HHS Public Access

Author manuscript

J Dig Dis Hepatol. Author manuscript; available in PMC 2017 February 13.

Published in final edited form as:

J Dig Dis Hepatol. 2016 ; 2016(5): .

Association between Hepatitis C Virus Infection, p53 Phenotypes, and Gene Variants of Adenomatous Polyposis Coli in Hepatocellular Carcinomas

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Abstract

Objective—To investigate the clinical value of p53 codon 72 single nucleotide polymorphisms (SNPs) and variants of adenomatous polyposis coli (APC) in hepatocellular carcinomas (HCCs).

Methods—DNA and RNA from 51 HCCs and their matching, uninvolved liver tissues were analyzed for p53 mutations, and the methylation and expression of APC variants were determined. Proliferation of each HCC was assessed by Ki67 immunohistochemistry. The results were correlated with the demographic and clinicopathologic features and patient survival.

Results—Of 51 HCCs, 12% exhibited missense p53 mutations. SNP analysis of p53 codon 72 demonstrated the highest prevalence of the Arg/Arg (56%) phenotype, followed by Arg/Pro (33%) and Pro/Pro (11%). Four of five cases with the Pro/Pro phenotype were African Americans (AAs). All five cases with the Pro/Pro phenotype had hepatitis C virus (HCV) infections, a high Ki67 index, and lower median survival (15.5 months) compared to those with Arg/Arg or Arg/Pro phenotypes (32 months). The overall frequency of APC methylation was 31%, which was found predominantly in Caucasians. There was lower mRNA expression of APC variants-2 and -3 in both HCCs and corresponding adjacent, uninvolved liver tissues as compared to APC variant-1. The expression of APC variant-3, but not variants-1 and -2, was lower in HCCs relative to uninvolved tissues. Expression of all APC variants was lower in HCCs with APC methylation

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Competing Interests: None

Financial disclosures: None

Author contributions: Leona N. Council, Chandrakumar Shanmugam, and Venkat R. Katkoori contributed equally to this work; Leona N. Council, Chandrakumar Shanmugam, Venkat R. Katkoori, Martine J. Heslin, Nirag C. Jhala and Upender Manne designed the research; Leona N. Council, Chandrakumar Shanmugam, and Venkat R. Katkoori performed the research; Leona N. Council, Chandrakumar Shanmugam, Venkat R. Katkoori, Alex Hanna, and Upender Manne analyzed the data; and Leona N. Council, Chandrakumar Shanmugam, Venkat R. Katkoori, Esther A. Suswam, and Upender Manne wrote the manuscript.

relative to HCCs without APC methylation, and low expression of APC variant-2 was associated with the Pro/Pro phenotype.

Conclusions—These findings suggest that, for AA patients with HCCs, the p53 Pro/Pro phenotype and low expression of APC variant-2 are associated with aggressive tumor behavior, HCV infection, and poor clinical outcome.

Keywords

Hepatocellular carcinoma; APC; p53 codon 72 polymorphism; HCV; Survival

Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide [1–5]. In the United States, there were 26,190 new cases and 19,590 deaths due to HCCs in 2011 [6]. Both the incidence and mortality of this disease are increasing [7,8] primarily due to hepatitis C virus (HCV) infections [3,9]. Worldwide, hepatitis B virus (HBV) and HCV infections, cigarette smoking, and/or heavy alcohol consumption are factors contributing to HCCs [10]. Although surgery is useful in some cases, cirrhosis in the adjacent, uninvolved liver tissue predisposes patients to metachronous HCCs. Thus, there is a need for biomarkers that can be used in assessment of the prognosis and in treatment decisions.

The pathogenesis of HCCs involves genetic alterations; irrespective of etiology, there is loss of p53 function [11]. The p53 mutational spectra vary with geographic regions, based on etiological differences and host susceptibility. People in sub-Saharan Africa and China, who have endemic exposure to aflatoxin B1 (AFB1) have a higher mutation frequency at codon 249 in p53 [12]. In the USA and Europe, p53 mutations with no specific hotspot occur in HCCs, either along with or after loss of heterozygosity (LOH) of p53 [13]. For HCCs, p53 mutations correlate with disease recurrence and poor prognosis [14]. In addition, the HBx gene of HBV is integrated into the host genome and binds to p53 to suppress its DNA binding and transcription as well as p53-mediated apoptosis [15]. Although the HCV genome does not integrate into the host genome, it suppresses the transcriptional activity of the p53 promoter [16]. The codon 72 polymorphism of p53 results in Pro/Pro and Arg/Arg phenotypes, with the Pro/Pro phenotype having less apoptosis-inducing potential and higher cancer cell proliferation [17,18]. p53 codon 72 polymorphisms are associated with risk of various cancers, including those of the liver [19,20]. The p53 codon 72 Pro/Pro phenotype is associated with increased risk of tumor progression and poor patient survival for non–small cell lung cancers [21] and colorectal cancers (CRCs), particularly for (AAs) [22].

The adenomatous polyposis coli (APC) protein functions as a tumor suppressor by forming a tetramer with β-catenin, conductin (a negative regulator of Wnt pathway), and serine-threonine glycogen synthase kinase-3β, which enhances β-catenin degradation, thereby preventing signal transduction to downstream transcription factors [23]. This function is altered due to mutations [24] and/or LOH [25] in the APC gene, and/or to APC promoter hypermethylation [26]. Unlike for tumors of other anatomic sites, mutations of the APC gene have not been identified in HCCs; however, APC promoter hypermethylation is

frequent [27]. The present investigation focused on assessing the clinical value of the p53 codon 72 polymorphism, APC promoter hypermethylation, and expression of APC variants together in HCCs.

Materials and Methods

Patients and tissues

This study was approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB). Medical records, including surgical pathology reports, were reviewed by two gastrointestinal pathologists (LNC & CS). From 137 HCC patients who had undergone transplantation or resection as a curative procedure between 1991 and 2009, formalin-fixed, paraffin-embedded (FFPE) tissue samples were obtained for 51 cases. None of these patients had received pre-surgical chemo- or radiation therapy. Diagnosis for chronic HCV infection was assessed based on the anti-HCV antibody status of the patients. The pathologists also reviewed hematoxylin and eosin-stained slides for pathologic features. HCCs were graded as well, moderate, or poorly differentiated [28], and staged by the criteria of the American Joint Commission on Cancer [29].

This patient population consisted of 15 AAs and 34 non-Hispanic Caucasians (CAs); for 2 patients, racial/ethnic information was missing. All patients were followed by the UAB tumor registry until their death or the date of the last documented contact within the study time frame. The registry also ascertained this information directly from patients or living relatives and/or from physician charts and validated it against State Death Lists. The registry updated information every 6 months; follow-up ended in December 2011.

p53 gene mutational analysis

DNA was extracted from 51 HCCs and their corresponding uninvolved liver tissues by a modified deparaffinization protocol [30]. Of these, 48 DNA samples were analyzable for p53 mutations (exon 4 through 9) by the polymerase chain reaction (PCR) and direct sequencing using exon-specific primers, as described in our previous report [22]. In brief, the PCR products were purified by electrophoresis on 3% agarose gels. The purified products were sequenced with an ABI 3100 sequencer. Compilation and sequence analyses were accomplished with LASERGENE (DNA STAR Inc., Madison, WI) software, which allows direct analysis of sequenc-ing electrophoretograms for detection of duplex sequence signals at each position to identify mutations/polymorphisms. Nucleotide changes in each exon sequence were confirmed by sequencing the opposite strand.

Methylation status of APC gene

DNA methylation status in the CpG island of the APC gene promoter was determined by chemical modification of the unmethylated, but not the methylated, cytosines to uracil, and subsequent PCR using primers specific for either the methylated or the modified unmethylated DNA [31]. Primer sequences of APC were, for the unmethylated reaction, 5′-gtgttttattgtggagtgtgggtt-3′ (forward primer) and 5′-ccaatcaacaaactcccaacaaa-3′ (reverse primer) and, for the methylated reaction, 5′-tattgcggagtgcgggtg-3′ (forward primer) and 5′-tcgacgaactcccgacga-3′ (reverse primer). Briefly, 1 μg of DNA was modified and purified

with an imprint DNA modification kit (Sigma-Aldrich, Saint Louis, MO). Controls without DNA were performed for each set of primers. Portions of each PCR reaction (10 μL) were loaded onto 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

APC gene expression analysis

Total RNA from 51 HCCs and matching uninvolved tissues was extracted using RNeasy Kits (Qiagen Life Sciences, Valencia, CA), and 1 μg of RNA was reverse transcribed. For each HCC and matching uninvolved tissue, the expression levels of three previously identified transcript forms of the APC gene (variant-1, NM_001127511.2; variant-2, NM_001127510.2; and variant-3, NM_000038.5) [32]were determined by quantitative real-time PCR (qRT-PCR) with transcript-specific primers. Briefly, PCR was performed with SYBR green reagent supermix (Bio-Rad laboratories, Hercules, CA) in a final volume of 25 μL consisting of 0.5 μL of each primer (5 pmoles), 12.5 μL of 2× supermix containing the reaction buffer, Fast-start Tag DNA double strand-specified SYBR green I dye, 7.0 μL of nuclease-free water, and 5 μL of the cDNA template. Incubation was at 95°C for an initial denaturation, followed by 45 cycles of 15-sec denaturations at 95°C, and annealing and extension at 60°C for 30 sec. The PCR reactions, performed in sets of four, were accomplished with an i-Cycler real-time PCR system (Bio-Rad). To exclude non-specific amplification, PCR products were subjected to melting curve analysis. For each patient, means of APC gene transcripts and β-actin gene copy numbers were calculated, and ratios of these means (APC/β-actin) were generated.

Immunohistochemistry

Sections (5-μm) representative of uninvolved and tumor tissue were cut from FFPE blocks and mounted on Superfrost/Plus slides (Fisher Scientific; Pittsburgh, PA). In brief, sections were melted at 60°C for 1.5 hr and incubated overnight at 37°C prior to staining. Sections were then deparaffinized in xylene, rehydrated in graded alcohols, and transferred to a Tris-buffer bath (0.05M Tris base, 0.15 M NaCl, and 0.01% Triton X-100, pH 7.6). Heat-induced antigen retrieval was performed with a pressure cooker at 15 psi for 10 min with EDTA buffer (0.01 M, pH 9). Each section was treated with 3% H_2O_2 for 5 min to quench endogenous peroxidase and incubated with 3% goat serum at room temperature for 1 hr to reduce nonspecific immunostaining. Tissue sections were incubated with antihuman Ki67 primary monoclonal antibody (clone SP6, dilution 1:1200; Lab Vision, Fremont, CA) raised in rabbits. Sections to which the primary antibody was not applied were utilized as negative controls. Secondary detection was accomplished with a horseradish peroxidase-labeled anti-rabbit antibody for 40 min (Jackson Immuno Research, West Grove, PA). Diaminobenzidine tetrachloride supersensitive substrate kits (ScyTek, Logan, UT) were used to visualize the antibody–antigen complex. Each section was counterstained with hematoxylin, dehydrated with graded alcohols, and washed with xylene before mounting with coverslips. Slides were independently examined and scored by two pathologists (LNC & CS). For discrepancies in individual scores, the pathologists re-evaluated these together to reach a consensus before combining the final individual scores. As described in previous studies [33], only tumors with distinct nuclear immunostaining in ≥10% of the cells were considered as positive.

Statistical analyses

Chi-square analyses were used to correlate the alterations of p53 and APC genes with clinical and pathological variables. P values of < 0.05 were considered statistically significant.

Results

Study cohort characteristics

The median age of the study cohort was 57 (range, 13–81) years; 35 were male, and 16 were female (Table 1). Most HCCs were associated with HCV infection (37 of 51, 73%) and alcohol use (32 of 51, 63%). The median survival of the cohort was 30 months (range, 2–146 months).

p53 mutations and codon 72 polymorphism

The overall incidence of missense point mutations of p53 (Table 2) was low (6 of 48, 12%). Missense point mutations, however, were common in CAs (4 of 6), and almost all of these patients had HCV infection (Table 2). CAs with point mutations had shorter median survival (22.5 months) relative to those with wild-type p53 (34 months) (data not shown).

The sequence traces representing the Arg/Arg, Arg/Pro, and Pro/Pro phenotypes of p53 codon 72 in HCCs are shown in Figure 1. In these analyses, the Arg/Argphenotype had the highest prevalence (27 of 48, 56%) followed by Arg/Pro (16 of 48, 33%) and Pro/Pro (5 of 48, 11%) (Table 3). Four of five patients with the Pro/Pro phenotype were AAs (χ^2 p = 0.028). All AA patients with the Pro/Pro phenotype had high Ki67 indices (Figure 2). Poorly differentiated tumors were most prevalent in Pro/Pro phenotype (2 of 5, 40%) (χ2 p = 0.016) and less prevalent in patients with Arg/Arg (1 of 27, 4%) or Arg/Pro (1 of 16; 6%) phenotypes (Table 3). All patients with the Pro/Pro phenotype had HCV infections (χ^2 p = 0.048) (Table 3). Furthermore, AAs with the Pro/Pro phenotype had lower median survival (15.5 months) relative to those with Arg/Arg or Arg/Pro (32 months) phenotype (data not shown).

APC gene methylation and mRNA expression

APC gene methylation was evident in 16 of 51 (31%) HCCs, predominantly in CA patients (χ^2 p=0.024) (Table 4). We also de-termined the differential mRNA expression of APC variants in the HCC samples. Among the APC variants, variant-3 (average copy number ratio 0.21) had lower expression levels in HCCs and in their corresponding uninvolved tissues relative to variant-1 (aver-age copy number ratio 2.7) and variant-2 (average copy number ratio 1.1) (Figure 3A). The expressions of all three APC variants were lower in HCCs that exhibited APC promoter methylation compared to unmethylated HCCs (Figure 3B). In HCCs with Pro/Pro phenotypes, there was low expression of APC variant-2 (χ^2 p=0.048) (Table 3).

Discussion

In these studies, we assessed the clinical value of codon 72 polymorphism of p53 in association with APC gene methylation and expression of APC variants in HCCs. Most patients exhibiting the Pro/Pro phenotype were AAs, and their tumors showed aggressive features, such as high proliferation and poor survival. In contrast, p53 mutations were common in CAs. Moreover, all patients with the Pro/Pro phenotype had HCV infections. Methylation of the APC gene was frequent in CAs but not in AAs, and low expression of APC variant-2 was associated with the Pro/Pro phenotype of p53 codon 72. Thus, the Pro/Pro phenotype is associated with low expression of APC variant-2, aggressive tumors, and short survival, particularly for AA patients.

For HCCs, p53 mutations exhibit geographical variations, reflecting varied risk and etiological factors [19,20,34,35]. As determined in the present investigation, there was a low frequency of missense point mutations (12 %), which occurred mostly in CAs. This was in contrast to a higher incidence of p53 mutations (37%) in Chinese HCC patients [34], but similar to a low p53 mutation frequency in Caucasian patients from Italy (13%) [35]. In related studies, a high incidence of missense mutations at p53 codon 249 was found in geographic regions with high AFB1 exposure, unlike the United States, where AFB1 exposure is rare [12]. Our study found p53 codon 249 mutation in only one patient. This geographical discrepancy in p53 mutations may be due to variations in etiological factors and/or individual susceptibility based on race/ethnicity. For many cancers, including HCCs, p53 mutations are associated with poor prognoses [36,37]. Our results demonstrated a shorter median survival for patients with p53 mutations, particularly CAs; however, due to the low frequency of p53 mutations in tumors of AA patients, a prognostic value for these mutations could not be demonstrated.

The codon 72 SNP of p53 results in Pro/Pro and Arg/Arg phenotypes. These phenotypes are distinct in that the Arg/Arg phenotype, owing to its localization in mitochondria, induces apoptosis 15-fold more than the Pro/Pro phenotype [17]. These phenotypes also differ in their biological and biochemical activities [38]. In our study, the Arg/Arg phenotype had the highest prevalence (56%) in HCCs, followed by Arg/Pro (33%) and Pro/Pro (11%). This is in concordance with other reports for HCCs in which the prevalence of the Arg/Arg and Pro/Pro phenotypes was 54.2% and 13.5%, respectively [39]. In our study, most patients exhibiting the Pro/Pro phenotype were AAs, whose tumors had higher proliferation rates and who had a shorter median survival. Similarly, for CRCs, the Pro/Pro phenotype, relative to the Arg/Arg phenotype, was fre-quent in AAs and was associated with aggressive tumors and a poor prognosis [22]. Thus, the Pro/Pro phenotype at codon 72 of p53 is a race-specific, molecular prognostic marker for AA patients with CRCs [22] and HCCs. These and other studies aid in identify-ing unique germline alleles/allelic combinations that modify can-cer risk and/or cancer progression. Furthermore, the differences between racial/ethnic groups illustrate the effect of different life-styles and/or exposures to environmental factors [40].

Compared to HBV, HCV infections induce inflammation and can lead to cirrhosis and to HCC [41,42]. HCV infections suppress the transcriptional activity of the p53 promoter [16]. Gene expression studies of HCV-related cirrhosis reveal upregulation of pro-inflammatory,

pro-apoptotic, and proproliferative genes [43]. Furthermore, induction of the HCV core protein in primary hepatocytes during senescence, immortalization, and anchorage-independent growth involves pathways that regulate cellular growth, immune response, oxidative stress, and apoptosis [37]. In our study, all HCC patients exhibiting the Pro/Pro phenotype had HCV infections. Furthermore, the Pro/Pro phenotype was associ-ated with increased risk of HCC in HCV-negative cases [19,44]. Together, these results suggest that the risk of HCC from the Pro/Pro phenotype is influenced by a chronic immune response against HCV, which may either conceal [45] or exacerbate the effect of the Pro/Pro phenotype [46], especially in AA patients with chronic liver disease, for which HCV is the most common cause [47].

The APC gene is involved in transduction of Wnt-signaling [23,52,53]. Unlike in CRCs, APC mutations are rare in other gastrointestinal malignancies, including HCCs [12], hence, we did not perform APC mutational analyses. However, APC promoter hypermethylation, a common phenomenon in HCCs, is reported in 53 to 100% of HCCs and in 27% of cases of liver cirrhosis [27,48]. In our study, 31% of HCCs showed APC hypermethylation, with CA patients having a higher frequency relative to AAs. The disparity in frequency of APC methylation between CAs and AAs, as shown for CRCs, may be due to differences in dietary factors, such as alcohol consumption [49].

Lower expression of tumor suppressor genes, either due to the influence of allelic loss or epigenetic mechanisms, is common in malignancies, including HCCs [26,27,50]. The present study is first to demonstrate lower mRNA expression of APC variants-2 and -3 in both HCCs and corresponding adjacent uninvolved liver tissues as compared to variant-1. In addition, there were lower levels of APC variants-2 and -3 in HCCs with APC promoter methylation as compared to HCCs without methylation. This suggests that APC methylation alters the expression of APC variants -2 and -3. Since splice variants of untranslated leader exons are associated with alternative promoter usage, resulting in differential gene expression with respect to cell or tissue type, efficient translation of the mRNA depends on differing leader exons and the methylation status of the involved gene [51]. The usage of alternative promoters in the regulation of APC variant expression may explain the observed differential expression of APC variants. These results demonstrate that low expression of APC variant-2 is associated with the Pro/Pro phenotype. Future studies of HCCs should identify the location of alternative promoters or genetic and epigenetic events involved in the control of expression of these APC variants and their relationship with the Pro/Pro phenotype of p53. In the current study, samples were collected from transplants or resections; hence, there is a possibility to have more favorable outcomes than those observed in the general population. However, the present results form a basis for large retrospective population-based studies or prospective studies to validate these findings.

Conclusions

We conclude that the Pro/Pro phenotype at codon 72 of p53 is associated with aggressive tumor features and poor clinical outcomes for HCCs, particularly for AA patients. Differential expression of APC variants in HCC versus uninvolved liver tissue, especially lower expression of APC variant-2 and methylation status of the APC promoter in HCCs, is

associated with the Pro/ Pro phenotype. These correlations should be validated in larger studies. Analysis of p53 codon 72 polymorphisms, APC variants, and APC promoter methylation, together with other confounding factors, would aid in understanding the aggressiveness of HCCs, particularly for AAs, and would contribute to design of new treatment strategies.

Acknowledgments

We thank Dr. Donald L. Hill, Division of Preventive Medicine, University of Alabama at Birmingham, AL, for his critical review of this manuscript.

Funding sources: This study was supported jointly by a Charles Barkley Award through the UAB Minority Health Disparities Research Center and a pilot grant from the UAB Center for Clinical and Translational Science (5UL1 RR025777) to Dr. Leona N. Council, and in part by grants from the NIH (3U54-CA118948, R01-CA98932) to Dr. Manne.

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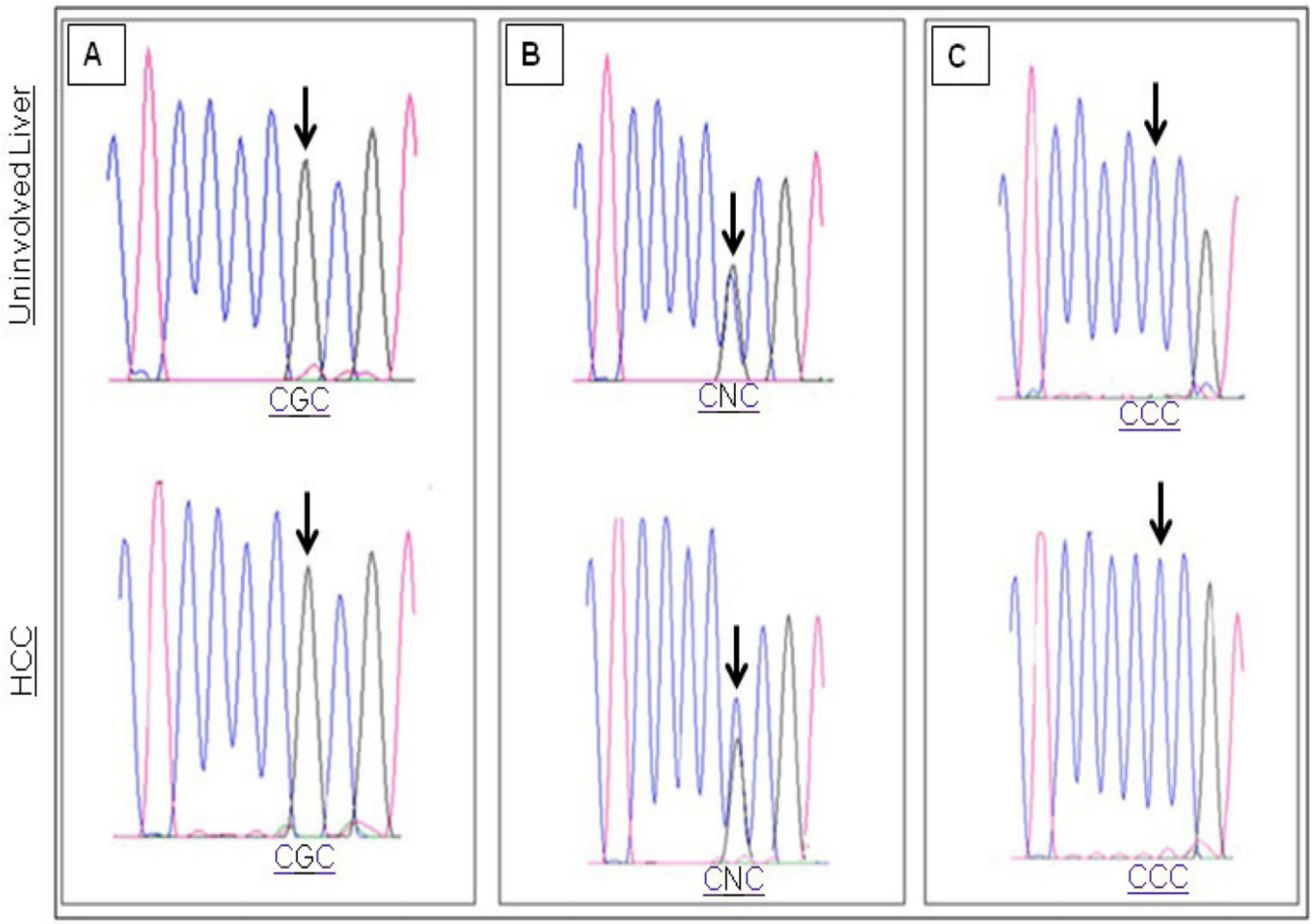

Figure 1. Genotypic analysis for codon 72 of the p53 gene

Direct sequencing analysis of the p53 gene at codon 72 for HCCs and uninvolved tissues demonstrating a nucleotide change (G>C). (A) homozygous for G/G (Arg/Arg phenotype), (B) heterozygous for G/C (Arg/Pro phenotype), (C) homozygous for C/C (Pro/Pro phenotype).

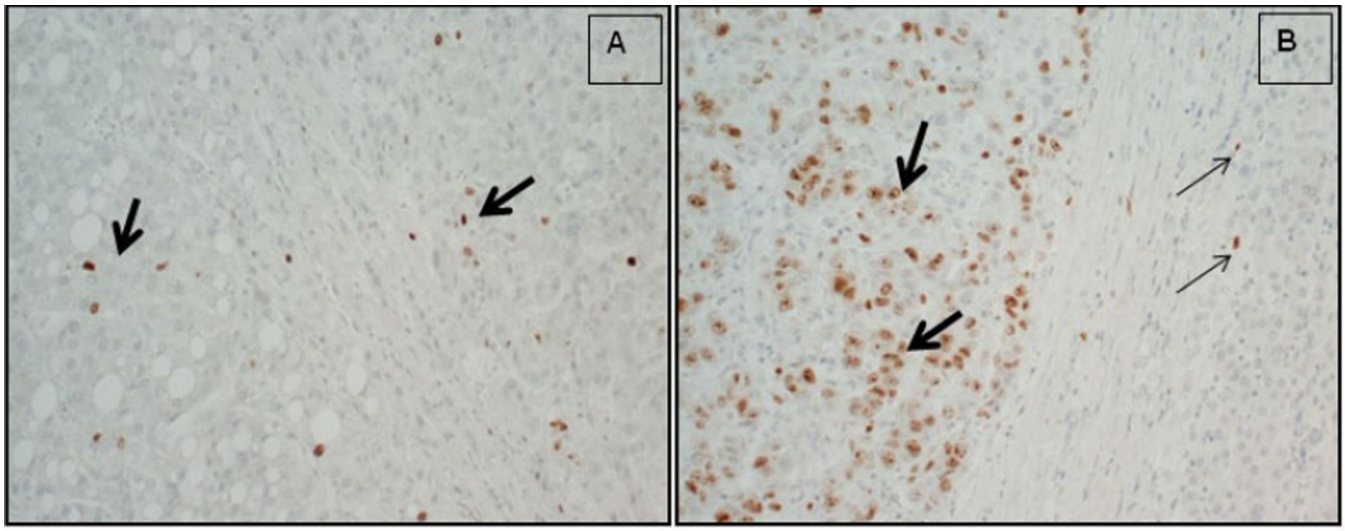

Figure 2. Ki-67 immunohistochemistry of HCCs based on p53 codon 72 phenotypes

Nuclear positivity (thick arrows) for Ki67 demonstrating low and high proliferative index in HCCs with Arg/Arg (A, 200 μm) and Pro/Pro (B, 200 μm) phenotypes. Note occasional positivity (thin arrows) in the adjacent uninvolved tissues (B).

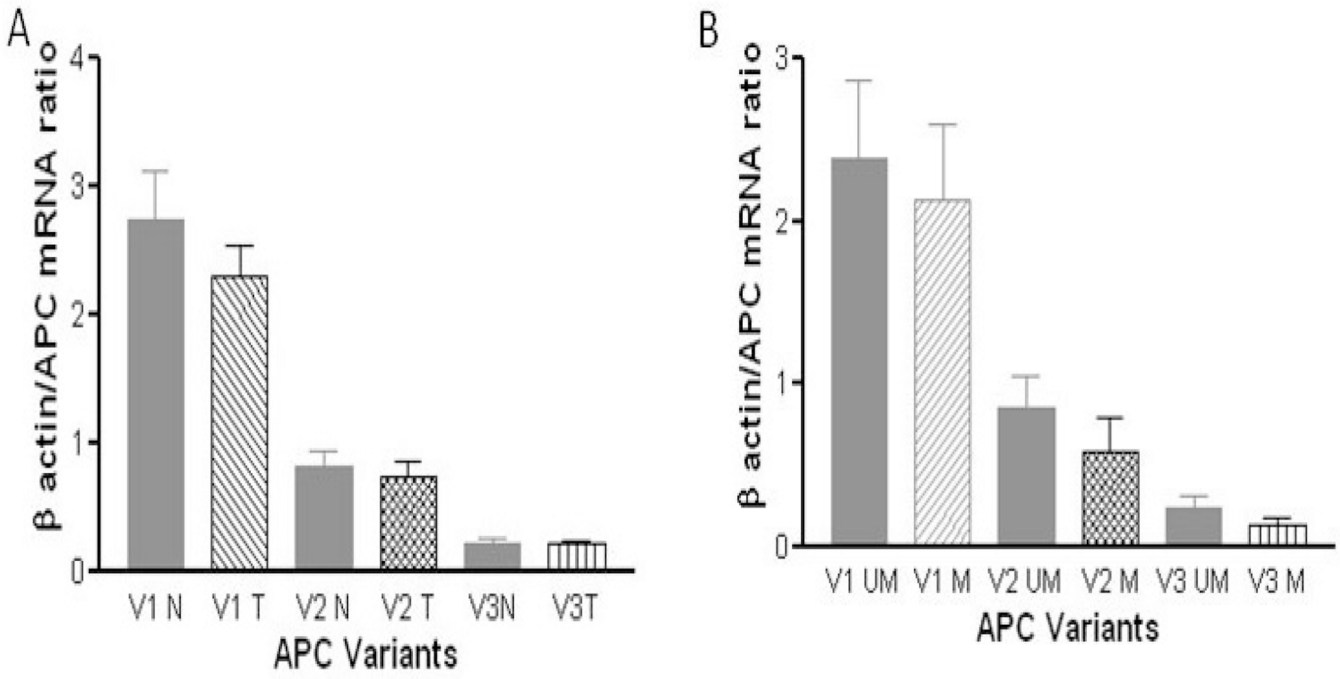

Figure 3. Expression of APC variants and its association with APC methylation in HCCs and their adjacent, uninvolved liver

(A) mRNA expression of APC variants in HCCs and uninvolved tissues. Variant 3 (V3) demonstrated lower levels of expression in HCCs (T) and their corresponding, uninvolved tissues (N) relative to variants 1 and 2 (V1 & V2). (B) mRNA expression of APC variants in HCCs based on APC promoter methylation status. The APC variants 2 and 3 (V2 & V3) demonstrated lower expression in HCCs exhibiting APC methylation (M) as compared to HCCs without APC methylation (UM).

Table 1

Clinicopathological features of the HCC study cohort (N= 51).

Variable	Number of patients (%)
Age group (years)	
< 50	7 (14)
≥ 50	44 (86)
Gender	
Female	16 (31)
Male	35 (69)
Ethnicity[a]	
African Americans (AAs)	15 (29)
Caucasians (CAs)	34 (67)
HCV Status	
Positive	37 (73)
Negative	14 (27)
Tumor stage[a]	
I	35 (69)
II	11 (21)
III	3 (6)
IV	1 (2)
Tumor differentiation	
Well	21 (41)
Moderate	26 (52)
Poor	4 (8)
Alcohol use[a]	
No	17 (33)
Yes	32 (63)

[a]Information is missing on race/ethnicity and alcohol usage for two cases, and tumor staging for one case.

Table 2

Details of p53 alterations and HCV infection.

Case no.	Mutation at codon	Race/ethnicity	Nucleotide change	Amino Acid change	Mutation type	Codon 72 phenotype	HCV status
5	308	CA	GCA>TCA	Ala>Ser	Missense	Arg/Arg	HCV
19	291	CA	CGC>TGC	Arg>Cys	Missense	Arg/Pro	None
24	235	CA	AAC>TAC	Asn>Tyr	Missense	Arg/Arg	HCV
25	249	NA	AGG>AGT	Arg>Ser	Missense	Arg/Arg	None
26	111	CA	CTG>CTA	Leu>Leu	Silent	Arg/Arg	HCV
27	246	CA	ATG>GTG	Met>Val	Missense	Arg/Arg	HCV
28	121	CA	TCT>TCA	Ser>ser	Silent	Arg/Arg	HCV
32	219	AA	CCC>GCC	Pro>Ala	Missense	Pro/Pro	HCV
45	88	CA	GCC>GCT	Ala>Ala	Silent	Arg/Arg	HCV

CA- Caucasian; AA- African American; HCV- Hepatitis C Virus; NA- Information not available

Table 3

Association between p53 polymorphisms and clinicopathological characteristics.

Variable	Number of patients (N=48)			χ^2 P-value
	Arg/Arg	Arg/Pro	Pro/Pro	
	27 (56%)	16 (33%)	5 (11%)	
Age group (years)				
< 50	4 (15)	3 (19)	0 (0)	0.583
≥ 50	23 (85)	13 (81)	5 (100)	
Gender				
Female	8 (30)	6 (38)	2 (40)	0.822
Male	19 (70)	10 (62)	3 (60)	
Ethnicity[a]				
African Americans	5 (19)	5 (31)	4 (80)	0.028
Caucasians	20 (74)	11 (69)	1 (20)	
HCV status				
Positive	21 (78)	8 (50)	5 (100)	0.048
Negative	6 (22)	8 (50)	0 (0)	
Tumor stage[a]				
I	17 (81)	13 (65)	2 (40)	0.295
II	7 (19)	1 (24)	3 (60)	
III	2 (0)	1 (9)	0 (0)	
IV	1 (0)	0 (0)	0 (0)	
Tumor differen-tiation				
Well	13 (48)	4 (25)	3 (60)	0.016
Moderate	13 (48)	11 (69)	0 (0)	
Poor	1 (4)	1 (6)	2 (40)	
Alcohol use[a]				
No	9 (33)	6 (38)	1 (20)	0.718
Yes	17 (63)	9 (56)	4 (80)	
APC variant expression[a]				
Variant-1				
High	11 (41)	9 (56)	4 (80)	0.226
Low	16 (59)	7 (44)	1 (20)	
Variant-2				
High	12 (44)	12 (75)	1 (20)	0.048
Low	15 (56)	4 (25)	4 (80)	
Variant-3				

Variable	Number of patients (N=48)			χ^2 P-value
	Arg/Arg	Arg/Pro	Pro/Pro	
	27 (56%)	16 (33%)	5 (11%)	
High	10 (37)	8 (50)	3 (60)	0.525
Low	17 (63)	8 (50)	2 (40)	
Mortality				
Alive	24 (89)	14 (88)	4 (80)	0.915
Dead	3 (11)	2 (12)	1 (20)	

[a] Information is missing on race/ethnicity and alcohol usage for two cases and tumor staging for one case.

[b] high (median value of β-actin/APC mRNA ratio greater than corresponding normal) expressors

Table 4

Association between APC methylation status and clinicopathological characteristics.

Variable	Number of patients (N=51)		χ^2 P-value
	Methylated	Unmethylated	
	APC	APC	
	16 (31%)	35 (69%)	
Age group (years)			
< 50	3 (19)	4 (11)	0.48
≥ 50	13 (81)	31 (89)	
Gender			
Female	5 (31)	11 (31)	1.0
Male	11 (69)	24 (69)	
Ethnicity[a]			
African Americans	1 (6)	14 (40)	0.024
Caucasians	13 (81)	21 (60)	
HCV status			
Positive	13 (81)	24 (69)	0.346
Negative	3 (19)	11 (31)	
Tumor stage[a]			
I	13 (81)	22 (65)	0.489
II	3 (19)	8 (24)	
III	0 (0)	3 (9)	
IV	0 (0)	1 (2)	
Tumor differentiation			
Well	8 (50)	13 (37)	0.321
Moderate	8 (50)	18 (51)	
Poor	0 (0)	4 (12)	
Alcohol use[a]			
No	3 (19)	14 (40)	0.151
Yes	12 (75)	20 (57)	
Mortality			
Alive	14 (88)	28 (88)	0.643
Dead	2 (12)	4 (12)	

[a]Information is missing on race/ethnicity and alcohol usage for two cases, and tumor staging of one case.