



Association between TP53 and MDM2 Gene Polymorphisms and Risk of Hepatocellular Carcinoma in Hepatitis C Virus among Egyptian Populations

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Authors' contributions

This work was carried out in collaboration among all authors. Author AMZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SMH and MMEB managed the analyses of the study. Author RAHAEW managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: The Murine double minute 2 (MDM2) gene is overexpressed in several human tumors. The oncogenic potential of MDM2 is partially explained by inhibition of the activity of the tumor suppressor protein P53 (negative regulator of the P53 tumor suppressor protein). A single nucleotide polymorphism (SNP) in the promoter region of MDM2 gene (T to G exchange at nucleotide 309) and TP53 gene (codon 72 exon 4, rs1042522 encoding either C or G) have been independently associated with increased risk of several cancer types. Few studies have analyzed the role of these polymorphisms in the development of hepatocellular carcinoma among Egyptian patients with chronic hepatitis C virus infection.

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Methods: The study consisted in the comparison of the genotype distribution of TP53 and MDM2 SNP309 in 100 viral hepatitis C-related hepatocellular carcinomas (HCC) cases and 100 controls without HCC matched for age, gender and ethnicity. PCR-RFLP (restriction fragment length polymorphism) and real time PCR methods were used to determine the genotype at the MDM2 SNP309T>G locus and TP53 rs1042522.

Results: Overall, our results indicate that frequencies of TP53 alleles (C and G) were not significant different between HCC cases and healthy controls (p=0.093) (Odds Ratio, OR=1.361,95% Confidence Interval, 95% CI=0.949 – 1.951). A significant increase of MDM2 SNP309 G/G and T/G genotypes were observed among HCC cases (Odds Ratio, OR=4.868, 95% Confidence Interval, 95% CI= 2.873 – 8.251).

Conclusions: Our finding suggest that people who have G allele increase the risk by 4.868 folds for developing HCC among Egyptian patients, consequently the MDM2 309T>G polymorphism is an important modulator of hepatocellular carcinoma development in Egyptian patients.

Keywords: Genetic polymorphisms; hepatocellular carcinoma; MDM2 SNP309; tumor suppressor protein P53 codon 72.

ABBREVIATIONS

HCC: Hepatocellular Carcinoma; MDM2: Mouse Double Minute-2; HCV: Hepatitis C Virus; TP53: Tumor Suppressor Protein P53 Codon 72.

1. INTRODUCTION

Hepatitis C virus (HCV), is one of the leading causes of chronic liver disease. Hepatocellular carcinoma (HCC) is a major complication associated with HCV infection, with significant mortality and morbidity rates [1]. Egypt holds the highest record of HCV infection globally with almost 15% of the population, which is the most important risk factor in developing liver cancer including HCC in Egypt [2]. Hepatocellular carcinoma (HCC) is the most prevalent type of liver malignancy and a main cause of tumor-related mortality. According to global cancer statistics from 2012, HCC is the second leading cause of cancer-related deaths in men and the sixth in women, worldwide, and the HCC incidence is still rising globally [3]. The overall 5-year survival rate is very low because of high aggressiveness of the cancer and the limited therapeutic options. The most effective therapies as resection or liver transplantation only work in patients at early stage of the disease. HCC in most cases occurs in the chronically inflamed

and damaged liver [4]. HCC represents the sixth most common cancer worldwide.

In Egypt ranks the third and 15th most populous country in Africa and worldwide, respectively. In Egypt, the health authorities consider HCC as the most challenging health problem [5].

There are many risk factors that play an important role in the development of HCC. These factors are summarized in Table 1.

There are conflicting reports about the effect of HCC screening and detection at an early stage on both the cure rate and the overall survival (OS) [6,7]. Globally, Screening of HCC in high risk patients as chronic hepatitis C (CHC) patients, the most used method is combination of ultrasound and serum level of alpha fetoprotein (AFP) [8,9]. The disadvantages of ultrasound are failure of detection of small tumors, as for the AFP serum level, it has disadvantages as low sensitivity and specificity so due to poor adherence to screening, HCC mortality worldwide is increasing in 80% of small tumor [10].

Table 1. Risk factors for HCC development

Environmental-related risk factors	Host/genetic-related risk factors
❖ Infectious as HCV and HBV	❖ Host-related risk factors as gender, ethnicity, obesity, diet and autoimmune hepatitis.
❖ Non-infectious as Alcohol abuse and smoking tobacco	❖ Genetic-related risk factors as Alpha 1 antitrypsin deficiency, hereditary hemochromatosis (iron overload) and family history.

In Egypt, a national screening campaign was started by the Egyptian Ministry of Health (MOH) in 2018 to combat high HCV prevalence in Egypt by 2020 [11,12]. All screened participants with confirmed HCV infection are enrolled in government subsidized treatment program using direct acting antiviral (DAA) drugs; sovaldi. However, a nationwide campaign for HCC surveillance is still not available. Many studies showed conflicting results regarding the outcome of DAA treatment and HCC recurrence exit. Given the size of the HCV and HCC problems in Egypt, the HCV treatment program could yield important results on the efficiency of HCV treatment using DAA therapy on HCC risk in the near future [13,14].

The tumor suppressor P53 is a phosphoprotein barely detectable in the nucleus of normal cells, located on chromosome 17p13.1, and it encodes a 53-kDa nuclear phosphoprotein which plays a central role in safeguarding the integrity of the human genome [15]. Upon cellular stress, particularly that induced by DNA damage, P53 can arrest cell cycle progression, thus allowing the DNA to be repaired; or it can lead to apoptosis. In cancer cells, this protein is no longer able to control cell proliferation, resulting in inefficient DNA repair and the emergence of genetically unstable cells [16,17].

MDM2 oncoprotein is a direct negative regulator for the P53 tumor suppressor protein, which accounts for 50% of human cancers if mutated, deleted or with loss of function [18]. Remarkably, P53 transactivates the MDM2 gene, thus establishing a negative feedback loop that helps maintain P53 in check in nonstresses cells [19]. Additionally, a naturally occurring G to T sequence variation (single nucleotide polymorphism-SNP309) in the promoter-enhancer region of the MDM2 gene. A genetic polymorphism of MDM2 gene can enhance the binding of activator Sp1 general transcription factor to this promoter region and increase MDM2 gene transcription [20]. formation of transcriptionally inactive P53-MDM2 complexes and alteration of the P53 pathway. These observations showing an oncogenic function for the variant SNP309 [21].

Association between TP53 codon 72 and MDM2 gene SNP309 have been previously investigated in Moroccan and Italian populations in which they associated with development of HCC in patients with chronic hepatitis C, and Korean patients with chronic hepatitis B [22–24].

In present study, we evaluated the distribution of the TP53 codon 72 genotypes and MDM2 SNP309 in a series of hepatocellular carcinoma patients with HCV infection and compared them with healthy controls without any previous diagnosis of any type of cancer matched for age, gender and ethnicity, to provide information about the risk associated with MDM2 SNP309 and TP53 in a rarely studied population of patients with HCC.

2. MATERIALS AND METHODS

2.1 Patients and Healthy Controls

The current study carried out on 100 Hepatocellular Carcinoma (HCC) patients with confirmed chronic HCV and 100 healthy control group without any previous diagnosis of any type of cancer. Patients with any other types of cancer, who have a history with any other type of cancer and who have any other type of viruses as Hepatitis B Surface Antigen virus (HbsAg) or human immunodeficiency viruses (HIV) were excluded from the study.

Genomic DNA was extracted from EDTA whole blood using column method with the PureLink® Genomic DNA Mini Kits for purification of genomic DNA (Invitrogen™, USA, catalog Number K182001) according to the manufacture instructions. The steps for DNA extraction included blood cell lysis, DNA binding, washes, and elution.

2.2 TP53 Codon 72(rs1042522) Genotyping

Genotyping and allelic discrimination of SNP rs17849079 was performed using a TaqMan® SNP ready-made genotyping assay and TaqMan® Genotyping Master Mix, on the 7500 Fast Dx Real-Time PCR System from Applied Biosystem (Applied Biosystems Inc. 850 Lincoln Centre Drive Foster City, CA 94404 USA). PCR primers used to detect SNP TP53 gene in HCC patients and controls are:

- Forward primer sequence to: (5'-GAC CAC TAT GTT TAA GGA AG-3').
- Reverse primer sequence: (5'-TGA CTC ACC TAC TTT CCC AC-3').

The thermal profile conditions were 94°C for 2 min; 50 cycles with denaturation at 94°C for 15 sec, annealing at 62°C for 30 sec, extension at 72°C for 1 min, finally hold at 4°C for infinite.

2.3 MDM2 Promoter SNP309 (T/G) Genotyping

The MDM2 SNP309 was determined by polymerase chain reaction (PCR) based-restriction fragment length polymorphism (PCR-RFLP) method then analysis of amplified products. The DNA fragment that contained the MDM2 SNP309 was amplified by PCR using the forward primer (5'-GGG AGT TCA GGG TAA AGG-3') (primer number L4419F09 designed by Invitrogen Thermo Fisher Scientific) and reverse primer (5'- GAC CAG CTC AAG AGG AAA-3') (primer number L4419F10 designed by Invitrogen Thermo Fisher Scientific). The thermal cycler conditions were 95°C for 2 min; 35 cycles with denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min, finally hold at 4°C for infinite. A single band at 174 bp for the restriction of the PCR products before restriction digestion. Subsequently, 10 µl of the PCR product was digested with 1.5µl of MspA1I restriction enzyme (Biolabs, New England L0151610), 7µl 10X NE Buffer and 31.5µl autoclaved distilled water. The 309-bp amplified product was digested overnight (3hrs and up) with MspA1I at 37°C. After digestion, the fragments were electrophoresed on 3% agarose gel and visualized by UV light after ethidium bromide staining. MDM2 SNP309 T allele was not cleaved by MspA1I endonuclease and had a single band of 174 bp. The MDM2 SNP309 G allele was cleaved by MspA1I and had two fragments of 126 pb and 48 pb. The MDM2 SNP309 G allele was cleaved by MspA1I and had two fragments of 126 pb and 48 pb. The MDM2 SNP309 heterozygous had three bands of 174, 126 and 48 bp.

2.4 Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level ($p < 0.05$). Chi-square test (χ^2) used for comparisons between different groups regarding the gender. Hardy-Weinberg equilibrium is a law which states that allele and genotype frequencies in a population will remain constant from generation to generation in absence of other evolutionary influence like mutation. Odd ratio (OR) Used to calculate the ratio of the odds and

95% Confidence Interval of an event occurring in one risk group to the odds of it occurring in the non-risk group. Confidence interval (CI) used to determine the association between HCC risk and genotypes. $P < 0.05$ was considered statistically significant in all tests.

3. RESULTS

The current study carried out on 100 HCC patients with confirmed chronic HCV and 100 healthy control group without any previous diagnosis of any type of cancer. In HCC patients included 63 Males (63%) and 37 females (37%), while control consisted of 52 males (52%) versus 48 females (48%), and no statistically significant difference was detected between the two groups as regards the gender ($p=0.067$).

Mean age of HCC patients was 57.51 ± 10.78 years compared to the controls 56.52 ± 11.47 years for control group. No statistically significant difference was detected between the two studied groups as regards the age ($p=0.464$) (Table 2).

The frequencies of TP53 codon 72 (rs1042522) genotypes has been estimated using an allele specific PCR that specifically detects either the TP53 proline (C allele) or arginine (G allele).

The frequencies of TP53 codon 72 (rs1042522) genotypes were found it consistent with Hardy-Weinberg equilibrium in both control and Patients group ($\chi^2=3.305$; $p=0.069$; and $\chi^2=1.692$; $p=0.193$, respectively).

TP53 gene (rs1042522) genotype and allele frequencies in HCC patients and healthy controls are shown in (Table 3). Overall, A slightly higher frequency of arginine allele (G) was observed in HCC cases compared to the healthy controls, however, no statistically significant difference was detected between the two-allele frequency ($p=0.093$).

The frequencies of MDM2 SNP309 genotypes has been analyzed using a PCR-RFLP based assay that specifically detects the frequencies of MDM2 SNP309 T/T, T/G and G/G genotypes among the HCC patients and healthy controls (Fig. 1).

Hardy-Weinberg analysis was performed for MDM2 SNP309 genotype frequencies and found it consistent with Hardy-Weinberg equilibrium in both control and Patients ($\chi^2=0.978$; $p=0.323$; and $\chi^2=0.859$; $p=0.354$, respectively).

The relative frequencies of the T allele and G allele were 67.5% and 32.5% in HCC patients and 91% and 9% in control group respectively. So, the minor allele G was significantly more than frequent in HCC patients than controls ($p < 0.001$) (Table 4).

According to Table 4, considering the allele frequency of MDM2 SNP309 in patients and control group it was shown that people who have G allele increase the risk by 4.868 folds for developing HCC (OR=4.868, 95% CI= 2.873 – 8.251).

Table 2. Comparison between the studied groups according to demographic data

Variable	HCC patients (n = 100)		Controls (n = 100)		Test of sig.	p
	No.	%	No.	%		
Sex						
Male	63	63.0	52	52.0	$\chi^2 =$ 3.343	0.067
Female	37	37.0	48	48.0		
Age (years)						
Min. – Max.	17.0 – 77.0		20.0 – 80.0		t=	0.464
Mean ± SD.	57.51 ± 10.78		56.52 ± 11.47		0.734	
Median	58.50		58.0			

p: value for comparison between the studied group χ^2 : Chi square test *t*: value for Student t-test

Table 3. Comparison between the two studied groups according to TP53 (rs1042522) allele and genotyping frequencies

	HCC patients (n = 100)		Controls (n = 100)		χ^2	p	OR	95% C. I
	No.	%	No.	%				
P53								
CC®	40	40.0	43	43.0	0.248	0.618	1	-
CG	43	43.0	51	51.0	1.720	0.190	0.906	0.546 – 1.505
GG	17	17.0	6	6.0	6.981*	0.008*	3.046	1.185 – 7.826
Allele frequency								
C®	123	61.5	137	68.5			1	-
G	77	38.5	63	31.5	2.830	0.093	1.361	0.949 – 1.951

χ^2 : Chi square test; C.I.: Confidence interval; *p*: *p* value for comparison between the studied groups;

*: Statistically significant at $p \leq 0.05$; OR: Odds Ratio

Table 4. Comparison between the studied groups according to MDM2/RFLP SNP309 genotyping frequencies

Variable	HCC patients (n = 100)		Controls (n = 100)		χ^2	p	OR	95% C. I
	No.	%	No.	%				
MDM2/RFLP								
TT®	47	47.0	82	82.0	33.679*	<0.001*	1	-
TG	41	41.0	18	18.0	15.870*	<0.001*	3.974	2.203 – 7.168
GG	12	12.0	0	0.0	13.043*	<0.001*	42.777	2.561 – 714.4
Allele frequency								
T®	135	67.5	182	91.0			1	-
G	65	32.5	18	9.0	39.626*	<0.001*	4.868	2.873 – 8.251

χ^2 : Chi square test; *p*: *p* value for comparison between the studied groups; *: Statistically significant at $p \leq 0.05$;

OR: Odds Ratio; C.I.: Confidence interval

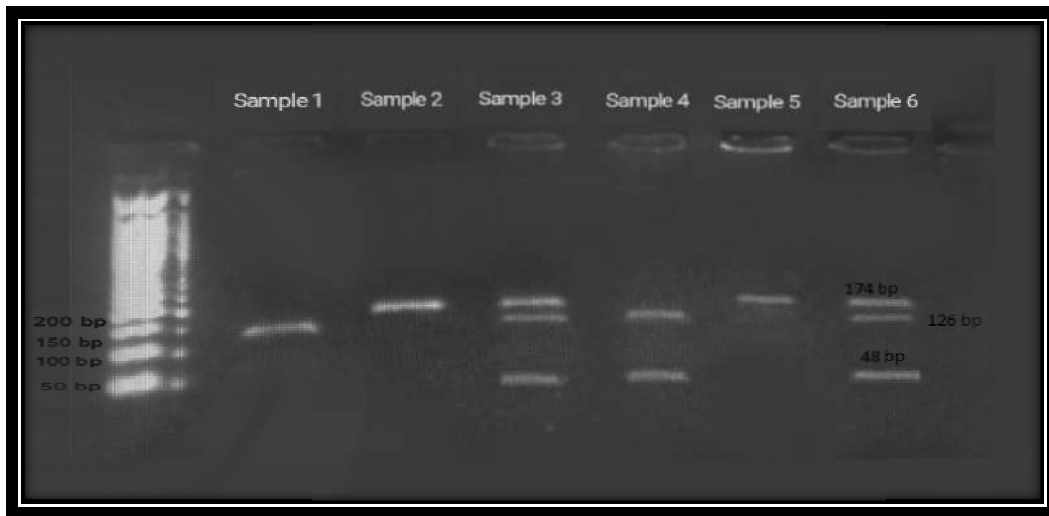


Fig. 1. PCR amplification followed by restriction fragment length polymorphism (PCR-RFLP) to determine MDM2 SNP309 polymorphism

MDM2 SNP309 T allele was not cleaved by MspA1I endonuclease and had a single band of 174 bp as samples 1, 2 and 5 (TT genotype). The MDM2 SNP309 G allele was cleaved by MspA1I and had two fragments of 126 pb and 48 pb as sample 4 (GG genotype). The MDM2 SNP309 G allele was cleaved by MspA1I and had two fragments of 126 pb and 48 pb The MDM2 SNP309 heterozygous (TG genotype) had three bands of 174, 126 and 48 bp as samples 3 and 6

4. DISCUSSION

MDM2 protein negatively regulates the P53 tumor suppressor protein, it binds to the N-terminal transactivation domain of P53, thereby inhibiting its transcriptional activity and its growth regulatory function, so the MDM2 gene is overexpressed in several human tumors [25]. Recently a SNP309 in the promoter region of MDM2 has been shown to be associated with both hereditary and sporadic cancers in humans. Several studies have reported the association between the TP53 codon 72 and MDM2 G variant SNP309 polymorphisms and increased risk for several types of cancer as gastric [26], breast [27], lung [28], and bladder cancers [29], but few evaluated the association of both these polymorphisms in viral-related liver cancer and none in hepatocellular carcinoma from Egyptian patients.

In the current study, we investigated the association between the TP53 codon 72 and MDM2 SNP309 T>G polymorphisms as a risk factor for hepatocellular carcinoma in patients with chronic HCV infection. The analysis of 100 HCC patients with chronic HCV infection and 100 matched healthy controls showed that the functional of polymorphism in the MDM2 promoter had a significant impact on the risk of developing HCC in Egyptian patients. The frequencies of TP53 codon 72 (rs1042522) and

MDM2 SNP309 genotypes were found it consistent with Hardy-Weinberg equilibrium in both control and Patients group ($\chi^2=3.305$; $p=0.069$; and $\chi^2=1.692$; $p=0.193$), ($\chi^2=0.978$; $p=0.323$; and $\chi^2=0.859$; $p=0.354$) respectively. The results also showed that, the distribution between the two studied groups according to TP53 (rs1042522) allele and genotyping frequencies were no statistically significant difference detected between the two-allele (C and G) frequency ($p=0.093$, OR=1.361, 95% CI=0.949 – 1.951). We found also that, the allele frequency of MDM2 SNP309 in patients and control group it was shown that people who have G allele increase the risk by 4.868 folds for developing HCC (OR=4.868, 95% CI= 2.873 – 8.251), compared with MDM2 SNP309 which have T allele. Given the role of MDM2 in cancer formation, patients which carry the G allele might expect having heightened expression of MDM2 over a lifetime are at higher risk for developing HCC.

In this study the frequencies of MDM2 SNP309 TG heterozygous (41%) and GG homozygous (12%) genotypes were significantly higher among HCC cases compared to healthy controls ($p<0.001$ for both genotypes). On other hand, the homozygous MDM2 SNP309 TT genotype in HCC was lower (47%) than that observed in controls (82%). The possible role of MDM2 SNP309 polymorphism in HCC development has

been analyzed in few other geographical regions. In Eastern Asia MDM2 SNP309 G allele has associated with higher risk of HCC in Japanese patients with chronic hepatitis C [30], in Taiwanese patients infected with HBV or HCV infection [31]. Similarly, in Moroccan (Northern Africa) was found that the GG genotype of MDM2 SNP309 is significantly associated with an increased risk of HCC [23]. Similarly, in Italy a significant increase of MDM2 SNP309 GG genotype was observed among HCC cases [24]. Similarly, the MDM2 SNP309 G allele was found to enhance the risk of viral associated HCC in Turkish patients (Western Asia) [32]. The study by Bond et al. (using samples of breast cancers and soft-tissue sarcomas) showed that MDM2 protein levels in homozygous and heterozygous SNP309 G are on average 4-fold and 1.9 fold higher, respectively, than homozygous MDM2 SNP309 TT, which mean the GG genotype of SNP309 has strong effect on tumorigenesis on humans [25]. Furthermore, Jin et al. (2011) performed a meta-analysis on these studies, including a total of 738 cases and 1062 controls, to evaluate the reliability of the associations of MDM2 SNP309 G allele with HCC. The OR reached 1.57 (95%CI:1.36-1.80) for G allele compared to T allele, which indicated that this SNP may be used to predictive molecular marker for the screening of high risk of HCC [33].

The proper regulation of MDM2 levels has been shown to be critical for P53 tumor suppression, and even a modest change in levels could affect the P53 pathway and increase the risk of cancer. MDM2 binds directly to inhibits P53 by regulating its location, stability and ability to activate transcription [34]. On other hand, in HCV-positive HCC patients, high prevalence of G alleles of the SNP309 indicate on the p53 function could have been indirectly suppressed by the heightened MDM2 levels and increase tumor development.

In Egyptian population, we also studied the effect of SNP309 and TP53 on the demographic data in HCC patients, In HCC patients with genotypes TT included 29 Males (61.7%) and 18 females (38.3%), while TG genotypes had 28 males (68.3%) versus 13 females (31.7%), while GG genotypes had 6 males (50%) versus 6 females (50%). No statistically significant difference was detected between the 3 genotypes as regards the gender ($p=0.247$). Mean age of HCC patients was 57.51 ± 9.71 years in TT genotypes, while TG genotypes had mean age 58.05 ± 11.82 years, while GG genotypes had mean age 55.67

± 11.29 years. No statistically significant difference was detected between the 3 genotypes as regards the age ($p=0.638$).

In HCC patients with genotypes CC included 25 Males (62.5%) and 15 females (37.5%), while CG genotypes had 28 males (65.1%) versus 15 females (34.9%), while GG genotypes had 10 males (58.8%) versus 7 females (41.2%). No statistically significant difference was detected between the 3 genotypes as regards the gender ($p=0.807$). Mean age of HCC patients was 57.10 ± 9.70 years in CC genotypes, while CG genotypes had mean age 57.77 ± 11.34 years, while GG genotypes had mean age 57.82 ± 11.99 years. No statistically significant difference was detected between the 3 genotypes as regards the age ($p=0.909$).

5. CONCLUSION

In conclusion, our results indicate an association between MDM2 SNP309 GG genotype and risk of hepatocellular carcinoma in hepatitis C virus among Egyptian population. Conversely, the TP53 polymorphism at codon 72 showed not to be a potential risk for development of HCC with chronic hepatitis C virus among Egyptian patients.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

All patients and healthy control patients' legal guardians were asked to give an informed consent after explaining the nature, steps, and aim of the study.

ETHICAL APPROVAL

This study was conducted after approval of department of gastroenterology and hepatology and the medical ethics committee Alexandria Faculty of Medicine.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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