



## Assessment of Tp53 Polymorphism in Hepatocellular Carcinoma with Hepatitis C Virus Infection

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**Abstract: Purpose:** The current study aims to clarify the role of tumor protein (TP53) gene single nucleotide polymorphism (SNP) G-C at codon 72 in the development of hepatocellular carcinoma (HCC) among patients with chronic hepatitis C, a step towards an efficient tool for early detection. **Methods/ patients:** We conducted this study on 20 patients with hepatocellular carcinoma (HCC) on top of HCV infection (group I); 10 patients with chronic liver disease on top of HCV infection whom age and sex were matched, serving as patient control (group II); and 10 healthy control subjects who are age and sex-matched with no history of hepatic complaints (group III). TP53 G-C gene polymorphism of codon 72 was tested by polymerase chain reaction (PCR), followed by restriction enzyme digestion (PCR-RFLP). **Results:** There was a highly statistically significant difference in TP53 G-C gene polymorphism between the three investigated groups ( $p < 0.01$ ). **Conclusion:** Detection of TP53 gene SNP G-C at codon 72 is highly recommended in chronic liver disease patients especially in known cirrhotic cases on top of HCV infection to detect HCC patients in earlier stages.

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**Keywords:** HCC, TP53, HCV, case-control study

### 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, and the third most frequent cause of cancer-related death as well. HCC is actually the most common cancer in 13 countries including Egypt [1, 2].

The main risk factors for HCC are hepatitis B virus (HBV) or hepatitis C virus (HCV) chronic infection, aflatoxin-contaminated foodstuffs, heavy alcohol intake, obesity, and type-2 diabetes, where these factors vary in incidence from one region to another [3]. In most high-risk HCC areas like China and Eastern Africa, the main causes are chronic HBV infection and aflatoxin exposure, whereas, in other countries like Japan and Egypt, HCV infection is likely the predominant cause [4].

The association of HCV with the development of HCC is well documented. HCC is often attributed to the inflammatory effects of chronic hepatitis and fibrogenesis. [5]. Hepatocellular carcinogenesis includes different genetic alterations that eventually lead to a malignant transformation of the hepatocyte [6]. Multiple mechanisms are involved in such process including gene transcription, chromosomal stability and cell differentiation [7]. In HCC, the process of

activation of protooncogenes to oncogenes, when identified, is associated with a poor prognosis [8].

HCC is usually asymptomatic for much of its natural history, and it is one of the cancers that benefit from screening programs for patients at risk for HCC including patients with virally induced liver cirrhosis. The main aim of screening programs is to identify the presence of cancer where early detection offers the opportunity of favorably impact patient outcomes. HCC when detected in an early stage could be managed surgically. Liver resection could be associated with 5 years survival rates of about 50 to 70% in well-selected patients [9].

TP53 is a tumor suppressor gene that plays a vital role in the protection the integrity of the human DNA. The loss of its function has been proposed to be the most important step in multistage hepatocarcinogenesis [10].

The single nucleotide polymorphism (SNP72) is variation of the G-C at the second position of codon 72 in exon 4, leading to Pro72 or Arg72 protein variants. TP53 codon 72 polymorphism is SNP in exon 4, causing expression of proline (CCC) or arginine (CGC) residues, greatly affecting the function of TP53 to induce apoptosis. It is associated with an

increased risk of HCC as well as breast, bronchogenic, esophageal, and urinary bladder carcinomas [11].

Referring back to the fact that HCC has a poor prognosis, mainly due to late diagnosis and deficient a highly active treatment to date, there is a serious need to identify novel diagnostic markers for the screening, early detection of the disease, as well as exploring innovative therapeutic approaches [12].

The current study expected to investigate the role of TP53 gene single nucleotide polymorphism (SNP) G-C at codon 72 in the development of HCC among patients with chronic hepatitis C.

## 2. Subjects and methods

The current study is a case-control study that we conducted in the period from September 2017 to January 2018 on forty individuals, divided into three groups. We recruited Group I and II from the Department of Tropical Medicine and Infectious diseases and outpatients' clinics.

**Group I**, included twenty (20) patients with hepatocellular carcinoma (HCC) on top of HCV infection, who had been recently diagnosed as having HCC by triphasic CT plus or minus fine-needle aspiration. These patients were newly diagnosed HCC, untreated, and free from other cancers.

**Group II**, included ten (10) patients with chronic liver disease on top of HCV infection whose age and sex were matched with the group I subjects.

**Group III**, included ten (10) age- and sex-matched, apparently healthy control subjects with no history of hepatic complaints and with normal liver function tests and normal abdominal ultrasound.

The study was consistent with the Declaration of Helsinki. All participants gave their informed consent to enter the study. The study has been approved by the ethical committee of the Faculty of Medicine, Ain Shams University.

### Collection of blood samples

Six milliliters of venous blood was withdrawn from each subject under complete aseptic condition and were handled as follows: four milliliters were collected in two separate K3 ethylene diamine tetraacetate (EDTA) tubes, 2ml in each one. The first one was stored at -20°C for deoxyribonucleic acid (DNA) extraction for the detection of TP53 gene polymorphism at codon 72 for all individuals in the study, whereas the second one was centrifuged at 3000 rpm for 10 minutes and the plasma was collected, aliquoted and stored at -80°C until used for polymerase chain reaction (PCR) for detection of HCV positive patients. Two milliliters of blood was placed in sterile vacutainers with a clot activator and was left to clot for 30 minutes and then centrifuged at 3000 rpm for 10 minutes and sera were used for

immediate assay of routine liver function tests, markers of viral hepatitis and serum AFP. Frozen samples were brought to room temperature and allowed to thaw only before analysis. Hemolyzed samples were discarded, repeated freezing and thawing was avoided.

### Genotyping of TP53 G-C polymorphism at codon 72 (Arg72Pro):

Genomic DNA was extracted from EDTA-anticoagulated peripheral whole blood by a DNA purification kit (QIAamp DNA blood midi kits). QIAamp DNA Blood midi kits contain QIAGEN Protease for sample lysis. After lysis, the lysate is loaded onto the QIAamp spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation, while impurities are effectively washed away using two different wash buffers; AW1 and AW2 in two centrifugation steps. The first step to removes proteins, metabolites, and other contaminants and the second step is to remove residual protein and salt. Finally, ready to use DNA can be eluted using elution buffer.

Polymerase chain reaction (PCR) was conducted by addition of 10 µL of DNA concentrate, 25 µL of master mix (chemically modified Maxima hot start Taq DNA polymerase, optimized hot-start PCR buffer, Mg<sup>2+</sup> 2, and dNTPs) (Thermoscientific, 168 Third Avenue, Waltham, MA, USA), 1 µL of each forward and reverse primers (Applied Biosystems, USA) and the final volume was completed to 50 µL by nuclease-free water (Thermoscientific, 168 Third Avenue, Waltham, MA, USA). Polymerase chain reaction (PCR) amplification as following: initial heat activation at 95° C for 5 min. Followed by 35 cycles of amplification were done using (Thermocycler-Biometra analytic Jena Company) as following DNA denaturation at 95° C for 30 s. Annealing at primer-specific annealing temperature (94° C for 30s and 55° C for 60 s. Extension at 72° C for 60 s, then after the 35 cycles. Final extension at 72° C for 10 min was done. PCR amplification was performed with the following primers: Forward primer (5' TGAGGACCTGGTCCTCTGACT 3') and reverse primer (5' AAGAGGAATCCCAAAGTTCCA 3').

Polymerase chain reaction (PCR) products were digested with the FastDigestPstI restriction enzyme (New England Bio Labs, UK). The following reaction components were combined at room temperature in the following order: 17 µL nuclease free water, 2 µL buffer, 10 µL of DNA concentration, 1µL of the restriction enzyme. The components were mixed gently, spun down, and incubated at 37°C for 60 minutes. Finally, the reaction mixture and the DNA ladder were loaded on 2% agarose gel, and the DNA fragments were separated by electrophoresis (100

volts for 30 minutes) using (Cleaver scientific LTD). The digestion products were identified as: 414 bp single band for the homozygous mutant type Pro/Pro (CC).161 and 263 bp two bands for the wild type Arg/Arg (GG) and 414, 263 and 161 bp three bands for the heterozygous mutant type Arg/Pro (GC).

#### Statistical analysis:

Data analysis was done using Statistical Package for Special Sciences (SPSS) software computer program Version (V. 22.0, IBM Corp., USA, 2013).

Data were expressed descriptively as Mean ( $\bar{X}$ )  $\pm$  standard deviation (SD) for quantitative parametric data, median and interquartile range (IQR) for quantitative non-parametric values, and as percent for qualitative data. The comparison between more than two independent groups' means was done by using analysis of variance (ANOVA) test for parametric data and Kruskal Wallis test for non-parametric data. Chi-square test was used for comparison between independent groups as regards the categorized data. P-Value > 0.05 was considered non-significant, P-Value

$\leq 0.05$  was considered significant and  $P < 0.01$  was considered highly significant for all tests.

### 3. Results

The results of the present study are presented in Tables 1 to 7 and Figures 1 to 4.

Comparisons between HCC versus patients' control group, HCC versus healthy control group and patients' control group versus healthy control group regarding different studied laboratory parameters using Mann-Whitney U-test revealed a statistically highly significant difference between the compared groups regarding all studied parameters ( $p < 0.01$ ) as presented in **Tables 1 and 2**.

As regards Arg72Pro TP53 genotypes (**Figures 1 and 2**) and allele frequency, descriptive statistics and statistical comparison were done between the three studied groups using the Chi-square test as shown in **Table 3**. They revealed a statistically highly significant difference between the three groups regarding the studied parameters ( $p < 0.01$ ).

**Table 1:** Descriptive and statistical comparison between HCC and healthy control groups regarding different studied laboratory parameters using Mann-Whitney U-test:

		Healthy Control group N= 10	HCC group N= 20	Test value Z	P-value
ALT (IU/L)	Median (IQR)	14 (12 – 17)	138.5 (100.5 – 220)	-4.401	< 0.01
AST (IU/L)	Median (IQR)	14 (13 – 17)	138 (103.5 – 172)	-4.404	< 0.01
Total Bilirubin (mg/dL)	Median (IQR)	0.5 (0.3 – 0.7)	4.6 (3.3 – 8)	-4.403	< 0.01
Direct Bilirubin (mg/dL)	Median (IQR)	0.15 (0.1 – 0.2)	3.15 (1.3 – 5.3)	-4.417	< 0.01
Albumin (g/dL)	Median (IQR)	4.75 (4.2 – 5.1)	1.65 (1.35 – 2.05)	-4.403	< 0.01
AFP (ng/mL)	Median (IQR)	4 (3 – 5)	1300 (935 – 1965)	-4.408	< 0.01

N: number Z: Mann-Whitney U-test  $P < 0.01$ : Highly significant difference

**Table 2:** Descriptive and statistical comparison between HCC and patients' control groups regarding different studied laboratory parameters using Mann-Whitney U-test:

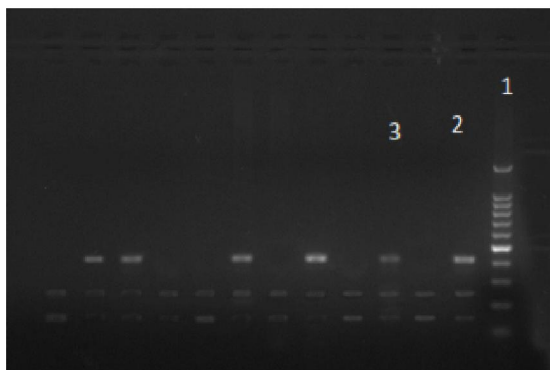
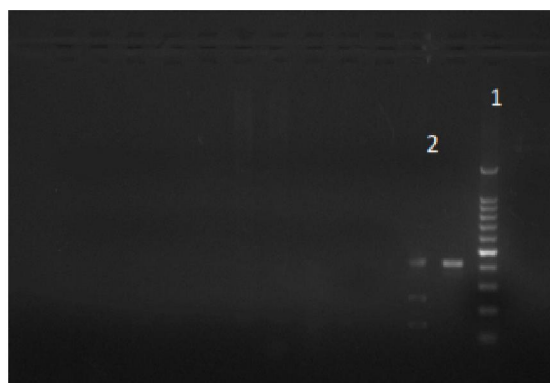
		Patients' control group N= 20	HCC group N = 20	Test value Z	P-value
ALT (IU/L)	Median (IQR)	55 (50 – 80.5)	138.5 (100.5 – 220)	-4.573	< 0.01
AST (IU/L)	Median (IQR)	75.5 (56.5 – 91)	138 (103.5 – 172)	-4.586	< 0.01
Total Bilirubin (mg/dL)	Median (IQR)	2.9 (2.2 – 4.95)	4.6 (3.3 – 8)	-2.414	< 0.01
Direct Bilirubin (mg/dL)	Median (IQR)	1.1 (1 – 3.15)	3.15 (1.3 – 5.3)	-2.271	< 0.01
Albumin (g/dL)	Median (IQR)	2.8 (2.4 – 2.95)	1.65 (1.35 – 2.05)	-4.718	< 0.01
AFP (ng/mL)	Median (IQR)	78.5 (74 – 89)	1300 (935 – 1965)	-5.413	< 0.01

Z: Mann-Whitney U-test  $P < 0.01$ : Highly significant difference

**Table 3:** Descriptive and statistical comparison between the three studied groups regarding Arg72Pro Tp53 genotypes and allele frequency using Chi-square test:

Gene polymorphism	Healthy Control group N = 10		Patients' control group N = 20		HCC group N = 20		Test value	P-value
	N	%	N	%	N	%		
Homozygous mutant type (CC)	1	10.0%	3	15.0%	7	35.0%	X <sup>2</sup> = 10.636	< 0.01
Heterozygous mutant type (GC)	4	40.0%	8	40.0%	12	60.0%		
Wild type (GG)	5	50.0%	9	45.0%	1	5.0%		
C allele	6	30.0%	14	35%	26	65%	X <sup>2</sup> = 9.823	< 0.01
G allele	14	70.0%	26	65%	14	35%		

X<sup>2</sup> = Chi-square test      P < 0.01: Highly significant difference

**Fig 1:** gel electrophoresis shows lane 1 = ladder, lane 2 = heterozygous mutation (Arg/Pro) with bands at 414 bp, 263 bp & 161 bp and lane 3 = wild type (Arg/Arg) with bands at 263 bp and 161 bp**Fig 2:** gel electrophoresis shows lane 1 = ladder, lane 2 = homozygous mutation (Pro/Pro) with single band at 414 bp**Table 4:** Statistical comparison between HCC and healthy control group regarding Arg72Pro Tp53 genotypes and allele frequency using Chi-square test:

Gene polymorphism	Healthy Control group N = 10		HCC group N = 20		Test value	P-value
	N	%	N	%		
Homozygous mutant type (CC)	1	10.0%	7	35.0%	X <sup>2</sup> = 8.813	< 0.01
Heterozygous mutant type (GC)	4	40.0%	12	60.0%		
Wild type (GG)	5	50.0%	1	5.0%		
C allele	6	30.0%	26	65%	X <sup>2</sup> = 6.562	< 0.01
G allele	14	70.0%	14	35%		

X<sup>2</sup> = Chi-square test      P < 0.01: Highly significant difference

**Table 5:** Statistical comparison between HCC and patients' control group regarding Arg72Pro Tp53 genotypes and allele frequency using Chi-square test:

Gene polymorphism	Patients' control group N = 20		HCC group N = 20		Test value	P-value
	N	%	N	%		
Homozygous mutant type (CC)	3	15.0%	7	35.0%	X <sup>2</sup> = 8.800	< 0.01
Heterozygous mutant type (GC)	8	40.0%	12	60.0%		
Wild type (GG)	9	45.0%	1	5.0%		
C allele	14	35%	26	65%	X <sup>2</sup> = 7.200	< 0.01
G allele	26	65%	14	35%		

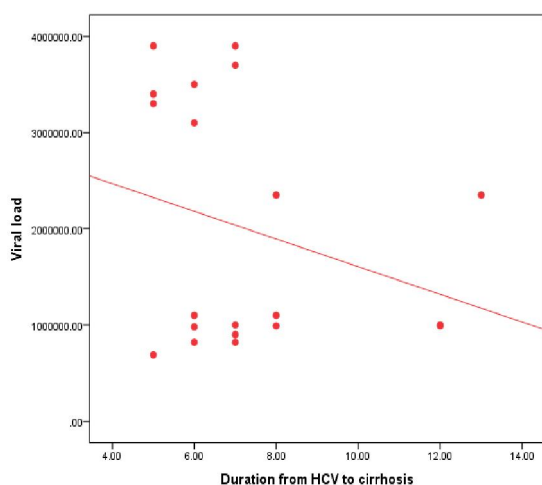
X<sup>2</sup> = Chi-square test      P < 0.01: Highly significant difference

**Table 6:** Statistical comparison between healthy control and patients' control group regarding Arg72Pro Tp53 polymorphism and allele frequency using Chi-square test:

Gene polymorphism	Healthy Control group N = 10		Patients' control group N = 20		Test value	P-value
	N	%	N	%		
Homozygous mutant type (CC)	1	10.0%	3	15.0%	X <sup>2</sup> =0.161	> 0.05
Heterozygous mutant type (GC)	4	40.0%	8	40.0%		
Wild type (GG)	5	50.0%	9	45.0%		
C allele	6	30.0%	14	35%	X <sup>2</sup> =0.150	> 0.05
G allele	14	70.0%	26	65%		

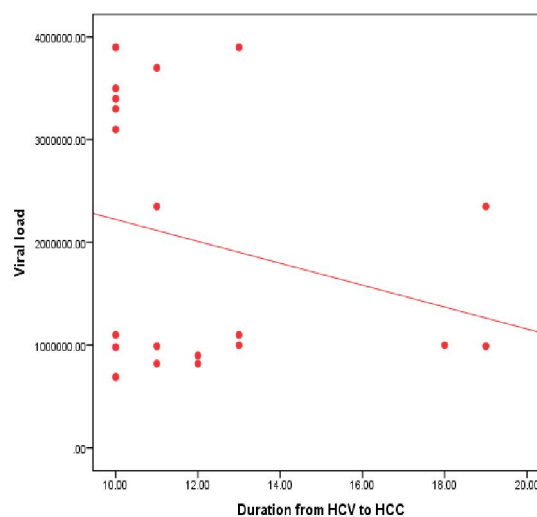
X<sup>2</sup> = Chi-square test      P >0.05: Non significant difference

Also, statistical comparisons of HCC versus patients' control group, HCC versus healthy control group and patients' control group versus healthy control group regarding the same parameters using Chi-square test revealed that there is a statistically highly significant difference between HCC versus patients' control group, HCC versus healthy control group studied parameter (p<0.01) in **Tables 4 and 5** and showed no significant statistical difference in patients' control group versus healthy control group as regards the same parameters (p > 0.05) as shown in **Table 6**.

**Fig 3** Correlation between viral load and duration from HCV infection to cirrhosis in HCC patients having Arg72Pro Tp53 polymorphism

Independent t-test for parametric data and Mann-Whitney test for non-parametric data were used in descriptive and statistical comparison within HCC group (GC versus CC) which revealed a significant statistical difference regarding duration from HCV to cirrhosis and duration from HCV to HCC (p<0.01) and a statistically non-significant difference as regards viral load (p > 0.05). One case was reported as wild type (GG) and had a viral load of 3700000.00 IU/ml,

the duration from HCV to cirrhosis was 13 years (**Figure 3**) and the duration from HCV to HCC was 13 years (**Figure 4**).

**Fig 4** Correlation between viral load and duration from HCV infection to HCC in HCC patients having Arg72Pro Tp53 polymorphism

#### 4. Discussion

Hepatocellular carcinoma (HCC) is usually asymptomatic in its earlier stages which makes the early diagnosis a crucial issue for a better prognosis. Researchers continuously work worldwide to find out an early sensitive and specific marker for HCC diagnosis [13]. The imaging-based diagnosis is relatively inaccurate for small tumors [14]. Histopathological examination of tumor biopsy is considered a reliable tool in the diagnosis of HCC, but tumor seedling is a recognized complication [15]. The most widely used serological marker for diagnosis of HCC is AFP, however, its sensitivity is limited ranging from 41 to 65% [16].

Searching for a more reliable diagnostic marker for HCC, many studies have identified TP53 Arg72Pro polymorphism to have an important role in the development of HCC [10, 17, 18].

Our study revealed that the Arg/Arg variant (wild type GG) was more frequent in the healthy control group than in patient's control group and than in HCC group, while Arg/Pro variant (heterozygous mutant type GC) was more frequent in HCC than in the comparable groups (patient's control group and healthy control group) and Pro/Pro variant (homozygous mutant type CC) was more frequent in HCC than in comparable groups. These results agree with those of previous studies who reported that the frequency of the Pro/Pro variant was significantly higher in HCC patients than in the healthy control group and Arg/Arg variant was significantly higher in the healthy control group [19,20,21].

Regarding the TP53 Arg/Pro variant, the present study revealed that the Arg/Pro variant is more frequent in the HCC group than in the comparable groups (patient's control group and healthy control group). These findings agree with Peng and colleagues [21].

The current study shows that the C allele is more frequent in HCC than in comparable groups (patients' control group and healthy control group), while the G allele is more frequent in healthy control group and patients' control group than in HCC group. Our results agree with those of Hu and colleagues [20] and Ezzikouri and colleagues [22] who reported that the C allele is more frequent in HCC than in the healthy control group, while G allele was more frequent in the healthy control group. These findings can be explained by the fact that TP53 gene regulates a large number of genes (>100 genes) that control numerous key tumors suppressing functions, such as cell cycle arrest, DNA repair, senescence and apoptosis so that the activation of TP53 often leads to apoptosis of injured cells, while TP53 inactivation facilitates tumor progression.

The Arg/Arg and Pro/Pro variants differ in binding activity, transcriptional activation, apoptosis induction, and cell cycle arrest. Arg/Arg variant has a DNA-binding transcription factor function that activates other tumor suppressor genes (e.g. p21, MDM2, GADD45, Bax) that are required for the regulation of cell cycle progression or apoptosis in response to DNA damage. Also, TP53-Arg72 variant is more effective in inducing apoptosis and protecting cells from cancer development than the TP53-Pro72 variant due to its ability to be localized at the mitochondrial membrane to regulate the release of cytochrome C into the cytosol which plays a pivotal role in the activation of caspase-3 and apoptosis induction which explains that the TP53-Pro72 variant might be a weaker tumor suppressor than its TP53-

Arg72 which is an accepted mechanism of tumorigenesis [23].

Although TP53-Pro72 binds weaker than TP53-Arg72 to the positive regulatory protein, PIN1 (peptidyl-prolyl cis-trans isomerase), it interacts more readily with the inhibitory protein iASPP (Inhibitor of apoptosis-stimulating protein), so it has a weaker apoptotic potential than TP53-Arg72. This difference could explain why the TP53-Pro variant may increase susceptibility to HCC. Moreover, the TP53-Arg72 variant was found to suppress the transformation of primary cells to a higher degree, compared with the TP53-Pro72 variant [24].

On the contrary, Cai and colleagues had disagreed with these findings as they reported a non-significant association to be identified between TP53 codon 72 polymorphism and HCC. Their possible explanations were that interaction between genetic factors and environment exposure (smoking, alcohol intake, HBV, and HCV) significantly increased the risk of HCC, not genetic factors alone [25].

Our study also found that there are significant differences regarding duration from HCV infection to cirrhosis and duration from HCV infection to HCC in the HCC group of patients, which were shorter in the Pro/Pro variant than in Arg/Pro and Arg/Arg variants. Also, it revealed a significant difference regarding duration from HCV to cirrhosis within the patient's control group, which was shorter in the Pro/Pro variant than in Arg/Pro and Arg/Arg variants. The possible explanation of this result is due to HCV proteins reaction with the TP53 gene and modulation of its biological functions. It has been reported that non-structural (NS3) protein of HCV contains a serine protease and an RNA helicase, protease cleaves the genome-encoded polyprotein, and helicase is required for both genome replication and virus assembly [26].

It was reported that exon 4 polymorphism, which encodes for arginine in codon 72 (TP53 Arg), confers more sensitivity to degradation by HCV NS3 protein and this process plays a role in cell malignant transformation and deformation [27]. Also, it is assumed that the core protein (central) of HCV is also included in malignant cell transformation, as it reacts with p21 and loses its function as a cell cycle regulator. Also, p21 is induced by Tp53, so TP53 polymorphisms may also affect p21 induction leading to malignant hepatocyte transformation [27]. On the contrary Ezzikouri and colleagues disagreed with this as they demonstrated that there is no association between TP53 Arg72Pro and enhanced risk of HCC in HCV positive subjects. One explanation is that the effect of the Pro allele may be masked by chronic HCV infection [22].

In the present study, there is a non-significant difference in the HCV viral load among Arg/Arg,

Arg/Pro and Pro/Pro variants within HCC groups and patients' control group. However, in this context Noh and colleagues had reported that HCV RNA titer may be considered to be an independent risk factor affecting the development of HCC in HCV-infected patients [28]. The disagreement between our finding and their finding can be possibly explained by the fact that serum level of HCV RNA is a dynamic parameter in patients with chronic hepatitis C, so it could not give the complete picture of the association of the severity of the liver disease based on the serologic fluctuation of viral load.

### Conclusion and Recommendations:

This case-control study confirmed that the Pro/Pro (C/C) genotype and the Pro (C) allele of TP53 codon 72 are associated with increased risk of HCC in HCV-infected patients. It is recommended to add TP53 gene SNP G-C at codon 72 assessment to the current standard tests for diagnosis of HCC as a new, less invasive and screening tool with significant accuracy. This, in turn, could greatly improve the ability to identify such patients and thus could allow them to benefit from earlier treatment. Limitations of our study are the small sample size and that it does not cover different geographic areas. So, we recommend that further studies should be conducted on a larger scale in different geographical areas.

### Conflicts of interest:

The authors declare that they have no conflict of interest.

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