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Predictive Markers for Hepatocellular Carcinoma Development in Patients with Chronic Hepatitis C Virus Genotype4a

Ebtsam R. Zaher*♦, Abeer A. Ghazal, Wael A. Ellabban***, Mona El-Deeb****, Lamyaa Al-Ghaleed*****

*Radiation Sciences Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
**Microbiology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
***Queen's Medical Center, Durham, UK
****Clinical Pathology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
*****Internal Medicine Department, Medical Research Institute, Alexandria University, Alexandria, Egypt

Abstract

Background: Egypt has the highest prevalence of hepatitis C virus worldwide. Monitoring hepatitis C-infected patients for hepatocellular carcinoma development is an important clinical issue to diagnose these patients during the potentially curable early-stage of disease. This study aims to evaluate the role of N-terminal procollagen III, matrix metalloproteinase-2, tissue inhibitor of matrix metalloproteinase-1, alpha-fetoprotein, and conventional liver function tests as predictors of hepatocellular carcinoma development upon long-term follow-up of non-responding hepatitis C virus patients.

Methods: The study included 850 treatment-naïve hepatitis C virus genotype 4a adult patients; after treatment, 360 achieved sustained viral response while 490 did not. Non-responding patients had a 5-year rate for hepatocarcinogenesis of 8.4% and a 10-year rate of 27.5%. N-terminal procollagen III, matrix metalloproteinase-2, tissue inhibitor of matrix metalloproteinase-1, alpha-fetoprotein, and conventional liver function tests were evaluated in all patients before and after treatment, and after hepatocellular carcinoma development. The study also included a group of 50 hepatocellular carcinoma patients who were negative for hepatitis C and hepatitis B viruses, and a group of 50 healthy subjects as controls.

Results: The non-responders had significantly higher age, stage, grade, viral load, alanine aminotransferase, and aspartate aminotransferase than responders. Also N-terminal procollagen III, matrix metalloproteinase-2, tissue inhibitor of matrix metalloproteinase-1, and alpha-fetoprotein were significantly higher in non-responders; after treatment they decreased in responders. In non-responders they remained higher than the control. The most significant risk factors for hepatocellular carcinoma development in non-responding hepatitis C virus patients were male gender and increased age, stage, grade, aspartate aminotransferase, N-terminal procollagen III, and tissue inhibitor of matrix metalloproteinase-1. Patients with viral-hepatocellular carcinoma were of significantly lower age, higher grade, stage, γ-glutamyl-transferase, N-terminal procollagen III, and matrix metalloproteinase-2 than non-viral hepatocellular carcinoma patients. Percent positive N-terminal procollagen III, tissue inhibitor of matrix metalloproteinase-1, and alpha-fetoprotein were significantly higher in viral hepatocellular carcinoma patients.

Conclusion: Data suggest that high N-terminal procollagen III and tissue inhibitor of matrix metalloproteinase-1levels after treatment might be particularly important as markers of hepatitis C virus-non-responding patients who are at higher risk of developing hepatocellular carcinoma, especially in older males with high stage and grade liver disease. However, studies of larger scale are needed to verify this suggestion.

Keywords: HCV, Hepatocellular carcinoma (HCC), PIIINP, MMP-2, TIMP-1, AFP, Predictive marker

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♦Corresponding Author:
Ebtsam R. Zaher, PhD
Medical Research Institute, Alexandria University
165 Eh-Horreya Avenue, El-Hadara, Alexandria, Egypt
Tel: 002-01125808505
002-03-4283543
Email: ebtsam.zaher@alexu.edu.eg

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Introduction

Worldwide, hepatocellular carcinoma (HCC) is the fifth most diagnosed cancer in men and second most frequent cause of cancer death. In women, it is the seventh most diagnosed cancer and the sixth cause of cancer death.[1] Throughout the past 2 decades, HCC global incidence has increased.[2,3] This increase is partly due to hepatitis C virus (HCV) infection, which is considered an established risk factor for HCC.[4]

Hepatitis C virus infection is a crucial health problem with approximately 130 million infected people worldwide, leaving approximately 3% of the world's population chronically infected. The future burden is expected to increase at least 3-fold by 2020.[4] In Egypt, the reported prevalence of HCV is the highest worldwide at an average of 13.8%, with a range from 9% to 28%. The predominant subtype is HCV genotype 4 (HCV-4), which represents more than 80% of HCV infections. The major clinical consequences of chronic HCV infection involve liver cirrhosis along with prospective complications of end-stage liver disease or primary liver cancer, but the definite mechanism is unknown.[5,6]

Monitoring HCV infected patients for HCC is an important clinical issue in order to diagnose patients during the potentially curable, early-stage of this disease. Most relevant is the regular ultrasound (US) check-up and serum alpha-fetoprotein (AFP) levels. Although both are relatively efficient for large tumors, their specificity is low, especially against a background of chronic hepatitis.[7] Many proteins are suspected to participate in the process of HCC carcinogenesis and are possible candidates for surveillance markers of HCC development in HCV patients. Matrix metalloproteinases, together with their inhibitors, are among these candidates.

The aim of the current study is to evaluate the role of N-terminal procollagen III (PIIINP), matrix metalloproteinase-2 (MMP-2), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), AFP, and conventional liver function tests as prognostic markers of HCV treatment and predictors of HCC development in long-term follow-up of HCV patients.

Subjects and Methods

From March 2006 to July 2008, we enrolled a cohort of 850 treatment-naïve chronic HCV-4 adult patients in this study. The local Ethics Committee approved the study and all enrolled patients provided an informed written consent.

Before treatment, each patient underwent a liver biopsy and determination of HCV genotype. All patients had detectable serum HCV RNA and received antiviral treatment with interferon (IFN) combined with Ribavirin for 24 weeks. At week 24, subjects with persistent viremia were enrolled as "non-responders". Subjects with undetectable HCV RNA were enrolled as "responders" and completed a 48-week course of combination antiviral treatment. Patients in the responder group who encountered an on-treatment or post-treatment virological relapse were also enrolled as "non-responders". During the study period, we evaluated the HCV patients by physical examination; hematological and liver biochemistry tests measured at weeks 24 and 48; and levels of HCV RNA assessed at weeks 4, 12, 24, 48, and 72. Non-responding patients were monitored for a mean period of 112 months (90-136 months) by US and AFP levels at 6-month intervals to check for the presence of HCC. Cases suspected of HCC were referred for clinical evaluation and treatment planning. The HCC diagnosis was based on clinical criteria of progressive hepatomegaly, increasing pattern of AFP, US and/or CT reports of lesions, and pathologic confirmation of disease.

Exclusion criteria comprised the following: any causes of liver diseases other than HCV (autoimmune, hepatitis B virus, HIV, HAV, hereditary); decompensated liver cirrhosis; autoimmune, thyroid or hematological diseases; pregnancy or breast feeding; poorly controlled Diabetes mellitus; renal failure; uncontrolled hypertension; ischemic heart disease in the last 6 months or congestive heart failure; CNS trauma; stroke which required medication; major psychiatric condition; malignancy; or previous treatment with IFN-α.

Blood samples were collected from all 850 patients at presentation, after 48 weeks of treatment,

Table 1. Characteristics of control and patient groups and comparison of responder to non-responder patients.

Parameter		Control	HCV patients (n=850)		*P*-value
			Responders	Non-responders	
Number (%)		50	360 (42.4)	490 (57.6)	
Males (%)		36 (72.0)	233 (64.7)	369 (75.3)	0.073
Females (%)		14 (28.0)	127 (35.3)	121 (24.7)	
Age (years)		45.9±13.2	41.9±10.4	48.7±9.8	0.021
Stage (Ishak)		-	2.4±1.4	4.4±1.8	0.000
Grade (CPT)	I	-	183 (50.8%)	145 (29.6%)	
	II		166 (46.1%)	196 (40.0%)	0.010
	III		11 (3.1%)	149 (30.4%)	

P-value: Significant when comparing responders to non-responders.; CPT: Child-Turcotte-Pugh score

and after the onset of HCC. In addition, we obtained blood samples from 50 HCC patients who were negative for both HCV and HBV; and from 50 normal healthy volunteers matched for age and sex as the patient group to set the cut-off values for the assayed parameters. Serum was immediately isolated, aliquoted, and stored at -80°C.

Viral serological markers

HCV antibodies were detected with an ELISA Murex anti-HCV (version III). Hepatitis B virus surface antigen (HBsAg) was detected with an ELISA kit (Abbott Murex Diagnostic Division).

Detection of hepatitis C virus (HCV)-RNA viral load by real-time PCR (RT-PCR)

We used 140 µL of serum for HCV RNA extraction according to the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) instructions, followed by RNA amplification using the TaqMan Universal PCR Master Mix (AB Applied Biosystems) with a single pair of PCR primers located in the 5`UTR in a 25 ml reaction volume. The reaction mixture was incubated at 48°C for 30 min for reverse transcription followed by AmpliTaq activation at 95°C for 10 min, then 45 cycles of two-step PCR amplification that included denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min with end point fluorescence detection.

Determination of hepatitis C virus (HCV) genotypes

We used the VERSANT HCV Genotype 2.0 Assay (LiPA 2.0; Innogenetics, Ghent, Belgium distributed by Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) to identify the HCV genotypes. The 5'UTR region and the core region of HCV were amplified by real-time PCR (RT-PCR) with biotinylated primers. The labeled amplicon was allowed to hybridize and mounted on a strip. After stringent washing, streptavidin labeled with alkaline phosphatase was used to trace the hybridized products. BCIP/NBT chromogen substrate formed a purple/brown precipitate that resulted in a visible banding pattern on the strip. The probe reactivity patterns were interpreted using the chart provided by the manufacturer.

Liver function tests

Biochemical tests were performed on anautoanalyzer, the Konelab 30i system, using reconstituted freeze-dried forms of the multi-analyte calibrators. Total and direct serum bilirubin, albumin (Alb), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), and alkaline phosphatase (ALP) levels were measured in all samples.

Serum biochemical assays

Alpha-fetoprotein was evaluated in all samples by an Immunoradiometric Assay (IRMA) Kit (BioSource Europe S.A., Nivelles, Belgium) and monoclonal antibody-coated tubes according to the manufacturer's instructions. This kit had a lower detection limit of 0.5 IU/ml. We assayed PIIINP using a quantitative radioimmunoassay (RIA) kit, UniQ PIIINP RIA (Orion Diagnostica, Espoo, Finland), which measured the intact N-terminal

Table 2. Biochemical parameters in responders and non-responders compared to controls, compared to each other, and the effect of treatment (significant at $P \leq 0.05$).

Parameter	Controls	Hepatitis C virus (HCV) patients					
		Responders		Non-responders			
		Mean±SD	*P*1/*P*2	Mean±SD	*P*1/*P*2	*P*3	*P*4
Viral Load (×10^6 IU/ml)	-						
Before treatment		1.6±1.5		2.5±2.4		0.032	0.025
After treatment		ND		1.3± 1.9		0.001	
			*P*2=0.001		*P*2=0.037		
ALT (U/l)	19.9±5.8						
Before treatment		60.2±30.7	*P*1=0.000	90.3±36.1	*P*1=0.000	0.039	0.115
After treatment		36.5±27.0	*P*1=0.002	56.5±34.8	*P*1=0.000	0.000	
			*P*2=0.011		*P*2=0.034		
AST (U/l)	21.1±4.7						
Before treatment		58.8±39.8	*P*1=0.000	85.8±41.3	*P*1=0.000	0.017	0.013
After treatment		34.6±31.7	*P*1=0.010	67.4±37.7	*P*1=0.000	0.008	
			*P*2=0.037		*P*2=0.087		
ALP (U/l)	40.1±12.9						
Before treatment		86.7±51.5	*P*1=0.000	85.4±54.8	*P*1=0.000	0.164	0.075
After treatment		48.7±27.9	*P*1=0.078	73.7±41.2	*P*1=0.000	0.004	
			*P*2=0.002		*P*2=0.338		
GGT (U/l)	25.4±13.6						
Before treatment		45.7±22.0	*P*1=0.000	52.9±21.3	*P*1=0.000	0.180	0.000
After treatment		26.6±14.1	*P*1=0.449	48.3±22.7	*P*1=0.000	0.021	
			*P*2=0.000		*P*2=0.618		
Total bilirubin (mg/dl)	0.55±0.54						
Before treatment		1.2±0.85	*P*1=0.000	1.9±1.33	*P*1=0.000	0.033	0.047
After treatment		0.8±0.67	*P*1=0.020	1.2±0.98	*P*1=0.000	0.045	
			*P*2=0.010		*P*2=0.091		
Alb (g/dl)		4.4±0.7					
Before treatment		4.1±1.2	*P*1=0.317	3.7±1.5	*P*1=0.210	0.741	0.764
After treatment		4.2±0.9	*P*1=0.257	3.9±1.4	*P*1=0.336	0.518	
			*P*2=0.451		*P*2=0.689		
Platelets (×10^9/l)	187.6±164.7						
Before treatment		167.6±164.5	*P*1=0.441	131.1±133.0	*P*1=0.043	0.037	0.491
After treatment		175.1±149.8	*P*1=0.555	154.9±135.7	*P*1=0.067	0.072	
			*P*2=0.629		*P*2=0.15		
PIIINP (µg/l)	3.1±1.2						
Before treatment		16.7±4.9•	*P*1=0.000	24.5±7.3•	*P*1=0.000	0.043	0.481
After treatment		7.8±4.1•*	*P*1=0.000	16.7±5.9•*	*P*1=0.000	0.012	
			*P*2=0.000		*P*2=0.000		
MMP-2 (ng/ml)	204±29.6						
Before treatment		610.0±198.4•	*P*1=0.000	832.0±201.4•	*P*1=0.000	0.024	0.006
After treatment		356±159.7•*	*P*1=0.027	679.5±207.0•	*P*1=0.017	0.000	
			*P*2=0.000		*P*2=0.075		
TIMP-1 (ng/ml)	267.3±79.4						
Before treatment		914.8±410•	*P*1=0.000	1076±564•	*P*1=0.000	0.559	0.000
After treatment		685±359•*	*P*1=0.000	738±435•	*P*1=0.085	0.760	
			*P*2=0.021		*P*2=0.174		
AFP (IU/ml)	2.7±2.1						
Before treatment		8.8±6.5•	*P*1=0.000	25.2±12.0•	*P*1=0.000	0.005	0.082
After treatment		4.3±4.0•*	*P*1=0.019	6.2±5.2•*	*P*1=0.000	0.095	
			*P*2=0.038		*P*2=0.000		

•:P1 significance compared to the control.; *: P2 significance when comparing before versus after treatment.; P3 significance when comparing responders versus non-responders.; P4 significance of the effect of treatment within responders versus non-responders.; PIIINP: N-terminal procollagen III; MMP-2: Matrix metalloproteinase-2; TIMP-1: Tissue inhibitor of matrix metalloproteinase-1; AFP: Alpha-fetoprotein; Alb: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase

Table 3. Logistic regression analysis of hepatocellular carcinoma (HCC) risk factors among hepatitis C virus (HCV) non-responding patients.

Parameter		Relative risk (RR)	*P*-value	95% CI®
Gender	Female	1		
	Male	5.6	0.012	3.0-12.3
Age (years)	>30⊙	1		
	30-50	5.1	0.001	2.5-15.6
	>50	15.4	0.001	6.5-50.5
Stage (Ishak score)	<3⊙	1		
	≥3	2.6	0.027	1.7-3.9
Grade (CPT)	I⊙	1		
	II and III	3.2	0.001	2.1-4.9
Viral load	<1×106 IU/ml⊙	1		
	≥1×106 IU/ml	1.3	0.248	0.7-2.4
AST	<40 U/ml⊙	1.6		
	≥40 U/ml	1	0.037	0.8-1.17
AFP	<10 IU/ml⊙	1		
	≥10 IU/ml	1.4	0.076	0.7-2.7
PIIINP	<10 µg/l⊙	1		
	≥10 µg/l	4.9	0.000	3.3-7.5
MMP-2	<500 ng/ml⊙	1		
	≥500 ng/ml	1.1	0.472	0.6-3.8
TIMP-1	<400 ng/ml⊙	1		
	≥400 ng/ml	1.7	0.047	0.9-3.4

⊙: Reference category; ®: 95% confidence interval (CI); PIIINP: N-terminal procollagen III; MMP-2: Matrix metalloproteinase-2; TIMP-1: Tissue inhibitor of matrix metalloproteinase-1; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein

propeptide of type III procollagen. The kit had a measurement range of 1-50 µg/l with a detection limit of 0.3 µg/l.

Matrix metalloproteinase-2 was measured by a Quantikine® Human MMP-2 Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN, USA) for the quantitative determination of the MMP-2 concentration in serum according to a quantitative sandwich enzyme immunoassay technique. The minimum detectable limit of the kit is 0.05 ng of MMP-2/ml. Tissue inhibitor of matrix metalloproteinase-1 was measured by the Quantikine® Human TIMP-1 Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN, USA) using a solid phase ELISA for the quantitative determination of TIMP-1 concentration in serum. The minimum detectable dose of TIMP-1 is typically less than 0.08 ng/ml.

For all measurements, samples with concentrations higher than the largest calibrated concentration were re-assayed at a 1:10 dilution.

Statistical analysis

Statistical analysis was executed using the Statistical Package for the Social Sciences (SPSS) software version 22.0 (SPSS Inc., Chicago, IL, USA). For description of variables, we used frequency and percent for qualitative variables, and the mean and standard deviation for quantitative variables. The Mann-Whitney test was used to study statistical significance in the median of

Table 4. Characteristics of patients with hepatitis C virus associated hepatocellular carcinoma (HCV-associated-HCC) and non-viral HCC.

Parameter	Viral-HCC (n=41)	Non-viral-HCC (n=50)	*P*-value
Age (years)	49.9±7.6	60.7±8.0	0.000
Tumor size (cm)	6.3±4.2	5.6±3.3	0.201
Hist. grade (n, %)			
I	4 (9.8)	8 (16)	0.036
II	20 (48.8)	30 (60)	
III	17 (41.4)	12 (24)	
CPT class (n, %)			
I	16 (39)	22 (44)	0.015
II	14 (34.2)	20 (40)	
III	11 (26.8)	8 (16)	
Hepatomegaly (n, %)	27 (65.9)	22 (44.0)	0.029
Echogenicity Normal	7 (17.1)	18 (36)	0.013
Hypoechoic	34 (82.9)	38 (66)	
Cirrhosis (n, %)	39 (95.1)	44 (88)	0.284
Portal vein thrombosis (n, %)	24 (58.5)	34 (68)	0.091
Vascular invasion (n, %)	19 (46.3)	28 (56)	0.182
ALT (U/l)	73.5±53.6	65.4±25.8	0.095
AST (U/l)	96.7±52.4	103.6±28.7	0.113
GGT (U/l)	209.3±159.4	98.4±31.5	0.031

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase

quantitative variables between groups. The relative risk (RR) with its 95% confidence interval (CI) was used to assess the risk of HCC among those with positive biochemical parameters relative to those whose biochemical parameters were negative. Relative risk of value 1 indicated no risk, whereas a RR of more than 1 indicated an increased risk.

We constructed receiver operating characteristic (ROC) curves using levels of post-treatment PIIINP, MMP-2, TIMP-1, AFP, and the controls to select a cutoff value for each parameter. Accuracy of a biochemical parameter was indicated by its area under the curve (AUC). For the AUC to be significant, its 95% lower CI should be above 0.50. All tests were 2-sided and the *P*-value was set at 0.05. All of the parameters showed significant asymptotic significance, which indicated their potential as predictors of HCC. N-terminal procollagen III showed the highest AUC (0.84) and an optimal cutoff of 10µg/l. Tissue inhibitor of matrix metalloproteinase-1 had an AUC of 0.79 and optimum cutoff of 400 ng/ml; MMP-2 had an AUC of 0.78 and optimum cutoff of 500 ng/ml; and AFP had an AUC of 0.72 with an optimum cutoff of 10 IU/ml.

We established another ROC curve to differentiate between HCV-associated HCC patients and non-viral HCC patients. The new cutoffs were: PIIINP (20 µg/l), TIMP-1 (400 ng/ml), MMP-2 (500 ng/ml), and AFP (200 IU/ml).

Results

The study included 950 subjects allocated into 3 groups. The first group included 850 HCV patients referred for IFN-based therapy and observed for 48 weeks to evaluate response to viral treatment, followed by long-term follow-up of non-responders for hepatocarcinogenesis. Of patients, 360 (42.4%) achieved sustained viral response (SVR) and were classified as responders. There were 490 (57.6%) patients who still had high blood viral titers after treatment and were classified as non-responders. Table 1 lists the characteristics of the control and patient groups. The male/female ratio in the non-responder group was higher than in the responder group, but was not statistically significant. The non-responding patients had significantly higher age (*P*=0.021),

Table 5. Patients' percent with PIIINP, MMP-2, TIMP-1 and AFP < or ≥ their respective cutoff values in patients with hepatitis C virus associated hepatocellular carcinoma (HCV-associated-HCC) and non-viral HCC at presentation.

Parameter	HCC-HCV	HCC	*P*1 *P*2
PIIINP (µg/l)	41.2±25.6	26.8±14.4	0.014
% patients <20	64.9	35.1	0.032
% patients ≥20	36.0	64.0	
MMP-2 (ng/ml)	762.6±332.0	628±264.6	
% patients <500	42.8	57.2	0.046
% patients ≥500	55.7	44.3	0.182
TIMP-1 (ng/ml)	960.2±487.3	814.8±442.0	0.138
% patients <400	58.8	41.2	0.012
% patients ≥400	34.2	65.8	
AFP (IU/ml)	638.4±465.0	518.5±326.7	0.086
% patients <200	67.6	23.4	0.002
% patients ≥200	30.9	69.1	

*P*1 significance when comparing mean values in HCC-HCV versus HCC.; *P*2 significance when comparing percent </≥ cutoff in HCC-HCV versus HCC.; PIIINP: N-terminal procollagen III; MMP-2: Matrix metalloproteinase-2; TIMP-1: Tissue inhibitor of matrix metalloproteinase-1; AFP: Alpha-fetoprotein

stage (*P*=0.000), and grade (*P*=0.010) compared to responders.

Table 2 lists the serum biochemical parameters in control subjects and responder and non-responder patients (mean±SD). The level of significance when comparing the responders and non-responders to controls and with each other, in addition to the trend for response to treatment in both are also shown. There was a significantly higher viral load in non-responders compared to responders. The trend of response to treatment significantly differed in both. The ALT, AST, ALP, GGT, and bilirubin levels were significantly higher than controls in both responder and non-responders before treatment. Although the platelet counts in responders did not differ from the controls, it was significantly lower in non-responders. Albumin was not significantly different in responders or non-responders compared to controls.

After treatment, serum ALT, AST and bilirubin significantly decreased but remained higher than the control levels, and higher in non-responders than responders. Serum ALP and GGT decreased in responders to normal levels after treatment but remained unchanged in non-responders. Platelet count and ALB did not show any variation in response to treatment either in responders or non-responders. There was no significant difference between the two groups.

The change in response to treatment in responders significantly differed from non-responders in viral load, AST, and GGT. However, the change in ALT, ALP, bilirubin, ALB, HB, and platelet count in response to treatment was not different between the two groups.

There were significantly higher mean levels of PIIINP, MMP-2, TIMP-1, and AFP compared to controls before and after treatment. Although most decreased after treatment, they remained significantly higher than the control levels. Non-responders had significantly greater mean PIIINP levels before (24.5 µg/l) and after (18.2 µg/l) treatment compared to responders whose mean PIIINP levels were 16.7 µg/l before treatment and 7.8 µg/l after treatment. Alpha-fetoprotein showed a similar pattern. There were significantly higher mean AFP levels in non-responders (8.8 IU/ml) compared to responders (25.2 IU/ml) before treatment. The AFP levels significantly decreased after treatment in both non-responders (4.3 IU/ml) and responders (6.2 IU/ml).

Mean MMP-2 levels significantly decreased after treatment in responders, from 610 ng/ml to

356 ng/ml. Tissue inhibitor of matrix metalloproteinase-1 levels also significantly decreased after treatment in responders, from 914 ng/ml to 685 ng/ml. There was a non-significant decrease in non-responders from 832 ng/ml before treatment to 679.5 ng/ml after treatment for MMP-2 and from 1076 ng/ml before treatment to 738 ng/ml after treatment for TIMP-1. Although MMP-2 levels in responders were significantly different from non-responders, both before and after treatment, TIMP-1 levels did not significantly differ in responders or non-responders either before or after treatment. The change due to treatment was significantly different in responders compared to non-responders in MMP-2 and TIMP-1, but not in PIIINP or AFP.

The 5-year rate of hepatocarcinogenesis in non-responding HCV patients was 8.4%, or 41cases of HCC from 490 non-responders. These patients consisted of 36 males and 4 females with a mean age of 49.9 years. The 10-year rate of HCC development was much higher (27.5%).

We applied the logistic regression model to all variables which were statistically significant by univariate analysis as predictors of risk for HCC development in HCV non-responding patients. Significant predictors in the regression model accounted for 72.8% of the HCC outcome. According to the Hosmer-Leme show test, the observed data did not significantly differ from predicted values in the models. Thus, the model accurately fit the observed data (x2=0.352, *P*=1).

Table 3 lists the contributing factors to HCC development as predicted from the regression model, their RR, significance, and 95% CI. The most noticeable of these variables were male gender (*P*=0.012) with an RR of 5.6, older age with an RR of 5.1 in 30-50 year-old patients (*P*=0.001) and 15.4 in patients older than 50 years (*P*=0.001). Higher stage (*P*=0.027), grade (*P*=0.001), and AST≥40U/ml (*P*=0.037) in HCV patients were also significant factors that contributed to HCC development. Of the studied parameters, PIIINP≥10 µg/l was a significant risk factor (*P*=0.000) with an RR of 4.9, as well as TIMP-1≥400 ng/ml (*P*=0.047) with an RR of 1.7. All other parameters–viral load (*P*=0.248), AFP≥10 IU/ml(*P*=0.076), and MMP-2≥500 ng/ml (*P*=0.472) were not of statistical significance as risk factors for hepatocarcinogenesis.

Patients with HCC based upon HCV infection were compared to patients with HCC without viral infection. Table 4 represents the characterization of each group. Patients with HCV-associated-HCC had significantly lower mean age (*P*=0.000), higher histological grade (*P*=0.036), and CPT class (*P*=0.015) compared to non-viral-HCC patients. Viral-HCC patients also had a higher prevalence of hepatomegaly (65.9%) compared to non-viral HCC patients (44%, *P*=0.029), hypoechoic echogenicity (82.9% versus 66%, *P*=0.013), and significantly higher levels of GGT (*P*=0.031). However, tumor size (*P*=0.201), incidence of cirrhosis (*P*=0.284), portal vein thrombosis (*P*=0.091), vascular invasion (*P*=0.182), and levels of ALT (*P*=0.095) and AST (*P*=0.113) were not significantly different in the two HCC patient groups.

Table 5 lists the mean levels and the percent below and above the cut off for PIIINP, MMP-2, TIMP-1, and AFP in viral-and non-viral-HCC patients. There was a significantly higher mean level for PIIINP in viral-HCC than non-viral-HCC (*P*=0.014). This result was above 20 µg/l in a significantly higher proportion of viral-HCC than non-viral-HCC patients (*P*=0.032) with an odds ratio of 4.9. Although the MMP-2 mean level was significantly higher in viral-HCC patients compared to non-viral-HCC (*P*=0.046) patients, the percent of cases above 500 ng/ml did not significantly differ in the two groups (*P*=0.182). Tissue inhibitor of matrix metalloproteinase-1 (*P*=0.138) and AFP (*P*=0.086) mean levels were not significantly different in viral-HCC and non-viral-HCC, but the percent of cases above 400 ng/ml for TIMP-1 or 200 IU/ml for AFP were significantly higher in viral-HCC (*P*=0.012) than non-viral-HCC patients (*P*=0.002). The odd ratios were 1.8 for TIMP-1≥400 ng/ml and 4.3 for AFP≥200 IU/ml.

Discussion

Egypt has a large prevalence of HCV in addition to high morbidity and mortality from

chronic liver disease, cirrhosis, and HCC. The Egyptian Demographic Health Survey (EDHS) examined the presence of HCV antibody in representative specimens from urban and rural populations of all 27 Egyptian governorates. They found an overall prevalence for positive HCV antibody of 14.7%.[8] The HCV subtypes found in Egypt are strongly homogenous, mostly 4a, which suggests an epidemic spread of HCV. The high prevalence of HCV in Egypt can be explained on basis of the former practice of parenteral therapy for schistosomiasis. The large reserve of chronic HCV infection founded during these campaigns may still be responsible for the high prevalence of HCV morbidity in Egypt,[9] though the antischistosomal campaigns concluded in the early 1980s. Hence, the present state of HCV in Egypt might be the consequence of mass antischistosomal therapy in addition to new infections after that era since HCV comprises more than 30% of the annual reported acute hepatitis cases. Blood transfusion has been a frequent route for HCV-4 transmission in Egypt until 1993, after which blood and blood products were screened for HCV.[10]

The HCV strain is one of the main independent factors that influences the effect of antiviral therapy. Genotypes 1, 2, and 3 are common throughout the U.S.A. and Europe, and have become the focus of much interest and research. The management and clinical presentation of infections that emanate from these viral genotypes has advanced rapidly. In contrast, genotype 4 represents more than 80% of HCV infections in the Middle East and Africa. Although HCV-4 is the cause of approximately 20% of chronic HCV cases worldwide, it has not been the subject of widespread research. Consequently, the features and management strategies for patients infected with genotype 4 are not as advanced as those for genotypes 1,2, and 3.[11]

Studies have reported higher grading and/or staging scores of fibrosis in patients with chronic HCV-4 compared to HCV genotypes 1, 2 and 3,[12] which may underlie the worse prognosis and higher prevalence of complications. Data from the National Cancer Registry of Egypt, the National Cancer Institute, and the Middle East Cancer Consortium show a close association between HCC and HCV-4. Moreover, the distribution of HCC in Egypt closely parallels that of HCV-4.[11]

Hepatitis C virus treatment responses are determined by an altered virological parameter rather than a clinical end-point. End-of-treatment response is defined by undetectable virus at the end of either a 24-week or 48-week course of therapy, which does not precisely predict an SVR but is mandatory for SVR.[13] The HCV patients are usually treated by the combination of pegylated interferone plus weight-based ribavirin administered for 48 weeks, which appears to be the optimal regimen with reported SVR rates of 26.7%-82% in HCV-4.[14,15]

The present study included 850 HCV patients who referred for 48 weeks of combined IFN-based therapy. Of these, 42.4% achieved SVR and 57.6% still had high blood viral titers. These rates were within the lower range reported for HCV-4 treatment and agreed with a report by Alfaleh et al.[15] although others reported much higher SVR rates.[16]

Although female sex was reported as a favorable factor for HCV treatment outcome,[17] in the current study, we observed no significant difference in the male/female ratio between responders and non-responders. Non-responders had significantly higher age, stage, and grade. Laboratory tests for routine evaluation of HCV patients included a panel of liver tests (Alb, total bilirubin, AST, ALT, and ALP), prothrombin time, and CBC. Compared to responders, non-responders had significantly higher viral load, ALT and AST, along with significantly lower platelet counts. This finding agreed with most published data.[15,16,18] Treatment affected most of the measured parameters, although the effect sometimes varied in responders and non-responders. The trend of change in AST and GGT in response to treatment significantly differed in these groups, which could mean that AST and GGT might be better indicators of the treatment impact.

Responding patients also had significantly lower mean levels of PIIINP, MMP-2, TIMP-1, and AFP. Mean levels of PIIINP and AFP significantly diminished after treatment in responders and non-responders. However, mean levels of MMP-2 and TIMP-1 significantly decreased after treatment in responders but not in non-responders. The change due to treatment was significantly different in responders compared to non-responders in MMP-2 and TIMP-1 levels, but not for PIIINP or AFP. There is not much data currently available about the change in these parameters in response to therapy to compare our results with. Kasahara et al.[19] have reported that serum levels of MMP-2 was a predictor for no response and the MMP-2 to TIMP-1 ratio in serum might be used to predict response to IFN in HCV patients.[19] Our results have confirmed the findings of their study.

Hepatocellular carcinoma can be reliably cured if recognized prior to vascular invasion. Hence, early detection of HCC confers the best opportunity for a curative treatment.[20] AFP and liver US are the most extensively used tools for HCC screening. Alpha-fetoprotein sensitivity and specificity are largely dependent on the chosen cut-off value. The efficiency of US when used for screening greatly differs according to the experience of the examiner and the technology used. However, periodic screening of all HCV patients as a high risk population is not cost-effective.[21] An appropriate recognition of the population at greater risk of acquiring HCC by using a predictive marker(s) would make it worthwhile.

Achieving SVR has been shown to dramatically affect HCC risk as reported in multiple retrospective studies of patient cohorts mostly treated with IFN-based therapies. These studies consistently showed significant reduction of the HCC incidence in SVR patients to a degree that approached the non-viral population. That might be partly attributed to the anti-carcinogenic properties of Interferon.[22] This finding ruled out patients who attained SVR as a high-risk population, leaving non-responders for further selection.

According to our data, the 5-year rate for HCC in non-responding HCV patients was 8.4%, whereas the 10-year rate increased to 27.5%. These elevated percentages might reflect the severity of HCV-4. However, varying data have been reported. In a study by Hirakawa et al.,[23] the reported 5-year rate of hepatocarcinogenicity was 1.5% in HCV patients, which was considerably lower than the rate reported in the current study.[23] This difference might be attributed, in part, to the fact that they calculated the percent of hepatocarcinogenicity from all HCV patients instead of only the non-responding group. However, a leading research by Ikeda et al.[24] reported rates of carcinogenesis in the primary cohort of 28.9% at the fifth year and 54.0% by the tenth year.[24] Their reported carcinogenesis rates were much higher than that noted in the current study. These studies and many others have reported 5- and 10-year rates of HCC development in HCV genotypes 1, 2 and 3. To the best of our knowledge, the current study reported, for the first time, rates of hepatocarcinogenesis in HCV-4.

When searching for predictors of HCV-related HCC, the most appealing candidates seem to be those which participate in matrix remodeling, including the matrix proteins, MMPs and TIMPs. A previous study has reported that deregulation of extracellular matrix (ECM) related elements may strongly indicate the aberrant morphology of dysplastic nodules considered to be primary precancerous lesions.[25] We expected the most relevant to be PIIINP, MMP-2 and TIMP-1, along with AFP (the standard serum marker for HCV and HCC), and conventional liver function tests. We assessed these parameters before and after HCV treatment and at presentation with HCC.

Assessment of predisposing factors to HCC in chronic HCV-patients showed that several factors were considered to be risk factors. We determined that male gender was a risk factor that posed a 5.6-times higher risk in males than in females. Male gender was proposed as a risk factor in several other reports.[23-26] Studies suggested that HCC might be a hormone-responsive neoplasm.

Androgen receptor (AR) had a more intense expression in HCC than in non-tumor liver tissue; in the presence of androgens, the HCV core protein raises AR-mediated transcriptional activity. Subsequent studies suggest that the HCV core protein serves as a positive regulator in AR signaling, which would provide further insight into oncogenic potential in HCC development in people infected by HCV.[26,27]

Older age was a significant high risk factor with a 5.1-fold higher risk for HCV patients 30-50 years of age and a 15.4-fold risk for HCV patients above the age of 50. This finding agreed with other studies that reported age as an important risk factor, although with varying risk ratios.[28,29] The impact of age might result from the fact that HCV core could induce spontaneous, persistent, and age-dependent activation of PPARα, which might contribute to HCC.[28,29] Higher stage and grade of liver disease in HCV infection were also significant factors that contributed to HCC development with RR of 2.6 and 3.2, respectively.

Levels of PIIINP≥10 µg/l and TIMP-1≥400 ng/ml were significant risk factors with RR of 4.9 and 1.7, respectively. However, MMP-2 levels >500 ng/ml were not a significant risk factor for HCC. Both PIIINP and TIMP-1 are among the ECM proteins involved in cirrhosis, which is the main risk factor for HCC in chronic HCV patients.[30] Although HCC progression has been reported in chronic HCV patients without cirrhosis, it is very rare.[31] In patients with HCV-related cirrhosis, the risk of HCC increases with time in an approximately linear manner, between 2% and 8% yearly.[30,32] Therefore, matrix proteins involved in cirrhosis could probably play a part in HCC evolution.

The fundamental step in the pathophysiology of liver cirrhosis is the balance between ECM deposition and removal. During cirrhosis, hepatic stellate cells undergo activation and proliferation along with a phenotypic switch to secret excess matrix proteins, followed by matrix degradation initiated by MMPs, which are inhibited by TIMPs.[33] Many studies have explored these markers as potential noninvasive tools to predict fibrotic changes. The increase in PIIINP as a main component of ECM material along with an increase in TIMP-1, which inhibits ECM degradation, might play a crucial role in fibrogenesis that causes cirrhosis and ultimately, HCC.[33-36] However, it has been reported that HCC arising in the setting of cirrhosis appears 20-30 years after the initial insult to the liver.[36] The development of HCC over a much shorter time in HCV patients might be due to the effect of viral oncogenic factors.

In the current study, AFP levels ≥10 IU/ml were not a significant risk factor for the development of HCC. This observation contradicted numerous reports that indicated AFP levels greater than a cutoff of either 10 or 20 IU/ml were risk factors and predictors of HCC development in HCV patients.[24, 37]

Thus, the group of chronic HCV patients expected to be at higher risk of developing HCC might include non-responding patients of male gender, older age, stage>3, grade >1, and AST levels >40 U/ml. Screening for PIIINP levels >10 µg/l and TIMP-1 levels >400 ng/ml could identify patients at higher risk for development of HCC at a particularly early phase.

A comparison of viral-HCC with non-viral-HCC is expected to show major differences that may reflect the different mechanisms of carcinogenesis in the two types of HCC. Patients with viral-HCC were significantly younger than non-viral-HCC patients, which indicated that viral-HCC took a much shorter time to develop. This would establish the association of the virus with carcinogenesis. HCV-associated tumors were of significantly higher histological grade and CPT class, which indicated a more aggressive type of tumor, confirmed by the fact that they also presented with higher prevalence of hepatomegaly and hypo-echogenicity. They had significantly higher levels of GGT.

We observed significantly higher mean levels of PIIINP in viral-HCC compared to non-viral-HCC. A significantly higher proportion of viral-HCC patients had PIIINP levels >20 µg/l compared to non-viral-HCC patients. In addition,

MMP-2 was significantly higher in viral HCC patients compared to non-viral HCC patients, however the proportion of viral HCC patients with MMP-2 levels >500 ng/ml did not differ from the non-viral HCC patients. Mean levels of TIMP-1 and AFP were not significantly different in viral HCC compared to non-viral HCC patients. However, the proportion of patients with viral HCC who had TIMP-1 or AFP levels above their cutoff values were more. This profile might indicate a more important role of ECM remodeling components in viral-HCC than in non-viral-HCC. However, this hypothesis would need to be further confirmed in a wider scale study.

In conclusion, the current data have suggested that after treatment of chronic HCV patients, elevated levels of PIIINP (>10 µg/l) and TIMP-1 (>400 ng/ml) might be important as markers for non-responding patients at higher risk of developing HCC, especially in older males with high stage and grade liver disease. However, larger scale studies would be needed to verify this suggestion.

Conflict of Interest

None declared.

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