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Background & Aims: Telomerase activation is the earliest event in hepatocellular carcinoma (HCC) development. Thus, we aimed to elucidate the role of telomere length maintenance during liver carcinogenesis.

Methods: Telomere length was measured in the tumor and non-tumor liver tissues of 1,502 patients (978 with HCC) and integrated with *TERT* alterations and expression, as well as clinical and molecular (analyzed by genome, exome, targeted and/or RNA-sequencing) features of HCC. The preclinical efficacy of anti-*TERT* antisense oligonucleotides (ASO) was assessed *in vitro* in 26 cell lines and *in vivo* in a xenograft mouse model.

Results: Aging, liver fibrosis, male sex and excessive alcohol consumption were independent determinants of liver telomere attrition. HCC that developed in livers with long telomeres frequently had wild-type *TERT* with progenitor features and *BAP1* mutations. In contrast, HCC that developed on livers with short telomeres were enriched in the non-proliferative HCC class and frequently had somatic *TERT* promoter mutations. In HCCs, telomere length is stabilized in a narrow biological range around 5.7 kb, similar to non-tumor livers, by various mechanisms that activate *TERT* expression. Long telomeres are characteristic of very aggressive HCCs, associated with the G3 transcriptomic subclass, *TP53* alterations and poor prognosis. In HCC cell lines, *TERT* silencing with ASO was efficient in highly proliferative and poorly differentiated cells. Treatment for 3 to 16 weeks induced cell proliferation arrest in 12 cell lines through telomere shortening, DNA damage and activation of apoptosis. The therapeutic effect was also obtained in a xenograft mouse model.

Conclusions: Telomere maintenance in HCC carcinogenesis is diverse, and is associated with tumor progression and aggressiveness. The efficacy of anti-*TERT* ASO treatment in cell lines revealed the oncogenic addiction to *TERT* in HCC, providing a preclinical rationale for anti-*TERT* ASO treatment in HCC clinical trials.

Lay summary: Telomeres are repeated DNA sequences that protect chromosomes and naturally shorten in most adult cells because of the inactivation of the *TERT* gene, coding for the telomerase enzyme. Here we show that telomere attrition in the liver, modulated by aging, sex, fibrosis and alcohol, associates with specific clinical and molecular features of hepatocellular carcinoma, the most frequent primary liver cancer. We also show that liver cancer is dependent on *TERT* reactivation and telomere maintenance, which could be targeted through a novel therapeutic approach called antisense oligonucleotides.

Keywords: Liver; Cancer; Chronic liver disease; Fibrosis; TERT; Telomeres; Antisense oligonucleotides; Treatment.
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Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and the fourth leading cause of cancer-related death worldwide.[1] HCC develop more often in males (male:female =

3:1), and most often in patients with severe chronic liver disease related to various risk factors, including HBV and HCV infections, most prevalent in Eastern countries, or excessive alcohol consumption, obesity and metabolic syndrome, highly frequent etiologies in Western countries.[2] The risk of HCC increases progressively with the severity of the underlying chronic liver disease and 90% of patients develop HCC on the background of cirrhosis. During this process, HCC result from the malignant transformation of hepatocytes or their progenitors. Genomic studies on HCC tumors have shown an important molecular diversity, with 11 major signaling pathways recurrently altered in combination.[2,3] This high genomic complexity and the lack of identified oncogenic addiction could explain why HCC tumors are usually resistant to targeted therapies.[4]

At the preneoplastic steps, chronic liver diseases lead to chronic inflammation and production of reactive oxygen species resulting in increased cell turnover.[5] During this process, telomeres, the repeated DNA sequences at the extremities of each chromosome, are essential to protect the genomic integrity of hepatocytes. Telomere length is under the control of the telomerase complex composed of *TERT* (telomerase reverse transcriptase), *TERC* (telomerase RNA component) and various proteins such as the shelterin components *TRF1*, *TRF2*, *TIN2*, *RAP1*, *TPP1*, and *POT1*.[6] Since the liver is a quiescent organ, telomerase is not physiologically expressed in most adult human hepatocytes. In this context, hepatocytes undergo progressive telomere shortening during chronic liver injury and inflammation,[5,7] contributing to tumor initiation through chromosomal instability.[8] However, in the absence of telomerase reactivation, tumor progression is usually impaired because in proliferative hepatocytes, chromosome erosion induces replicative senescence.[9] Consequently, telomerase reactivation is a key event at the early step of liver tumor development and has been observed in more than 90% of HCC.[10,11] It is the most frequently altered pathway in HCC, mainly through *TERT* overexpression caused by a somatic activating promoter mutation, or less frequently by viral HBV or adeno-associated virus-2 (AAV2) insertion or chromosome rearrangements.[12–15] In rare HCCs, an alternative lengthening of telomeres (ALT) mechanism has been suggested but remains to be demonstrated.[16,17]

The role of telomere maintenance in the progression and aggressiveness of HCC is debated. Telomere length has been associated with tumor aggressiveness and patient prognosis in several cancers, but data in HCC coming mostly from Asian populations are conflicting.[17] Since telomere maintenance is altered frequently and early in HCC, it is an appealing therapeutic target. However, no therapy that efficiently inactivates telomerase has been validated in HCC, while oncogenic addiction to telomerase activation remains to be demonstrated.

Herein, we investigate the natural history of telomere alteration and telomere maintenance activation, in relation to the underlying non-tumor (NT) tissue and to tumor progression and aggressiveness, in HCC. We also explore how tumor hepatocyte proliferation is dependent on telomerase activation and how to target this process in a therapeutic setting using preclinical models of HCC.

Patients and methods

Frozen liver tumor and NT tissues were collected from 1,502 patients after informed consent in 25 academic hospitals in France (LICA-FR cohort, Table S1), the study was approved by the local Ethics Committee (CCPPRB Paris Saint-Louis IRB00003835). A total of 1,148 HCC tumors were sequenced with whole-genome sequencing (WGS, n = 46) or whole-exome sequencing (WES, n = 210), RNAseq (n = 185) and/or targeted sequencing of 31 genes using Sanger or Miseq as previously described.[12,18,19] Copy number analysis was performed as previously described[12] and manually corrected with GAP tool.[20]

Estimation of telomere length from WGS data was performed using the TelSeq pipeline[21] (n = 132, Fig. S1A, Table S1). Based on Cawthon's method,[22] we adapted a quantitative PCR method (TeloPCR) using correction for amplification efficiency[23] to measure telomere length in the whole series of 2,650 samples (supplementary methods, Fig. S1A,B, flow-chart in Fig. S2).

Transcriptomic profiling was performed in 893 out of 1,148 tumor samples using RNAseq (n = 185), and/or quantitative PCR analysis of 190 genes (n = 893[24,25]). *TERT* expression fold-change (FC) was calculated *vs.* normal liver level of expression. Tumors were classified in the G1-G6 classification as previously described for the LICA-FR cohort.[26] We used an in-house adaptation of the gene set enrichment analysis (GSEA) method.[27] Single-sample GSEA scores were calculated as gene set variation analysis (GSVA) enrichment scores, using the GSVA package.[28]

Twenty-six liver tumor cell lines derived from HCC (n = 24) and hepatoblastoma (n = 2) were previously characterized with WES and RNAseq[4] (supplementary methods, https://lccl.zucmanlab.com). To inactivate *TERT* expression, 3 different antisense oligonucleotides (ASOs) targeting *TERT* and 2 scrambled ASOs (sequences in the supplementary methods) were provided by Ionis Pharmaceuticals after a negative test of toxicity in mice. Each experiment was performed in triplicate and reproduced at least 2 times for most of the cell lines. The 12 cell lines showing the highest ASO efficiency were then treated with the 3 anti-*TERT* ASOs, 2 scrambled ASOs or no treatment until proliferation arrest, as assessed by an MTS assay in triplicate. Immunofluorescence was quantified using Operetta CLS™High-Content Analysis System (PerkinElmer).

Animal studies were performed in female immunodeficient nude BALB/c-Foxn1$^{nu/nu}$ mice (Charles River), aged 5 weeks at beginning of experiments (Authorization n°2015082610 113065.01, Ethics Committee Paris-Nord C2EA 121). The SNU-878 cell line was subcutaneously xenografted by injecting 5x10^6 cells resuspended in 50 µl of Matrigel™ (Corning) into both flanks. Once xenografts reached 200 mm^3 volume (median 28 days for engraftment), mice were randomized to receive either 100 µg ASO-09 or scrambled ASO-CC1 (control) diluted in 50 µl PBS, injected in each tumor every 48 hours.

Statistical analyses were performed with R version 3.3.2 (http://www.R-project.org), Bioconductor version 3.4 and GraphPad Prism (version 7.0).

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Telomere attrition in the liver is related to age, fibrosis, male sex, and alcohol consumption

We measured the mean telomere length in NT liver samples from 1,502 patients. 76% of patients had chronic liver diseases of varying severity (F0-F1 40%, F2-F3 21% and F4 39%), mainly related to excessive alcohol consumption (n = 495, 37%), HCV infection (n = 279, 23%) or HBV infection (n = 190, 15%); among them, 1,076 patients developed HCC (detailed in Table S1). The

mean NT liver telomere length was 5.91 kb (standard deviation 1.17 kb). We observed age-related telomere attrition ($p = 2x10^{-21}$; Fig. 1A) that was significant in livers with or without chronic liver disease (Fig. S3A) and was faster in males than females (significantly in patients >40 years-old) (Fig. 1B).

Telomere attrition was also associated with severity of fibrosis even after adjustment for age and sex (F0-F1 *vs.* F4, adjusted $p = 5.7x10^{-5}$; F2-F3 *vs.* F4, adjusted $p = 0.02$; Fig. 1C and Fig. S3B&C). Stratified analysis revealed that fibrosis severity was closely related to telomere shortening in the liver of males ($p = 1.65x10^{-5}$ for trend) and females ($p = 0.02$); it was also significant in patients below 30 ($p = 0.015$) or between 30 and 60 years old ($p = 3.87x10^{-5}$), but not in older patients. Interestingly, telomere attrition was more severe in patients with excessive alcohol consumption regardless of cirrhosis or sex (age and sex-adjusted $p = 0.002$; Fig. 1D and Fig. S3D) whereas chronic viral hepatitis, the metabolic syndrome, tobacco exposure or ethnicity did not modulate telomere length independently (Table S2). Overall, age, cirrhosis, and excessive alcohol consumption remained independently associated with shorter telomere length in multivariate analysis; in contrast, female sex was independently associated with longer telomere length (Fig. 1E).

Non-tumor liver telomere length associates with HCC diversity

We analyzed telomere length in 1,148 HCC samples from 978 patients mainly associated with excessive alcohol consumption (45%), HCV infection (25%), HBV infection (18%), metabolic syndrome (24%) or without underlying liver disease (9%) (see details in Table S1).

First, we evaluated the relationship between tumor telomere length and the paired NT liver telomere length (Fig. 2A, top panel). Overall, we observed that most of the HCCs that developed on livers with long telomeres harbored relative telomere shortening within the tumor, whereas the vast majority of HCCs that developed on livers with short telomeres harbored relative telomere elongation. Interestingly, both liver and tumor telomere length followed a 'Gaussian' distribution centered on a similar mean value of 5.65 kb *vs.* 5.70 kb in NT and tumor tissues, respectively (Fig. 2B).

Then, we compared patient and tumor characteristics of the HCCs developed on the longest *vs.* the shortest liver telomere length tertile (Fig. 2A, bottom panel). Patients with HCC developed on livers with long telomeres were more frequently female (23% *vs.* 12%, $p = 0.0004$), of African origin (13% *vs.* 6%, $p = 0.0007$), without significant fibrosis (F0-F1 30% *vs.* 17%, $p = 2x10^{-5}$), more frequently associated with hepatitis B (23% *vs.* 17%, $p = 0.04$) or without etiology (11% *vs.* 6%, $p = 0.009$). Interestingly, these HCCs were enriched in specific molecular alterations associated with liver progenitor features, such as the G1 transcriptomic subclass (13% *vs.* 4%, $p = 0.0003$), *BAP1* inactivation (6% *vs.* 0%, $p = 0.0003$), and *RPS6KA3* alteration (7% *vs.* 1%, $p = 0.002$) and higher *TERT* expression ($p = 0.02$). Moreover, these patients displayed higher levels of serum alpha-fetoprotein (AFP) (median 18 ng/ml *vs.* 8 ng/ml, $p = 0.0003$). Conversely, HCCs arising on livers with the shortest telomeres frequently developed in Europeans (92% *vs.* 82%, $p = 5x10^{-5}$), males (88 *vs.* 77%), and patients with cirrhosis (61 *vs.* 47%, $p = 0.0003$); excessive alcohol consumption (56 *vs.* 39%, $p = 6x10^{-6}$) or hemochromatosis (8 *vs.* 3%, $p = 0.003$) were more common etiologies, with

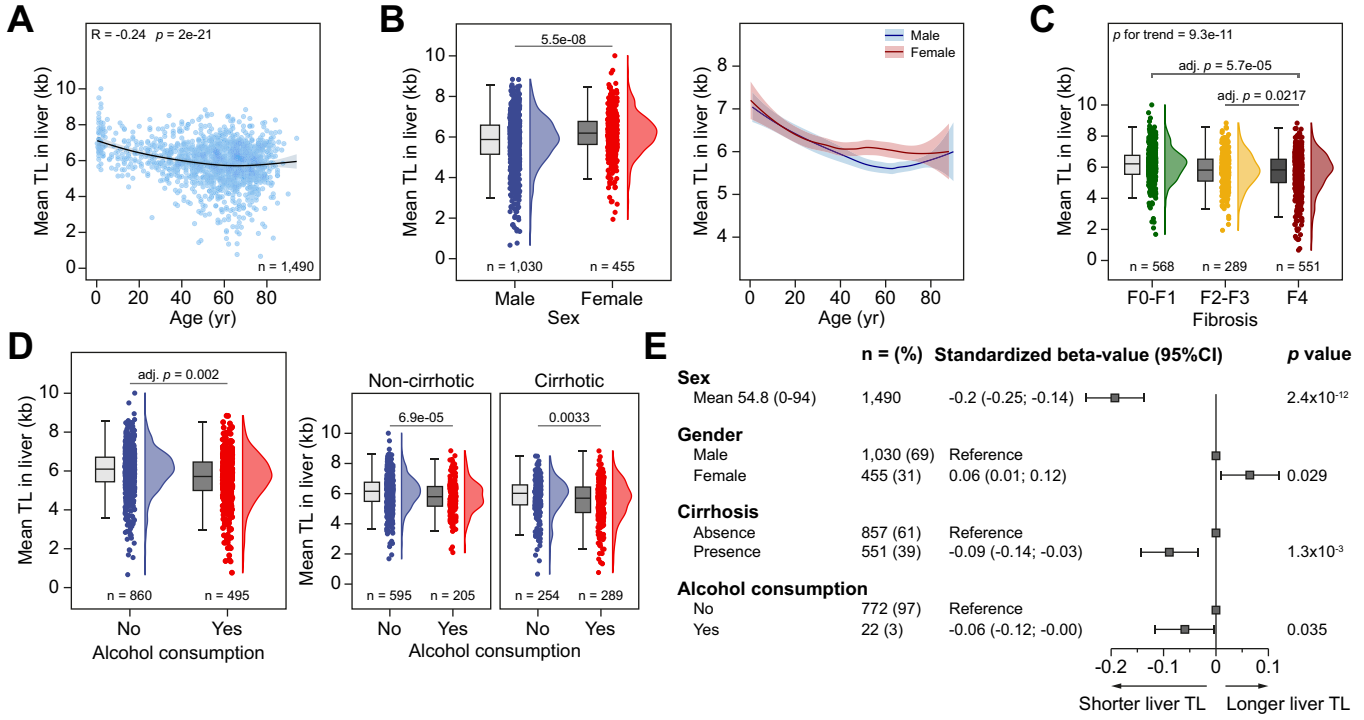

Fig. 1. Telomere attrition in the liver is related to age, fibrosis, male sex and alcohol consumption. (A) Evolution of non-tumor liver TL with age (Pearson correlation), (B) sex, (C) fibrosis METAVIR progression and (D) excessive alcohol drinking. Curve fitting (A-B) with Loess regression. Unadjusted comparisons (A-B) with Wilcoxon test, Adjusted (adj) comparisons (C-D) on age and sex with multiple linear regression, Spearman test for trend (C). (E) Forest plot of multivariate analysis (standardized beta-estimates with 95% CI; details in Table S2). TL, telomere length. (This figure appears in color on the web.)

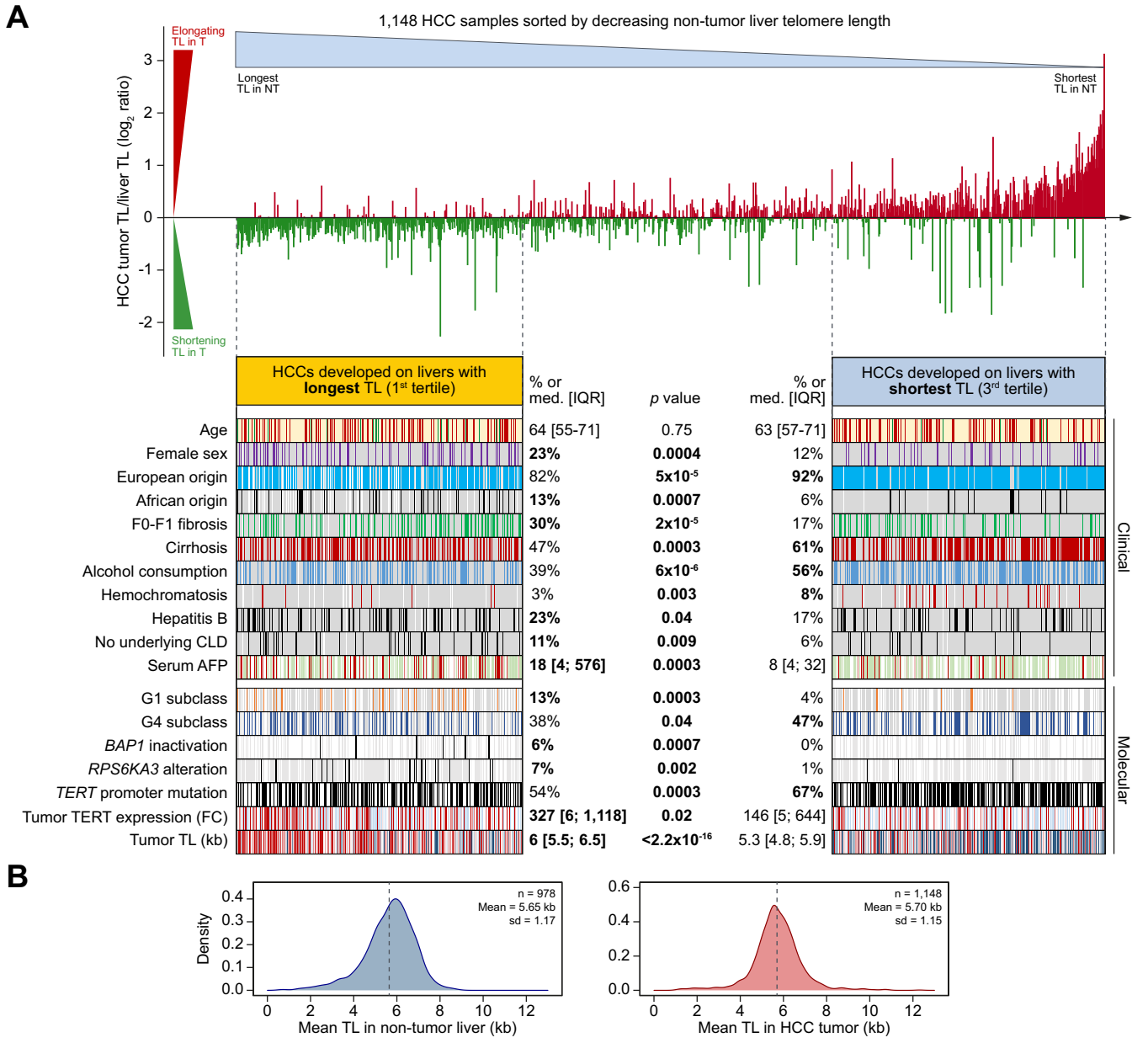

Fig. 2. Non-tumor liver telomere length associates with HCC diversity. (A) Top panel shows tumor (T)/non-tumor (NT) TL ratio in 1,148 HCC samples sorted from left to right by decreasing paired NT-TL. Green line to the negative values and red line to the positive values represent telomere shortening or elongating from NT to T, respectively. Bottom panel shows the clinical and molecular features of HCCs developed on the longest *vs.* shortest tertile of NT-TL, represented as heatmaps and compared with Wilcoxon or χ^2test. (B) Density distribution of NT-TL and T-TL in 1,148 HCCs developed in 978 patients. AFP, alpha-fetoprotein; CLD, chronic liver disease; HCC, hepatocellular carcinoma; NT, non-tumor; T, tumor; TL, telomere length. (This figure appears in color on the web.)

these HCCs more frequently belonging to the non-proliferative G4 subclass (47 *vs.* 38%, $p = 0.04$) and showing somatic *TERT* promoter mutations ($p = 0.0003$). We next sought to decipher the respective impact of *TERT* expression, *TERT* alterations and other tumor molecular features on tumor telomere length.

The mechanism of telomere maintenance in HCC associates with patient features and specific molecular alterations

First, we observed that 89.1% of HCCs harbored *TERT* mRNA overexpression (Fig. S4A, defined by a FC >10). Tumor telomere length was slightly related to the level of *TERT* mRNA expression (R = 0.1, $p = 0.0032$, Fig. S4B), and *TERT* mRNA expression did not correlate with relative telomere shortening or elongation. Moreover, tumor telomere length did not differ between HCCs with *TERT* overexpression compared to HCCs without *TERT* overexpression ($p = 0.15$).

To fully describe the somatic alterations driving *TERT* overexpression and determine whether they were associated with tumor telomere length, we analyzed 256 HCC sequenced by whole-exome (n = 210) or whole-genome sequencing (n = 46). Altogether, 78.5% of samples harbored *TERT* alteration (Fig. 3A, Table S3). *TERT* promoter mutations were the most frequent mechanism (n = 148; 57.8%), followed by chromosome 5p gain of *TERT* (copy number alteration [CNA], n = 23; 9%), chromosome

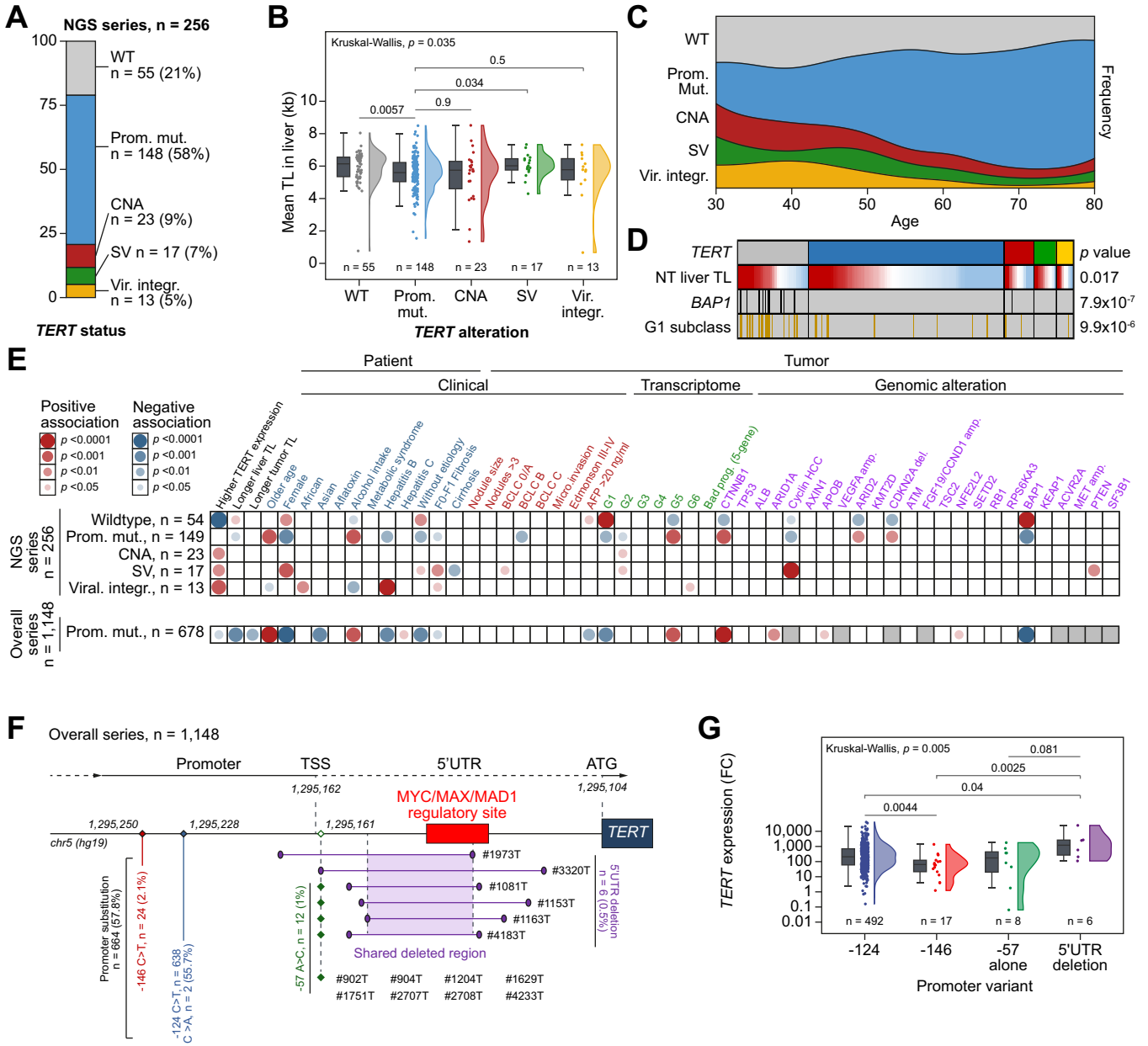

Fig 3. The mechanism of telomere maintenance in HCC associates with patient features and specific molecular alterations. (A) *TERT* alterations, (B) corresponding NT liver TL, (C) age-depend distribution, and (D) heatmap of *TERT and BAP1 gene* status, the liver progenitor G1 subclass and NT liver TL (Fisher's test for association with wild-type *TERT*). For A to D, NGS in 256 HCC (details in Table S3). (E) Mosaic plot summarizing the associations of *TERT* alterations with patient and tumor characteristics (Wilcoxon or χ^2 test, details in Table S4). (F) Summary of *TERT* promoter and 5'UTR alterations in 1,148 HCC by targeted sequencing and (G) *TERT* expression according to the promoter variant. Comparisons in (B&F) with Wilcoxon and Kruskal-Wallis test. CNA, copy number alteration; FC, fold change; NGS, next-generation sequencing; NT, non-tumor; SV, structural variations; TL, telomere length; TSS, transcription start site; UTR, untranslated region; WT, wild-type. (This figure appears in color on the web.)

structural variations (SV) involving *TERT* (n = 17; 6.6%) and HBV or AAV2 viral integrations in the *TERT* promoter (n = 13; 5.1%). *TERT* mRNA expression levels were closely related to the mechanism of alteration ($p = 7.8\text{x}10^{-11}$, Fig. S4B), with *TERT* promoter mutations showing the lowest level of expression, whereas HBV or AAV2 viral integration in the *TERT* promoter led to the highest level of *TERT* expression. *TERT* promoter mutations were associated with *CTNNB1* ($p = 1.6\text{x}10^{-4}$), *ARID2* ($p = 0.009$) and *CDKN2A* deletions ($p = 0.002$), while *TERT* SV were associated with the cyclin-HCC subtype ($p = 7.6\text{x}10^{-8}$) and *PTEN* deletion ($p = 0.001$, Fig. S5A&B).

Interestingly, the mechanisms of *TERT* alteration differed according to the level of telomere length in the corresponding NT liver (Fig. 3B & Fig. S4D). *TERT* promoter alterations were associated with shorter NT liver telomere length ($p = 9.6\text{x}10^{-5}$), whereas *TERT* wild-type tumors developed on livers with longer telomere length ($p = 0.017$). Accordingly, HCCs that developed in older ($p = 5.5\text{x}10^{-6}$) and male patients ($p = 2.8\text{x}10^{-7}$) were enriched in

TERT promoter mutations, whereas younger, female patients without significant fibrosis harbored *TERT* SV (exclusively observed in non-cirrhotic patients), CNA, viral integrations or no *TERT* alteration (Fig. 3C and Table S4).

Further exploring the *TERT* wild-type subgroup, we first observed that, strikingly, *TERT* and *BAP1* alterations (11/256, 4.3%) were mutually exclusive ($p = 5.8x10^{-8}$), and *BAP1* inactivated tumors only demonstrated weak *TERT* mRNA overexpression (Fig. S4E). Moreover, we observed a close association between the wild-type *TERT* genotype and longer NT liver telomeres, *BAP1* inactivation and the G1 transcriptomic subgroup of HCC (Fig. 3D-E & Fig. S4F).

Then, we analyzed 29 samples that displayed *TERT* mRNA overexpression (FC>10) and no *TERT* or *BAP1* alteration. Targeted sequencing of the promoter region of *TERT* revealed a novel 5'untranslated region (UTR) deletion involving the *MYC*/*MAX*/*MAD1* regulatory site in 1 sample (#1081T). Expanding analysis to the overall 1,148 HCC series showed recurrent small deletions of the *TERT* 5'UTR in 6 samples (ranging from 23 to 44 base pairs) with a minimally deleted region consistently affecting the *MYC*/*MAX*/*MAD1* regulatory site (Fig. 3E). Interestingly, all the deletions were co-occurring with A>C mutation (n = 4) or deletion (n = 2) of the -57 locus. -57 A>C mutation, adjacent to the transcription start site located at position -58, were found in 12 samples (1%). 5'UTR deletion significantly increased *TERT* mRNA expression compared to the other *TERT* promoter alterations, namely the classical hotspot mutations at -124 (55.7%) and -146 (2.1%) before ATG loci (Fig. 3F). Overall, neither the mechanism of *TERT* alteration, the promoter alteration variant nor the presence of *BAP1* mutations were significantly correlated to tumor telomere length (Fig. S4G), or to relative tumor shortening or elongation (Fig. S4H).

Thirteen tumors out of 246 HCCs with *TERT* expression data available (5.3%) – mostly developed in non-cirrhotic livers (11/13) – showed neither *TERT* overexpression nor *TERT* or *BAP1* alterations (Fig. S4I). Seven of these HCCs without *TERT* overexpression showed relative telomere elongation compared to the paired NT liver tissue. These tumors were frequently mutated for *TP53* (5/7), and displayed high chromosomal instability, but did not show mutations in *ATRX*, *DAXX* or *H3F3A* genes usually associated with the ALT mechanism of telomere maintenance. In contrast, the remaining 6 HCCs without *TERT* overexpression, *TERT* alteration nor telomere elongation, were mostly well differentiated (5/6), in female patients (4/6), developed on hepatocellular adenoma (2/6), without *TP53* alterations, and corresponded to less aggressive tumors.

Telomere elongation is associated with HCC aggressiveness

We then analyzed the relationship between HCC tumor telomere length and other molecular characteristics. We observed significant telomere elongation (Fig. S6A) and overall longer tumor telomeres (Fig. S6B) in *TP53* altered and aflatoxin-associated tumors, and in tumors belonging to the proliferative transcriptomic subclasses of HCC, suggesting that long tumor telomeres were a feature related to a progenitor/stem-cell phenotype.

In agreement, GSEA analysis of RNA-seq data from 185 HCCs within the next-generation sequencing series showed that *TERT* overexpression and pathways reflecting telomere elongation were associated with transcriptomic signatures associated with high tumor proliferation, stem-cell features, low cell differentiation and poor survival (Fig. 4A). Using quantitative reverse-transcription PCR analysis (n = 893), we further validated that both long tumor telomeres and high *TERT* expression related to high expression of genes involved in cell proliferation (*MKI67*, *CDC20*, and *RRM2*), transcription (*E2F5*, *TAF9* and *MYC*) and progenitor markers (*AFP*) (Fig. 4B and Fig. S6C).

These biological observations were also consistent with clinical characteristics in the whole series of 1,148 HCCs (Fig. 4C, and Table S5). Indeed, longer telomere length was observed in larger tumors ($p = 1.7x10^{-8}$), in multinodular HCC ($p = 2.7x10^{-4}$), displaying poor differentiation according to Edmondson grade III-IV ($p = 7.5x10^{-4}$), high serum AFP ($p = 1.4x10^{-6}$) and within the BCLC C stage ($p = 0.036$). Moreover, in patients with HCC treated by curative R0 resection (n = 578), the 10% of tumors with the longest telomere length showed shorter recurrence-free survival ($p = 0.006$) and disease-specific survival ($p = 0.009$, Fig. 4D and Table S6&7) compared to the rest of the patients with shorter telomere length. In multivariate analysis, long tumor telomere length remained an independent predictor of early recurrence risk ($p = 0.029$) and provided additional prognostic information among patients already harboring poor prognostic features such as BCLC C, Edmonson III-IV grade, *TP53* alteration or G3 transcriptomic subclass. (Fig. 4D, Fig. S6D and Table S6&7). Interestingly, *TERT* overexpression was not predictive of poorer survival, highlighting the predominant role of telomere length in enabling tumor aggressiveness.

***TERT* is a reliable therapeutic target in preclinical models**

Since telomere maintenance is predominantly driven by *TERT* activation in HCC, we aimed to test if HCC cells could be addicted to the oncogenic *TERT* activation and if inhibition of *TERT* expression could have an anti-tumoral effect in preclinical models of HCC. To this end, we evaluated the effect of ASOs specifically designed against *TERT* in cell lines derived from 24 human HCCs and 2 hepatoblastomas (detailed in the supplementary methods). *TERT* mRNA was over-expressed in all the cell lines at a variable level (ranging from 18.2 FC to $1.3x10^5$ FC), Cell lines harbored a *TERT* promoter mutation (n = 19), a HBV integration in *TERT* promoter (n = 2) or no *TERT* alteration (n = 5).

Treatment of the cells with 10 μM of 3 different *TERT* ASOs (see Methods) showed free uptake and an efficient extinction of *TERT* expression (≥50% reduction) in 16 out of the tested 26 cell lines (Fig. S7). Efficiency of *TERT* extinction was not related to the baseline level of *TERT* expression but it was higher in cell lines belonging to the recently described CL3 subclass of liver cancer cell lines,[4] associated with high cell proliferation and poor differentiation.

Then, we showed in the 12 cell lines with the highest *TERT* extinction that treatment with all 3 anti-*TERT* ASOs induced cell proliferation arrest in all tested cell lines after a variable duration of treatment ranging from 3 to 16 weeks compared to no treatment and 2 scrambled control ASOs (Fig. 5A&5B, Fig. S8A). *TERT* mRNA expression levels or baseline telomere length were not associated with the time to proliferation arrest. Finally, stopping anti-*TERT* ASO treatment after proliferation arrest in the SNU-398 cell line showed that effects of *TERT* mRNA extinction on proliferation arrest were reversible (Fig. S8B) and that proliferation was dependent on *TERT* expression.

We selected 2 cell lines with different timing of cell arrest, SNU-398 (5 weeks) and Mahlavu (16 weeks), but similar molecular features (both showed similar baseline *TERT* expression

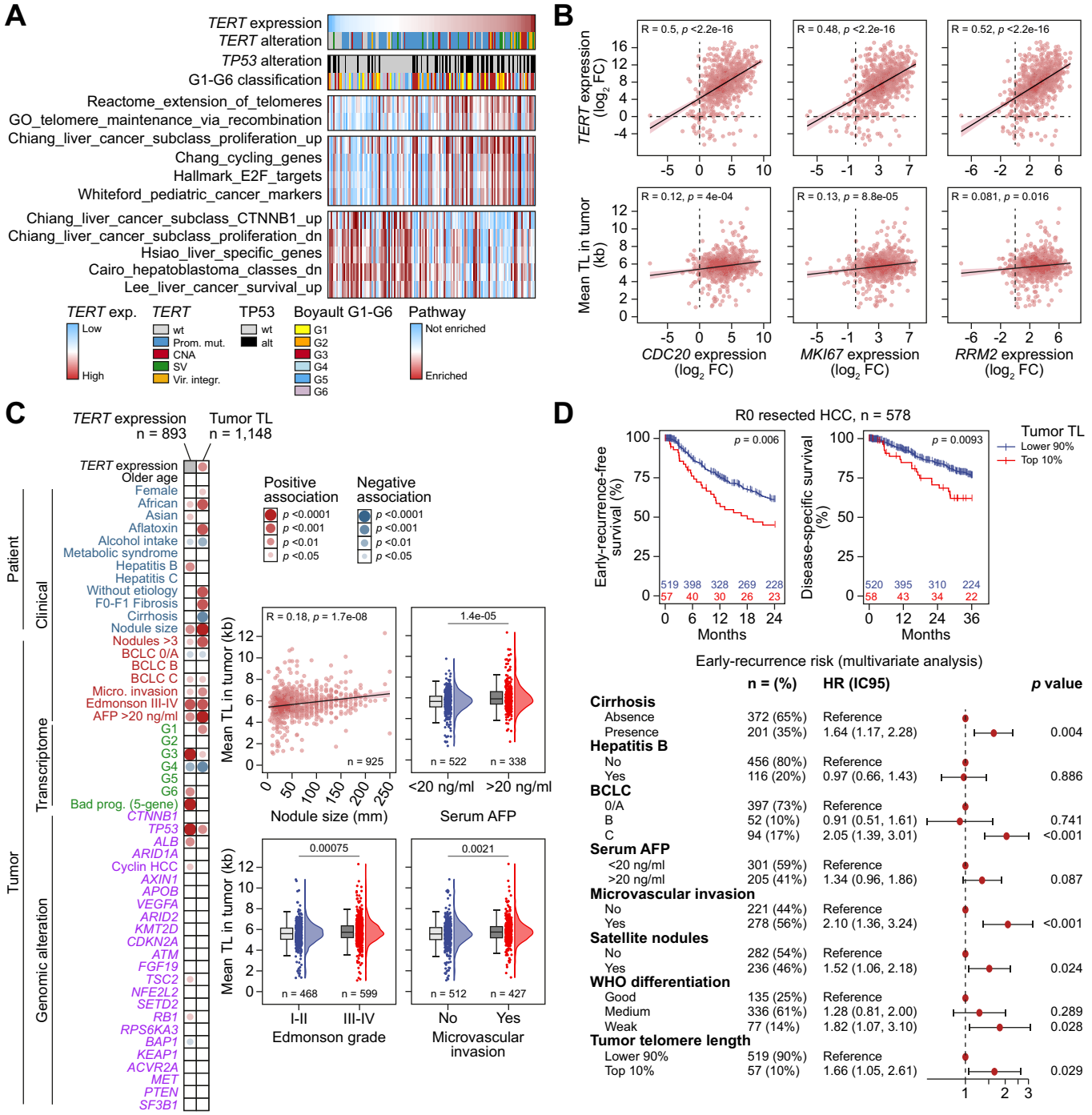

Fig. 4. *TERT* overexpression and long tumor telomeres are associated with HCC tumor aggressiveness. (A) Heatmap of single-sample GSEA analysis (n = 185 with RNA-Seq) sorted from left to right by increasing *TERT* expression. (B) Correlation of *TERT* expression and tumor TL with proliferation markers (Spearman correlation, n = 893 with quantitative PCR) and (C) patient and tumor features (Wilcoxon or χ^2 test, details in Table S5). (D) Kaplan-Meier curves (*top*) and multivariate Cox regression survival analysis (*bottom*) according to the percentile of tumor TL after curative R0 resection of HCC (n = 578, details in Table S6&7). FC, fold change; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; RNA-Seq, RNA sequencing; TL, telomere length. (This figure appears in color on the web.)

and telomere length, *TP53* and *TERT* promoter mutations), to study the phenotypical changes occurring after prolonged *TERT* knockdown. In both cell lines, *TERT* expression was consistently knocked down. We observed progressive telomere attrition that coincided with increased expression of DNA damage markers, accumulation of phosphorylated histone H2Ax foci and apoptotic markers such as cleaved caspase 3 staining and TUNEL assay, and decreased EdU incorporation (Fig. 5C&D and Fig. S9A). In addition, we analyzed the transcriptomic modifications following *TERT* inhibition with ASO. Interestingly, at the time of maximal anti-proliferative effect, we observed an increased expression of several genes of the IL6/JAK/STAT pathway (such as *IL6*, *CCL5*,

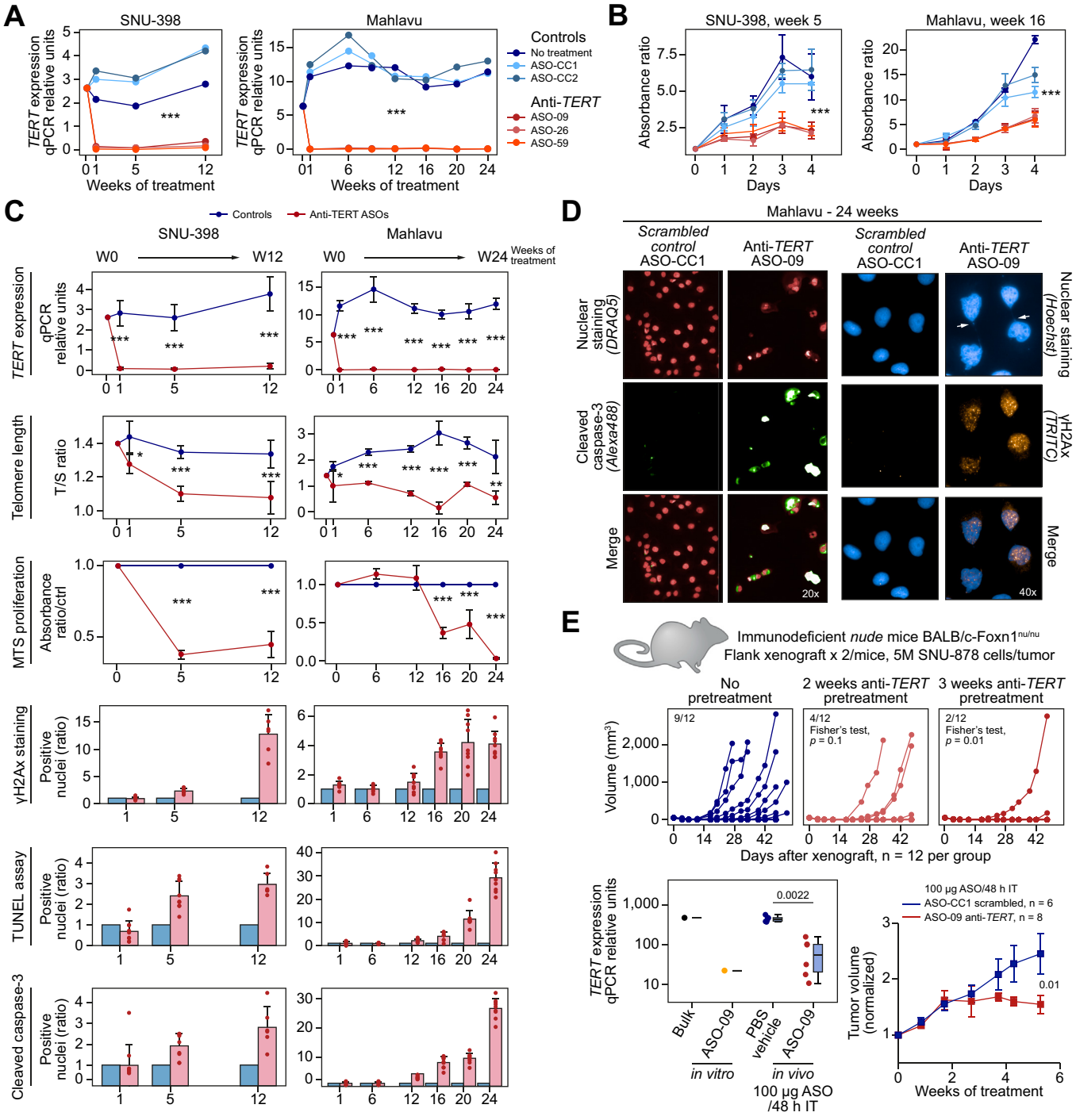

Fig. 5. ***TERT* is a reliable therapeutic target in preclinical models of HCC.** *In vitro* treatment of SNU-398 and Mahlavu HCC cell lines with anti-*TERT* (ASO-09, ASO-26 and ASO-59) or 2 *scrambled* (ASO-CC1 and ASO-CC2) antisense oligonucleotides or no treatment (NT) control. (A) *TERT* expression, (B) MTS cell viability, and (C) merged results for, *from top to bottom*, *TERT* expression, telomere length, MTS cell viability, DNA damage (γH2Ax) and apoptosis activation (TUNEL, cleaved caspase-3). For (A, B, C), mean ± standard deviation, normalized in (C) on mean of controls for MTS assay, γH2Ax, TUNEL and cleaved caspase-3 (all comparisons between merged anti-*TERT vs.* controls using multiple t-test with false discovery rate (FDR) approach; q-values *<0.25, ** <0.05, ***<0.01; see also Fig. S9). (D) Representative immunofluorescence of Mahlavu cells after 24 weeks. White arrows: anaphasic bridges due to chromosome end-to-end fusion. (E) *In vivo* experiments with anti-*TERT* ASO in a cell line xenograft model. Engraftment rate (*top*) after *in vitro* pretreatment, *TERT* expression (*lower left*, Wilcoxon test) and tumor growth (*lower right*, mean normalized on individual day 0 volume ± standard deviation, Wilcoxon test) in flank tumors after IT treatment. ASO, antisense oligonucleotide; HCC, hepatocellular carcinoma; IT, intratumoral; TL, telomere length. (This figure appears in color on the web.)

REG3A) and of genes associated with angiogenesis (such as *FLT1*/*VEGFR2* or *SPP1*/osteopontin, Fig. S9B).

We further explored the effect of *TERT* knockdown on tumor invasiveness *in vivo,* by performing subcutaneous xenografts of SNU-878 cell line in nude BALBc/FOXn1$^{-/-}$ immunodeficient mice, after increasing durations of *in vitro* anti-*TERT* ASO pre-treatment (Fig. 5E). Non pre-treated cells induced tumor formation in 9 out of 12 injection sites, whereas cells pre-treated *in vitro* for 2 weeks to 3 weeks with anti-*TERT* ASO showed reduced tumor formation, respectively in 4/12 (p = 0.1 Fisher's exact test) and 2/12 (p = 0.01 Fisher's exact test) injection sites. Finally, we tested the anti-proliferative capacity of anti-*TERT* ASO *in vivo* (Fig. 5E). Intratumor delivery of 100 μg of anti-*TERT* ASO-09 every 2 days was effective to decrease *TERT* expression in xenografted tumors compared to PBS injection (p = 0.002), and we observed that 5 weeks of intra-tumor treatment with anti-*TERT* ASO-09 was able to induce a significant arrest in tumor growth compared to scrambled ASO-CC1 (p = 0.01). We did not observe toxicity related to *TERT* ASO treatment, in particular mice weight was stable and organ histology did not change.

Discussion

In the present study, analysis of the natural history of telomere length evolution has elucidated the key role of telomere maintenance in the development of HCC on chronically injured liver tissues. We further demonstrated that tumor cell proliferation depends on telomerase expression and this oncogenic addiction can be counteracted with ASOs directed against the expression of *TERT*, thus constituting a promising treatment for HCC.

The unprecedented statistical power provided by our large series of 1,502 patients allowed us to identify aging, sex, liver fibrosis severity and alcohol consumption as independent determinants of telomere shortening in liver tissues, both in subgroup and multivariate analyses. While cirrhosis has been previously associated with shortened telomeres in the liver,[29] the respective effect of age and chronic liver disease has been a matter of debate, mainly due to small sample sizes in previous studies.[30,31] Herein, we also showed that female sex was protective against aging-related telomere attrition in liver tissues, particularly after 40 years of age. This observation in the liver tissues is consistent with similar sex effect on telomere attrition recurrently identified in circulating leukocytes and recently validated in more than 100,000 individuals of the GERA cohort.[32] Although the mechanism of the protective effect of female sex remains to be identified in hepatocytes, it could partly explain the higher prevalence of HCC observed in males compared to females.[2] In contrast, whereas the role of alcohol consumption on telomere attrition in peripheral leucocytes is debated,[33,34] our study revealed excessive alcohol consumption as the unique risk factor for chronic liver disease independently associated with telomere attrition in liver tissues. Since oxidative stress is a major pathophysiological component shared by aging, chronic tissue injury and alcohol-related liver disease, it could account for a specific telomere shortening effect in hepatocytes.[35]

In other cancer types, the relation between telomere length in tumors and their corresponding NT tissue is highly variable,[36] but the role of chronic injury is predominant in the liver.[2] Indeed, our study shows that telomere length in the underlying NT liver is a major determinant of telomere elongation or shortening in the tumor. In most HCCs, tumor telomeres tend to converge towards a narrow biological range similar to the NT liver, suggesting that telomerase activation stabilize telomere length in a range allowing sustainable cell survival and proliferation. Also, the level of NT liver telomere length associates with variable mechanisms of telomerase activation in the tumor. In particular, HCCs developed on livers with shorter telomeres were enriched in *TERT* promoter mutations, in line with the previously published association with aging and its description as the earliest and most frequent driver event in HCCs developed in cirrhotic patients.[11,12,37]

Interestingly, tumors harboring liver progenitor features such as HCCs belonging to the G1 subclass or *BAP1* inactivated HCCs characterized by fibrolamellar-like features[25] showed less frequent *TERT* alteration. The patient and liver profile of these patients, *i.e.* females with low fibrosis stage, that show longer liver telomeres, could explain a delayed need for tumor telomere maintenance. Alternatively, *BAP1* inactivation may have a role in *TERT* expression through epigenetic regulation,[38],[38] but in our series, the weak level of *TERT* expression in this subgroup rather suggests that *BAP1* inactivation could be associated with an alternative mechanism of telomere maintenance that remains to be determined. Of note, in malignant pleural mesothelioma, a cancer with high prevalence of *BAP1* alterations, a similar exclusion between *BAP1* and *TERT* promoter alteration has recently been described by 2 independent groups,[39,40] suggesting a functional link between *BAP1* and telomere maintenance. In addition, we identified another subset of HCCs (3%) showing elongated telomeres despite no *TERT* or *BAP1* alteration nor *TERT* overexpression. These tumors could be candidates for an ALT mechanism,[41] as previously suggested in a smaller pathological study of HCC.[42] However, we identified no alterations previously involved in ALT (such as *ATRX* or *DAXX*) but enrichment in *TP53* alterations, as suggested by a pan-cancer analysis.[36]

Very aggressive HCCs were characterized by longer tumor telomeres together with massive *TERT* overexpression, frequent *TP53* alterations, poor differentiation, stem-cell features and G3 proliferative subclass of HCC. Here, long-term treatment with ASOs was efficient to silence *TERT* expression *in cellulo* and *in vivo* leading to cell proliferation arrest and tumor cell death. These results highlighted the critical role of *TERT* expression and telomere maintenance in tumor progression and cell survival. Of note, variations in the capacity of ASOs to suppress *TERT* mRNA expression have limited the test of their anti-proliferative potential to the more proliferative and less differentiated cells that showed free uptake. However, using GRN163L, a lipo-conjugated oligonucleotide targeting the template region of the telomerase complex, Djojosubroto and colleagues have shown in 3 well differentiated cell lines that telomerase inhibition could effectively induce telomere shortening and suppress growth rate *in vitro* and *in vivo*.[43] In accordance with our data, this shows that the antitumor effect could be achieved with specific telomerase inhibition in a wide variety of molecular subtypes of HCC. Telomerase targeting has been a promising anti-cancer strategy, and several inhibitors have shown results in early phase trials.[17,44] In HCC, a phase II trial testing telomerase-based peptide vaccination failed in advanced stages,[45] but, in our experience, prolonged anti-*TERT* treatment seems to be required to achieve tumor cell cytotoxicity. Consequently, our proof-of-concept study indicates that future trial designs will have to consider the delayed antitumoral effect achieved by *TERT* inhibition, and combination therapy might be mandatory to avoid initial uncontrolled growth.

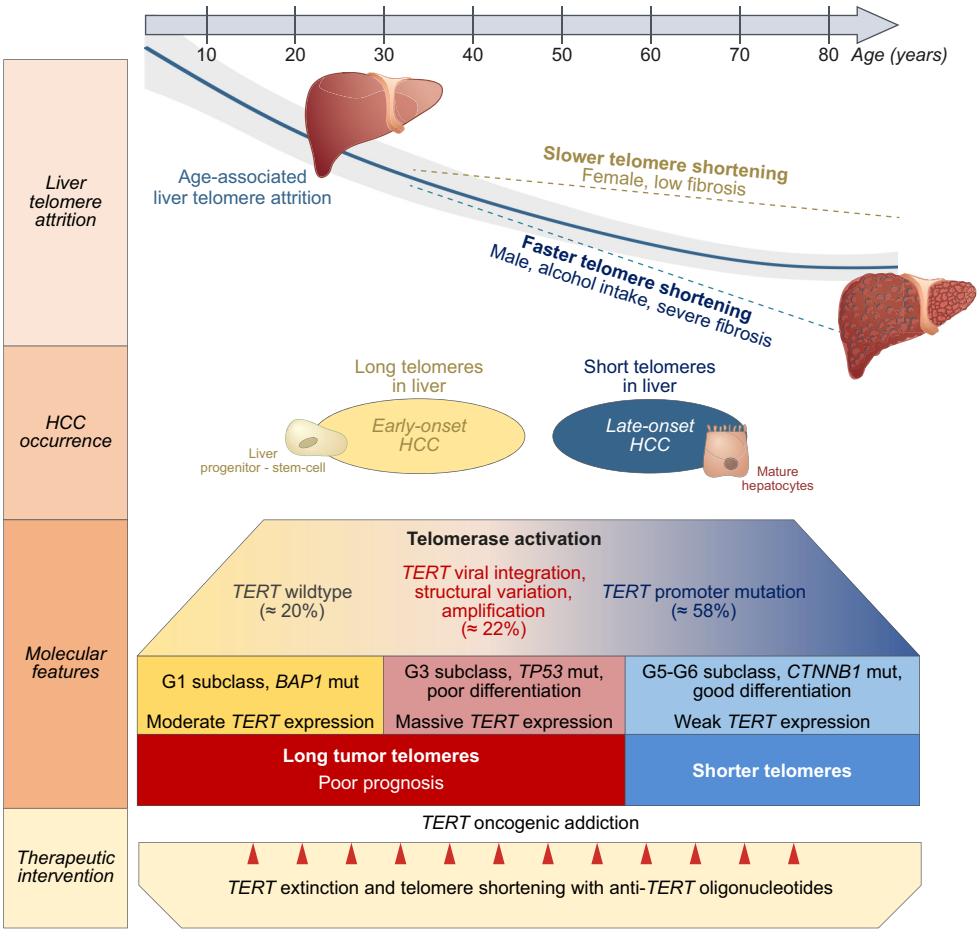

Fig. 6. Natural history of telomere length control during liver carcinogenesis: consequences for targeted treatment. Aging, fibrosis, sex and alcohol consumption independently modulate liver TL, which influences tumor molecular features, telomerase reactivation mechanism and TL. Longer tumor TL is associated with liver progenitor/stem-cell features (*BAP1*/*TP53* alterations, transcriptomic subclasses) and increased aggressiveness. Overall, telomerase reactivation occurs in ≈ 90% of HCCs and is an oncogenic addiction actionable with antisense oligonucleotides. HCC, hepatocellular carcinoma; TL, telomere length. (This figure appears in color on the web.)

Safety concerns around telomerase targeting, although theoretical, remain a valid issue, as telomerase is highly expressed in tissues with high cell turnover such as bone marrow. In our experience, the reversibility of *TERT* extinction in cell lines after ASO interruption is reassuring when considering potential side effects. Moreover, to mitigate off-target effects, ASOs could be conjugated with tissue-specific ligands or tumor-specific ligands to increase specificity, mimicking the antibody-drug-conjugate strategy. ASOs have shown promising clinical results in orphan diseases such as Huntington's disease[46] or amyotrophic lateral sclerosis,[47,48] using *in situ* delivery and without systemic adverse events.

In conclusion, our study constitutes the first integrative analysis of telomere length, *TERT* alterations and expression in paired tumor and NT liver tissues (Fig. 6). This allowed us to associate the major role of the NT liver telomere attrition with specific patterns of telomere maintenance in HCC tumors. *TERT* reactivation is a feature of most adult HCCs and long telomeres are critical for HCC aggressiveness. Our study suggests that *TERT* activation meets the criteria of oncogenic addiction in HCC and could be actionable with ASOs, warranting further evaluation in clinical trials.

Abbreviations

AAV, adeno-associated virus; ALT, alternative lengthening of telomeres; ASO, antisense oligonucleotide; CNA, copy number alteration; FC, fold-change; GSEA, gene set enrichment analysis; GSVA, gene set variation analysis; HCC, hepatocellular carcinoma; NT, non-tumor; RNAseq: RNA-sequencing; SV, structural variations; UTR, untranslated region; WES, whole-exome sequencing; WGS, whole-genome sequencing.

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Conflict of interest

Youngsoo Kim and A. Robert MacLeod are part of Ionis Pharmaceuticals, Carlsbad, CA, USA.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: JCN, SR, JZR. Acquisition of data: MN, AF, ALV, BN. Provided samples and study materials: JFB, GA, NG, VP, CG, JC, GM, YK, ARM. Analysis and data interpretation: MN, SC, TZH, QB, SR, JZR. Manuscript writing: MN, JZR and all. Obtained funding: JCN, SR, JZR.

Data availability statement

We declare that all data involved in this study are available in the article along with the supplementary materials.

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Supplementary data

Supplementary data to this article can be found at https://doi.org/10.1016/j.jhep.2020.11.052.

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Author names in bold designate shared co-first authorship

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