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.

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 (AFBI), a fungal mycotoxin, which contaminates foods that may act in synergy with chronic HBV infection. Exposure to AFBI 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/β-catenin 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±3.1 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 RNAlater 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-Cl (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 precipititation in pure cold ethanol. DNA was resuspended in 10 mmol/l Tris-Cl (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 the 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
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\text{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.

**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    | ACAAAAGTGCCCACGCGCACGCGAG              |
|              | hTERT REV    | CTGGCGTCCCTGCAACCCTGG                  |
| TP53 exon 7  | TP53 exon 7 FWD | GCGCACTGGCCTCATCTTGT                   |
|              | TP53 exon 7 REV | GGGTACGGCAAGCCAGAG                    |
| TP53 exon 4  | TP53 exon 4 FWD | CGGGTCCTCTGACGTGCTTCTT                |
|              | TP53 exon 4 REV | AGGCATTGAAGTCTCATGGA                  |
| CTNNB1 exon 3| CTNNB1 exon 3 FWD | GGTATTGGAAGTATACCATAC         |
|              | CTNNB1 exon 3 REV | CTGGTCCGTCGTATTGAGC               |

**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. χ² 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.
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 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 A>G) 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 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 AFBI, 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 AFBI) 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 carcinogenic process, and confirming them as excellent candidate biomarkers for early tumor detection or monitoring.

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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 the study was performed 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.

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