Review

Mutations in TP53, CTNNB1 and PIK3CA genes in hepatocellular carcinoma associated with hepatitis B and hepatitis C virus infections☆☆☆

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide. Hepatocarcinogenesis is a multistep process mainly associated with persistent infection with hepatitis B (HBV) or C (HCV) viruses and always involving the accumulation of genetic alterations over decades of chronic liver disease. Mutations in TP53 and CTNNB1 genes are considered the cancer drivers for HCC development with variable frequencies depending on the etiology. Here we present a comprehensive review evaluating somatic mutations in TP53 and CTNNB1 genes in HBV- and HCV-related HCCs. Moreover, we report the mutational analysis of TP53 (exons 4–9) and CTNNB1 (exon 3) as well as PIK3CA (exon 9) genes in HCC from Southern Italy. The overall mutation frequency of TP53 and CTNNB1 was 33.3%, while hotspot variations in PIK3CA were completely absent. CTNNB1 mutations were significantly associated with young age (P = 0.019) and moderately/poorly differentiated HCV-related HCC (P = 0.015). The extended analysis of genetic alterations will help to identify molecular markers for liver cancer prevention, diagnosis and treatment of HBV and HCV-associated liver cancer.

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Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; TP53, tumor protein p53 gene; CTNNB1, human β-catenin gene; PIK3CA, phosphatidylinositol 3-kinase catalytic subunit.

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1. Introduction

Hepatocellular carcinoma (HCC), with 748,000 cases and 695,000 deaths in 2008, is the most common liver malignancy as well as the third cause of death from cancer worldwide [1]. The regions of higher incidence are Eastern and South-Eastern Asia as well as Middle and Western Africa, with age standardized rates (ASR) ranging from 12.2 to 24.0 per 100,000 persons. In Europe, a remarkable North–South geographic gradient has been observed, with ASRs ranging from 3.8 per 100,000 men in Northern to 9.8 per 100,000 men in Southern Europe [1]. The highest incidence of HCC has been reported in men living in Southern Italy with a peak of 34.8 cases per 100,000 in the Campania region [2].

The majority of HCC cases are attributable to hepatitis B (HBV, 54%) and C (HCV, 31%) viruses, although a substantial geographic variation exists in the world. In Africa and Asia, where HBV is endemic, more than 60% of liver cancers are related to HBV infection, 20% are due to HCV and the remainder are caused by alcohol abuse and dietary exposure to aflatoxins. Conversely, in the United States, Europe, Egypt and Japan, more than 60% of HCCs are associated with HCV, 20% with HBV and the rest are mainly attributable to excessive alcohol consumption and liver metabolic diseases [3].

Both HBV and HCV, despite their distinct virologic features, are preferentially hepatotropic, not directly cytopathic, able to subvert the innate and adaptive immunity by several mechanisms and persist long into hepatocytes [4,5]. Chronic infections give rise to a complex, multistep process of hepatocarcinogenesis, lasting more than 30 years, that encompass prolonged inflammation, hepatocyte regeneration, fibrosis, cirrhosis, dysplasia and finally HCC [6–8]. Cirrhosis is characterized by reduced hepatocyte proliferation, fibrosis, destruction of liver cells and occurrence of dysplastic nodules in around 15% of cirrhotic patients [9]. During this process the molecular interaction between hepatitis viral products and the host cell machinery, with or without other environmental factors, contributes to the accumulation of a variety of genetic alterations which begin in preneoplastic lesions and increase in dysplastic hepatocytes and HCC [9]. Increased frequency of genetic damages may provide a progressive selective growth advantage of hepatocytes with a malignant phenotype leading to the development of phenotypically and genetically heterogeneous HCCs.

2. Chromosomal instability and genetic alterations in HBV- and HCV-related liver cancer

Chromosomal aberrations and genome instability are more common in HBV than in HCV-related HCCs. Structural genetic alterations, such as amplifications and deletions, develop slow during chronic hepatitis and increasingly in cirrhotic nodules and liver tumors [9]. Comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analyses of HCC DNA have shown the occurrence of chromosomal instability in more than 80% of HBV-associated tumors. The most distinctive events include gain of chromosomes 1q, 5, 6p, 7, 8q, 17q and 20; as well as loss of chromosomes 1p, 4q, 6q, 8p, 13q, 16, 17p and 21 [9–12]. The gain of 8q24 locus copy number, together with overexpression of c-Myc located on this region, has been significantly associated with HBV, HCV, and alcohol abuse but not with cryptogenic HCCs [13], suggesting that liver tumors can be classified in genetically homogeneous subclasses with different etiologies.

HBV sequences are found often integrated into the DNA of HCC as well as non-cancerous liver cells of chronic HBV carriers in associations with several genetic alterations [14–17]. Whole-genome deep sequencing showed that HCCs harbored large numbers of clonally-integrated HBV genomes, while autologous non-tumor tissues contained low-frequency viral integration sites scattered across the human genome, indicating that such events occur during the early stage of tumorigenesis [18]. HBV integration is accompanied by altered expression of human genes located near the integration sites, by formation of viral–human fusion genes, and changes in DNA copy number possibly introduced by genomic instability at viral–human junctions. Independent studies have reported recurrent HBV integrations within or upstream of the telomerase reverse transcriptase (TERT) gene in several HCC cases [19,20]. Other recurrent integration sites were found to affect cellular genes, such as FAR2, ITPR1, IRAK2, MAPK1, MLL2 and MLL4 genes, potentially providing cell growth advantages [20–24]. Recently, Sung et al. reported that HBV integration breakpoints frequently affect the 1800-bp region containing the viral enhancer, the X gene and the core gene [20]. This breakpoint usage may facilitate the formation of chimeric viral–human fusion genes, corrupting cellular tumor suppressors or imposing cis-regulatory effects on the expression of downstream cellular genes. Moreover, among 26 pairs of HCC and normal tissue samples having integrated HBV, only one breakpoint was shared between pairs of tumor and non-tumor DNAs [20]. These results strongly support the hypothesis that HBV contributes to the hepatocarcinogenesis by introducing numerous genetic lesions in different hepatocytes, some of which determine structural and functional alterations of genes relevant for cell cycle control.

HCV-related pathogenic processes, leading to viral cirrhosis and HCC, are much less known [25]. HCV is a completely cytoplasmic-replicating virus, maintaining its genome as an episome associated with endoplasmic reticulum. HCV causes chronic hepatitis in more than 80% of infected subjects, versus the 10% persistence of HBV infected patients; and it is 10 to 20-fold more efficient than HBV in promoting liver cirrhosis [5]. HCV pathogenic activity has been related mainly to the core protein and the non-structural proteins NS3 and NS5A, which are able to promote cell survival and growth, and contribute to the oncogenic transformation of infected hepatocytes [26]. Moreover, HCV proteins may concur indirectly to the genetic instability of infected cells through the suppression of DNA repair mechanisms, induction of DNA breaks, enhancement of the mutation frequency and chromosome rearrangements [27]. The non-structural HCV protein NS3 has both protease and helicase activities, which can directly contribute to genomic instability [28].

3. Mutations of tumor suppressor genes and oncogenes in HBV- and HCV-related hepatocellular carcinoma

During the past 20 years significant efforts have been made to define the patterns of somatic mutations affecting key pathways in HBV- and HCV-associated liver tumors. In particular, non-synonymous nucleotide changes have been commonly detected in TP53 (above 30%), CTNNB1 (19%) and AXIN1 (14%) genes, while homozygous deletions and epigenetic silencing have been identified in CDKN2A and CDKN2A alternate open reading frames (11.5%). More recently the application of new powerful tools, such as whole-genome or exome sequencing analysis, has confirmed most of previously known mutations in TP53 and CTNNB1 genes in HCC but also identified novel genetic alterations in genes critical for cell proliferation and tumor development [29]. According to the Catalog of Somatic Mutations in Cancer (COSMIC), four tumor suppressor genes (TP53, ARID1A, AXIN1 and CDKN2A) and one oncogene (CTNNB1) are mutated in more than 10% of the tumors, whereas several other genes are less frequently altered (Fig. 1) [30]. In particular, among mutated tumor suppressor genes ARID1A, encoding for a chromatin remodeling SWI/SNF complex component, was recently shown to be inactivated in several tumor types, including gastric, ovarian and bladder carcinoma, but its involvement in HCC was not known before the application of next generation technologies [31]. Recurrent somatic mutations in ARID1A and ARID2 genes have been found in 16.8% and 5.6% of HCC cases, respectively [10]. ARID1A mutations were significantly more frequent in HCC related to alcohol intake (P = 0.002) than in tumors of other etiologies and showed a significant association with CTNNB1 mutations;
while mutations in ARID2, encoding for a protein belonging to the polybromo- and Brg1-associated factor (PBAF) complex, were less frequent but exclusive from ARID1A mutations (P = 0.05) [10]. Fujimoto et al. reported an overall mutation frequency of 50% in chromatin regulator encoding genes, including ARID1A, ARID1B, ARID2, MLL and ML3, in HCC associated with hepatitis B or C viruses infections. This observation is consistent with the high heterogeneity of genetic alterations in liver cancer resulting from aberrations in chromatin remodeling.

3.1. The spectrum of mutations in TP53 gene in HBV- and HVC-related HCCs

Mutations in the TP53 tumor suppressor gene are among the most common genetic alterations in many human malignancies including liver cancer [32–36]. Up to 90% of TP53 mutations are non-synonymous and determine single amino-acid changes primarily within the DNA binding domain region located between codons 125 and 300 [37]. Amino acid substitutions in the p53 protein often affect its tertiary structure resulting in a stable protein with increased half-life and reduced DNA-binding activity [38]. A mutated p53 protein may inactivate wild-type p53 proteins via the formation of mutant and wild-type dimeric structures which in turn prevent the formation of functional tetramers [39–41]. Moreover several p53 mutants acquire ex novo pro-oncogenic properties, defined as “gain of function”, which include enhanced cell proliferation, resistance to drugs, increased cell migration and invasion as well as promotion of neo-angiogenesis [42–46].

Several studies have demonstrated a link between exposure to specific carcinogens, induction of definite mutational events within the TP53 gene and development of specific cancers, leading to the notion of “mutagen fingerprints” [47,48]. Among these signatures the well documented associations are: 1) exposure to ultraviolet irradiation with tandem CC to TT transitions in non-melanoma skin cancers [49]; 2) tobacco smoking derived polycyclic aromatic hydrocarbons with G to T transversions at specific G:C base pairs of TP53 gene in lung cancer [50,51]; 3) dietary exposure to aristolochic acid with A to T mutation in Balkan endemic nephropathy [52]; 4) and most prominently the dietary exposure to aflatoxin B1 (AFB1) with G to T transversion at the third base of codon 249 (AGG to AGT) in liver tumors [53,54].

Up to 50% of HCC, from geographic regions with dietary exposure to AFB1 and high prevalence of HBV infection, contains G to T mutations at codon 249 (arginine to serine, R249S) of TP53 gene [55]. A recent study conducted in Thailand showed that TP53 R249S mutation occurs preferentially in liver tumors that develop in a context of HBV infection without concomitant liver cirrhosis, suggesting that TP53 R249S represents one of the critical hit in the development of liver cancer [56]. Importantly, the TP53 R249S can also be detected in circulating free DNA from patients with HCC, so that TP53 mutant DNA may be a biomarker of exposure to AFB1 and possibly early diagnosis of HCC [57]. It has been hypothesized that AFB1-induced TP53 R249S mutation is an early event in the hepatocarcinogenesis process and HBV chronic infection has a multiplicative effect on the risk of HCC [58]. The mutated protein p53 R249S, in fact, is able to form stable complexes with the viral oncoprotein HBx and to exert tumor promoting activities in infected hepatocytes. HBx, on the other hand, has been shown to enhance HBV replication and to exert multiple effects on a wide range of host factors involved in apoptosis, cell proliferation and DNA repair [60,61]. In addition, TP53 mutations in HBV-related HCC, particularly the hot spot mutations at codons 249 and 157, have been associated with the acquisition of the stem cell-like phenotype, tumor aggressiveness and poor prognosis [62].

In geographic regions with low dietary exposure to AFB1 codon 249 is rarely affected and, instead, a variety of somatic mutations in TP53 gene may occur as a late event in liver carcinogenesis [63,64]. This hypothesis is supported by the appearance of dedifferentiated cellular subpopulations following the occurrence of TP53 mutations within HCC [65], the occurrence of distinct mutations in nodule-in-nodule HCCs [66], presence of more severe cellular atypia in areas with LOH of TP53 within HCC [64], and finally the high rate of TP53 mutations in moderately to poorly differentiated HCC [67]. Moreover, the spectrum of TP53 mutations is very wide with 75% missense mutations scattered over 200 codons mainly throughout the central portion of the gene affecting the central DNA-binding domain of the encoded protein [35,37].

To identify specific patterns of TP53 nucleotide changes in HCC associated with different etiologies, we have reviewed data annotated in the TP53 database (http://www-p53.iarc.fr/) of the International Agency for Research on Cancer (IARC) and the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). All TP53 mutations identified in HCC samples were stratified according to HBV, HCV, AFB1-induced R249S TP53 status and geographical origin (Africa, Americas, Asia and Europe). As shown in Fig. 2, we observed an overall TP53 mutation rate significantly higher in Africa (35.1%), Asia (31.6%) and Europe (25.2%) than Americas (11.4%), (P < 0.0001). More than 18% of African HCC samples were mutated at codon 249 in TP53 gene confirming that a high proportion of mutations in African regions are due to AFB1 exposure. In order to evaluate the frequency of TP53 mutations not associated to AFB1, we have excluded HCC samples with the TP53 R249S mutation and then stratified the remaining tumors by etiology and geographical origin. Among HBV-related and TP53 R249S-unrelated HCCs we observed a high mutation frequency in Asia (23.2%) and Europe (24.5%) and a lower frequency in Africa (6.25%), consistent with the preponderant role of AFB1 in HBV-related liver tumorigenesis in African regions. In HCV-related and TP53 R249S-unrelated HCC, on the other hand, TP53 mutations were similarly frequent in Africa (24.2%), Asia (25.3%) and Europe (27.6%), suggesting a minor effect of AFB1 in the development of liver cancer associated with HCV infection.

The analysis of mutated nucleotides in TP53 gene showed that more than 60% of nucleotide changes were localized in non-transcribed strand in both HBV- and HCV-related HCCs, suggesting that a strand bias occurred during the mutation process probably due to the preferential repair of the transcribed DNA during transcription-coupled repair [68]. Moreover, the nucleotide change G to A and the mirror transition C to T were more abundant in HBV-related (36%) than in HBV-related HCC (25%) (Fig. 3). The G to A transition may result from deamination of methylated cytosines at CpG sites originating from spontaneous deamination of 5-methylcytosine into thymine [69]. This process is
enhanced by oxygen and nitrogen radicals, leading to a higher load of CpG transitions in cancers arising from inflammatory precursors such as Barrett’s mucosa or ulcerative colitis [70–74]. Chronic inflammation and the generation of an excess reactive oxygen species, which causes oxidative DNA damage or increases susceptibility to the acquisition of somatic mutations, may be responsible for the larger proportion of

Fig. 2. Frequency of TP53 mutations in all HCCs (% TP53 mut) stratified by HBV (as percentage of all HBV-related R249S–unrelated HCC: % HBV TP53 mut) and HCV (as percentage of all HCV-related R249S–unrelated HCC: % HCV TP53 mut) status as well as by AFB1-induced R249S mutation. Cases associated with unspecified hepatitis virus and of unknown etiology have been grouped in the undetermined (as percentage of all undefined and R249S–unrelated HCC: % und TP53 mut) group. HBV and HCV double infections were included in the HBV group.

Fig. 3. Mutational patterns of TP53 gene in HBV- and HCV-related HCCs from different geographical regions. Cases associated with unspecified hepatitis virus and of unknown etiology have been grouped in the undetermined (% und CTNNB1 mut) group.
TP53 mutations accumulating in HCV-related cancer [75]. Free radicals, in fact, reactive nitrogen or oxygen species, have been demonstrated to directly damage DNA and proteins, and indirectly perturb these macromolecules via lipid peroxidation in the formation of DNA repair protein complexes and transcriptional transactivation of DNA repair genes [76]. Nucleotide changes G to T (at sites other than codon 249) and C to A were found more frequently in HBV-related HCC (29%) than in HCV-related HCC (19%). As shown in Fig. 3, following geographical stratification, G>T transversion frequency remained higher in HBV-positive HCC (36% of all mutations in Americas and Europe; 28% in Africa and Asia) compared to HCV-positive tumors (11% Americas and Europe; 18% in Africa and Asia). The nucleotide changes A to T and T to A were also relatively frequent among African and Asian samples (20% in HBV-positive; 22% in HCV-positive), suggesting a possible genotoxic effect of agents such as vinyl chloride which has been shown to induce A to T transversion in animal models [77].

The effect of TP53 mutations on pathway deregulation has been studied by microarray analysis of genes differentially expressed in HCC with mutant or wild type TP53 gene. Okada et al. found 83 differentially expressed genes in TP53 mutant compared with wild type TP53 HCCs [78]. Among these genes, overexpression was described for cell cycle-related genes (CCNB2, BZW1) and cell proliferation-related genes (SSR1, ANX2, S100A10, and PTMA). These results together with data demonstrating that p53 mutations constitute an unfavorable prognostic factor, related to recurrence in HCC, suggest that mutant p53 tumors may have higher malignant potentials than those with wild type p53 [62,79].

3.2. CTNNB1 mutations in hepatocellular carcinoma

β-Catenin, encoded by CTNNB1 gene, is a crucial element of the canonical Wnt signaling pathway [80]. In the absence of Wnt, cytoplasmic β-catenin protein is phosphorylated at the N-terminal domain (serine 33, 37 and 45, threonine 41) by the Axin complex (composed of Axin, APC, CK1, and GSK3) and then ubiquitinated and degraded by β-Trcp ubiquitin ligase [81]. Activation of Wnt signaling induces the stabilization and translocation of β-catenin to the nucleus, where it associates with transcription factors of the TCF family and generates a functional complex able to transactivate several genes (MYC, CCND1, MT-CO2, and MMP7) involved in the cell cycle control and proliferation [82,83].

Aberrant activation of Wnt signaling and nuclear accumulation of β-catenin has been reported in several types of cancer, including HCC [84]. The most common mechanisms of β-catenin increase in HCC are mutations in CTNNB1 gene, identified in about 20–40% of liver cancers [85–87], and mutations in genes encoding for the components of the degradation complex of β-catenin including AXIN1 (3–16%) [75,173,175,176], and AXIN2 (3%) genes [86,88]. Most CTNNB1 mutations occur within the exon 3 of the gene, in a region encoding for the protein sequence containing the consensus sites for phosphorylation. Mutation of serine/threonine residues results in impaired Axin/APC/GSK3β mediated degradation of β-catenin and gain of oncogenic activity [89]. Several studies demonstrated a critical role of β-catenin mutations in hepatocarcinogenesis. In particular, CTNNB1 mutations are found to be associated with increased levels of glutamine synthetase, higher macrovascular and microvascular invasions and tumor size increase [90]. Moreover, Wnt/β-catenin signaling has shown to play an important role in oval cell activation, considered to be hepatic stem cells, and HCCs with stem cell signatures are shown to have a more aggressive behavior [91,92]. However the clinical implications of CTNNB1 missense mutations in terms of overall survival and disease recurrence have not yet been comprehensively evaluated.

Whole genome sequencing studies confirmed previous analyses showing that CTNNB1 is the most frequently mutated oncogene in HCCs [18]. Interestingly, a high frequency of CTNNB1 mutations has been shown in HCCs occurring in HCV-positive patients [10,19,93]. In particular, Guichard et al. analyzed the spectrum of mutations in CTNNB1, TP53 and several previously uncharacterized genes in HCC samples from a cohort of French patients [10]. They observed that CTNNB1 mutations were present in 11.4% of HBV-associated HCC, in 33.3% of HCV-related HCC and in 41.8% of alcohol-related HCCs [10]. Although CTNNB1 gene has been found mutated at lower frequency in HBV-positive tumors, it has been demonstrated that the Wnt/β-catenin pathway is always perturbed in HBV-related tumors by the direct action of HBx protein which decreases proteasomal degradation of β-catenin [94,95]. Interestingly mutations of CTNNB1 and TP53 gene appear to be largely mutually exclusive, suggesting that inactivation of either pathway is sufficient to induce cellular transformation [10]. Moreover, HCCs characterized by activation of Wnt/β-catenin pathway exhibit specific features such as high differentiation associated with a homogeneous microtrabeculo-acinar pattern, low-grade cellular atypia, and cholestasis [96]. These findings demonstrate an essential role of the Wnt signaling in hepatocarcinogenesis and suggest that targeting this pathway may be promising for therapeut options.

We have differentiated the CTNNB1 mutations annotated in COSMIC database according to HBV and HCV status and to geographical origin (Africa, Americas, Asia and Europe). As shown in Fig. 4, we observe an overall CTNNB1 mutation rate higher in Europe (25.2%) and Americas (21.8%) than Asia (15.9%). The single study performed in Northern Africa showed only 4 mutated cases in 42 (9.5%) HCCs. Among HCV-related HCC, CTNNB1 mutations were similarly distributed in HCC from Asia (28.3%) America (25.6%) and Europe (23.6%), suggesting the absence of an exogenous genotoxic factor diversely distributed in different geographic regions. On the other hand, frequency of CTNNB1 mutations in HBV-related HCCs is equal or below 10% in all studied regions (Asia 9.2%, America 10% and Europe 6.8%) with exception of Africa where stratification by hepatitis virus was not available.

During the last 10 years, microarray technology enabled the analysis of global gene expression in a large number of HCCs and the identification of tumors with specific signatures of pathway deregulation, including those specifically associated to CTNNB1 mutations [91,97–100]. Boyault et al. performed an unsupervised transcriptomic profile along with genetic analysis in 60 HCCs and they identified six subgroups of tumors (termed G1 to G6) with distinct clinical and genetic characteristics [101]. HCCs from groups G1 to G3 were chromosome instable whereas tumors from groups G4 to G6 were chromosome stable [101]. Interestingly, HCCs falling into G5 and G6 were highly mutated in CTNNB1 gene and Wnt pathway was activated. Tumors of G6 group, characterized by satellite nodules, show the higher activation of the Wnt pathway and E-cadherin underexpression [101].

Finally, mutations in CTNNB1 genes have been associated to down-regulation of miR-107, a small non-coding RNAs, which modulates the HNF1α gene involved in β-catenin regulation via downregulation of miR-375 [102]. Hence, liver tumors show also distinct miRNA expression fingerprints according to malignancy, risk factors as well as mutations in oncogenes and tumor suppressor genes.

3.3. PIK3CA gene mutation in HCC

Controversial reports have been published on the presence of somatic mutations in the exon 9 of phosphoinositide-3-kinase-catalytic-alpha (PIK3CA) gene. The PIK3CA gene has been found mutated in approximately 35.6% of HCC cases in China [103], in 28% of HCC in Italy [104] and in zero cases of HCC in Japan [105]. PIK3CA is an effector of the phosphatase and tensin homolog (PTEN)–AKT pathway affecting cell proliferation, apoptosis and angiogenesis. Knowledge of mutational status of PIK3CA gene is particularly relevant considering that several anticancer drugs, targeting PI3K/Akt pathway, have given promising preliminary results in human malignancies [106]. However, few studies have been conducted to evaluate the
occurrence of PIK3CA gene in HCC associated with different etiologies in different regions of the world and no clear conclusion can be drawn on the role of such gene in liver cancer.

4. TP53, CTNNB1 and PIK3CA gene mutation in HCC from Southern Italy

In the province of Naples (Southern Italy) 23.2% of men aged 65 years or older are HCV-positive and 61% of HCC cases are attributable to HCV infection [107,108]. Liver cancer develops in 1% to 3% of HCV-positive subjects in 20–30 years after they become infected, suggesting that besides HCV other factors may contribute to tumorigenesis [109–111]. We have evaluated the mutation frequency of TP53 (exons 5–8), CTNNB1 (exons 3) and PIK3CA (exon 9) genes in a series of HCV- and HBV-positive cases diagnosed with hepatocellular carcinoma from people living in the province of Naples.

Liver samples from patients with primary HCC (n = 67) and cirrhosis (n = 10) were analyzed for TP53, CTNNB1 and PIK3CA mutations. Gender, viral infection and tumor differentiation are described in Table 1. The majority of patients were males (78.7%), and the mean ages were 66.3 ± 9.3 (range 39–85) and 74.4 ± 7.3 (range 61–87) for males and females, respectively. The greater number of cases was HCV-positive cirrhotic patients with HCC.

The TP53 gene was altered in 9 out of 57 (15.8%) HCV-positive and in two out of 10 (20%) HBV-positive HCC cases. The most common nucleotide changes were missense mutations (n = 10) and in one case there was a single nucleotide deletion creating a frameshift (Table 2). The AFB1-related G to T transversion at codon 249 was not detected in any HCC sample.

Ten out of 57 (17.5%) HCV-positive and none of the HBV-positive HCC cases harbored nucleotide changes in CTNNB1 gene. Six mutations affected serine residues (Ser33, Ser37 and Ser45) (Fig. 5). No insertion or deletions were detected. CTNNB1 mutations were significantly

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**Table 1**
Distribution of mutations in TP53 and CTNNB1 genes according to the characteristics of HCC Patients.

| Characteristics | TP53 | | CTNNB1 | |
|-----------------|------|-----------------|--------|
| | No Mut | % | P | No Mut | % | P |
| Sex | | | | | | |
| Male (n = 53) | 8 | 15.1 | 0.686 | 9 | 16.7 | 0.675 |
| Female (n = 14) | 3 | 21.4 | 1 | 7.1 |
| Age | | | | | | |
| ≤60 (n = 13) | 3 | 23.1 | 0.473 | 5 | 30.8 | 0.019 |
| >60 (n = 54) | 8 | 14.8 | 5 | 9.3 |
| Grading* | | | | | | |
| G1 (n = 2) | 0 | 0.614 | 0 |
| G2 (n = 57) | 9 | 15.8 | 6 | 10.5 |
| G3 (n = 8) | 2 | 25.0 | 4 | 37.5 |
| pT | | | | | | |
| pT1 (n = 8) | 1 | 12.5 | 0.912 | 1 | 12.5 | 0.979 |
| pT2 (n = 33) | 6 | 18.2 | 5 | 12.1 |
| pT3 (n = 26) | 4 | 15.4 | 4 | 15.4 |
| No Tumor sites | | | | | | |
| Single (n = 41) | 7 | 17.1 | 0.856 | 4 | 11.1 | 0.169 |
| Multiple (n = 26) | 4 | 15.4 | 6 | 12.9 |
| Virus | | | | | | |
| HBV (n = 10) | 2 | 20.0 | 0.742 | 0 | 0.337 |
| HCV (n = 57) | 9 | 15.8 | 10 | 17.5 |

* Well differentiated (G1); Moderately differentiated (G2); Poorly differentiated carcinoma (G3).

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**Table 2**
TP53 and CTNNB1 mutations are mutually exclusive in hepatocellular carcinomas.

<table>
<thead>
<tr>
<th>ID</th>
<th>Grade</th>
<th>Etiology</th>
<th>TP53 mutation</th>
<th>CTNNB1 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-137</td>
<td>G2</td>
<td>HBV</td>
<td>134, TTT&gt;TTA Phe&gt;Leu; 275 TGT&gt;TAT Cys&gt;Tyr</td>
<td></td>
</tr>
<tr>
<td>LC-159</td>
<td>G2</td>
<td>HBV</td>
<td>274, GTT&gt;GTC</td>
<td></td>
</tr>
<tr>
<td>LC-061</td>
<td>G3</td>
<td>HCV</td>
<td>245, GCC&gt;GCA Gly&gt;Val</td>
<td></td>
</tr>
<tr>
<td>LC-132</td>
<td>G2</td>
<td>HCV</td>
<td>232, ATC&gt;AAC Ile&gt;Asn</td>
<td></td>
</tr>
<tr>
<td>LC-153</td>
<td>G2</td>
<td>HCV</td>
<td>226, Delta G (GCC&gt;GCG)</td>
<td></td>
</tr>
<tr>
<td>LC-156</td>
<td>G2</td>
<td>HCV</td>
<td>137, CTG&gt;CCA</td>
<td></td>
</tr>
<tr>
<td>LC-180</td>
<td>G2</td>
<td>HCV</td>
<td>220, TAT&gt;CAT Tyr&gt;His</td>
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</tr>
<tr>
<td>LC-181</td>
<td>G2</td>
<td>HCV</td>
<td>208, GAC&gt;GTC Asp&gt;Val</td>
<td></td>
</tr>
<tr>
<td>LC-187</td>
<td>G2</td>
<td>HCV</td>
<td>245, GGC&gt;GAC Gly&gt;Asp</td>
<td></td>
</tr>
<tr>
<td>LC-226</td>
<td>G2</td>
<td>HCV</td>
<td>311, AAG&gt;GGC Gln&gt;Asp</td>
<td></td>
</tr>
<tr>
<td>LC-237</td>
<td>G2</td>
<td>HCV</td>
<td>245, GCC&gt;GAG Gly&gt;Asp</td>
<td></td>
</tr>
<tr>
<td>LC-064</td>
<td>G3</td>
<td>HCV</td>
<td>35, ATC (Ile)&gt;TTT (Phe)</td>
<td></td>
</tr>
<tr>
<td>LC-117</td>
<td>G3</td>
<td>HCV</td>
<td>37, TCT (Ser)&gt;TAT (Tyr)</td>
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</tr>
<tr>
<td>LC-129</td>
<td>G2</td>
<td>HCV</td>
<td>37, TCT (Ser)&gt;TAT (Tyr)</td>
<td></td>
</tr>
<tr>
<td>LC-138</td>
<td>G2</td>
<td>HCV</td>
<td>45, TCT (Ser)&gt;TCT (Pro)</td>
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</tr>
<tr>
<td>LC-144</td>
<td>G2</td>
<td>HCV</td>
<td>34, GAA (Glu)&gt;GGA (Glu)</td>
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</tr>
<tr>
<td>LC-177</td>
<td>G3</td>
<td>HCV</td>
<td>33, TCT (Ser)&gt;TCT (Cys)</td>
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<td>HCV</td>
<td>33, TCT (Ser)&gt;GCT (Ala)</td>
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<td>LC-212</td>
<td>G2</td>
<td>HCV</td>
<td>34, GCA (Glu)&gt;GTA (Val)</td>
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<tr>
<td>LC-225</td>
<td>G3</td>
<td>HCV</td>
<td>37, TCT (Ser)&gt;TTT (Phe)</td>
<td></td>
</tr>
<tr>
<td>LC-235</td>
<td>G2</td>
<td>HCV</td>
<td>32, GAC (Asp)&gt;GAC (Glu)</td>
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</tr>
</tbody>
</table>
correlated with the younger age ($P = 0.019$) and poorly differentiated HCC histotype ($P = 0.015$).

The combined mutational pattern of CTNNB1 and TP53 genes showed that relevant proportions of nucleotide changes were C:G>T:A transitions (33%), A:T>G:C transitions (17%) and A:T>T:A transversions (17%) potentially arising from deamination of DNA bases. The other mutations were A:T>C:G (17%), C>G>A:T (12%) and C>G>C:G mutations (12%).

The exon 9 of PIK3CA gene was mutated at codon 518 (ACT>GCT, Ala>Thr) only in one out of 67 (1.5%) HCCs. Moreover, a rare synonymous polymorphism in PIK3CA gene at codon 495 (rs45455192, TCC>TCT, Ser/Ser) was observed with T/T homozygous (1.5%), C/T heterozygous (14.9%) and C/C homozygous (83.6%) genotypes of all analyzed liver tissues. There was no statistical significant difference in the frequency of this polymorphism between HCCs with different characteristics.

No mutations in TP53, CTNNB1 and PIK3CA genes were detected in DNA samples extracted from cirrhotic tissues.

5. Conclusions

There are two main etiological types of HCC, heterogeneously distributed in different geographical regions, owing to HBV or HCV infection [3]. Several studies have explored the molecular differences between them. The analysis of public available databases showed that TP53 mutations could be defined as the cancer drivers for both HBV-related and HCV-related HCCs due to the high mutation frequency of this tumor suppressor gene in the two types of tumors. In low-resource regions (mainly in Africa and Asia) AFB1 exposure represent the main genotoxic factors for TP53 R249S mutation and development of HCC in association with HBV infection. Mutations in CTNNB1 gene, on the other hand, are similarly frequent in HCV-related and non-viral related HCCs from different geographic regions, while are underrepresented in HBV-associated tumors.

The results obtained in our Italian HCC series show that somatic mutations in TP53 gene are similarly represented in HBV- (20%) and HCV-related (15.8%) HCC cases and comparable to that previously reported among HCC cases from Northern Italy (25%) and France (18%) [112]. Moreover, TP53 mutations were similarly distributed among the different patient groups, and no statistically significant correlation with sex, onset age and histological tumor grading was found. We observed no mutations at hot spot codons 249 indicating that AFB1 is not a relevant carcinogenic agent in HCC for patients from Southern Italy.

In agreement with the previous report [10], we found that mutations in exon 3 of CTNNB1 gene occurred exclusively in HCV-related HCCs (17.5%). All mutations were heterozygous, located between codon 32 and codon 45, and mutually exclusive with mutations in TP53 gene. The proportion of activating mutations in CTNNB1 gene was significantly lower compared to that observed among French HCC cases [10]. The lower mutation frequency may be due to the fact that all cases analyzed in our study were cirrhotic HCCs and CTNNB1 mutations were described to be particularly prevalent in non-cirrhotic HCC patients [90]. On the other hand, non-cirrhotic HCC are rarely found in the Italian population representing less than 2% of HCC cases [111]. A relevant observation from our study was that patients with HCC mutated in CTNNB1 gene were significantly younger or with moderately/poorly differentiated tumors compared to patients with wild-type CTNNB1.

Hot spot mutations in the exon 9 of PIK3CA were found in none of the HCC samples. This observation is in agreement with previous studies performed in HCC series from Japan and France showing that mutations in PIK3CA are rare and likely not a key event in liver carcinogenesis [10,105]. The controversial results concerning the high frequency of mutations in exon 9 of PIK3CA gene in HCC samples from China [103] and Southern Italy [104] might partially be explained by the confounding effect of PIK3CA pseudogene sequences located on chromosome 22 and sharing 98% homology with PIK3CA exon 9 [105,113]. The GT to C mutation at nucleotides 1658–1659 described in 15% of HCC by Colombino et al., was identified in 27.7% of HCC and autologous noncancerous liver by Tanaka et al. and shown to be a PIK3CA pseudogene sequence [105]. Therefore, particular attention should be paid on the interpretation of data related to hot spot mutations in PIK3CA gene.

In conclusion, our analysis suggests that TP53 and CTNNB1 genes together contribute to define distinct subgroups of viral-related HCC. Further studies will be needed to define whether stratification of HCC patients by TP53 and CTNNB1 mutational status might be predictive of prognosis and relevant for patient tailored therapy.
6. Materials and methods

6.1. Patient and tissue samples

Liver biopsies from 57 HCV-positive, 10 HBV-positive, along with 10 cirrhotic liver tissue samples were obtained with informed consent from patients treated at the liver unit of the INT “Pascale” in Naples. Patients were classified according to Child–Pugh score into A (n = 56), B (n = 9) and C (n = 2). Tumor size and number of tumor nodules were determined by routine imaging (i.e., computed tomography or magnetic resonance imaging). Each liver biopsy was divided in two sections: the first section was stored in RNA Later at −80 °C (Ambion, Austin, TX), the second was subjected to histopathologic examination. Only liver biopsies histological confirmed to be hepatocellular carcinoma or cirrhotic tissue were included in the study.

The 67 HCCs were divided into three groups according to their histological grade: well differentiated (G1) HCC (n = 2), moderately differentiated (G2) HCC (n = 57) and poorly differentiated (G3) HCC (n = 8) according to the criteria of Edmondson and Steiner [114].

Genomic DNA was extracted according to published procedures [115]. Tissue samples were digested by proteinase K treatment (150 μg/ml at 56 °C for 2 h) in 100–500 μl of lysis buffer (10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 150 mM NaCl, 1% SDS), followed by DNA purification with phenol–chloroform–isoamyl alcohol (25:24:1) extraction and ethanol precipitation in 0.3 M sodium acetate (pH 4.6). The study protocol was approved by the local ethical review board.

6.2. TP53 codon 4–9 mutational analysis

The analysis of TP53 gene in exons 4–9 was performed using specific oligonucleotides and amplification protocols according to the IARC guidelines (http://www-p53.iarc.fr/Download/TP53_DirectSequencing_IARCppdf.pdf). All PCR reactions were undertaken using 10 to 100 ng genomic DNA in 50-μl reaction mixture following the IARC amplification procedures. All samples with sufficient amount of DNA were subjected to bidirectional direct sequencing analysis by Primm Srl Laboratories (Milan, Italy).

6.3. CTNNB1 exon 3 and PIK3CA exon 9 mutational analysis

CTNNB1 exon 3 was amplified using the oligonucleotides Beta-F (5′-CCCAAGACATAGTCTGATTTGGTGGAGACGCGG-3′) and Beta-R (5′-GCTCCGATTCTGGGATTATTTGAACT-3′) able to generate a 310 bp fragment which covers hot spot codons 33, 37, 41 and 45. PIK3CA exon 9 was amplified using the oligonucleotides PIK3-9-F1 (5′-TGGTCTTGTT GTTGGCTAA) and PIK3-9-R1 (5′-CTTACCTGTGACTCCATAGA) generating a 410 bp fragment encompassing the hot spot codons 542 and 545 and designed to avoid amplification of the PIK3CA pseudogene. PCR reactions were performed in 50 μl reaction mixture containing 50 to 100 ng of target DNA, 5 pmol of each primer, 2.5 mM MgCl2, 50 mM of each dNTP and 5 μl Hot Master buffer and 1 μl of Hot Master Taq DNA Polymerase (5 Prime GmbH, Hamburg, Germany). DNA was amplified in a Perkin-Elmer GeneAmp PCR System 9700 thermal cycler with the following steps: an initial 1-min denaturation at 94 °C, followed by 45 amplification cycles of 58 °C for 30 s, 72 °C for 30 s, 94 °C for 30 s and a 1-min final annealing at 58 °C followed by 5-min elongation at 72 °C. All samples were subjected to bidirectional direct sequencing analysis.

6.4. Statistical analyses

A Fisher’s exact test or Yates corrected $\chi^2$ test was used, as appropriate, to compare the proportions of cases mutated in TP53 or CTNNB1 genes among patients stratified by sex, age, tumor grading and hepatitis virus infection. All analyses were performed with Epi Info 6 Statistical Analysis System Software (6.04d, 2001, Centers for Disease Control and Prevention, USA). Differences were considered to be statistically significant when P values were less than 0.05.


