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High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions

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Somatic mutations activating telomerase reverse-trancriptase promoter were recently identified in several tumour types. Here we identify frequent similar mutations in human hepatocellular carcinomas (59%), cirrhotic preneoplastic macronodules (25%) and hepatocellular adenomas with malignant transformation in hepatocellular carcinomas (44%). In hepatocellular tumours, telomerase reverse-transcripase- and *CTNNB1*-activating mutations are significantly associated. Moreover, preliminary data suggest that telomerase reverse-trancriptase promoter mutations can increase the expression of telomerase transcript. In conclusion, telomerase reverse-trancriptase promoter mutation is the earliest recurrent genetic event identified in cirrhotic preneoplastic lesions so far and is also the most frequent genetic alteration in hepatocellular carcinomas, arising from both the cirrhotic or non-cirrhotic liver.

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Hepatocellular carcinoma (HCC) is a highly heterogeneous disease at the clinical, pathological and molecular levels. More than 80% of HCC develop from a cirrhotic liver after exposure to specific risk factors mainly represented by hepatitis B (HBV) and C viral infections, alcohol intake, obesity and rare genetic diseases[1]. The most frequent genetic alterations identified in HCC are mutations of *TP53* and *CTNNB1* (15–40%)[2]. Recent studies using whole-exome sequencing have revealed recurrent mutations in new driver genes involved in the chromatin remodelling (*ARID1A* and *ARID2*), in the ras/raf/map kinase (*RPS6KA3*) and the oxidative stress (*NFE2L2*) pathways[3,4]. However, no recurrent somatic mutations were described in cirrhotic macronodules, the principal preneoplastic lesion for HCC development[5]. In 20% of cases, HCCs are developed in the absence of cirrhosis and, rarely, they result from the malignant transformation of hepatocellular adenoma (HCA). Among these benign tumours, adenoma harbouring *CTNNB1*-activating mutations are more at risk of malignant transformation leading to the development of an HCC in normal liver[6]. However, *CTNNB1* mutations alone are not sufficient for the development of a malignant tumour[7]. Telomerase reverse-trancriptase (*TERT*), coding for telomerase, is also known to be involved in tumorigenesis: rare *TERT* germline-inactivating mutations are associated to cirrhosis in human[8,9] and telomere-deficient mTerc $-/-$ mice are prone to develop HCC but reactivation of telomerase is required for tumour progression[10–13].

Recently, frequent somatic mutations in the *TERT* (coding for telomerase reverse transcriptase) promoter have been reported in melanoma[14,15] and in several other tumour types[16]. These mutations created a potential binding site for ETS (E-twenty six)/ternary complex factor (TCF) transcription factors and are predicted to increase promoter activity and *TERT* transcription. In this study, we searched for *TERT* promoter mutations in a large panel of hepatocellular tumours. We identified *TERT* promoter mutations as the most frequent somatic genetic alterations in HCC and as the first gene recurrently mutated in cirrhotic preneoplastic lesions. Moreover, *TERT* promoter mutations were involved at the last step of malignant transformation of HCA.

Results

***TERT* promoter mutations in HCCs**. We sequenced the promoter region of *TERT* using direct Sanger technique in 305 HCCs related to various risk factors and surgically resected in two French university hospitals (Table 1)[17]. We found recurrent somatic mutations in 179 HCCs (59%) at the two hot spots previously described (Supplementary Table S1)[14–16]. Among 24 hepatocellular cell lines tested, *TERT* promoter mutations were identified in 15 cases (63%; Supplementary Table S2). The most frequent mutations were located at -124 bp from the ATG start site, they consist in G to A (-124G$>$A, 166 cases, 93%) or G to T (-124G$>$T, two cases, 1%) substitutions (Fig. 1a and Supplementary Table S1). The second hot spot was situated at -146 bp from the ATG and characterized by G to A substitutions (-146G$>$A, 11 cases, 6%; Fig. 1a and Supplementary Table S1). The mutations create a typical ETS/TCF-binding sequence, as previously described[14,15,18]. We confirmed that all mutations were somatic and mutations at the two hot spots were mutually exclusive. In contrast to melanoma, we did not found any tandem GG$>$AA mutations, a hallmark of ultraviolet-induced mutagenesis. In addition, mutations at -146 bp were significantly less frequent in HCC (6% of all *TERT* promoter mutations) than that in melanoma (46% of all *TERT* promoter mutations, $P<0.0001$, Fisher's exact test)[14,15]. Together, these data suggest that the somatic mutations in the *TERT* promoter are the most frequent genetic alterations in human HCC (Fig. 1b).

Among the 305 screened HCCs, we observed that *TERT* promoter mutations were more frequent in men ($P=0.001$, χ^2-test), in patients with low serum levels of alpha-fetoprotein ($P=0.01$, χ^2-test), in small tumours of <5 cm ($P=0.01$, χ^2-test), non-related to HBV ($P<0.0001$, χ^2-test) and mutated for *CTNNB1* ($P<0.0001$, χ^2-test, Fig. 1b and Table 1). Interestingly, *TERT* expression assessed by quantitative reverse transcription (RT)–PCR was increased in HCC harbouring *TERT* promoter mutations compared with normal liver, cirrhosis and HCA ($P=0.0007$, Mann–Whitney test, Fig. 2a). Within the malignant liver tumours, *TERT* expression was increased (fold change tumour/normal liver>10) in almost all HCCs exhibiting *TERT* promoter mutation (92%), whereas HCCs non-mutated for *TERT* promoter overexpressed less frequently *TERT* messenger RNA (80%; $P=0.007$, Fisher's exact test).

***TERT* promoter mutations in cirrhotic preneoplastic lesions**. To search for early genetic events in preneoplastic lesions, we screened for *TERT* promoter mutations in 69 cirrhotic tissues and 20 cirrhotic macronodules with or without dysplasia (13 and 7 cases, respectively, Supplementary Table S3). We identified a somatic *TERT* promoter mutation in five macronodules (25%) with (three cases) or without (two cases) dysplasia, whereas none of the cirrhotic tissues were mutated. Accordingly, we showed that *TERT* promoter mutations in macronodules were associated with an increased transcription of telomerase, whereas *TERT* was not overexpressed in cirrhosis and non-mutated macronodules ($P=0.004$, Mann–Whitney test, Fig. 2a and b). Moreover, we screened 15 of these cirrhotic macronodules for mutations in 10 genes that are the most frequently mutated in HCC (*CTNNB1*, *ARID1A*, *ARID2*, *RPS6KA3*, *NFE2L2*, *TP53*, *KRAS*, *PIK3CA*, *AXIN1* and *CDKN2A*), and no somatic variants were identified. Altogether, these results identified *TERT* promoter mutation as the first recurrent genetic alteration in cirrhotic preneoplastic macronodules. In addition, it revealed *TERT* as the earliest genomic event currently identified in the multistep process of liver carcinogenesis on cirrhosis (Fig. 1b).

***TERT* promoter mutations in malignant transformation of HCA**. Next, we screened 60 typical HCAs of various molecular subclasses without signs of malignant transformation, and no mutations were identified in *TERT* promoter (Supplementary Table S4). In contrast, in a series of 16 HCAs with malignant transformation ('nodule in nodule' or HCA with HCC foci) we found *TERT* promoter mutations in seven cases (44%), all associated with a *CTNNB1*-activating mutation (Supplementary Table S5). These data show that somatic mutations of the *TERT* promoter are not required for clonal benign hepatocellular tumorigenesis but they are critical for malignant transformation of HCA in addition to *CTNNB1* mutations (Fig. 1b). Finally, in other HCC developed in normal liver, *TERT* promoter mutations are frequent (55%, Fig. 1b) and they are also significantly associated with *CTNNB1*-activating mutations. Interestingly, β-catenin were previously identified as an activator of *TERT* in embryonic stem cell and in intestinal hyperplasia developed in mice[19]. Here in HCA mutated for *CTNNB1* and without *TERT* promoter mutation, we did not observe an increased transcription of *TERT* (Fig. 2c). In another study, Park *et al.*[20] identified telomerase as an activator of the Wnt/β-catenin signalling in cooperation with BRG1, a SWI/SNF-related chromatin remodelling protein[21]. In the present study, in HCC demonstrating a *TERT* promoter mutation and without

Table 1 | Characteristics of the patients according to presence or absence of *TERT* promoter mutations ($n=305$).

Variable	Overall series ($n=305$)	TERT promoter mutated $n=179$ (59%)	TERT promoter non-mutated $n=126$ (41%)	P-value
Age				
>60 years*	187/305 (61%)	117 (65%)	70 (55%)	0.08
Gender				
Male*	242/305 (79%)	154 (86%)	88 (70%)	0.001
Aetiology				
HCV*	68/301 (26%)	49 (28%)	19 (15%)	0.03
HBV*	67/303 (22%)	26 (15%)	41 (33%)	<0.0001
Alcohol*	118/300 (39%)	80 (46%)	38 (31%)	0.03
Haemochromatosis*	19/299 (6%)	12 (7%)	7 (6%)	0.84
Miscellaneous*	13/291 (5%)	7 (4%)	5 (4%)	0.74
Unknown*	46/291 (16%)	22 (13%)	24 (20%)	0.14
Tumour size				
<5 cm*	127/303 (42%)	87 (49%)	40 (32%)	0.01
Tumour number				
Single*	212/304 (70%)	118 (66%)	94 (75%)	0.39
Vascular invasion				
Microvascular*	161/300 (54%)	91 (52%)	70 (56%)	0.7
Macrovascular*	46/300 (15%)	28 (16%)	18 (14%)	0.94
Differentiation				
Edmonson I–II*	143/295 (48%)	83 (48%)	60 (49%)	0.70
Edmonson III–IV*	152/295 (52%)	91 (52%)	61 (51%)	
Metavir score (non-tumour liver)				
F0–F1*	106/299 (35%)	58 (33%)	48 (38%)	0.21
F2–F3*	83/299 (28%)	45 (26%)	38 (30%)	
F4*	110/299 (37%)	71 (41%)	39 (32%)	
Preoperative AFP				
>20 ng ml^{-1}*	123/274 (45%)	60 (38%)	63 (54%)	0.01
G1-G6 classification				
G1*	22/286 (8%)	9 (5%)	13 (11%)	0.12
G2*	22/286 (8%)	13 (8%)	9 (8%)	
G3*	53/286 (18%)	37 (22%)	16 (14%)	
G4*	93/286 (32%)	49 (28%)	44 (38%)	
G5*	62/286 (22%)	41 (24%)	21 (19%)	
G6*	34/286 (12%)	22 (13%)	12 (10%)	
CTNNB1				
Mutated*	101/304 (33%)	75 (42%)	26 (21%)	<0.0001
TP53				
Mutated*	60/302 (20%)	34 (19%)	26 (21%)	0.73
Events				
Median follow-up (months)†	34/260 (17–56)	33 (16–54)	36 (22–58)	0.13
Tumour-related death <5 years‡	92/260 (35%)	57 (37%)	35 (33%)	0.26
Overall recurrence <5 years‡	138/260 (53%)	84 (55%)	54 (50%)	0.28

AFP, alpha-fetoprotein; HBV, hepatitis B; TERT, telomerase reverse-trancriptase.
*Expressed as number (%) and analysed using the χ^2-test (group size of >5) with Yates' continuity correction or Fisher's exact test (group size of ≤5) except for multiple variable comparisons (χ^2-test two sided). *P*-values were adjusted for multiple comparisons using Monte-Carlo resampling. †Expressed in months (median, 25th and 75th percentile) and analysed using the Kruskall–Wallis test. ‡Patients treated by surgical curative resection (R0) with available follow-up were included in the survival analysis ($n=260$), and survival analysis was performed using the log-rank test.

CTNNB1 mutation we did not observe a significant increase of expression in the β-catenin target genes (*GLUL* and *LGR5*) compared with HCC without *TERT* and *CTNNB1* promoter mutations (Fig. 2d). These results suggested that in tumour hepatocytes, β-catenin may not control directly *TERT* transcription and *vice versa*.

Discussion

In this study, we identified *TERT* promoter mutations in 59% of HCC and in 25% of cirrhotic macronodules with or without dysplasia. It is the most frequent somatic genetic alterations in HCC and also the first recurrent gene somatically mutated in preneoplastic cirrhotic lesions. In addition, classical driver genes

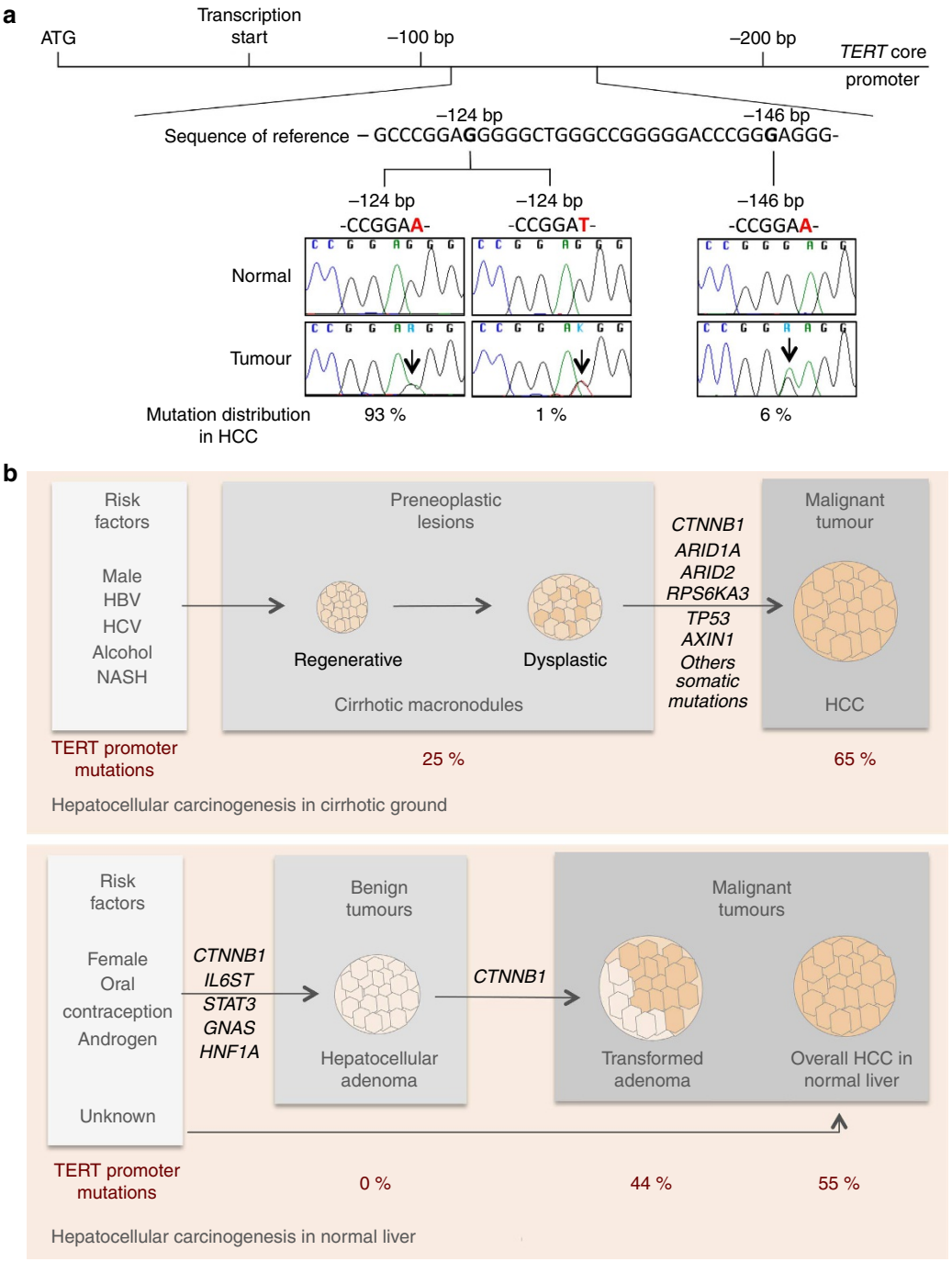

Figure 1 | Somatic mutations of the *TERT* promoter in human liver carcinogenesis. (**a**) Mutation spectrum of the *TERT* promoter in 179 human HCCs: substitution at two hot spots (− 124 and − 146 bp from the ATG start site, g.1,295,228 and g.1,295,250, respectively) that create a new ETS-/TCF-binding sequences. Distribution (%) of the 179 mutations along the *TERT* promoter (59% of all HCCs) is indicated. Mutations are represented on the + strand of DNA. (**b**) *TERT* promoter mutations in the multistep process of liver carcinogenesis. Percentage of *TERT* promoter mutations is reported at each step of liver carcinogenesis. On cirrhosis, 5/20 (25%) cirrhotic macronodules (three with dysplasia and two without dysplasia) and 71/110 (65%) HCCs were mutated for *TERT*. On normal liver, no mutations of the *TERT* promoter (0%) were identified among 60 HCAs; in contrast, 7/16 (48%) of malignant transformation of HCA and 58/106 (55%) of HCCs without signs of malignant transformation from HCA exhibited mutations of the *TERT* promoter. Other genes most frequently mutated in HCC and HCA are indicated.

the most frequently mutated in liver carcinogenesis (*CTNNB1*, *ARID1A*, *ARID2*, *RPS6KA3*, *NFE2L2*, *TP53*, *KRAS*, *PIK3CA*, *AXIN1*, *CDKN2A*)[3] were identified only in HCC and not in dysplastic macronodules. This finding suggests that mutations of the *TERT* promoter are the earliest genetic events described so far in the multistep process of carcinogenesis on cirrhosis. In contrast, in benign tumorigenesis in the normal liver, we have previously described several driver genes recurrently mutated including *HNF1A*, *CTNNB1*, *IL6ST*, *GNAS* or STAT3 (ref. 6). Moreover, HCA harbouring a *CTNNB1* mutation have a higher risk of malignant transformation[7,22]. We found *TERT* promoter mutations in malignant transformation of HCA but not in

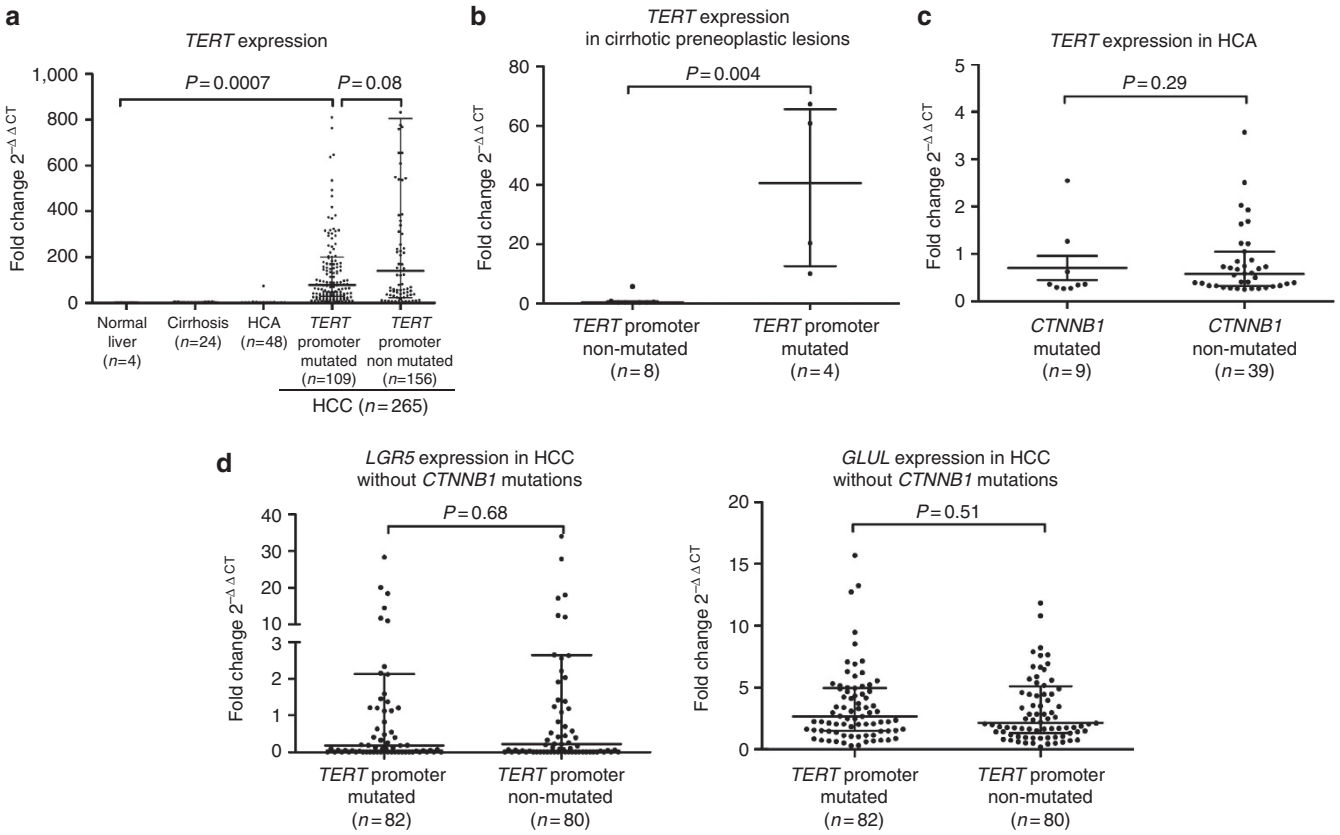

Figure 2 | *TERT* transcript expression according to mutation status of the *TERT* promoter. (**a**) *TERT* messenger RNA (mRNA) is not expressed in 4 normal livers, 24 cirrhosis and 48 HCAs, whereas it is overexpressed in 156 HCCs mutated and 109 HCCs not mutated for the *TERT* promoter. (**b**) *TERT* transcript was overexpressed in all mutated cirrhotic macronodules ($n=4$) when compared with non-mutated macronodules ($n=8$). (**c**) *TERT* mRNA was not overexpressed in HCA with *CTNNB1* mutations ($n=9$) compared with HCA without CTNNB1 mutations ($n=39$). (**d**) β-catenin target genes (*GLUL* and *LGR5*) were not overexpressed in HCC harbouring *TERT* promoter mutations without *CTNNB1* mutations ($n=82$) compared with HCC lacking both *TERT* promoter and *CTNNB1* mutations ($n=80$). *TERT, GLUL and LGR5* mRNA levels were measured using quantitative RT-PCR. All the results were normalized with the mean of normal liver tissues (see Methods section). Results were reported in median with interquartile range and compared using non-parametric Mann–Whitney test. Differences were considered significant when the *P*-value was <0.05.

classical HCA without dysplasia. It underlines that *TERT* promoter mutations are involved at the last step of malignant transformation in association with *CTNNB1* mutations. This association between *TERT* promoter and *CTNNB1* mutations was also found in HCC. Altogether, these data suggested that *TERT* promoter mutations and activation of the Wnt/β-catenin pathway could cooperate to promote malignant transformation. Previous studies have shown *in vitro* and in the mice model that *TERT* could regulate *CTNNB1* expression and conversely that β-catenin could bind to TERT promoter and participate to control TERT expression[19–21]. In our study, we showed that *CTNNB1*-activating mutations alone did not increase *TERT* transcription in HCA. In addition, HCC with *TERT* promoter mutations without *CTNNB1* mutations did not exhibit a Wnt/β-catenin activation. Consequently, we did not find a direct transcriptional relationship between *TERT* and Wnt/β-catenin pathway in benign and malignant liver tumours. However, the limited numbers of cases and the different histological subtypes (HCA and HCC) analysed restrain this conclusion, and the role of *TERT* and *CTNNB1* mutations in tumorigenesis remains to be functionally investigated[15,16]. As the functional consequences of *TERT* promoter mutations on transcription are still unclear, we have also investigated the link between *TERT* promoter mutation and *TERT* transcription in liver tissues. We showed an increase in the transcription of *TERT* in cirrhotic macronodules carrying *TERT* mutations compared with macronodules without mutations that will require additional validation in a larger series of samples. Interestingly, both HCC with *TERT* promoter mutations and HCC without mutations exhibited an increase in *TERT* transcription. It suggests that telomerase reactivation is a hallmark of HCC possibly explained by *TERT* promoter mutations but also by alternative mechanisms[23,24]. In particular, the lower rate of *TERT* promoter mutations in HBV-related HCC could be explained by the frequent insertion of HBV DNA in the *TERT* promoter known to induce telomerase transcription[25,26].

In conclusion, *TERT* promoter mutations are the most frequent somatic genetic alterations in human HCC arising both from cirrhosis and the normal liver. They are early major events in tumorigenesis occurring at preneoplastic stages in cirrhosis and in malignant transformation of HCA in association with WNT/β-catenin pathway activation.

Methods

Selection of patients. This study was approved by our local institutional review board committee (CCPRB Paris, St Louis, 1997 and 2004). All patients gave their informed consent according to the French law[17]. Tumours samples were frozen following the liver resection for tumour in French University hospitals. We included a total of 401 liver samples including 305 HCCs, 60 typical HCAs, 16 HCAs with signs of malignant transformation and 20 cirrhotic preneoplastic nodules. Patients and tumours features are detailed in Table 1 for HCC, Supplementary Table S3 for cirrhotic preneoplastic lesions, Supplementary Table S4 for HCA without malignant transformation and in Supplementary Table S5 for HCA with malignant transformation. In addition, we studied a series of 24 hepatocellular cell lines

(Supplementary Table S2). We also included a series of 69 cirrhotic tissues of various aetiologies (alcohol $n=19$, HBV $n=14$, HCV $n=31$, haemochromatosis $n=3$, other aetiologies $n=2$). Tumour and non-tumour liver samples were frozen immediately after surgery and conserved at $-80\,^{\circ}C$. Tissue samples from the frozen counterpart were also fixed in 10% formaldehyde, paraffin-embedded and stained with haematoxylin and eosin and Masson's trichrome. The diagnosis of liver tumours was based on established histological criteria[27,28] by expert pathologists (JC and PBS).

Sequencing. After amplification using additives dimethylsulphoxide 5%/glycerol 5% (annealing 62 °C), the *TERT* promoter was sequenced by Sanger's direct sequencing using forward 5′-(CAGCGCTGCCTGAAACTC)-3′ and reverse 5′-(GTCCTGCCCCTTCACCTT)-3′ primers[15]. All mutations were confirmed by sequencing a second independent amplification product on both strands and the somatic or germline status was assessed by sequencing the corresponding non-tumour liver. All HCAs were screened for *CTNNB1*, *IL6ST*, *HNF1A*, *GNAS* and *STAT3* mutations (Supplementary Table S6) and results were already reported[7,29–31]. All HCCs were screened for *CTNNB1* and *TP53* mutations and results were already reported[32]. Preneoplastic cirrhotic lesions (regenerative macronodules, low-grade dysplatic macronodules and high-grade dysplastic macronodules) were screened for *CTNNB1*, *ARID1A*, *ARID2*, *RPS6KA3*, *NFE2L2*, *TP53*, *KRAS*, *PIK3CA*, *AXIN1*, *CDKN2A* mutations (Supplementary Table S6)[3].

Quantitative RT–PCR. Expression was assessed by quantitative RT–PCR in duplicate using TaqMan (Applied Biosystems) gene expression assay (TERT Hs00972656_m1, GLUL Hs00374213_m1 and LGR5 Hs00173664_m1). The relative amount of RNA was calculated with the 2-delta delta CT method[32]. Gene expression was normalized with the RNA ribosomal 18S, and the level of expression of the tumour sample was compared with the mean level of the gene expression in normal liver tissues and expressed as an *n*-fold ratio.

Statistical analysis. Continuous/quantitative data were compared using the Kruskall–Wallis or Mann–Whitney test. Dichotomous data, along with independence and measures of association, were analysed using the χ^2-test (group size of >5) with Yates' continuity correction or Fisher's exact test (group size of ≤ 5) except for multiple variable comparisons (χ^2-test two sided). *P*-values were adjusted for multiple comparisons using Monte–Carlo resampling. All reported *P*-values were two tailed, and differences were considered significant when the *P*-value was <0.05. Statistical analysis was performed using the R software.

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Author contributions

J.C.N., M.M. and C.P. designed, analysed and verified the sequencing data; J.C. and P.B.S. provided samples and pathological reviewing; L.C., C.B. and A.L. provided samples and clinical information; J.C.N. and J.Z.R. designed and coordinated the overall study; all authors contributed to writing the manuscript.

Additional information

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Corrigendum: High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions

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The original version of this Article contained a typographical error in the spelling of the author Jessica Zucman-Rossi, which was incorrectly given as Jessica Zucman Rossi. This has now been corrected in both the PDF and HTML versions of the Article.