Correlation between Hepatocellular Carcinoma and Hepatitis C genotypes and their role in Hepatocarcinogenesis

Nadia El-Deeb[1]; Muhammad El-Saadani[2]; Ashraf El-Enbaby[1]; El-Sayed Hafez[3]; Amr El Saify[1]

(1) Clinical Oncology and Nuclear Medicine Dept., Faculty of Medicine, University of Alexandria
(2) Biochemistry Dept., Faculty of Sciences, University of Alexandria
(3) Molecular Pathology Dept., Mubarak City for Scientific Research and Technology Applications

✉ *Corresponding Author: Dr. Ashraf El-Enbaby*
Assistant Professor of Clinical Oncology and Nuclear Medicine, Faculty of Medicine
Alexandria University, Egypt
Email: elenbaby@hotmail.com

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Abstract

Background: Hepatitis C virus (HCV) is known to be a major risk factor for the development of hepatocellular carcinoma (HCC).
Aim of the work: to correlate HCV genotypes among HCV positive cases of HCC with the clinicopathological profiles of patients, and to assess if there is characteristic pattern of the virus that may accelerate oncogenesis.
Patients and methods: A prospective study; 60 patients; two groups: **Group I:** 30 patients: HCV with superadded HCC. **Group II:** 30 patients: HCV without superadded HCC (control); recruited from Alexandria University hospitals, Egypt. Confirmation of HCV infection and virus RNA extraction were done. The extracted HCV RNA was transformed to complementary DNA (cDNA) using reverse transcription PCR. INNO-LiPA HCV II was used to identify the genotype spectrum of the 60 samples.
Results: Most of HCC patients were in the 6th decade, males, of rural residence, in stage II (BCLC). Serum GGT was superior to AST and ALT in detecting deterioration in liver functions, suggesting that it could be used as a sensitive biochemical marker for development of HCC in patients with HCV. All 60 HCV-RNA positive samples (100%) were genotype 4. Sequence Analysis of 5-untranslated region of HCV (5UTR) was done to 8 RNA PCR extracts to check for a specific pattern of HCV genotype 4 in HCC patients. No mutations were detected in HCC group characterizing the virus. However; two different sequences (one from each group) were gathered in one ancestor and can be considered as a subgenotype from Genotype 4 in Egyptian patients.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors that carry a poor prognosis worldwide. In Egypt, it is the second most common malignancy in males and the fifth in females.[1,2]

The incidence of HCC varies widely according to geographic location. High incidence regions (more than 15 cases per 100,000 population per year) include sub-Saharan Africa, the People's Republic of China, Hong Kong, and Taiwan.[3] Over 40 percent of all cases of HCC occur in China, which has an annual incidence of 137,000 cases[4]. North and South America, most of Europe, Australia and parts of the Middle East are low incidence areas with fewer than three cases reported per 100,000 population per year. However, the incidence in the United States has increased during the past two decades, possibly due to a large pool of people with longstanding chronic hepatitis C [5,6].

Males are far more likely to develop HCC than females. The disparity is more pronounced in high incidence regions, where males are affected 2.1 to 5.7 times more frequently than females. [7]

The majority of HCCs occur in patients with chronic liver disease or cirrhosis; at a mean age at presentation between 50 and 60 years. [8,9] In sub-Saharan Africa, however, the mean age of presentation of HCC is decreasing, with a mean age of 33 years. [10]

Hepatitis C virus (HCV) causes more than 90% of parenterally transmitted non-A, non-B hepatitis (NANBH).[11] In more than 85% of HCV infections, chronic hepatitis is established and may slowly progress to worsening stages of fibrosis and cirrhosis and ultimately may lead to the development of HCC.[12,13]

In Egypt, approximately 13.8% of the population is infected, and 10% with chronic HCV. [14] The prevalent genotype in Egypt is type 4, with the presence of other genotypes. [15] In Europe and USA, HCV-1a and -1b, HCV-2a and -2b, and HCV-3a are the most common subtypes. Types 5 and 6 have been identified in South Africa and Hong Kong, respectively. [16]

Hepatitis C virus has a high rate of mutation during replication, and it exists in the bloodstreams of infected persons as complex distributions of mutants known as viral quasispecies, which fluctuate during the course of the disease, mainly as a result of immune response. [17,18]

Clinical and epidemiologic studies suggest that HCV is more hepatocarcinogenic than HBV, as the frequency of HCC development among HCV-induced cirrhosis is much higher than that of HBV-induced cirrhosis [19]

Unlike HBV, HCV is an RNA virus that does not integrate into the host genome. Instead, host-viral protein interactions seem to be the major pathways of hepatocarcinogenesis. The proteins widely reported to be associated with HCV-mediated hepatocarcinogenesis are core, NS3 and NS5A proteins, which have all been shown to inhibit p21WAF1 tumor suppressor expression post-transcriptionally.[20-23]

Aim of the work

In Patients having HCV infection, with and without superadded HCC, we aimed at identifying HCV Genotypes Spectrum, investigate their role in hepatocarcinogenesis and to correlate HCV Genotypes and viral loads to clinical and biochemical profiles of the patients.

Patients and Methods

A prospective study including 60 patients in two groups: **Group I:** 30 patients with HCV with superadded HCC. **Group II:** 30 patients with HCV without superadded HCC (control group). Approval by Ethics Committee of Faculty of Medicine, Alexandria University was obtained.

Patients were recruited from Clinical Oncology, Hepatology and Internal Medicine departments, Alexandria University hospitals, Faculty of Medicine, University of Alexandria. Eligibility Criteria included patients 20-70 years old; any stage of HCC; and signed informed consent.

All Patients were subjected to: History taking and complete physical examination, abdominal ultrasonography, triphasic CT of the liver (done to all patients in Group I and 3 patients from Group II), laboratory and biochemical investigations: serology for HCV infection using anti-HCV IgG 3rd generation ELISA kit, complete blood picture (CBC), liver transaminases (ALT, AST and GGT), serum bilirubin and Serum albumin.

HCC (for patients in Group I) was diagnosed according to EASL criteria,[24] all had radiological evidence of HCC in either CT or US or both, and alpha feto protein (AFP) level above 400 µg/l. Seven patients had liver biopsy to confirm diagnosis. Barcelona Clinic Liver Cancer (BCLC) Group system was used for staging.[25]

Patients in both groups were subjected to HCV Viral RNA extraction using Qiagen Kit. Viral loads were determined by real time PCR. HCV genotyping was tried using Multiplex-PCR with the specific primers for different HCV genotypes; however it did not work, so we worked using INNO-LiPA II kit. Results were interpreted using INNO-LiPA charts.

Randomly selected eight samples (4 from each group) were subjected to cloning and sequencing of PCR-amplified fragments of 5' Untranslated Region (5'UTR) through the following steps:

a. Expected PCR-amplified fragments were excised from the agarose gel and purified using Qiagen Gel Extraction kit (Qiagen, Germany).

b. Cloning and subcloning into an eukaryotic vector as a step towards sequencing was done using TOPO TA Cloning® Kit for Sequencing.

c. Plasmid DNA was isolated using QIA Spin miniprep kit (Qiagen, Germany). Plasmid DNA was sequenced in both directions using BigDye Sequencing Kit and ABI 377 DNA sequencer (ABI, USA).

To compare the resulted 5'UTR sequences, pairwise and multiple DNA sequence alignment were carried out using CLUSTALW (1.82). Bootstrap neighbor-joining tree (Saitou and Nei, 1987) was generated using MEGA 2.1 (Kumar et al., 2001) from CLUSTALW alignments.

Results

Demographic data were comparable in both groups regarding age, sex and residence. Mean age was 56.2 years in group I and 57.3 years in group II. Only few patients in both groups were below 30 years (10% and 6.7% respectively). Sex distribution showed obvious male predominance. Group I included 80% males while group II included 83.3%. Majority of patients were from rural areas (63.3% in Group I and 56.7 % in group II). (Table 1).

Table 1: Demographic data.

	Group I		Group II	
	No.	%	No.	%
Age (in years)				
< 30	3	10.0	2	6.7
30 – < 40	7	23.3	10	33.3
40 – 50	14	46.7	11	36.7
60 or more	6	20.0	7	23.3
Range	28 – 67		27 – 68	
Mean	56.2		57.3	
S.D.*	13.65		11.85	
T- value	0.85			
p - value	0.34			
Sex				
Male	24	80.0	25	83.3
Female	6	20.0	5	16.7
X^2	0.11			
p	0.738			
Residence				
Rural	19	63.3	17	56.7
Urban	11	36.7	13	43.3
X^2	0.11			
p	0.738			

*Standard deviation

History of blood transfusion was comparably present in both groups (36.7% in group I and 30% in group II). On the other hand, history of schistosomiasis and clinical jaundice (>2 mg/dl) were only represented in group I (33.3% and 20 % respectively).

At time of sampling, serum AFP level was significantly higher in group I as a level of 400 µg/l or above was an inclusion criterion for this group. The mean in group I was 520.6 µg/l versus 8.69 µg/l in group II. AST, ALT, GGT and bilirubin levels were also significantly higher in group I; while serum albumin was significantly lower. (Table 2).

All patients in group I (30 cases) had high GGT, 84% had high ALT, and 56% had high AST levels.

Table 2: Laboratory findings at time of sampling.

	Group I	Group II
AFP (µg/l) Mean	520.6	8.69
p	0.0001	
AST (U/L) Mean	165.6	42.6
p	0.0001	
ALT (U/L) Mean	175.65	45.8
p	0.0001	
GGT (U/L) Mean	130.0	60.6

p	0.001	
Albumin (g/dl) Mean	3.18	4.25
p	0.048	
Total bilirubin (mg/dl) Mean	1.68	0.85
p	0.002	

Table 3 shows Triphasic CT findings in group I where 60% of patients with HCC presented with 2 lesions. The mean size of lesions was 5.6 cm. Intra-abdominal lymph nodes were evident in around 13 % of patients. Half of the patients had cirrhosis; almost third of them had hepatic fibrosis; while 16.7% had mixed cirrhosis and fibrosis.

Table 3: Triphasic CT findings in group I patients.

	No.	%
Number of lesions		
1	7	23.3
2	18	60.0
3	5	16.7
Size of lesions (in cm)		
< 2	2	6.7
2 – < 4	6	20.0
4 – < 6	15	50.0
6 or more	7	23.3
Range	2-8	
Mean	5.61	
S.D.	2.09	
Abdominal LNs involvement		
Yes	4	13.3
No	26	86.7
Co-morbid liver disease		
Fibrosis	10	33.3
Cirrhosis	15	50.0
Mixed	5	16.7

Most of HCC patients were grouped under B in Child Pugh classification (54.9%); most of them were staged as BCLC stage B (56.6 %). (Table 4).

Table 4: Child Pugh classification and BCLC staging for group I patients.

	No.	%
Child classification		
Child A	8	25.6
Child B	16	54.9
Child C	6	19.5
BCLC staging		
Stage A	3	10.0
Stage B	17	56.6
Stage C	8	26.7
Stage D	2	6.7

HCV viral loads were found to be significantly higher in HCC group, with a mean of 4.23 x 10^5 compared to a mean of 8.78 x 10^4 in the control group; taking into account that all patients did not receive systemic antiviral treatment. Moreover, viral loads quantity tended to be higher in more advanced stages of HCC. (Table 5)

Table 5: Correlation between viral loads and HCC stage in group I patients.

Viral loads	BCLC stage			
	A	B	C	D
1-9.99 x 10^4 or less	3	-	-	-
1-9.99 x 10^5	-	13	4	1
1-9.99 x 10^6 or more	-	4	4	1

Genotyping of HCV was attempted for all 60 patients using INNO-LiPA HCV II and results were interpreted using INNO-LiPA HCV II interpretation chart. All samples showed genotype 4 with no subgenotypes detected. (Figure 1).

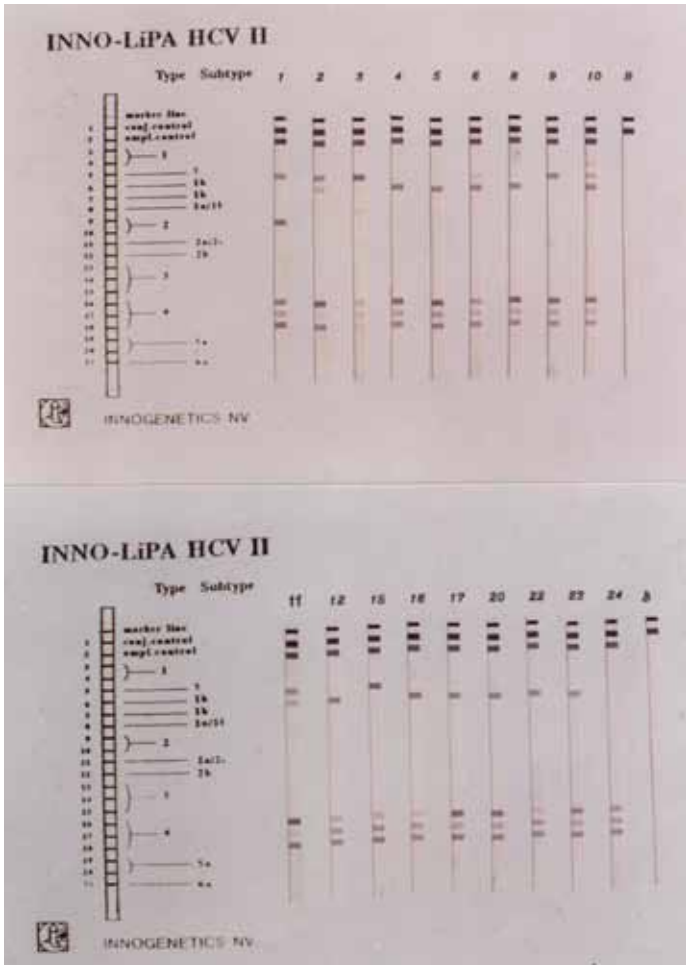

Fig 1: HCV Genotyping by INNO-LiPA HCV II showing Genotype 4 in all samples

Nucleotide sequencing of 8 HCV 5'UTR (5'Untranslated Region) random samples (4 from each group) was performed; sequences 1-4 from group I and sequences 5-8 from group II. The 8 nucleotide sequences were identified and registered on GeneBank under the following accession numbers: HCV1.sqn HCV1 HQ228205, HCV1.sqn HCV2 HQ228206, HCV1.sqn HCV3 HQ228207, HCV1.sqn HCV4 HQ228208, HCV1.sqn HCV5 HQ228209, HCV1.sqn HCV6 HQ228210, HCV1.sqn HCV7 HQ228211, HCV1.sqn HCV8 HQ228212 (***ncbi.nlm.nih.gov***).

Comparison between the 4 isolates of each group was done. Samples showing similarity less than 97% were considered as subgenotypes. Isolate HCV4 from group I as well as isolate HCV6 from group II showed similarity

with the other three isolates ranging from 87 % to 96%. This means that each sample was considerably different from the other three; perhaps denoting a new subgenotype.

Multiple sequence alignment of the 4 samples from each group was done. For group I, minor differences between samples 1, 2 and 3 were noted, while the three samples were different considerably from sample 4. Similarly, minor differences between samples 5,7 and 8 of group II were noticed, while they were different considerably from sample 6.

Phylogenetic analysis of the 4 DNA sequences of HCV 5'UTR from group I using Mega 4 program showed that the four examined 5 UTR regions were divided into two main groups having one ancestor. Group one contained samples HCV1, 2 and 3, while group 2 contained only sample HCV4. The same was observed in group II samples; group one contained samples HCV5, 8 AND 7, while group 2 contained only sample HCV6. This means that sample 4 and 6 are different considerably from the other 3 samples in each group.

To confirm the results, comparison of the 8 samples together showed that isolates 4 and 6 were different from the other 6 isolates, while showing high similarity to each other. Alignment of the 8 samples together shows that isolates 4 and 6 are different from the other 6 isolates, and show high similarity to each other.

The examined 5'UTR of the different 8 patients' samples were grouped into two main groups. Group one consisted of 6 sequences (HCV 1,2,3,5,7 and 8), while, the second group consisted of only two sequences (HCV 4 and 6). Group one was further divided into two main subgroups, subgroup one contains (HCV2, 8, 5, 7 and 1) and subgroup 2 included only one UTR (HCV3). This denotes that the two samples 4 and 6 may represent subgenotypes from genotype 4.

In conclusion; no significant pattern was characteristic to one group over the other; the 5'UTR from both groups were similar and there were no evident mutations present in group I characterizing the virus. Two sequences (sample 4 from group I and sample 6 from group II) showed similarity less than 97% with the other 6 sequences, while they show high similarity to each other and gathered in one ancestor in phylogenetic analysis. These two sequences can be considered a subgenotype from Genotype 4 that is present in Egyptian patients. Howerever, larger trials are needed to confirm these results.

Discussion

The mean age of patients in group I with HCC was 57 years, which is similar to the age incidence in Western Europe and Asia (50-60 years),[8,9] while in sub saharan Africa the mean age of incidence is 33 years[10].

The majority of cases in both groups of our study were males (80%), from rural origin (60%). This high incidence of HCV and HCC in males from rural areas may be attributed to schistosomiasis treatment given by I.V. injection (tarter emetic) that was used for a considerable period of time in many rural areas of Egypt when the use of disposable syringes was unknown.

Bruno et al. [26] found that old age and male sex are independent risk factors for the development of HCC in anti-HCV positive cirrhotic patients. Tsai et al.[27] found that male cirrhotic patients developed HCC more frequently than did female cirrhotic patients, but the difference was not significant, and the frequency of HCC development was higher in those older than 50 years. Their results agree with ours.

History of blood transfusion was present in 36% of patients in group I and 27% in group II. This showed that blood transfusion may be a leading cause of transmission of HCV especially in the era before efficient routine laboratory screening of blood.

Comparable to our results, Kiyosawa et al. showed that 94.4% of patients with HCC in their series (54 cases) were positive for anti-HCV and in 42% of patients (21 cases) history of blood transfusion was documented. [28] On the other hand, Darwish et al.[29] found no association between blood transfusion and HCV seroprevalence.

Thirty two percent of patients in group I had past history of schistosomiasis with anti-schistosomal parentral therapy. This agrees with results by Darwish et al.[29] which showed that schistosomiasis was significantly associated with HCV infection. Moreover, Abdel Wahab et al. found that 54.1% of patients with HCC were positive for anti-HCV; of them 32.9% had schistosomal infection.[30]

In the present study, laboratory investigations were used to assess the progression of liver disease. There was significant difference between the two groups regarding liver transaminases (ALT and AST), Gamma-glutamyl transferase (GGT), and alpha fetoprotein (AFP) levels. All patients in group I (30 cases) had high GGT, 84% had high ALT, and 56% had high AST levels. This showed that GGT may be the most sensitive test for assessment of deterioration in liver status and perhaps the development of HCC in patients with HCV.

Tarao et al.[31] demonstrated the association between high serum ALT level and more rapid development of HCC in patients with hepatitis C associated cirrhosis as 71.4% of patients in the high ALT group compared to 25% of patients in low ALT group developed HCC over a follow up period of more than 5 years. [31]

BCLC staging system was used to stage patients with HCC in group I. Most of the cases (56.6 %) were Stage B, 26.7% of cases stage C, 10% stage A and 6.7% stage D. Our results are comparable to Ikeda et al. multivariate analysis of HCC patients where most of the patients (51%) were in stage B.[32] This shows that early (stage A) HCC is rarely detected (due to lack of symptoms) as the case with late stages (as the symptoms must have appeared).

The recognition of specific genotypes of HCV is useful in studying the epidemiology, clinical manifestations and pathogenesis of disease. Sequential studies for the detection of the emerging of HCV mutants during the course of the disease may be necessary to understand the role of genotype variants in disease progression.[33]

Data regarding the role of viral genotypes in predicting long-term sequelae are contradictory and incomplete. Most efforts have focused on a comparison between those infected with genotype 1 and genotype 2 [34,35] or genotype 1b and 1a,[36] finding the former to be associated with more severe disease than the latter. Despite the fact that HCV genotype 4 is sometimes found in Europe, this genotype is the predominant one in the Middle East and Africa, excluding the republic of South Africa. [37-39]

In the present study we used INNO LiPA HCV II for genotyping of patients blood samples from all patients in both groups. It allows the discrimination of six HCV genotypes from 1 to 6 and 17 subgenotypes; 1a, 1b, 2a, 2b, 2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4f, 4h, 5a, 6a. Our results revealed that all patients in both groups (100 %) had genotype 4 HCV with no recognized subgenotypes.

Yates et al. found that 96% (74 of 77) of the Egyptian HCV strains among HCC cases were genotype 4.[40] This is consistent with report by McOmish et al.,[37] who found that genotype 4 is the predominant in Egypt.

Other authors reported the prevalence of genotype 4a in Egypt; Halim et al. (9%),[41] and Angelico et al.(12%)[42] In the former study the genotype was identified by type specific primers PCR technique, while in the latter study they used the first version of INNO-LiPA HCV, which is a line probe assay that discriminates five HCV genotypes (1-5) and 8 subgenotypes; 1a, 1b, 2a, 2b, 3a, 3b, 4a and 5a.

In our study, viral loads of HCV which indicate the activity of viral replication were significantly higher in group I (HCC patients) and increased with the stage of the disease. The loads in more advanced stages were higher

than in early stages and considerably higher than loads in control group which indicates that the virus gets more aggressive as the condition deteriorates. These results agreed with Kato et al. [43] study where the Viral loads of patients with late stages of HCC (C and D) were significantly higher than earlier stages (A and B).

Importance of Viral loads comes from being a variable quantity during disease progression; it can be the focus of larger trials testing its use as a predictor for malignant transformation in patients with HCV.

Sequence Analysis of 5-untranslated region of HCV (5UTR) was done to 8 RNA PCR extracts (4 samples from each group) using BigDye Sequencing Kit and ABI 377 DNA sequencer (ABI, USA), to check for a specific pattern of the 5UTR of HCV (genotype 4) in the HCC patients that is different from the control group. Our aim was to check if there is a detectable mutation in the HCC group HCV that may be a risk factor for acceleration of development of HCC.

Phylogenetic analysis and sequence alignments of the 8 samples were plotted based on Nucleotides and deduced aminoacid sequences using CLUSTALW (1.82).The result of the comparison of the 8 DNA sequences from both groups showed that the 5'UTR were similar and there was no mutation present in group I characterizing the virus, different from group II.

However, two sequences (sample 4 from group I and sample 6 from group II) showed similarity less than 97% with the other 6 sequences while showing similarity of 97% or more to each other; they were gathered in one ancestor. This indicates that these two sequences may be considered as a new subgenotype from Genotype 4 that is present in Egyptian patients. Confirmation of such results needs more research with larger number of patients

Conclusions

Genotype 4 is the predominant genotype of HCV in the studied patients. Viral loads of HCV may be used to monitor development of HCC. Subtypes of HCV genotype 4 identified need further work on larger scale. Serum GGT rather than AST and ALT can be used as a sensitive biochemical marker of hepatic dysfunction in patients with HCC. Further studies with large number of patients are needed to confirm these findings.

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