH–HCC development involves the building up of triglyceride in hepatocytes, which causes steatosis in insulin-resistant conditions, making liver cells more vulnerable to oxidative stress, ATP depletion and endotoxin attacks, then inflammation, fibrosis, cirrhosis, and finally NASH-HCC [18, 20]. The idea of the two-hit hypothesis leading to NASH-HCC has now developed into the "multiple-hit" hypothesis, which postulates that a combination of dietary factors, genetic predispositions, insulin resistance, adipose tissue hormones, dymicrobime and epigenetic modifications [18, 21]. In addition to having a high risk rate of mortality, NASH-HCC patients usually require liver transplantation posing serious public health concerns [22–26]. One-third of the world's population is expected to be affected by NASH-HCC, with projected incidence to rise up to 56% in the next ten years [26, 27]. The multiple driving risk factor NASH-HCC makes it challenging to develop generic screening guidelines for NASH-HCC without cirrhosis, suggesting the necessity of a patient-specific risk assessment based on risk variables that have been identified [28]. The progression of NAFL to NASH-HCC may be significantly reduced by treating its risk factors, such as obesity, type 2 diabetes, and dyslipidemia, as well as regulating a sedentary lifestyle and bad eating habits. On the other hand, the effect posed by somatic drivers genetic risk factors are difficult to treat due to their origin in the organism's molecular blueprint. Fortunately, early identification and understanding of their roles and mechanisms in the progression of NAFL to NASH-HCC may provide important treatment strategies in clinical settings.

Risk factors of NASH-HCC

Poor dietary habits

Certain dietary regimens have been proven to have a positive effect on NASH-HCC development. Particularly, the Mediterranean diet which is rich in monounsaturated fatty acids and high-density lipoproteins that reduces risk factors for metabolic syndrome related to NASH-HCC [29–31]. However, typically diet-related visceral obesity (diet high in fructose, fat, and cholesterol) is associated with NAFL and NASH-HCC development [32]. Recent study on mice by Hymel et al. [32] revealed that unhealthy diet composition appears to drive the healthy liver progression to NASH-HCC rather than the organismal effects of obesity. This study is the only relevant study associated with poor feeding habit and NASH-HCC development as no study on human yet. However, about half of a decade, European Guidelines Association for the Study of the Liver, (2016), recommended daily protein intake of 1.2–1.5 g/kg of body weight/day for all with NASH-related cirrhosis and modest caloric reductions of 500–800 kcal/day in combination with a physical activity/exercise programme in obese patients [33, 34]. This recommendation could reduce the burden of obesity caused by unhealthy eating and may ultimately contribute to reducing the progression of healthy liver to NASH-HCC.

Type 2 diabetes

Type 2 diabetes is significantly reported as one of the risk factors for NASH-HCC in various populations. However, the underlying mechanisms by which T2D promotes NAFLD spectrum and NASH-HCC inclusive is/are still not completely understood [35]. Some studies revealed an increase in the risk of developing NASH-HCC by T2D patients. For instance, Bril and Cusi [36] reported that NAFLD spectrum occurs in high prevalence among type 2 diabetes patients (up to 70–75%) and are at higher risk of developing NASH-HCC and twofold to fourfold increased risk of developing severe liver-related complications (cirrhosis, liver failure, and HCC). Recently, Castera et al. [37] proved 58% higher risk of developing HCC in patients with NASH compared to patient having other aetiologies. In fact, some epidemiological evidence shown that there is a bidirectional correlation between NAFLD/NASH-HCC and type 2 diabetes where NAFL occurs before and/or speeds up the development of type 2 diabetes [38]. However, it is unclear whether NAFL drives T2D, or if hyperglycemia/hyperinsulinemia pushes NAFL towards an advanced stage such as NASH-HCC indicating that the pathological processes are most likely interwined [39]. One most recent meta-analysis comprised of 395 studies on the association of the incidence and prevalence of NAFL/NASH-HCC and T2D reported

the significant influence of T2D in NAFLD/NASH-HCC pathologies [40].

Obesity

NASH-HCC pathogenesis is influence by obesity particularly central obesity. Recent studies indicate that central obesity, usually measured by either waist circumference or waist-to-hip ratio, is more essential in the pathogenesis of NAFLD/NASH-HCC than generalised obesity [41, 42]. In addition to having a higher chance of developing NAFLD, obese people are also more likely to advance to NASH-HCC [43]. South Asian cities have the highest prevalence of generalised obesity, according to a cross-sectional study conducted across four geographic regions [44]. This could significantly contribute to the overall statistics on the NAFDL and NASH-HCC in the region surpassing other regions across the globe.

Dyslipidemia

Dyslipidemia is clinically manifested as increased in serum triglyceride and low-density lipoprotein cholesterol levels and decreased high-density lipoprotein cholesterol levels [45]. Dyslipidemia in NASH-HCC is characterised by increased low-density lipoprotein (LDL) cholesterol, decreased HDL cholesterol, and increased serum triglycerides [46, 47]. The global prevalence of dyslipidemia and hypertriglyceridemia among NAFLD/NASH-HCC patients was estimated to be 72.1% and 83.3%, respectively [48]. This may associate with overall NAFLD pathogenesis and NASH- HCC development together with other risk factors such as T2D, poor diet and sedentary life styles further necessitating more studies on NASH-HCC studies in diverse populations.

Sedentary life style

Sedentary life style is another important emerging NASH-HCC risk factor that affects both work class individual as well as non-working class individuals including children who mostly engaged in watching movies over an extended period of time. The prevalence of NAFLD in western world and Asian countries is high, which is presumably due to several factors such increasingly sedentary lifestyle coupled with as socioeconomic growth, urbanisation, and poor health awareness [49, 50]. A sedentary lifestyle is a key risk factor that can significantly influence NASH-HCC. Research and awareness creation on sedentary life and NASH-HCC pathogenesis has potentials in reducing its burden in both studied population and underrepresented regions across the globe.

Dysmicrobiome

Microbiome consortiums are receiving research attention uncovering their roles in disease pathogenesis, potential diagnostic markers, and therapeutics. Changes in microbiome compositions can influence chronic human diseases, and the efficiency of therapies has driven efforts to develop microbiota-centred therapies such as first and next-generation probiotics, prebiotics, postbiotics, microbiota editing, and faecal microbiota transplantation [51].

Gut microbiota is the second largest microbiota in the human body and has a significant impact on human health recent evidence suggests that dysbiosis may be associated with the development of NAFLD/MAFLD [52]. The GM and their by-products influence the progression of NAFLD/NASH to NASH-HCC through modulating variable factors including the rate of gut epithelial permeability, endogenous alcohol formation, choline metabolism, bile acids metabolism as well as the liberation of proinflammatory cytokines [53, 54]. It will be too complex to account for the types of organisms that impact NASH-HCC development due to the large number of identified organisms and diverse species. To mention a few, one animal-based study showed that endotoxemia caused by *P.gingivalis* was found to alter glucose/lipid metabolism that may contribute to the progression of the MAFL/NAFL to NASH-HCC [55]. Interestingly, understanding GM might aid in inventing innovative approaches such as the sequencing of GM genes along with the application of machine learning would help in the isolation of crucial diagnostic biomarkers and identification of therapies associated with NASH-HCC/MASH-HCC [56].

Epigenetic aspect of NASH-HCC

Epigenetics simply refers to the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence [57, 58]. The common types of epigenetic processes are DNA methylation, histone modifications, and small non-coding RNA modulations. Most of these epigenetic processes are reversible. This feature renders epigenetics to be a promising diagnostic and therapeutic tool for the treatment of a wide range of diseases [59]. NASH-HCC is currently one of the emerging HCC and attracts researchers to establish innovative diagnostic, prognostic, and therapeutic approaches. One area of interest is the potentialities and implications of epigenetics landscapes in NASH-HCC patients. Epigenetic processes such as DNA methylation, histone modifications, and microRNAs are becoming recognised as risk factors associated with the development and progression of NAFL to NASH-HCC. Their dysregulations appeared to be potential therapeutic options and non-invasive biomarkers [60]. Recent studies have shown that DNA methylation, which was directly associated with NAFLD's

inflammatory grading, lipid synthesis, and oxidative stress, plays a crucial role in the initiation and progression of NAFL to NASH-HCC [61]. Some basic observatory studies on the aspect of epigenetics and NAFLD/NASH-HCC pathogenesis provided literal knowledge, however, much needs to be uncovered to have a deep understanding of their mechanisms. For instance, Pirola et al. [62] characterised NASH-HCC patients based on identified severity enhanced by hepatic DNMT levels which induced methylation on NADH dehydrogenase 6 (MT-ND6) in comparison to patients with simple steatosis. Another interesting study identified HDAC8 as a key player in NASH/MASH-HCC development by driving oncogenic pathways and chromatin modifications [63]. Contrarily, over ten years back study on HDAC8 knocked down showed significant reverses of IR and decreased tumorigenicity associated with NAFLD/MASLD [64]. This study contradicted most of the reported pathogenic effect of the HDAC8 modifications, but lack sufficient explanation on the mechanism which hinders general conclusion. Additionally, research has demonstrated that the deacetylation of histone H4 lysine 16 leads to the suppression of cell death-related genes, contributing to the initiation of NAFL and its progression to NASH-HCC [65]. Another study reported that altered expression and function of histone acetyltransferase and histone deacetylase enzymes upregulates hepatic metabolism and cellular transformation in NAFLD spectrum [66]. Furthermore, methylation of some genes has been identified as potential blood biomarkers for the diagnosis of liver fibrosis in NAFLD pathogenesis, such as CISTR, IFT140, and RGS14 [67]. Studies on the roles of epigenetics modification in NASH-HCC are at infancy stage, this necessitates more research in this context to uncover the impact of epigenetic landscapes especially on the relevant genes associated with NASH-HCC development, diagnosis, and therapeutic targets.

MicroRNA (miRNAs) roles in NASH-HCC

miRNAs deserve special discussion among the epigenetics processes impacting NASH-HCC due to its unique features. They are the dominant type of small non-coding RNA with approximately 22nt length which are regulators of protein expression from through degradation of targeted mRNA [68]. The remarkable stability of miRNAs renders them potential as novel targets and non-invasive biomarkers for various diseases like NAFLD/NASH-HCC [69]. Dysregulated miRNAs can disrupt the balance between lipid accumulation and clearance, exacerbating inflammatory responses, and promote fibrogenesis, thus advancing the severeness of NAFL from simple steatosis to more complex NASH-HCC [70]. This agrees with the fact that some evidence supported the role of miRNAs in the epigenetic deregulation of metabolic processes in NAFL, NASH, and NASH-HCC [71]. Furthermore, hepatic and circulating miRNA signatures are related to all hallmarks of NAFL, up to NASH-HCC [72, 73]. MiR-122 is the most abundant miRNA in human hepatocytes and has been proposed by several studies as a potential biomarker of NASH and NASH-HCC severity [73]. Contrarily, several reports showed an opposite association in the liver, with hepatic miR-122 expression downregulated in NAFL and NASH, both in a human patient and animal models [74, 75]. Recent studies reported that miR-19a, miR-19b, miR-21, miR-125b, miR-192, and miR-375 are upregulated in NAFL/NASH and NASH-HCC conditions than the normal control individuals [76]. In contrast to this study, a decade ago Takaki et al. (2014) reported that a low level of miR-122 has been pointed out as a direct inducer of NASH-HCC [77]. A recent study by Fernández-Tussy et al. [78], on the role of hepatic miR-33 during the progression of MASLD and the development of MASH-HCC shown suppression of hepatic miR-33 may be an effective therapeutic approach to impacts the development of NASH-HCC/MASH-HCC.

Genetic mutations and development of NASH-HCC

Understanding the somatic driver genetic landscape associated with NASH-HCC has advanced significantly in recent years. Numerous genetic variations and expression of somatic genes are found to link to the pathophysiology of NAFLD and NASH-HCC severity. The development of NASH-HCC is significantly impacted by genetic predispositions and expression status, especially presence of certain somatic genes pathogenic SNPs and their dysregulation. The prevalent of those SNPs and expression of the variant proteins could render individuals to be more susceptible to the development of NASH-HCC. The overexpression of metabolic pathways related genes, especially those related to metabolisms of lipids, carbohydrates and metals in the liver, are linked to certain SNPs in specific genes. The most extensively studied of these genes and their variants are patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*), membrane-bound O-acyltransferase domain-containing 7 (*MBOAT7*), hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*), and glucokinase regulatory protein (*GCKR*) genes. In addition to the SNPs of mentioned metabolically associated genes, most recent research focus on driver mutations in genes associated with cellular growth and development predisposing individuals to the development of NASH-HCC. Among the related driver genes that are very common in NASH-HCC cohorts with significant mutation prevalence are *CTNNB1*, *TP53*, *ACVR2A*, and *TERT* promoter SNPs [12, 79–81]. Among

these, genes TERT promoter mutation is the most prevalent (Fig. 1).

Telomerase and *TERT* subunit complex

TERT promoter mutations have recently been the research subject because of their critical role in the NASH-HCC pathogenesis. Telomerase is a multi-enzyme complex made up of the Telomerase Reverse Transcriptase (*TERT*) subunit, the Telomerase RNA Component (*TERC*), and other auxiliary proteins which include Telomeric Repeat-Binding Factor 1 (TRF1), Telomeric Repeat-Binding Factor 2 (TRF2), TRF1-Interacting Nuclear Protein 2 (TIN2), Repressor/Activator Protein 1 (RAP1), Telomere Protection Protein 1 (TPP1), and Protection of Telomeres 1 (POT1) which aid in telomere synthesis and length maintenance [82]. The TERT subunit catalyses the insertion of the DNA sequences (TTA GGG) to newly replicated telomeres, whereas TERC subunit supplies the template for creating repeated DNA sequences (TTAGGG). These two subunits work together to prevent telomere length from degrading during traditional cell division [83].

TERT gene description

The *TERT* gene has 41 kb nucleotides located on the p arm of chromosome 5 (5p15.33) with 16 exons and 15 introns [84–86]. It is located on the antisense strand of chromosome 5; this feature may have an impact on its telomere's end length maintenance ability. Its functional active coding sequence (CDS) starts 80 base pairs upstream from the transcription start site (TSS). The *TERT* gene is also called Telomerase Protein 2 (TP2) and Chromosome 5q Telomere Maintenance Mechanism 9 (CMM9). *TERT* gene transcribes two mRNA variants, which encode the TERT protein subunit with 1,132 amino acids sequence having two major isoforms [87]. The fully expressed *TERT* subunit possesses four domains, namely, the N-terminal essential domain (TEN), the reverse transcriptase domain (RT), the RNA-binding domain (TRBD), and the C-terminal extension (CTE) [88–91]. Each of these domains contributes differently to enable overall *TERT* subunit catalytic activity. The N-terminal domain attaches to the shorter end of the Telomere, the RT domain catalyses the addition of DNA repeats using TERC as a template, the TRBD binds to the *hTERC* subunit, while the CTE is involved in protein folding and the regulation of *TERT* activity. Since the earliest metazoans, the structural arrangement of *TERT* has remained remarkably stable throughout evolution [92, 93]. High conservation of the *TERT* gene and low genetic diversity may be significantly impacted by even small changes at the *TERT* locus, creating a chance to affect telomere biology [88, 94]. The *TERT* gene structure and its expressed catalytic subunit are depicted in Fig. 2 below.

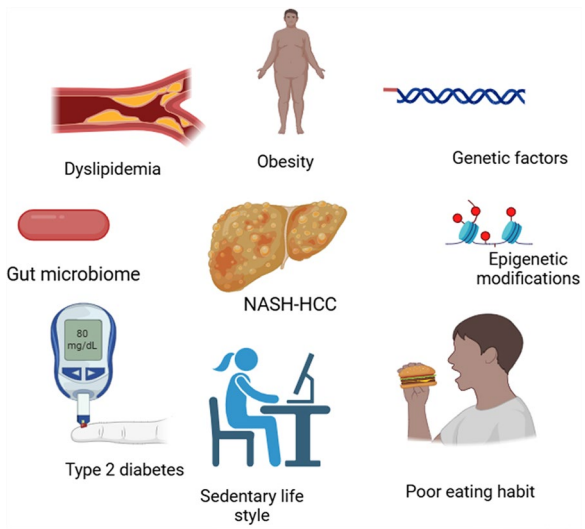


Fig. 1 Risk factors of NASH-HCC (Created with BioRender.com)

TERT catalytic subunit biology in normal cells

Telomere's length gradually shortens in normal cells after each cycle of cell division; this shortening initiates signals that cause the cell to either cease dividing or undergo apoptosis [95]. Telomerase maintains telomere length by incorporating the telomere DNA repeat (TTAGGG) [87]. There are notable differences in *TERT* activity levels between malignant and normal cells. *TERT* subunit activity is tightly regulated in healthy adult somatic cells, which correlates to reduced telomerase activity. This lower activity acts as a signal to initiate apoptotic pathways [96]. Most adult cells, including mature hepatocytes, typically have decreased *TERT* activity after each cycle of normal cell division [97].

The lower activity of *TERT* in the majority of cell types encourages a limited cell cycle and aids in the natural ageing process. Long-term cellular activity, genomic integrity, and telomere stability depend on *TERT* activity, which limits the possibility of further proliferation [98]. TERT activity, however, is markedly increased in cells that divide quickly, such as those lining the lungs, gastrointestinal system, bone marrow, and foetal tissues [99].

TERT catalytic subunit biology in cancer cells

Unlike in normal cells, *TERT* activity is unregulated in cancerous cells. The maintenance of telomere ends is highly efficient in cancerous cells due to the constant addition of DNA repeats (TTAGGG). Sustaining telomere length results from the constant insertion of DNA repeats at telomeric ends, allowing cancer cells to

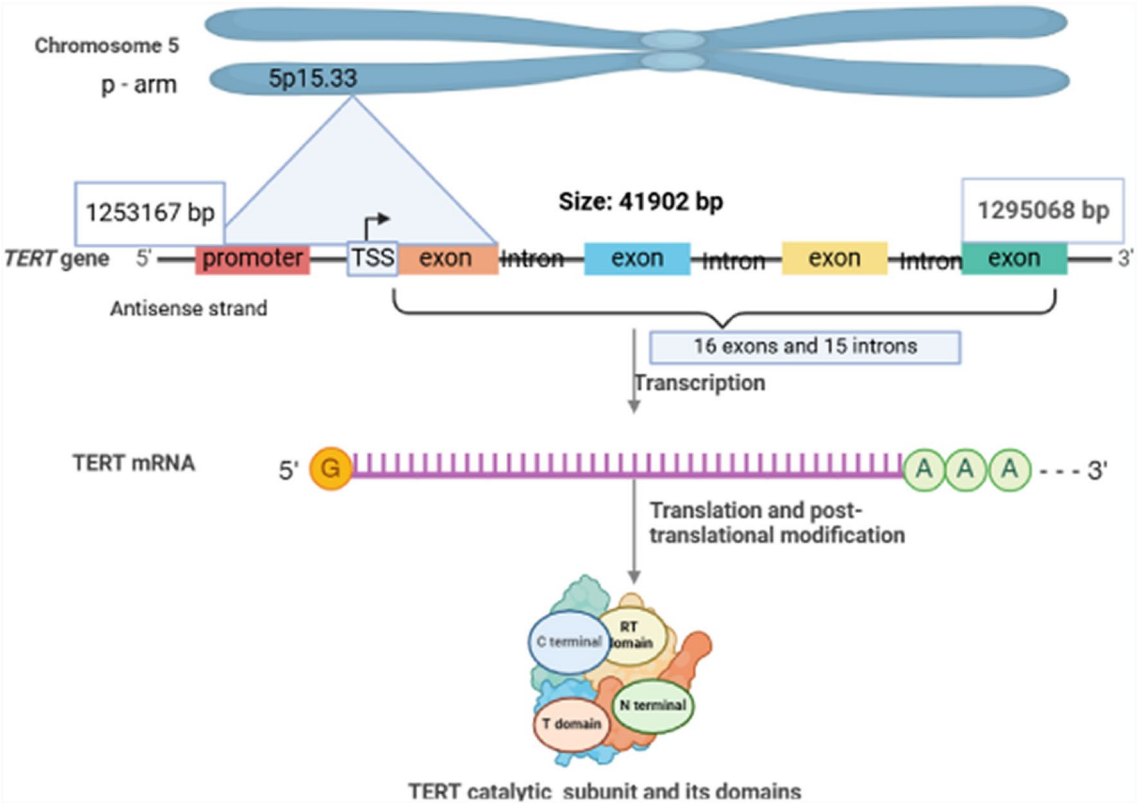


Fig. 2 Structure of *TERT* gene and its protein domains (Created from BioRender.com)

replicate indefinitely and become immortal. This feature plays a crucial role in the development and spread of cancer. Reactivated telomerase enables cells to evade telomere shortening, which results in cell immortalisation and the accumulation of genetic abnormalities that may cause cancer development [100]. Over two decades ago, *TERT* has become a crucial prospective molecular diagnostic biomarker and therapeutic target due to its additional functions in cancer progression beyond telomere maintenance [101]. Telomere dysfunction paradoxically contributes to cancer by inducing chromosomal rearrangements, even while it first inhibits tumour growth by triggering DNA-damage signalling pathways [99].

Mechanisms by which overexpressed TERT subunit drives carcinogenesis

TERT subunit stimulates oncogenesis in cancer cells via telomere-dependent and independent pathways and mechanisms [102]. Cellular immortalisation and malignant transformation depend on the maintenance of telomere length is mostly accomplished by *TERT* promoter mutations, increase in its copy number, translocation, overexpression, telomerase activation, epigenetic modifications, and alternative lengthening of telomeres (ALT) [100–106]. *TERT* promoter mutations impair telomere-TERT interactions, attract epigenetic activators, displace negative regulators, and produce new transcription factor binding sites [107]. A robust study of 31 cancer types revealed that 73% of the cases had *TERT* overexpression [108].

The normal *TERT* promoter region and its transcription factors

Numerous transcription factors are involved in the activation of the *TERT* promoter, which is highly regulated in normal cell. *TERT* promoter region is rich in guanine and cytosine (GC-rich), lacks TATA and CAAT boxes but has E-boxes that interact with transcriptional enhancers, repressors and GC-boxes that bind the zinc finger transcription factor Sp1 [84, 93, 102]. Through transcription factors such as Myc, oestrogen, SP1, nuclear factor kappa B (NF-κB), p53, activator protein 1 (AP-1), and E2F, these elements closely regulate *TERT* expression in normal cells [106]. However, tumourmany of these factors exhibit abnormal expression in tumour cells, leading to uncontrolled activation of *TERT* and overexpression of other carcinogenic pathways [107, 109–111].

Mutations in the *TERT* promoter

The *TERT* gene is essential for cellular growth, development, and ageing, and its expression is mostly controlled at its promoter region. The two most common *TERT* promoter mutations known as C228T and C250T SNPs are located at positions − 124 (G > A) and − 146 (G > A) upstream of the ATG translation start site, respectively [112, 113]. Several studies have reported that the mutation at the -124 bp hotspot, located in the gene's core promoter region, is more prevalent than that at the -146 bp hotspot [79, 107, 113–121]. Cong et al. and Wick et al. [84, 85] investigated the role of *TERT* promoter mutations in cancer cell immortality with transient transfection experiments using *TERT* promoter-luciferase reporters. This study revealed that the *TERT* promoter is inactive in both normal and transformed pre-immortal cells, while it is activated in immortal cells. Furthermore, *TERT* promoter mutations allow escape from cellular senescence by facilitating ectopic expression of *TERT* in various cancer types [122].

The normal *TERT* promoter region and its transcription factors

Numerous cancers have mutant *TERT* promoters that cause overexpression by recruiting transcription factors that are typically not involved in *TERT* expression regulation. This leads to aberrant telomerase activation [123–127]. The wild-type *TERT* promoter region lacks the new binding sites for transcription factors like E-twenty-six (ETS) and GA-binding protein alpha (GABPA), whose binding upregulates *TERT* gene overexpression [128, 129]. Lombardo et al. [130] found an activator protein 2 (AP2) consensus sequence with novel mutation, − 297 (C > T), that is recognised and bound by the AP2 transcription factor in 7.5% of HCC cases, which further activates oncogenesis. Several TERT promoter's SNPs from HCC patients were recently identified with no known effects. Some may have advantageous or detrimental consequences on the variant carrying them. This suggests further studies that may justify the impacts of the novel TERT promoter mutation other than the categorised ones (Fig. 3).

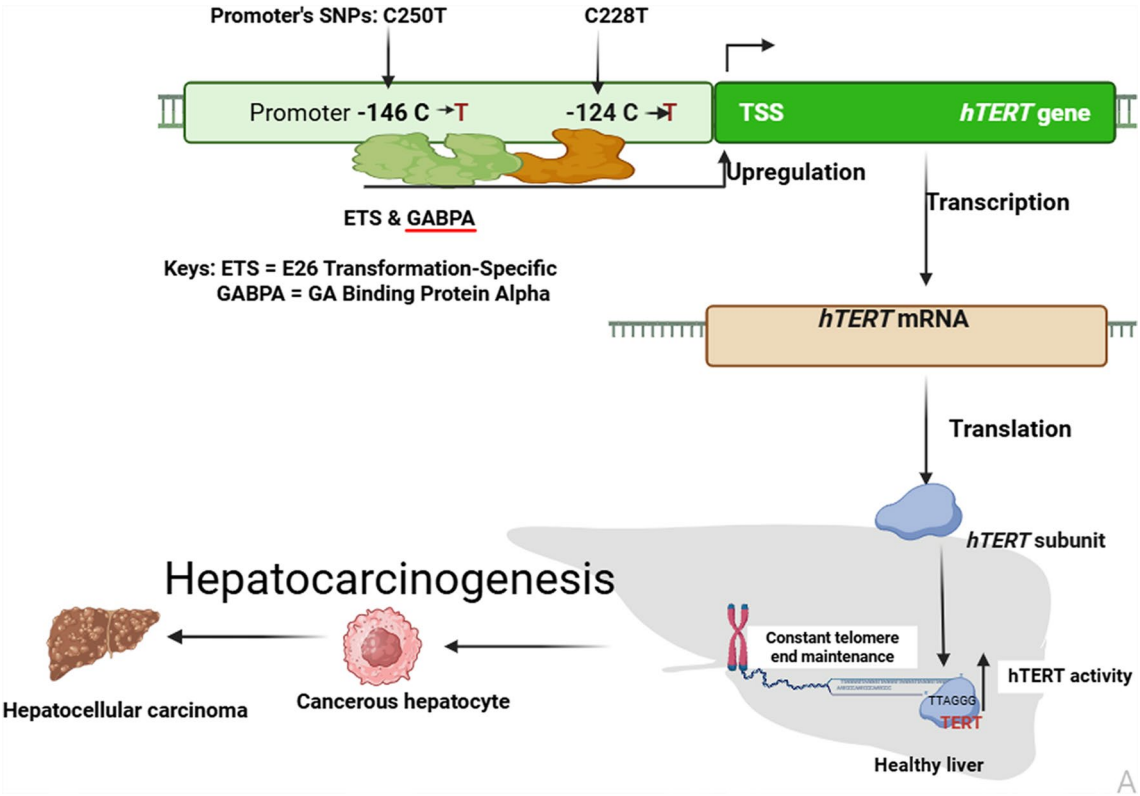


Fig. 3 Proposed mechanism by which (C228T) and (C250T) promote carcinogenesis of healthy to NASH-HCC (Created from BioRender.com)

TERT promoter and novel transcription factor binding sites

Numerous cancers have mutant *TERT* promoters that cause overexpression by recruiting transcription factors that are typically not involved in *TERT* expression regulation. This leads to aberrant telomerase activation [26, 122, 125]. The wild-type *TERT* promoter region lacks the new binding sites for transcription factors like E-twenty-six (ETS) and GA-binding protein alpha (GABPA), whose binding upregulates *TERT* gene overexpression [127, 128]. Lombardo et al. [130] found an activator protein 2 (AP2) consensus sequence with novel mutation, −297 (C > T), that is recognised and bound by the AP2 transcription factor in 7.5% of HCC cases, which further activates oncogenesis. Several SNPs from HCC patients were recently identified with no known effects. Some may have advantageous or detrimental consequences on the variant carrying them. This suggests further studies that may justify the impacts of the novel TERT promoter mutation other than the C228T and C250T.

Prevalence of *TERT* promoter mutations in different types of cancers

Different cancers have been found to harbour mutations in the *TERT* promoter region, which plays a crucial part in the early phases of tumour growth by reactivating and increasing TERT subunit activity [131, 132]. The *TERT* promoter mutation was first identified in cutaneous melanoma [133]. Huang et al. and Killela et al. [114, 134] uncovered variations in the frequency of *TERT* promoter mutations by tumour type, ranging from 64 to 80% in melanoma, ~ 84% in glioblastoma, ~ 65% in bladder cancer, and 32–45% in HCC. On the other hand, intestinal cancer [135], acute myeloid leukaemia, non-Hodgkin's lymphoma [136, 137], testicular cancer [138] and some types of breast cancer [139] are among the tumours that have a low prevalence of *TERT* promoter mutations in most studies.

TERT promoter mutation prevalence in HCC

TERT promoter mutations are thought to be early events in the development of HCC and are commonly observed in both low- and high-grade dysplastic nodules [134]. Somatic *TERT* C228T and C250T TERT promoter's mutations are the most common mutations in HCC with a prevalence ranging from 30 to 60%, indicating its well-defined landscape [140]. Environmental factors like aflatoxin B1 (AFB1) and high alcohol intake impact the geographic variability associated with predisposition by *TERT* promoter mutation and prevalence of HCC [141]. Several studies on C228T and C250T associated with HCC mainly focussed on major risk factors of HCC, such as hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol-related, reflecting their historical significance in HCC development. For instance, a comprehensive exome sequencing study conducted by the International Cancer Genome Consortium (ICGC) [142] states that C228T and C250T in over 68% of HCC cohorts are independent of the ancestry of the patients. While, Jiao et al. [143] reported that C228T and C250T as the most prevalent genetic mutations in HCC cases, with a mutation rate above 60%, contrasting other types of cancers with lower prevalence. Contrarily, Pezzuto et al. [141] identified C228T and C250T in HCV-related HCC cohorts with a prevalence of 44.8% in the US and 69.7% in Asia; prevalence was higher than in HBV-related HCC in the US (21.4%) and Africa (45.5%). While the above studies categorised the HCC-related aetiologies, some research did not adequately specify the aetiologies, particularly in terms of the inclusion and exclusion criteria for the study cohort selection. For example, Lee et al. [120] found that C228T and C250T were more prevalent in HCV-related HCC infection (83.3%) compared to alcohol-related HCC and other unspecified risk factors. Nault et al. [79] reported C228T and C250T frequencies in HCC of 44–59% without specifying the underlying risk factors. Furthermore, a comprehensive literature review by Pezzuto et al. [141] on the frequency and geographical distribution of C228T and C250T in 1,939 primary HCC cases across four continents reported higher prevalence from Europeans (56.6%) and Africans (53.3%) compared to Asians (42.5%) and Americans (40%).

Mutations in the *TERT* promoter in NASH-HCC cases

Currently, few researches focused on comparison of prevalence of C228T and C250T in HCC patients from different aetiologies. This could result from less attention given to NAFLD spectrum and NASH-HCC, respectively, compared to other traditional aetiologies such as viral and alcohol-related HCCs. More studies are needed to identify the link between TERT promoter mutations and NAFLD development and progression to NASH-HCC, since NASH-HCC is currently the fastest-growing risk factor of HCC worldwide. The necessity for more studies on *TERT* promoter mutations C228T and C250T (SNPs) in NASH-HCC cohorts is realised after observing the recent studies that showed the importance of SNP variation and potentially impacting the increased global prevalence of NASH-HCC which, coupled with the lack of a universally established molecular diagnostic approach for early NASH-HCC detection. In contrast, well-established risk factors like hepatitis B virus (HBV) and hepatitis C virus (HCV) have several established serological and molecular methods of detection, highlighting the necessity of predictive biomarkers for NASH-HCC. Recently,

Akuta et al. [131] found that the *TERT* C228T mutation is more common in the Japanese population with NAFLD than in HBV and HCV infections. These studies imply that mutations in the *TERT* promoter region play an essential role in NASH-HCC development.

In contrast, Pinyol et al. [144] reported no significant difference in the prevalence of the *TERT* C228T mutation between patients with NASH-HCC and those with HBV, HCV, and alcoholic-related HCC in the Spanish population. By considering the importance of comparative studies, more studies are needed to facilitate the validation of diagnosis methods for NASH-HCC using TERT promoter SNPs.

Prevalence and impact of *TERT* promoter mutations C228T and C250T (SNPs) in NASH-HCC.

NASH-HCC is currently a leading cause of chronic liver disease posing serious public health concerns across the globe [24–26]. One-third of the world's population is expected to be affected by NASH, with projected incidence to rise to 56% in the next ten years [27]. According to Estes et al. [145] almost a decade ago, Markov model analysis predicted that NAFLD-NASH-HCC in the US will increase by 122% in 2030. This predicted incidence may also be applicable to regions with a high prevalence of NASH-HCC. Studies on *hTERT* C228T and C250T SNPs and NASH-HCC development is emerging with potentials of offering important molecular insights into its pathogenesis and support informed diagnosis strategies both in laboratory and clinical settings. For instance, a recent mutational profiling of NASH-HCC tumours by Pinyol et al. [144] on 80 NASH-HCC and 125 NASH samples using expression array and whole exome sequencing found *TERT* promoter (56%), to be higher than CTNNB1 (28%), TP53 (18%) and (ACVR2A) (10%) as the most frequently emerging somatic mutated genes from NASH-HCC patients. This study suggested TERT promoter mutation as the potential diagnostic marker for NASH-HCC. Another study by Ki Kim et al. [146] from Japanese patients with NAFLD-HCC using targeted sequencing of 26 genes, including 12 recurrently mutated genes from whole-exome sequencing and 14 typical HCC-associated genes and found *hTERT* promoter mutations in 9 out of 11 NASH-HCC cases. Donati et al. [147] found that Italian NAFLD-HCC cohorts significantly enriched *hTERT* mutations. Lombardo et al. [130] also identified C228T variants of the *hTERT* promoter in 41.8% of a Southern Italian NAFLD-HCC cohort. Additionally, Pezzuto et al. [148] detected and quantified TERT promoter C228T mutation with concordance rate between droplet digital PCR (ddPCR) (63.6%) and Sanger sequencing (52.1%) among NASH-HCC subtype patients. This study suggested the sensitivity of the methods as well as suggested the mutation may represent a prognostic signature in the NASH-HCC subtype of HCC. Recently Zhao et al. [149] reported that sgRNA-guided CjABE efficiently converts the mutated TERT promoter −124 C > T to −124 C in HCC cells and underscores the potential to treat NASH-HCC inclusive among real HCC patients. These novel findings not only suggest the potentiality of −124 C > T for mediation but also a potential diagnostic approach [150]. Most of these studies included HCCs from multiple aetiologies, such as alcoholism and viral infection without accurate categorization. This is associated with lack of stringent selection criteria using the underlining aetiology. These limitations further necessitate only NASH-HCC hTERT promoter's mutation-based studies to provide a comprehensive understanding of their molecular relationships and practical applicability in clinical setting (Table 1).

Practical potentials of hTERT promoter mutations C228T and C250T (SNPs) for NASH-HCC diagnosis in clinical settings

Currently, there is a high unmet clinical need for appropriate diagnostic, prognostic, and therapeutic options to tackle the emerging NASH-HCC epidemic [151]. The current gold standard is imaging tools used in diagnostic screening and prevention have limited sensitivity and accuracy, expensive for patients, invasiveness of the diagnostic surgical procedures as well as risk of complication [152].

The diagnosis of NASH-HCC is often made sometimes incidentally when routine laboratory tests show abnormal liver biochemical tests or imagining tests show hepatic steatosis or hepatomegaly [153]. The imaging modalities include ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI) with

Table 1 Available studies on identified *TERT* promoter mutations C228T and C250T (SNPs) from NASH-HCC and NAFLD patients

Population	Disease	Mutation identified	Key finding	References
Japanese	NAFLD	C228T	C228T might be an essential factor of liver carcinogenesis from NAFLD	[146]
Italians	NASH-HCC	C228T	C228T could serve as a predictive biomarker for NASH-HCC	[148]
Spanish	NASH-HCC	C228T& C250T	C228T and C250T are mutational signatures that characterises NASH-related HCC	[144]
Japanese	NAFLD	C228T	C228T is one of the predictor prognosis markers after surgical resection for liver cancer	[131]

ultrasonography as the most widely used as it is the least expensive and most available modality in NASH-HCC diagnosis [153]. Ultrasound is typically used for patients with cirrhosis, but the quality can be inadequate for HCC screening if patients are obese, a feature of most NAFLD/NASH patients [154]. Ultrasound usage is also dependent on the operator and its sensitivity may vary depending on the severity of fibrosis [154].

At present, there are no validated non-invasive molecular biomarkers available to allow for better NASH-HCC risk prediction [151, 152]. Although, alpha-fetoprotein (AFP) has been used for HCC surveillance as a gold standard in clinical practice, however, it has limited sensitivity and specificity for HCC detection, and a proportion of patients with advanced HCC do not have significant AFP secretion [155]. This suggests the difficulties in establishing broad guidelines for diagnosing NASH-HCC patients at an early stage who do not have fibrosis and or cirrhosis; instead, each patient should be assessed individually based on the clinical presentation [28]. Currently, there have been studies dedicated to finding molecular signatures and biomarkers to allow for better NASH-HCC risk prediction [152].

The ultimate goals of biomarker establishment are to implement it in clinical settings. However, establishing molecular biomarkers for clinical diagnosis is challenging due to strict validations of their efficiency, sensitivity, reliability, reproducibility and to some extent affordability by the patients. To date, no study has been validated to be a NASH-HCC biomarker in clinical settings. However, some studies suggested that the hTERT promoter is a potential biomarker for NASH-HCC diagnosis from HCC patients. For instance, a recent study by Hirai et al. [156] revealed that among the 130 NASH-HCC patients examined, 71 patients (54.6%) were positive for TERT promoter mutations in ctDNA, of which 64 patients were C228T and 10 were C250T. This study revealed that hTERT promoter mutations in ctDNA were associated with short survival and could be a valuable biomarker for predicting the prognosis of patients with advanced NASH-HCC. Another clinical-based study by Pinyol et al. [144] revealed that in mutational profiling of NASH-HCC tumours, the TERT promoter represented (56%), as the most frequently mutated gene among the set of genes studied, suggesting it to be a potential biomarker for NASH-HCC diagnosis. Another study by Quaas et al. [157] over a decade, reported that mutations in TERT promoter were up to (65%), making it the most prevalent among the studied genes, suggesting that the findings may help in translating of basic practical knowledge of HCC biology into clinical practice. Although this study did not explicitly focus on NASH-HCC patients, however, NASH-HCC patients were considered in the general HCC patients. Another HCC unclassified etiological study by Schulze et al. [158] identified *hTERT* promoter mutations alongside specific mutational signatures as a risk factor of HCC which will be useful in designing clinical trials for targeted therapy. TERT promoter mutation C228T may be a useful prognostic marker to identify patients at high risk of distant recurrence of cancer [159]. However, clinical data for the C250T mutation are still limited, with no evidence up to date to confirm its prognostic significance [159]. Despite the limited and clear NASH-HCC patient selection criteria applied by the previous studies, those studies suggested the potentiality of TERT promoter mutations as diagnostic biomarker. More speNASH-HCC studies are needed with only patients clinically diagnosed with NASH-HCC. Furthermore, strict selection criteria when recruiting the NASH-HCC patients could significantly assist for tailoring clinical-based research on *hTERT* promoter mutation and NASH-HCC disease.

Clinical potential of hTERT mRNA as a diagnostic biomarker for NASH-HCC

It is well established that cancer is a genetic disease where several genetic variations appear to alter the expression patterns of the affected gene [160]. Gene expression is a process where the genotype gives rise to the phenotype by quantifying the amount of transcribed mRNA present in a biological system [161]. Gene expression analysis is crucial for the identifying of genes and understanding their effects on cellular processes [162]. Considering proteins as the final products of gene expression responsible for determining the phenotypes and biological processes, their expression level detection can be used for cancer diagnosis, prognosis, and treatment prediction in a clinical setting [160]. However, gene expression studies are very sensitive and mostly conducted by quantifying messenger RNA (mRNA). This is due to the stability and efficiency of mRNA which appears to be a key regulator in controlling the expression of many proteins [163]. Dysregulation of mRNA stability has been associated with human diseases, including cancer, inflammatory disease, and Alzheimer's [163].

Furthermore, changes in mRNA expression profile in disease, and its correlation with clinical parameters, serve as clinically relevant biomarkers [164]. Hence, accurate determination of the mRNA levels is critically important in describing the biological, pathological, and clinical roles of genes in health and disease conditions [164]. Alwine et al. [165] earlier developed the RNA-based gene expression studies assay using the Northern Blot method. Since then, there has been progress in this field. Today more sophisticated techniques are developed for gene expression studies which

include qPCR, RT-PCR, expression microarrays, RNA sequencing (RNA-Seq) among others [166]. Understanding the expression landscape associated with NASH-HCC is significantly advancing.

Biomarkers greatly help detect HCC at an early stage, providing an avenue to identify people at high risk for its development [143]. Recent research has progressively investigated the use of mRNA in tumour, such asHCC diagnostics and treatment as a promising approach to gene therapy [159]. Taking advantage of the transcribed gene as reflection of mRNA level, its detection can provide important information about the baseline transcription levels of gene activity in both healthy and cancer conditions. Alpha-fetoprotein (AFP) has historically been the main molecular marker for HCC, although, it has not been sufficient for early-stage diagnosis [167]. Since the human *hTERT* gene is suppressed in healthy somatic cells and activated in cancer cells, this feature makes it a potential hallmark of cancer development [168]. Over a decade, El-Mazny et al. [169] found that serum hTERT mRNA correlates with tumour growth and has higher sensitivity and specificity than standard AFP for early HCC diagnosis. Miura et al. [170] demonstrated the clinical importance of serum *hTERT* mRNA in diagnosing HCC by developing a quantitative detection method. More studies on serum *hTERT* mRNA levels in NASH-HCC are required to prove that expression level of *hTERT* mRNA is a reliable biomarker for NASH-HCC.

Association of C228T and C250T SNPs and hTERT mRNA levels in NASH-HCC

hTERT gene expression level might be directly or indirectly influenced by the significant *hTERT* promoter mutation's upregulation effect as well as uncategorised and unknown SNPs. Studies on the precise genetic relationship between hTERT's C228T and C250T SNPs and hTERT mRNA levels in NASH-HCC cohorts are scarce. However, Lombardo et al. [130] found C228T and a novel unclassified SNP at − 297 bp location. After analysis of the identified mutation, he found more than a 300-fold relative increase in *hTERT* expression from NASH-HCC cohorts compared to healthy controls. Hence, evaluating the relationship between C228T and C250T and hTERT mRNA levels within the same patient's sample may provide invaluable biomarkers for early NASH-HCC detection. The delays that mostly come with the diagnosis of NASH-HCC using traditional diagnostic techniques like AFP, imaging, and tissue biopsy could be successfully addressed by coupling the detection of C228T and C250T SNPs with hTERT mRNA expression levels.

Future research directions

Considering the limited and infancy of studies on *hTERT* SNPs in NASH-HCC patients as well as its potentiality as a molecular diagnostic biomarker in clinical settings, more multi-ethnic and larger population—based researches are required to establish the statistics regarding the prevalence of C228T and C250T and their impact to the current surge of NASH–HCC across the globe. Most of the identified studies were on European and Asian populations limiting the generalisation of suggestions and hypotheses. Additionally, the multi-regional and tailored researches on C228T and C250T in future should capture the cohorts with well-established stages of NAFLD such as NASH, NASH-cirrhosis, and NASH-HCC. Such studies may shed light on the proportion of mutations at each stage as well as possibly offer crucial information about how these mutations affect the course of the NASH–HCC progression from NAFLD. Additionally, coupling the detection and validations of *hTERT* promoter mutation studies with serum *hTERT* mRNA quantification from NASH-HCC cohorts will be a crucial NASH-HCC diagnostic approach in a clinical setting. This strategy might be a breakthrough that may help in the early molecular identification of NASH-HCC, which may lessen the difficulties related to its late and poor diagnosis.

Conclusion

This review presented available studies on impact and potentials of using *TERT* C228T and C250T as an early diagnostic biomarker of NASH-HCC. However, more multi-ethnic and regional-based researches are required for its validation as a potential biomarker which may lessen the difficulties related to its poor diagnosis in clinical settings in nearest future. Finding *hTERT* promoter's C228T and C250T SNPs, possible novel SNPs and their effects on TERT gene mRNA expression levels as a potential diagnostic biomarker for NASH-HCC patients will not only provide its diagnostic biomarker but also facilitate the discovery of more targeted therapy.

Abbreviations

ACVR2A	Activin A receptor type 2A
AFP	Alpha-Fetoprotein
AFB1	Aflatoxin B1
ALT	Alternative lengthening of telomeres
AP-1	Activator protein 1
AP2	Activator protein 2
ATP	Adenosine triphosphate
bp	Base pair
CDS	Coding sequence
CMM9	Chromosome 5q telomere maintenance mechanism 9
CTE	C-terminal extension
CTNNB1	Catenin beta 1
E-box	Enhancer box
ETS	E-twenty-six

GABPA	GA-binding protein alpha
GC-rich	Guanine and cytosine rich
GCKR	Glucokinase regulatory protein
HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HSD17B13	Hydroxysteroid 17-beta dehydrogenase 13
ICGC	International Cancer Genome Consortium
kb	Kilobase
LAL	Lysosomal acid lipase
MASH	Metabolic dysfunction-associated steatohepatitis
MASLD	Metabolic dysfunction-associated steatotic liver disease
MBOAT7	Membrane-bound O-acyltransferase domain-containing 7
mRNA	Messenger ribonucleic acid
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
p arm	Short arm of a chromosome
PNPLA3	Patatin-like phospholipase domain-containing protein 3
POT1	Protection of telomeres 1
RAP1	Repressor/activator protein 1
RT	Reverse transcriptase domain
SNPs	Single nucleotide polymorphisms
Sp1	Specificity protein 1 (Zinc finger transcription factor)
TEN	N-terminal essential domain
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TIN2	TRF1-interacting nuclear protein 2
TP2	Telomerase protein 2
TP53	Tumour protein 53
TRBD	RNA-binding domain
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
TTAGGG	Telomere DNA repeat sequence

Author contributions
This manuscript is conceptualised by UUL, SH, SS. UUL conducted the literature search and initial manuscript drafting. ASH, SDS, SMS, KHT, RCS and MAN reviewed the manuscript and provided critical comments. All authors reviewed and approved the final manuscript before submission.

Funding
Not applicable.

Declarations

Competing interests
There are no competing interests.

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Received: 3 March 2025 Accepted: 1 June 2025
Published online: 21 June 2025

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