

Hepatitis C Virus Core Gene Polymorphism in Cases of Hepatocellular Carcinoma

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ABSTRACT: Hepatocellular carcinoma (HCC) is one of the common sequelae of hepatitis C virus (HCV) infection. Although HCV core protein displayed tumorigenic activities in cell culture and experimental animals, its clinical impact on HCC development in humans is still unclear. We aimed to map differences in the viral core gene sequences in cases (HCC patients) and controls (HCV patients without HCC). Direct sequencing of HCV core gene from 30 HCC patients and 30 controls was done to compare with each other. This study showed that sequences of HCV in patients with HCC differed from those of controls; specifically, one polymorphism at amino acid position 71 was particularly strongly associated with HCC as it was present in 33.3% of patients with HCC compared to only 6.7% of controls. These results highlight that HCV core gene sequence data might provide useful information about the risk of developing HCC.

KEYWORDS: Egypt, HCC, HCV, core gene, sequencing

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Introduction

Hepatitis C virus (HCV) is a globally prevalent pathogen with a strong burden on the health care system as a leading cause of death and morbidity.¹ Egypt reports the highest prevalence worldwide.² According to Egypt Health Survey 2015, the percentage of population aged 1–59 years positive on HCV antibody (chemiluminescence test) was 6.3%, and the percentage positive on HCV RNA test was 4.4%; moreover, in Alexandria, the percentages were 3.6% and 2.4%, respectively.³

This extraordinarily high prevalence results in an increasing incidence of hepatocellular carcinoma (HCC) in Egypt, which is now the second most frequent cause of cancer and cancer mortality.⁴ Upon infection with HCV, spontaneous clearance of the virus occurs in only 15%–20% of the infected, while remainders develop chronic infection.⁵ The main complications of HCV infection are severe liver fibrosis and cirrhosis, and 30%–50% of individuals with cirrhosis develop HCC.⁶ Several host factors such as male gender, older age, elevated α -fetoprotein (AFP) level, advanced liver fibrosis, and nonresponsiveness to interferon (IFN) therapy have been reported as important predictors of HCC development.⁷ On the other hand, it remains controversial as to whether HCV itself plays a direct role in the development of HCC. Experimental data suggest that HCV contributes to HCC

by modulating pathways that promote malignant transformation of hepatocytes. HCV core, NS3, and NS5A proteins were shown to be involved in a number of potentially oncogenic pathways in cell culture and experimental animal systems.⁸ Of the several HCV proteins reported to alter cellular growth,⁹ the core protein is the one most strongly associated with cellular transformation. Expression of the core protein enhances cell proliferation, DNA synthesis, cell cycle progression, cellular transformation, and liver cancer in experimental systems.¹⁰

The HCV core gene is a complex genetic region of the viral genome. HCV core is a highly conserved basic protein that makes up the viral nucleocapsid. HCV Core protein consists of the 1st 191 amino acids in HCV polyprotein. It contains two open reading frames, three confirmed RNA structures, and several additional putative RNA structures. Therefore, mutations in the core gene have the potential to alter the proteins encoded by the two open reading frames, the RNA secondary structures, and/or the RNA signals.^{11,12}

HCV sequences differ in patients with early-stage versus late-stage liver disease.¹³ It is not known whether the sequence differences are present because viruses with certain polymorphisms have enhanced potential to cause liver damage; consequently, viruses that carry these mutations are more



likely to be found in patients with liver cancer.¹⁴ Alternatively, the sequence differences arise because liver disease progression changes the cellular environment in which HCV replicates and selects for variants carrying polymorphisms that enhance survival in the damaged liver. HCV core genes differ in tumor versus nontumor samples of the same liver.¹⁵ Interestingly, analyses of full-length core gene sequences from serum indicate that there may be specific substitutions in the circulating HCV RNA of patients with HCC.¹⁶

Regardless of the events that give rise to HCV disease-specific polymorphisms, knowledge of these polymorphisms is potentially useful. If HCV mutations can be associated with liver disease status, then HCV sequencing can become a non-invasive and economical tool for obtaining information about the condition of a patient's liver and HCC risk.¹⁷ In addition, understanding the causal relationship between viral mutations and liver disease may suggest new targets for therapeutic intervention.¹⁷ This study examined the differences in the viral core gene sequences in HCC patients from those of control HCV patients without HCC to investigate the presence of specific core gene mutations associated with HCC risk.

Patients and Methods

A case-control study was carried out on 60 HCV-seropositive and HCV RNA-positive subjects selected from the Tropical Medicine Department in Alexandria Main University Hospital. Informed consent was taken from each patient participating in this study. The study was approved by the Research Ethics Committee at the Alexandria Faculty of Medicine. The research was conducted in accordance with the principles of the Declaration of Helsinki. Patients were divided into the following two groups: cases that included 30 chronic HCV patients with HCC and controls that included 30 chronic HCV patients free from HCC.

All relevant information was collected from each patient, including personal data (age, sex, etc) as well as health data (history of blood transfusion, history of shistosomiasis and anti-shistosomiasis treatment, previous surgical interference, and dentistry). All patients were negative for hepatitis B surface Ag (HBsAg) to exclude hepatitis B virus coinfection.

Five milliliters of blood was withdrawn from each patient, and the sera were separated and aliquoted and then stored at -80°C until use. Determination of liver function tests including, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total serum bilirubin, was performed using automated spectrophotometric apparatus.

HCV RNA extraction. RNA was extracted from 140 μL of serum using the QIAamp viral RNA mini kit (Qiagen Inc.) following the manufacturer's instructions. Briefly, samples were incubated with lysis buffer. After incubation, there were two washing steps, and the nucleic acids were eluted in a volume of 50 μL of elution buffer.

Detection of HCV-RNA viral load. HCV-RNA viral load was detected by real-time polymerase chain reaction

(PCR) with TaqMan assay using Artus HCV QS-RGQ PCR Kit based on the amplification and simultaneous detection of a specific region of the HCV genome. PCR was performed in 25 μL of reaction mixture containing 6 μL of HCV RG Master A and 9 μL of HCV RG Master B, and 10 μL of extracted RNA was added to bring the reaction to a final volume of 25 μL . The reaction took place under the following thermal profile: incubation at 50°C for 30 minutes to transcribe viral RNA to complementary DNA (cDNA) by reverse transcription (RT). This was followed by AmpliTaq gold activation for 95°C for 10 minutes, followed by 45 cycles of three-step PCR amplification, denaturation for 95°C for 30 seconds, followed by annealing at 50°C for one minute and extension at 72°C for 30 seconds, with end point fluorescence detection.

Amplification of the core gene. Transcription of RNA into cDNA was done using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamers.

Amplification of cDNA was done by nested conventional PCR reaction using the following primers: sense primer: 5'-GCTAGCCGAGTAGTGTTG-3', antisense primer: 5'-GATGTGRTGRTCGGCCTC-3' for the first-run PCR and sense primer: 5'-GGAGGTCTCGTAGACCGTGC-3', antisense primer: 5'-ATGTACCCCATGAGGTCCGGC-3' for the second-run PCR.

PCR was performed in 25 μL of reaction mixture containing 2 μL cDNA, 12.5 μL Maxima Hot Start Green PCR Master Mix (2 \times) (Thermo Scientific Fermentas), 0.5 μL (5 pmol) of forward and reverse primers, and H_2O for the first run and in 50 μL of reaction mixture containing 5 μL of first-run amplicon, 25 μL Master Mix, 1 μL (10 pmol) of forward and reverse primers, and H_2O for the second run.

The thermal profile was as follows: initial denaturation at 95°C for four minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, a 30-second annealing step at 50°C , and a 60-second extension step at 72°C , each with a final extension cycle at 72°C for 10 minutes for the first and second runs. DNA fragments were detected on 1% agarose gels with ethidium bromide staining.

Sequencing of the core gene. To search for the presence of mutations, sequencing of the core gene was performed. The PCR amplification products were purified using PureLink PCR purification kit (Invitrogen Life Technologies). Automated sequencing reactions were performed with the BigDye terminator cycle sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems) using the same primers that were used in the amplification of the core gene.

Sequencing results analysis. For determination of HCV genotypes and subtypes, the core sequencing results were analyzed using HCV BLAST free online tool from HCV sequence database. BioEdit Sequence alignment editor version 7.2.5 and ClustalW multiple alignment tool were used to align core nucleotide sequences from different patients and control isolates and convert them to amino acid sequences after toggle translation. The derived sequences were aligned

and compared. Genotype 4 published Reference Sequence with accession number (DQ988078) from NCBI (National Center for Biotechnology Information) was chosen to be aligned with the sequenced samples in order to determine the presence of mutations.

Statistical analysis. Statistical differences in the baseline parameters of HCC and control groups were determined by Student's *t*-test for numerical variables and Fisher's exact probability or chi-square tests for categorical variables. Likewise, statistical differences in viral mutations between HCC and control groups were determined by Fisher's exact probability test. Kaplan–Meier analysis was performed to estimate the cumulative incidence of HCC. The data obtained were evaluated by the log-rank test. Univariate and multivariate logistic analyses were performed to identify variables independently associated with HCC development. Variables with $P < 0.1$ in univariate analysis were included in a backward stepwise multivariate logistic regression analysis. The odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated. All statistical analyses were performed using SPSS v. 19 software. Unless otherwise stated, $P < 0.05$ was considered statistically significant.

Results

This study included 60 HCV-positive subjects: 30 HCV patients with HCC and 30 HCV patients free from HCC. The age of HCV patients with HCC ranged between 45 and 85 years, 70% of them were in the age group of 50+ years, while 30% were in the age group below 50 years. The age of HCV patients without HCC ranged between 20 and 58 years, 70% were in the age group below 50 years, while 30% were in the age group of 50+ years. The male-to-female ratio was 4:1 in the group of cases and 3:1 in the control group.

Distribution of HCV isolates in patients with and without HCC according to their HCV genotype and subgenotype using direct sequencing of HCV core gene. Amplification and direct sequencing of the core gene amplified product (Fig. 1) followed by analysis of the obtained sequences revealed that 73.3% of cases were of subgenotype 4a, followed by 13.3% subgenotype 4l, then subgenotypes 4n and 4o in equal percentage, 6.7%. Using the same technique

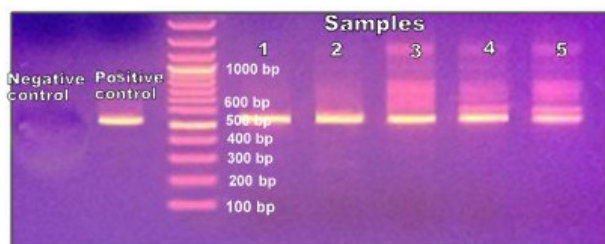


Figure 1. PCR products (core gene) were analyzed on a 2% agarose gel stained with ethidium bromide, compared to a 100-bp DNA ladder and visualized by ultraviolet light showing an expected band of 573 bp length.

for subgenotyping HCV amplicons, 90% of controls were of subgenotype 4a followed by 10% subgenotypes 4l, 4n, and 4o in equal percentage, 3.3% (Fig. 2).

Correlation between core protein sequence polymorphism and HCC development. HCV core protein sequences were obtained from all cases and controls (Fig. 3). Comparative sequence analysis revealed that in cases, 33.3% of the isolates showed no amino acid substitutions in the core region from that of the reference strain, while 66.7% of them did. On the other hand, in controls, 63.3% of the isolates showed no amino acid substitutions in the core region from that of the reference strain, while 36.7% of them did. This denotes that HCV core amino acid substitutions are significantly associated with cases than controls ($P \leq 0.05$, Table 1).

When the sequence patterns at positions 70 and 91 were examined, there was no significant correlation between these sequence patterns at positions 70 and 91 and HCC. On the other hand, a single mutation at position 71 was the only polymorphic factor within core protein that was significantly associated with HCC development. We found that 10 (33.3%) of 30 HCV isolates with HCC had P⁷¹, while only two (6.7%) of the 30 control isolates did ($P = 0.049$, Table 2).

For identification of independent factors correlated with HCC development by multivariate logistic regression analyses (Table 3). Keeping all other factors constant, AST, ALT, AST/ALT ratio, GGT, alkaline phosphatase, AFP, HCV core amino acid substitutions, and hepatic fibrosis score were evaluated as independent risk factors for HCC development. Regarding AST, the increased level by 1 unit/L was associated with increased risk for having HCC by about 13% (OR = 1.13). As for ALT, the increased level by 1 unit/L was associated with increased HCC by about 15% (OR = 1.15). Regarding AFP, the risk for having HCC among HCV patients was increased by 39 times upon one unit increase in the level of AFP (OR = 39.4). Regarding alkaline phosphatase, the increased level by 1 unit/L was associated with increased risk for having HCC by about 4% (OR = 1.037).

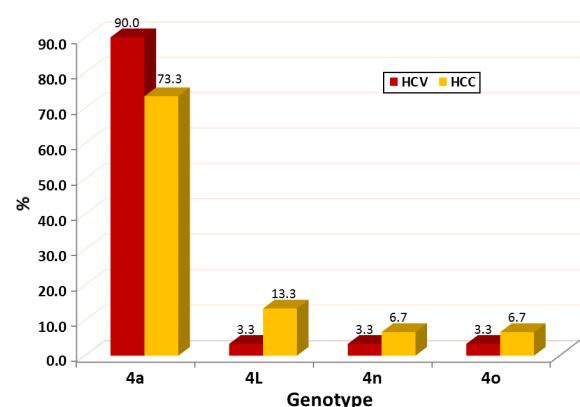


Figure 2. Distribution of HCV isolates in patients with and without HCC according to their HCV genotype and subgenotype using direct sequencing of HCV core gene.

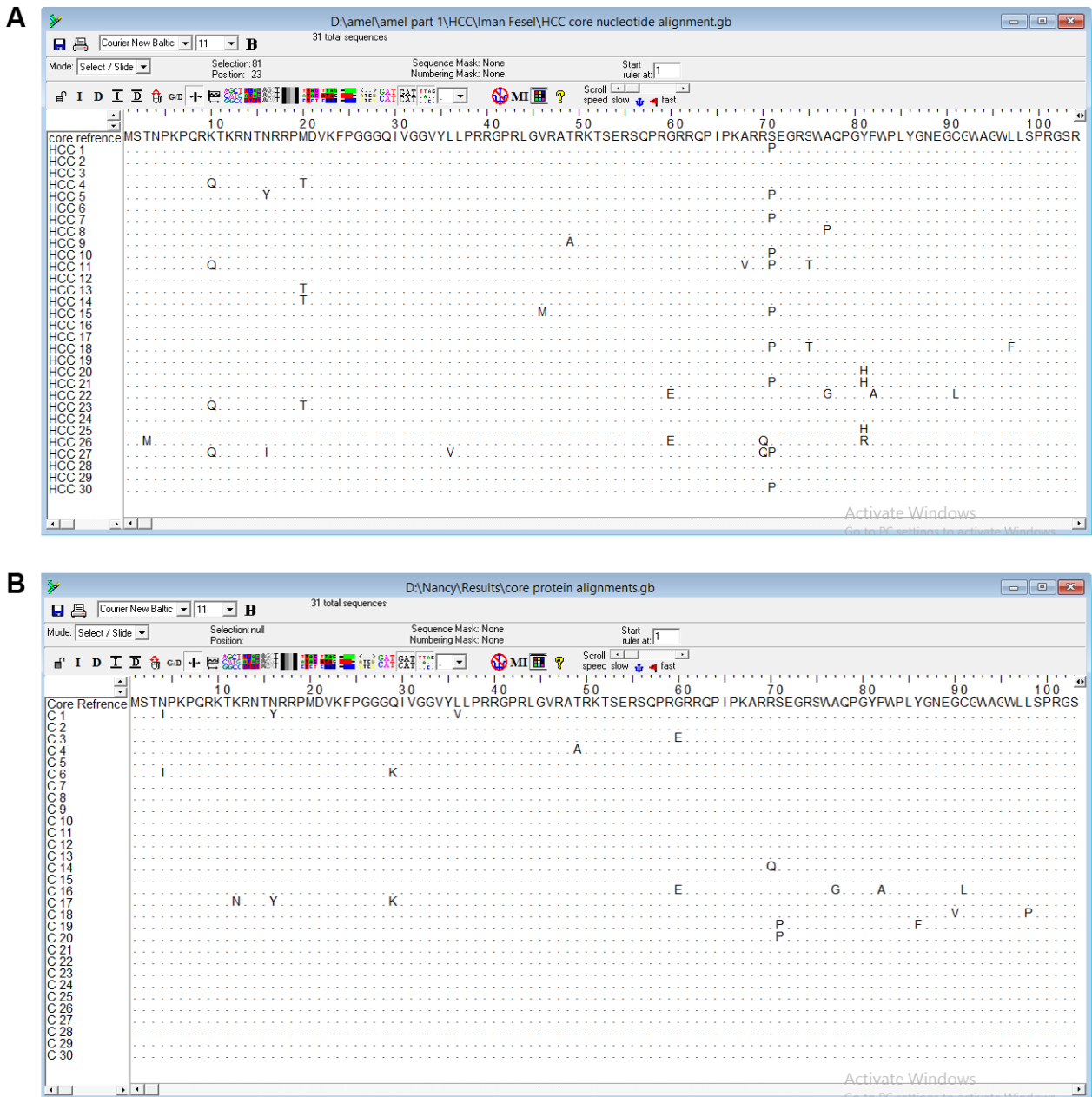


Figure 3. Core amino acid sequence alignment obtained from the 30 HCC cases (A) and the 30 HCV controls (B) showing amino acid substitutions at positions 70, 71, and 91 in the core region. Amino acid sequences were aligned with BioEdit. Reference sequence with accession number (DQ988078) is shown at the top.

Table 1. Comparison between HCV patients with and without HCC according to presence (mutant core) or absence (wild core) of HCV core amino acid substitutions using direct sequencing of HCV core region.

HCV CORE	GROUP				χ^2 (P)
	HCV +VE WITH HCC		HCV +VE WITHOUT HCC		
	NO	%	NO	%	
Wilde core	10	33.3	19	63.3	0.039*
Mutant core	20	66.7	11	36.7	
Total	30	100	30	100	

Note: *P < 0.05 (significant).

Table 2. Association between specific mutations in the HCV core region and HCC in HCV patients.

AMINO ACID POSITION (WILD-MUTANT)	GROUP				FEP
	HCV PATIENT WITH HCC		HCV PATIENT WITH NO HCC		
	NO	%	NO	%	
Position 70 (R-Q)	2	6.7	1	3.3	0.554
Position 71 (S-P)	10	33.3	2	6.7	0.049*
Position 91 (C-L)	1	3.3	1	3.3	1.000

Note: *P < 0.05 (significant).

Abbreviation: FEP, Fisher exact probability.

Table 3. Identification of independent factors correlated with HCC development by multivariate logistic regression model analyses.

FACTOR	B	SE	P	OR	95% CI FOR OR	
					LOWER	UPPER
AST	0.113	0.030	0.001*	1.13	1.054	1.184
ALT	0.127	0.030	0.001*	1.15	1.069	1.200
T.bilirubin	6.32	1.850	0.002*	25.1	9.345	184.300
AFP	3.65	1.98	0.042*	39.4	16.8	120.7
Alk. phosphatase	0.036	0.010	0.001*	1.037	1.017	1.057
Mutant HCV core	1.093	0.536	0.041*	2.983	1.044	8.527
Fibrosis	3.3	0.796	0.001*	20.91	4.354	98.736
Model significance	0.001*					
Classification accuracy	81.4%					
Pseudo R^2	0.33					

Abbreviations: *, significant value; B, regression coefficient; SE, standard error; OR, odds ratio; CI, confidence interval; Pseudo R^2 , pseudo R -squared.

Considering HCV core amino acid mutations, the increased number of HCV core amino acid mutations by a single amino acid mutation was associated with increased risk for having HCC by three times (OR = 2.983). Regarding hepatic fibrosis, the risk for having HCC among HCV patients was increased by 20 times upon unit increase in fibrosis score (OR = 20.91).

No significant relationship was found between HCV viral load and HCV core heterogeneity in both HCV patients with or without HCC (Table 4).

Discussion

HCC is one of the common long-term complications of HCV infection. The burden of HCC has been increasing in Egypt with a doubling in the incidence rate in the past 10 years. However, whether HCV itself plays a direct role in the development of HCC and whether all HCV isolates

Table 4. Distribution of HCV viral load in relation to HCV core amino acid substitutions in HCV patients with and without HCC.

VIRAL LOAD	GROUP				MCP
	HCV +VE WITHOUT HCC		HCV +VE WITH HCC		
	NO	%	NO	%	
$10^2 < 10^3$	2	6.7	3	10.0	0.874
$10^3 < 10^4$	2	6.7	3	10.0	
$10^4 < 10^5$	7	23.3	6	20.0	
$10^5 < 10^6$	10	33.3	9	30.0	
$10^6 < 10^7$	6	20.0	8	26.7	
$> 10^7$	3	10.0	1	3.3	

Abbreviation: MCP, Mont Carlo exact probability.

are equally associated with HCC development remain to be determined. HCV core, NS3, and NS5A proteins have been reported to affect a wide variety of potentially oncogenic pathways in cell culture and experimental animal systems.⁷ Ogata et al¹⁴ reported that HCV genotype 1b strains might be associated with HCC on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein. Giménez-Barcons et al¹⁸ reported that high amino acid variability within the NS5A of HCV might be associated with HCC in patients with HCV-1b-related cirrhosis.

In this study, we examined HCV core gene sequences to identify amino acid polymorphisms associated with HCC. Core genes in HCC group having characteristic mutations and significantly being more diverse than those of HCV core genes in the control group, also the significant association between the HCV core mutation at position 71 (core-p⁷¹), but not at position 70 nor position 91, and HCC development were the most striking results of our study.

Previous several studies in agreement with this study supported the relationship between characteristic mutations in HCV core gene and the occurrence of HCC. Although most of these studies were especially on amino acid positions 70 and 91, we could not detect any significant association between the HCV core mutation at position 70 or position 91 and HCC development. A previous study revealed that position 271, which is part of codon 91, that encodes either leucine (271U/C) or methionine (271A) in the genotype 1b core protein in more than 300 sequences from Japanese patients with high viral load genotype 1b HCV showed associations between methionine 91 (271A) and IFN resistance and HCC.¹⁹

In contrast, smaller direct sequencing studies from Japan involving 28 HCC and 15 control sequences showed an association between leucine 91 (271U/C) and HCC.^{16,20} The relationship between 271U/C and HCC risk may be clearer when the molecular changes that accompany mutations at position 271 are understood. Recent data indicate that the 271U/C polymorphism decreases the strength of a signal for the internal initiation of HCV protein synthesis. This modulation of a translation signal may be the key functional change that occurs as a result of mutations at position 271.²¹

As regards position 70 in contrast to our study, the sequence analysis in many previous studies revealed that Gln at position 70 of the core protein (core-Gln⁷⁰) was significantly associated with HCC development.²²⁻²⁴ On the other hand, a different study in agreement with our results revealed that sequence changes in codon 70 were not distributed differently between HCC and non-HCC GT1a and 1b carriers. Most importantly, for GT1a carriers, a panel of specific nucleotide changes in other codons was collectively present in all subjects with HCC, but not in any of the non-HCC patients.²⁵

Another study was done on 63 patients with HCC, and 188 patients with no HCC core gene nucleotides at each group were compared, certain polymorphisms were found to be associated with HCC. Upon analysis of these polymorphisms by logistic



regression, eight polymorphisms were significantly related to increased risk of HCC; these mutations could bring out four amino acid substitutions: K10Q, R70Q, M19L, and G161S.²⁶

Recently, a similar study determined four amino acid substitutions associated with increased HCC risk, and three of them were the same as our results (G209A, A271C/U, and G481A). Moreover, up to now, the G209A polymorphism has been thought to be related with both IFN/ribavirin (RBV) treatment resistance and HCC.^{17,27} No previous studies found significant association between the HCV core mutation at position 71 (core-p⁷¹), in HCV genotype 4, and HCC development that was a striking feature in our sequence analysis.

Our results revealed that all patients (100%) from both study groups belonged to genotype 4. Although HCV subgenotype 4l was reported in HCC cases four times more than in the control group, that difference was not statistically significant; also, no significant association was found between other HCV subgenotypes and HCC. On the other hand, Abdel-Hamid et al concluded in a study of 131 HCV RNA-positive subjects that the majority of HCV virus was of subtype 4a, but five other subtypes within genotype 4 were also observed. Interestingly, subtype 4o showed an association with HCC.²⁸

In this study, no significant difference between HCV viral load was detected between HCV patients with or without HCC. A study done by Park et al²⁹ agreed with us and concluded that HCV viremia strongly influences the occurrence of HCC without titer-dependence. Derbala and Amer³⁰ reported in their two case reports that the patients developed HCC in spite of viral clearance, suggesting that HCV per se could have a direct oncogenic effect that is not attributed to HCV viral load.

Conclusion

Although many host and viral factors contribute to the development of HCC, this study identified that polymorphisms in the HCV core gene were associated with increased HCC risk in a multivariable logistic regression model; also, HCV core amino acid p71 was significantly associated with HCC. It remains unclear whether these mutations are inherently oncogenic or if they are benign adaptations to the altered environment that exists in damaged livers. In either case, they are associated with HCC and thus may serve as clinical markers of HCC. Patients harboring HCV strains with these mutations may benefit from closer surveillance. Further studies are warranted to assess the diagnostic value of HCV sequencing in cirrhosis/HCC identification and to identify the molecular pathways underlying the association between certain HCV mutations and advanced liver disease and HCC.

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Author Contributions

This work was carried out in collaboration between all authors. Author MAAK participated in the planning of the study, suggested the idea of the study and shared in management of the literature searches and critical review of the manuscript. Authors AGES, MMR, and EFY designed the study, wrote the protocol, performed the laboratory work, performed the statistical analysis, managed literature searches, and wrote the first draft of the manuscript. Author KMAD recruited the cases and performed critical review of the manuscript. All authors read and approved the final manuscript.

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