Frequency of somatic mutations in *TERT* promoter, *TP53* and *CTNNB1* genes in patients with hepatocellular carcinoma from Southern Italy

DANIELE LOMBARDO[1,2*], CARLO SAITTA[1,3*], DOMENICO GIOSA[1,2], FRANCESCA CASUSCELLI DI TOCCO[1,2], CRISTINA MUSOLINO[1,2], GIUSEPPE CAMINITI[3], VALERIA CHINES[1,2], MARIA STELLA FRANZÈ[1,2], ANGELA ALIBRANDI[4], GIUSEPPE NAVARRA[5], GIOVANNI RAIMONDO[1,2] and TERESA POLLICINO[1,5]

[1]Division of Clinical and Molecular Hepatology; [2]Department of Clinical and Experimental Medicine; Departments of [3]Internal Medicine, [4]Economics and [5]Human Pathology, University Hospital 'G. Martino' of Messina, 98124 Messina, Italy

Received July 31, 2019; Accepted November 25, 2019

DOI: 10.3892/ol.2020.11332

Abstract. Somatic mutations in the TERT promoter and in the TP53 and CTNNB1 genes are considered drivers for hepatocellular carcinoma (HCC) development. They show variable frequencies in different geographic areas, possibly depending on liver disease etiology and environmental factors. TP53, CTNNB1 and TERT genetic mutations were investigated in tumor and non-tumor liver tissues from 67 patients with HCC and liver tissue specimens from 41 control obese subjects from Southern Italy. Furthermore, TERT expression was assessed by reverse transcription-quantitative PCR. Neither CTNNB1 mutations or TP53 R249S substitution were detected in any case. The *TP53* R72P polymorphism was found in 10/67 (14.9%) tumors, but was not found in either non-tumor tissues (P=0.001) or controls (P=0.009). TERT gene promoter mutations were found in 29/67 (43.3%) tumor tissues but were not found in either non-tumor (P<0.0001) or control liver specimens (P<0.0001). The most frequent mutation in the tumors was the known hot spot at -124 bp from the TERT ATG start site (-124G>A, 28 cases, 41.8%; P<0.0001). A new previously never reported TERT promoter mutation (at -297 bp from the ATG, -297C>T) was found in 5/67 (7.5%) tumors, in 0/67 (0%) non-tumor (P<0.0001), and in 0/41 (0%) controls (P=0.07). This mutation creates an AP2 consensus sequence, and was found alone (1 case) or in combination (4 cases) with the -124 bp mutation. The mutation at -124 and -297 bp induced a 33-fold (P<0.0001) and 40-fold increase of TERT expression levels, respectively. When both mutations were present, TERT expression levels were increased >300-fold (P=0.001). A new *TERT* promoter mutation was identified, which generates a *de novo* binding motif for AP2 transcription factors, and which significantly increases TERT promoter transcriptional activity.

Correspondence to: Professor Teresa Pollicino, Division of Clinical and Molecular Hepatology, University Hospital 'G. Martino' of Messina, Via Consolare Valeria 1, 98124 Messina, Italy
E-mail: tpollicino@unime.it

*Contributed equally

Key words: somatic mutations, TP53 gene, CTNNB1 gene, TERT promoter, hepatocellular carcinoma, activating protein 2 transcription factor

Introduction

Hepatocellular carcinoma (HCC) is one of the most challenging health problems worldwide. Currently, HCC is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related death globally (1). Although HCC is more prevalent in Asian and African nations, important evidence indicates that the incidence of HCC is rising in developed countries (1). The main risk factors for HCC development are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, and metabolic syndrome, including type 2 diabetes and non-alcoholic steatohepatitis (NASH) (2). Other cofactors, such as smoking and aflatoxin-contaminated food supplies are well-characterized contributors to HCC (1-3). Over the past decade, advances in genomic research have increased our knowledge of HCC molecular pathogenesis. However, the exact molecular mechanisms underlying the development of HCC are still unclear. Each HCC appears to be characterized by a distinctive pattern of somatic mutations in passenger and driver genes that accumulate overtime (4). Recent studies performing whole-genome or whole-exome sequencing have identified specific somatic mutations in driver genes that appear to contribute to tumor initiation and progression (4-6). The most frequently detected mutations affect the catalytic subunit of telomerase holoenzyme, the telomerase reverse transcriptase (TERT). These mutations are detected in 44-65% of HCC (7,8), representing the most frequent somatic genetic alterations in human HCC (9). *TERT* promoter mutations are associated with an increased expression of telomerase, which allow cells to acquire the

ability to overcome senescence and to become immortal (10). *TERT* mutations can be found in preneoplastic lesions and in early-stage HCCs (11,12). Thus, *TERT* promoter mutations correlate with tumor initiation, while mutations in other genes, such as *TP53*, *CTNNB1*, are associated with later stages of HCC progression, causing further genomic modification (12). It is of note that HBV and HCV infections have different impacts on the *TERT* gene (8,9). *TERT* promoter mutations have been more frequently found in HCV-related and alcohol intake-related HCC than in HBV-related HCC (9,13,14), where induction of telomerase overexpression is in some cases due to integration of HBV DNA sequences into the *TERT* promoter (8,15). Other driver genes frequently mutated in HCC belong to key signaling pathways of oncogenesis as the WNT/β-catenin pathway and the P53 cell cycle pathway (5,6). Indeed, somatic mutations in *CTNNB1* (coding for β-catenin) and *in TP53* tumor suppressor gene are recurrently detected in HCCs. Mutations in the *TP53* gene are detected in 12-48% of HCC, with high frequency in advanced tumors (6,16). The mutational spectrum in this gene has shown a strong molecular association between environmental carcinogen exposure and cancer. In Asia and Africa the increased risk of HCC is particularly related to the dietary intake of aflatoxin B1 (AFB1), a fungal mycotoxin, which contaminates foods that may act in synergy with chronic HBV infection. Exposure to AFB1 induces G: C to T: A transversions at the third base in codon 249 of TP53 leading to the R249S substitution (17,18). No other specific recurrent TP53 hotspot somatic mutations outside the R249S mutation have been identified in HCC (16). Concerning *CTNNB1*, somatic mutations in this gene have been found in 11-37% of HCC samples (6,16,19). *CTNNB1* mutations can be nucleotide substitutions or in-frame deletions in the consensus site targeted by the APC/AXIN1/GSK3B inhibitory complex (6,19,20). As a consequence this leads to the impairment of β-catenin degradation and Wnt signaling activation, which promotes cell motility, de-differentiation, and proliferation (20). *CTNNB1* and *TP53* mutations are frequently mutually exclusive, whereas *CTNNB1* mutations are associated with *TERT* mutations (11,21). Though recurrent somatic mutations in the *TERT* promoter region, in the exon 3 of *CTNNB1* gene, and in *TP53* gene have been recognized as common events in HCC they show variable frequencies in different geographic areas, depending on liver disease etiology and environmental factors (6,9,14,16,18,22).

In this study, we investigated the presence of *CTNNB1*, *TP53,* and *TERT* promoter mutations in paired tumor and non-tumor liver specimens from a cohort of HCC patients from Southern Italy, a geographic region with a high incidence of liver cancer (23-25).

Patients and methods

Patients. Frozen tumor and non-tumor liver specimens from 67 HCC patients (47 males and 20 females; mean age, 66.4±9 years) were analysed. Additionally, we studied frozen liver specimens from 41 control patients (19 males and 22 females; mean age, 49.2±13.1 years) with morbid obesity that underwent bariatric surgery and whose liver histology showed the presence of non-alcoholic fatty liver (NAFL) with no sign of steatohepatitis and fibrosis (26). The choice of a control group, which included people with simple hepatic steatosis and no sign of hepatic injury was due to the fact that patients who usually undergo liver biopsy are those that frequently show severe chronic liver disease, and as demonstrated by Nault *et al* (11), these patients may have already developed TERT promoter mutations in the liver. Indeed, TERT is considered as the earliest genomic event currently identified in the multistep process of liver carcinogenesis on cirrhosis. Forty (59.7%) of the 67 patients with HCC had HCV- and 3 (4.5%) had HBV-related liver diseases. Among the other 24 patients, 2 had alcohol-related liver disease and 22 had cryptogenic liver disease. Patients' characteristics are shown in Table I. Tumor and non-tumor liver tissues were obtained by surgical resection. Similarly, tissue specimens from obese subjects were obtained by bariatric surgery. Each liver specimen was stored in an appropriate volume of RNA*later* RNA stabilization reagent (Ambion) at -80˚C. The study protocol was approved by the Ethics Committee of the Messina University Hospital, and written informed consent was obtained from all patients.

PCR amplification and sequencing analysis. Exon 3 of *CTNNB1*, exons 4 and 7 of *TP53,* and the *TERT* promoter region were analysed by PCR amplification and Sanger's direct sequencing. DNA was extracted from the frozen liver specimens of each patient by standard procedures. In brief, tissue specimens were homogenized by digestion in 150 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 7.4), 10 mmol/l EDTA, 1% SDS and proteinase K (800 μg/ml) overnight at 37˚C. After extraction with phenol/chloroform, nucleic acids were precipitated in 2 volumes of pure, cold ethanol. Nucleic acids were then resuspended and digested with pancreatic ribonuclease (100 μg/ml), followed by extraction with phenol/chloroform and reprecipitation in pure cold ethanol. DNA was resuspended in 10 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EDTA. DNA concentration was measured using the ND-1000 spectrophotometer (NanoDrop Technologies) at 260 nm.

PCR amplification of the TERT promoter was carried out using additives (dimethylsulphoxide 5% and glycerol 5%) under the following conditions: 95˚C for 2 min and then 35 cycles of 95˚C for 30 sec, 62˚C for 40 sec, 72˚C for 50 sec. Amplification of *CTNNB1* exon 3 and *TP53* exons 4 and 7 were carried out under the following conditions: 95˚C for 5 min and then 35 cycles of 94˚C for 15 sec, 55˚C for 20 sec, 72˚C for 60 sec.

Nucleotide sequences of PCR products were determined using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The sequencing products were resolved in an automatic DNA sequencer (ABI PRISM 3500 Dx Genetic Analyzer; Thermo Fisher Scientific, Inc.). The oligonucleotide primers used for PCR amplification and sequencing of *TERT* promoter, of *TP53* exons 4 and 7, and of *CTNNB1* exon 3 are reported in Table II. Mutations detected in each genomic region analysed were confirmed by sequencing both DNA strands of a second independent PCR amplification product. The somatic or germline status of the mutations was assessed by sequencing the corresponding non-tumor liver tissue.

RNA extraction and TERT reverse transcription-quantitative PCR (qPCR). To evaluate the effect of the *TERT* promoter

Table I. Demographic, clinical, and genetic characteristics of the studied patients with HCC and the control subjects.

Characteristics	Patients with HCC (n=67)	Control subjects (n=41)	P-value
Age, years	66.4 (±9)	49.2 (±13.11)	<0.0001
Sex, Male/Female	47/20	19/22	0.013
Etiology			
HCV	39/67 (58.2%)	0/41 (0%)	
HBV	3/67 (4.5%)	0/41 (0%)	
HCV + HBV	1/67 (1.49%)	0/41 (0%)	
Alchool	2/67 (3%)	0/41 (0%)	
Unknown	22/67 (32.8%)	0/41 (0%)	
CTNNB1 mutations, exon 3 mutated	0/67 (0%)	0/41 (0%)	
TP53 mutations			
R249S	0/67 (0%)	0/41 (0%)	
R72P	10/67 (14.9%)	0/41 (0%)	0.009
TERT promoter mutations			
-124 (G>A)	28/67 (41.8%)	0/41 (0%)	<0.0001
-245 (G>A)	47/67 (70.1%)	22 /41 (53.7%)	0.08
-297 (C>T)	5/67 (7.5%)	0/41 (0%)	0.007

HCC, hepatocellular carcinoma.

Table II. Oligonucleotide primers used for PCR amplification and sequencing of *TERT* promoter, of *TP53* exons 4 and 7, and of *CTNNB1* exon 3.

Gene	Primer	Sequence 5'->3'
hTERT	hTERT FWD	ACGAACGTGGCCAGCGGCAG
	hTERT REV	CTGGCGTCCCTGCACCCTGG
TP53 exon 7	TP53 exon 7 FWD	GCGCACTGGCCTCATCTTG
	TP53 exon 7 REV	GGGTCAGCGGCAAGCAGAG
TP53 exon 4	TP53 exon 4 FWD	CTGGTCCTCTGACTGCTCTT
	TP53 exon 4 REV	AGGCATTGAAGTCTCATGGA
CTNNB1 exon 3	CTNNB1 exon 3 FWD	GGTATTTGAAGTATACCATAC
	CTNNB1 exon 3 REV	CTGGTCCTCGTCATTTAGCAG

mutations on gene transcription, real-time PCR quantification of TERT transcripts was performed in all tumor and non-tumor liver specimens as well as in liver tissue specimens from 20 patients with NAFL. RNA extraction was performed using the QIAzol reagent (Qiagen) following the manufacturer's instructions. Total liver RNA was resuspended in nuclease-free water and concentration was determined by spectrophotometry at 260 nm. To eliminate DNA contamination each sample was treated with RQ1 RNase-Free DNase (Promega Corporation) for 30 min, at 37˚C. Then RNA was reverse transcribed for first-strand cDNA synthesis by using AffinityScript Multi-Temp cDNA Synthesis kit (Agilent Technologies) and random examers. RNA reverse transcription was performed under the following conditions: 65˚C for 5 min, 42˚C for 5 min, 55˚C for 60 min and 70˚C for 15 min.

TERT expression was assessed using TaqMan Applied Biosystems gene expression assay (Hs00972656_m1) (Thermo Fisher Scientific, Inc.). The relative amount of RNA was calculated with the $2^{-\Delta\Delta Cq}$ method (27). TERT gene expression was normalized to internal control ribosomal 18S RNA (Hs99999901_s1), and the expression level in the tumor specimens was compared with the mean level of the gene expression in liver tissues from subjects with NAFL and expressed as an *n*-fold ratio.

Statistical analysis. All statistical analyses were performed using the SPSS 22.0 software package (SPSS Inc.) for Windows. Numerical data were expressed as mean and standard deviation (SD) and categorical variables as number and percentage. χ^2 test was used for comparison of categorical data. Analyses by the Kolmogorov-Smirnov test showed that TERT gene expression did not followed a normal distribution. Therefore, a non-parametric Kruskal-Wallis test was used for comparisons of mean values among the different groups,

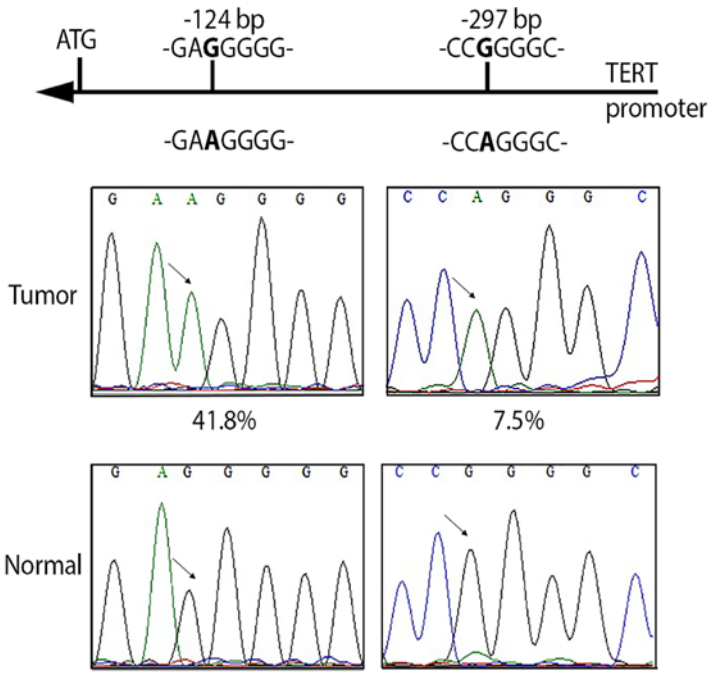

Figure 1. Somatic mutations of the TERT promoter in 67 human HCCs: Substitutions at the -124 and -297 bp from the ATG start site (g.1,295,228 and g.1,295,401, respectively) each creating a new binding motif, E-twenty-six-/ternary complex factor and activating protein 2, respectively. Distribution (%) of the 33 mutations along the TERT promoter (43.3% of all HCCs) is indicated. HCC, hepatocellular carcinoma.

followed by post-hoc testing using un-paired Mann-Whitney U tests with a Bonferroni-adjusted alpha level of 0.017. All tests were two-tailed. P<0.05 was considered to indicate a statistically significant difference.

Results

Sequencing analysis of CTNNB1, TP53, and the TERT promoter region. Sequencing analysis showed the absence of mutations in *CTNNB1* exon 3 as well as the absence of the R249S substitution in *TP53* gene both in patients with HCC and in the control cases. Interestingly, the functionally significant 215G>C polymorphism at codon 72 (R72P, rs1042522) of *TP53* gene, which has been associated with a higher risk of developing several different types of cancers (including oral, lung, thyroid, bladder, and liver cancer) (28-33), was found in 10/67 (14.9%) tumors, in 0/67 (0%) non-tumor tissues (P=0.001), and in 0/41 (0%) controls (P=0.009) analysed. The homozygous 215 G>C mutation leading to R72P was identified in 2 of 67 cases (2.9%). Heterozygosity of 215 G>C was revealed in 8 of 67 cases (11.9%).

Concerning the *TERT* promoter region, 28/67 (41.8%) tumors, 0/67 (0%) non-tumor tissues (P<0.0001), and 0/41 (0%) control tissues (P<0.0001) showed the recurrent somatic mutation at the previously described hot spot located at -124 bp (-124G>A) from the ATG start site of *TERT* gene (11,34-36) (Fig. 1), whereas the other described hot spot located at -146 bp (-146 bp G>A) from the ATG start site (11,34-36) was not detected in any of the HCC cases nor in the controls. Analogously, neither the HCC or control cases showed the mutation at -57 bp (-57 bp A>C) previously described in melanoma and in bladder cancer (35,37) or the tandem GG>AA mutation, a hallmark of ultraviolet-induced mutagenesis, described in melanoma (34,35). The rs2853669

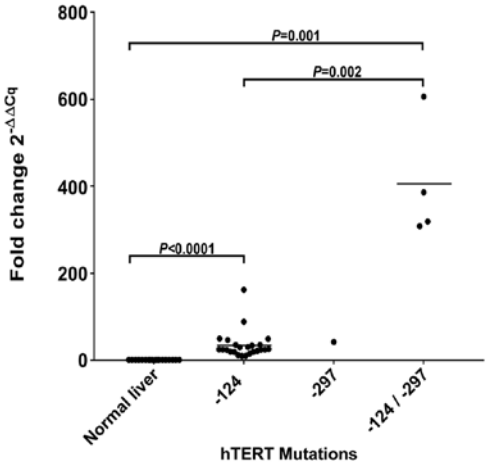

Figure 2. TERT transcript expression according to mutation status of the TERT promoter. All the results were normalized with the mean of normal liver tissues (see Methods section). Results were reported in mean and compared using Mann-Whitney U tests with a Bonferroni-adjusted alpha level of 0.017.

A>G single nucleotide polymorphism (SNP) located at -245 bp from the *TERT* ATG start site-able to modify the *TERT* expression levels induced by TERT promoter somatic mutations (38,39)-was found in 47/67 (70.1%) tumors, in 44/67 (65.7%) non-tumors (P=0.6), and in 22/41 (53.7%) controls (P=0.08).

Interestingly, in 5/67 (7.5%) tumors, in 0/67 (0%) non-tumor tissues (P<0.0001), and in 0/41 (0%) controls (P=0.07) analysed, a new mutation was identified in the *TERT* promoter, located at -297 bp (-297 C>T; G>A on opposite strand) from the ATG start site (Fig. 1). This mutation creates an activating protein 2 (AP2) consensus sequence (CCGGGGC>CCAGGGC) (40,41)

and was found alone (1 tumor tissue) or in combination (4 tumor tissues) with the -124 bp mutation.

Expression levels of TERT gene in tumor and non-tumor liver tissues. Real-time qPCR quantifications of *TERT* transcripts confirmed that within the tumors harboring the -124 bp mutation, *TERT* expression levels were significantly upregulated compared with control liver tissues (P<0.0001, fold change tumors/control livers=33). Interestingly, significantly higher levels of *TERT* expression were also found in tumors harbouring the -297 bp somatic mutation. In particular, the tumor specimen with the single mutation at -297 bp showed *TERT* expression levels 40-fold higher compared with control liver tissues. The 4 tumor specimens harbouring both the -124 and the -297 bp mutations showed that *TERT* gene expression was significantly further increased compared with control tissues (P=0.001; fold change tumors/control livers >300) and even when compared with tumors harboring the -124 bp mutation alone (P=0.002; fold change tumors with both -124 and -297 bp mutations/tumors with -124 bp mutation alone >11) (Fig. 2). In these 4 tumors *TERT* transcripts were significantly increased also when compared with tumors not mutated in the *TERT* promoter (P=0.001; fold change tumors/non-mutated tumors >8). The SNP rs2853669 associated modulatory effect on *TERT* expression was not detectable in tumors with or without *TERT* promoter somatic mutations.

Summarising, 29 (43.3%) of the 67 patients with HCC harboured the -124 bp and/or the -297 bp somatic mutation in tumor tissue. The underlying liver disease of the 29 HCC patients, was HCV-related in 18 (62%) cases, HBV-related in 2 (6.9%) cases, and cryptogenic in 9 (31%) cases (P=0.59). Therefore, *TERT* mutations were observed at similar frequencies in viral-related HCCs and in HCCs related to other causes of liver disease.

Amongst the other variables tested, there were significant differences in age distribution between patients with and without the -124 bp mutation. Patients with the -124 bp mutation were older (70.7±7.5 years) than those without the mutation (63.4±8.4 years, P=0.0008). No other variable was associated with the *TERT* somatic mutations.

Discussion

In this study, we analysed the mutational pattern of *TP53*, *CTNNB1*, and *TERT* promoter in tumor and non-tumor liver tissue specimens from patients with HCC and from control obese patients, all from Southern Italy. Interestingly, we detected no *CTNNB1* and TP53 R249S somatic mutations in any patients. *CTNNB1* mutations have been identified in about 20-40% of HCCs in previous studies (19,42-44), and this prevalence was shown to be higher in individuals with HCV-related HCC than in those infected by HBV (21). In our study population, HCC was related to HCV in 59.7% of the patients. Given the absence of *CTNNB1* somatic mutations in the studied HCCs it is possible that Wnt/β-catenin activation in our patients is induced independently of the *CTNNB1* genetic background as it has been shown for adrenal aldosterone producing adenomas (45,46).

Concerning the *TP53* gene, the absence of R249S somatic mutation in the analysed HCCs could be explained by the fact that all the studied patients were from a geographic area where there is no dietary exposure to the human liver carcinogen AFB1, and where the general prevalence of HBV infection is low (less than 1%). In *TP53* gene we detected the 215G>C polymorphism at codon 72 (R72P, rs1042522). It was found in 10% of the HCC specimens and in none of the non-tumor liver tissues and the control livers. Thus, the prevalence of this polymorphism was significantly higher in HCCs than in the control tissues. Our results are in accordance with previous studies showing that carriers of both the heterozygous and homozygous *TP53*-SNP72 genotypes are at a high risk of HCC development (31,47-49). Interestingly, it has been hypothesized that tumorigenesis, relying on *TP53* R72P, might play a role only in selected populations of patients living in low incidence geographic areas (50). Indeed, in areas of high HCC incidence (Far East and Southern Africa) the presence of potent risk factors (HBV infection and AFB1) able to induce major genomic alterations in the exposed populations may likely further reduce the weak oncogenic impact of the R72P SNP (50).

Concerning the *TERT* gene promoter, in this study we report for the first time the identification of a somatic mutation located at -297 bp (-297 G>A) from the ATG start site of the *TERT* gene. This mutation was detected in 7.5% of the studied tumor specimens but in none of either the paired non-tumor or control liver tissues. The nucleotide change in the sequence generates a *de novo* consensus binding motif for AP2 transcription factor (40,41) and real time PCR quantification revealed that *TERT* transcripts in the tumors harboring the -297 bp mutation were 1.2-fold higher than those expressed in tumors showing the -124 bp mutation, known to create novel consensus binding motifs for E-twenty-six (ETS) and ternary complex factor (TCF) transcription factors (35). Interestingly, tumors harboring both -124 and -297 bp mutations had more than 300-fold increase in *TERT* transcript levels compared with control tissues, thus clearly indicating that the combination of the 2 somatic mutations has a strong impact on the promoter activation and the up-regulation of *TERT* gene expression.

AP2 family genes have been implicated in a large number of tumors in various stages of tumorigenesis (51). Our data demonstrating the creation of a *de novo* AP2 binding site in *TERT* promoter indicate that *TERT* may become an AP-2 target gene, and this-possibly-not only in HCC but also in other cancer types, strengthening the crucial role of *TERT* promoter mutations and telomerase activation in the carcinogenetic process, and confirming them as excellent candidate biomarkers for early tumor detection or monitoring.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TP, GR, CS and GN conceived and designed the study. DL, GC, CM, VC and FCdT performed all the analyses. TP, DL, CS, DG and MSF were involved in the interpretation of all data. DL was involved in the preparation of the figures and tables. AA performed statistical analysis. TP wrote the manuscript and GR revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Messina University Hospital (reference no. 65-15), and written informed consent was obtained from all patients, parent or guardian. In addition, the procedures of this manuscript were in accordance with the Helsinki declaration of 1964 and its later amendments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
2. Kulik L and El-Serag HB: Epidemiology and management of hepatocellular carcinoma. Gastroenterology 156: 477-491.e1, 2019.
3. Laursen L: A preventable cancer. Nature 516: S2-S3, 2014.
4. Schulze K, Nault JC and Villanueva A: Genetic profiling of hepatocellular carcinoma using next-generation sequencing. J Hepatol 65: 1031-1042, 2016.
5. Watson IR, Takahashi K, Futreal PA and Chin L: Emerging patterns of somatic mutations in cancer. Nat Rev Genet 14: 703-18, 2013.
6. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M and Gores G: Hepatocellular carcinoma. Nat Rev Dis Primers 2: 16018, 2016.
7. Quaas A, Oldopp T, Tharun L, Klingenfeld C, Krech T, Sauter G and Grob TJ: Frequency of TERT promoter mutations in primary tumors of the liver. Virchows Arch 465: 673-677, 2014.
8. Totoki Y, Tatsuno K, Covington KR, Ueda H, Creighton CJ, Kato M, Tsuji S, Donehower LA, Slagle BL, Nakamura H, *et al*: Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. Nat Genet 46: 1267-1273, 2014.
9. Nault JC and Zucman-Rossi J: TERT promoter mutations in primary liver tumors. Clin Res Hepatol Gastroenterol 40: 9-14, 2016.
10. Rudolph KL, Hartmann D and Opitz OG: Telomere dysfunction and DNA damage checkpoints in diseases and cancer of the gastrointestinal tract. Gastroenterology 137: 754-762, 2009.
11. Nault JC, Mallet M, Pilati C, Calderaro J, Bioulac-Sage P, Laurent C, Laurent A, Cherqui D, Balabaud C and Zucman-Rossi J: High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions. Nat Commun 4: 2218, 2013.
12. Nault JC, Calderaro J, Di Tommaso L, Balabaud C, Zafrani ES, Bioulac-Sage P, Roncalli M and Zucman-Rossi J: Telomerase reverse transcriptase promoter mutation is an early somatic genetic alteration in the transformation of premalignant nodules in hepatocellular carcinoma on cirrhosis. Hepatology 60: 1983-1992, 2014.
13. Lee SE, Chang SH, Kim WY, Lim SD, Kim WS, Hwang TS and Han HS: Frequent somatic TERT promoter mutations and CTNNB1 mutations in hepatocellular carcinoma. Oncotarget 7: 69267-69275, 2016.
14. Pezzuto F, Buonaguro L, Buonaguro FM and Tornesello ML: Frequency and geographic distribution of TERT promoter mutations in primary hepatocellular carcinoma. Infect Agent Cancer 12: 27, 2017.
15. Kawai-Kitahata F, Asahina Y, Tanaka S, Kakinuma S, Murakawa M, Nitta S, Watanabe T, Otani S, Taniguchi M, Goto F, *et al*: Comprehensive analyses of mutations and hepatitis B virus integration in hepatocellular carcinoma with clinicopathological features. J Gastroenterol 51: 473-486, 2016.
16. Zucman-Rossi J, Villanueva A, Nault JC and Llovet JM: Genetic landscape and biomarkers of hepatocellular carcinoma. Gastroenterology 149: 1226-1239 e4, 2015.
17. Hussain SP, Schwank J, Staib F, Wang XW and Harris CC: TP53 mutations and hepatocellular carcinoma: Insights into the etiology and pathogenesis of liver cancer. Oncogene 26: 2166-2176, 2007.
18. Tornesello ML, Buonaguro L, Tatangelo F, Botti G, Izzo F and Buonaguro FM: Mutations in TP53, CTNNB1 and PIK3CA genes in hepatocellular carcinoma associated with hepatitis B and hepatitis C virus infections. Genomics 102: 74-83, 2013.
19. de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A and Perret C: Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci USA 95: 8847-8851, 1998.
20. Clevers H and Nusse R: Wnt/β-catenin signaling and disease. Cell 149: 1192-1205, 2012.
21. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, Calderaro J, Bioulac-Sage P, Letexier M, Degos F, *et al*: Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 44: 694-698, 2012.
22. Saitta C, Lanza M, Bertuccio A, Lazzara S, Navarra G, Raimondo G and Pollicino T: Evaluation of CTNNB1 and TP53 variability in patients with hepatocellular carcinoma and occult hepatitis B virus infection. Cancer Genet 208: 513-516, 2015.
23. Statistics INIo: http: //dati.istat.it/?lang=en, 2018.
24. Fusco M, Girardi E, Piselli P, Palombino R, Polesel J, Maione C, Scognamiglio P, Pisanti FA, Solmone M, Di Cicco P, *et al*: Epidemiology of viral hepatitis infections in an area of southern Italy with high incidence rates of liver cancer. Eur J Cancer 44: 847-853, 2008.
25. Dal Maso L, Lise M, Zambon P, Crocetti E, Serraino D, Ricceri F, Vercelli M, De Lisi V, Tagliabue G, Federico M, *et al*: Incidence of primary liver cancer in Italy between 1988 and 2002: An age-period-cohort analysis. Eur J Cancer 44: 285-292, 2008.
26. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM and Sanyal AJ: The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American association for the study of liver diseases. Hepatology 67: 328-357, 2018.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Mostaid MS, Ahmed MU, Islam MS, Bin Sayeed MS and Hasnat A: Lung cancer risk in relation to TP53 codon 47 and codon 72 polymorphism in Bangladeshi population. Tumour Biol 35: 10309-10317, 2014.
29. Wu B, Guo D and Guo Y: Association between p53 Arg72Pro polymorphism and thyroid cancer risk: A meta-analysis. Tumour Biol 35: 561-565, 2014.
30. Wang YC, Chen CY, Chen SK, Chang YY and Lin P: p53 codon 72 polymorphism in Taiwanese lung cancer patients: Association with lung cancer susceptibility and prognosis. Clin Cancer Res 5: 129-134, 1999.
31. Jia S, Tang W and Luo Y: p53 codon 72 polymorphism and hepatocellular carcinoma: A meta-analysis. Hepatol Int 7: 669-675, 2013.
32. Zeng XT, Luo W, Geng PL, Guo Y, Niu YM and Leng WD: Association between the TP53 codon 72 polymorphism and risk of oral squamous cell carcinoma in Asians: A meta-analysis. BMC Cancer 14: 469, 2014.
33. Vinagre J, Almeida A, Populo H, Batista R, Lyra J, Pinto V, Coelho R, Celestino R, Prazeres H, Lima L, *et al*: Frequency of TERT promoter mutations in human cancers. Nat Commun 4: 2185, 2013.

34. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L and Garraway LA: Highly recurrent TERT promoter mutations in human melanoma. Science 339: 957-959, 2013.
35. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, Kadel S, Moll I, Nagore E, Hemminki K, *et al*: TERT promoter mutations in familial and sporadic melanoma. Science 339: 959-961, 2013.
36. Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, Diaz LA Jr, Friedman AH, Friedman H, Gallia GL, Giovanella BC, *et al*: TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. Proc Natl Acad Sci USA 110: 6021-6026, 2013.
37. Giedl J, Rogler A, Wild A, Riener MO, Filbeck T, Burger M, Rummele P, Hurst C, Knowles M, Hartmann A, *et al*: TERT core promotor mutations in early-onset bladder cancer. J Cancer 7: 915-920, 2016.
38. Park CK, Lee SH, Kim JY, Kim JE, Kim TM, Lee ST, Choi SH, Park SH and Kim IH: Expression level of hTERT is regulated by somatic mutation and common single nucleotide polymorphism at promoter region in glioblastoma. Oncotarget 5: 3399-3407, 2014.
39. Ko E, Seo HW, Jung ES, Kim BH and Jung G: The TERT promoter SNP rs2853669 decreases E2F1 transcription factor binding and increases mortality and recurrence risks in liver cancer. Oncotarget 7: 684-699, 2016.
40. Eckert D, Buhl S, Weber S, Jäger R and Schorle H: The AP-2 family of transcription factors. Genome Biol 6: 246, 2005.
41. Williams T and Tjian R: Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev 5: 670-682, 1991.
42. Huang H, Fujii H, Sankila A, Mahler-Araujo BM, Matsuda M, Cathomas G and Ohgaki H: Beta-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C virus infection. Am J Pathol 155: 1795-1801, 1999.
43. Zucman-Rossi J, Benhamouche S, Godard C, Boyault S, Grimber G, Balabaud C, Cunha AS, Bioulac-Sage P and Perret C: Differential effects of inactivated Axin1 and activated beta-catenin mutations in human hepatocellular carcinomas. Oncogene 26: 774-780, 2007.
44. Pezzuto F, Izzo F, Buonaguro L, Annunziata C, Tatangelo F, Botti G, Buonaguro FM and Tornesello ML: Tumor specific mutations in TERT promoter and CTNNB1 gene in hepatitis B and hepatitis C related hepatocellular carcinoma. Oncotarget 7: 54253-54262, 2016.
45. Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, Samson-Couterie B, Zennaro MC, Plouin PF, Skah S, *et al*: WNT/β-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. Hum Mol Genet 23: 889-905, 2014.
46. Boulkroun S, Samson-Couterie B, Golib-Dzib JF, Amar L, Plouin PF, Sibony M, Lefebvre H, Louiset E, Jeunemaitre X, Meatchi T, *et al*: Aldosterone-producing adenoma formation in the adrenal cortex involves expression of stem/progenitor cell markers. Endocrinology 152: 4753-4763, 2011.
47. Yoon YJ, Chang HY, Ahn SH, Kim JK, Park YK, Kang DR, Park JY, Myoung SM, Kim DY, Chon CY and Han KH: MDM2 and p53 polymorphisms are associated with the development of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. Carcinogenesis 29: 1192-1196, 2008.
48. Zhu ZZ, Cong WM, Liu SF, Xian ZH, Wu WQ, Wu MC, Gao B, Hou LF and Zhu GS: A p53 polymorphism modifies the risk of hepatocellular carcinoma among non-carriers but not carriers of chronic hepatitis B virus infection. Cancer Lett 229: 77-83, 2005.
49. Zhu ZZ, Cong WM, Liu SF, Dong H, Zhu GS and Wu MC: Homozygosity for Pro of p53 Arg72Pro as a potential risk factor for hepatocellular carcinoma in Chinese population. World J Gastroenterol 11: 289-292, 2005.
50. Rebbani K, Marchio A, Ezzikouri S, Afifi R, Kandil M, Bahri O, Triki H, El Feydi AE, Dejean A, Benjelloun S and Pineau P: TP53 R72P polymorphism modulates DNA methylation in hepatocellular carcinoma. Mol Cancer 14: 74, 2015.
51. Kołat D, Kałuzińska Ż, Bednarek AK and Płuciennik E: The biological characteristics of transcription factors AP-2α and AP-2γ and their importance in various types of cancers. Biosci Rep 39: pii: BSR20181928, 2019.