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HCV Genotypes and Cellular Immune Response in Correlation to Liver Fibrosis

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The correlation between HCV genotypes and possible serum markers for clinical prediction of disease progression is still controversial. The diagnostic accuracy of serum markers (alpha-fetoprotein (AFP), tumor necrosis factor- alpha (TNF-α), and hyaluronic acid (HA) in assessment of hepatic fibrosis was evaluated in 130 treatment-naive CHC patients who had undergone liver biopsy. 70 healthy subjects were used as reference control. Patients who had laboratory test results of (AST, ALT AFP, TNF-α, HA) allowing the calculation of HA-to platelet ratio (HAPRI), AFP-to platelet ratio (AFPPRI) and TNF-to platelet ratio (TNFPRI) were included in this study. Serum HCV RNA positive patients were chosen for HCV genotype analysis using line probe assay. The degree of fibrosis scored according to the METAVIR staging system. ROC (receiver operating characteristics) curves of serum markers were used to predict liver fibrosis. In patients with HCV genotype 4 (n =56; 43.1), there was a significant increase (p<0.001) in the levels of serum markers and liver fibrosis, whereas, ninety (69.2 %) patients had significant fibrosis (F2-F4) and fifty four (41.5%) had cirrhosis (F4). Using diagnostic cut-off values of serum markers (HA, AFP, and TNF), significant fibrosis and cirrhosis could be correctly predicted in 74.6%, 73.1%, 75.3% for fibrosis and 83.1%, 61.5%, 43.85% for cirrhosis respectively. The data showed that HA, AFP, and TNF can accurately detect fibrosis in patients with different HCV genotypes and may use as non-invasive biomarkers in predicting severity of liver disease in patients with varying HCV genotype.

Key words: HCV Genotypes, Hyaluronic acid, AFP, TNF, Fibrosis index, Chronic Hepatitis.

Hepatitis C virus (HCV) is classified into multiple HCV genotypes and more than 50 subtypes which show distinct geographical and frequency distribution across the whole world[1-5].

The outcome of HCV infection is heterogeneous ranging from an asymptomatic self-limiting infection to liver cirrhosis and HCC. Recent studies have concluded that this difference appears to be dependent on the route of transmission and viral related characteristics[6,9]. HCV genotypes may be related to disease progression among patients with different disease outcomes[10].

Some studies have reported an association of tumor necrosis factor-α (TNF-α) and alpha-fetoprotein (AFP) in hepatitis C patients with chronic liver injury[11-14]. Also, it was reported that serum hyaluronic acid (HA) contributes significantly as biomarker to measure the deleterious outcome of chronic liver diseases. Its levels correlate with clinical severity and progression of liver damage in patients with chronic HCV infections[15-16].

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We rely on repeated liver biopsies with their associated risks, cost, and sampling errors to detect, grade, and monitor hepatic pathology in hepatitis C (HCV) infections and other chronic liver diseases[17-18]. It is difficult to justify serial liver biopsies to diagnose and monitor patients with chronic HCV when there are limited options for managing the disease, as is the usual case in Egypt[19].

Noninvasive reliable biomarkers for diagnosing and grading hepatic ûbrosis and to monitor outcome of treatment and the course of HCV infection are an active area of clinical interest[19-22].

The main objective of this study was to determine the existence of any correlation between HCV genotypes to different clinical serum markers; alpha-fetoprotein (AFP), tumor necrosis factor-alpha (TNF-α), and hyaluronic acid (HA) and evaluate the diagnostic accuracy of these biomarkers for the assessment of hepatic fibrosis in relation to liver biopsy in chronic hepatitis C (CHC) patients.

MATERIALS AND METHODS

Patients

This study included 200 adult subjects admitted to the outpatients of Gastroenterology Surgical Center, faculty of medicine, Mansoura University, Mansoura, Egypt. Out of these, 70 healthy individuals (50 men and 20 women, between 14 and 66 years of age) with a mean age of 38.6±7.4 were selected as controls from a population undergoing standard annual physical examination and biological measurements for medical insurance and 130 treatment-naive CHC patients who had undergone liver biopsy (100 men and 30 women, aged from11 to 64 years of age) with a mean age of 39±8.7 were included in this study. All gave their informed consent, which included undergoing a pretreatment of liver biopsy.

Patients Selection

The inclusion criteria were: Patients who abstain from alcohol abuse for more than 6 months; with a proven HCV viremia, HCV RNA positivity and genotype determinations were selected. Liver biopsy was taken from patients prior to antiviral therapy or any other antifibrotic therapy. Serum marker levels (such as AST, ALT AFP, TNF- α and HA) were performed on the day of biopsy or within 5 days after liver biopsy.

The exclusion criteria were: presence of HIV and/or HBV co-infection; other causes of chronic liver diseases; hepatocellular carcinoma and prior liver transplantation. Also, subjects with iron supplementation, overweight and obesity (BMI: ≥ 25 and ≥ 30 Kg/m2), previously received interferon therapy and insufficient liver biopsy were excluded from this study.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was reviewed and approved by ethical committee of Gastroenterology Surgical center, Faculty of medicine, Mansoura University. All subjects completed a structured questionnaire with questions regarding demographic data, and daily medication use. The demographics and baseline characteristics of patients and controls are presented in (Table 1). Venous blood samples from each patient were collected either before the administration of preoperative drugs on the day of biopsy or within 5 days after biopsy. Samples were given a coded study identiûcation number and were shipped frozen for analysis.

Methods

Virology

Diagnosis of chronic HCV was established by elevated alanine aminotransferase enzyme (ALT) levels in persons having HCV antibody (anti-HCV) by a third generation enzyme immunoassay (EIA) (Axsym HCV 3.0, Abbott Laboratories, and Chicago, IL) according to the manufacturer's instructions and HCV-RNA using an in-house direct reverse transcriptase polymerase chain reaction (RT-PCR) assay[21]. HCV genotypes were identified by reverse hybridization method using Line Probe assay (INNO-LiPA HCV II kit, Innogenetics, Swigdrecht, Belgium) according to the manufacturer's instructions.

Laboratory Determinations

Serum aspartate aminotransferase (AST) (Max Discovery) and alanine aminotransferase (ALT) (Bio Scientific Co., USA.) were measured on a Cobas Integra-analyser (Roche, Basel, Switzerland) by standard colorimetric methods. Also, blood platelet counts were performed using routine standard method. Serum hyaluronic acid (HA) was measured in an enzyme-linked immunosorbent assay (ELISA) using HA-binding

protein (Corgenix). Serum α -Fetoprotein (AFP) (R&D Systems, USA) and Tumor necrosis factor (TNF-α) (BD Biosciences, USA) were measured by a sandwich ELISA. The HA, TNF-œ and AFP indexes were calculated by dividing the patient's test results by platelet count ratio divided by 10^3 times 100 [23-25].

Histologic Data

After submitting an informed consent document, all patients underwent a percutaneous liver biopsy to ascertain the diagnosis and their stage of liver injury. Biopsies were obtained using an automatic 16-gauge tru-cut needle (biopsy gun). All biopsy cores of at least 1–1.5 cm length and encompassed at least three portal areas were considered suitable for interpretation[23,26]. Formalin-fixed, paraffin-embedded sections were stained with hematoxy-lin and eosin and with Masson's Trichrome. Slides were labeled with patient identification numbers and then reviewed and graded blindly by a senior pathologist, the mean length of liver biopsy and the number of portal tracts were assessed (including only the complete, intact portal tracts).The degree of fibrosis was scored according to the METAVIR system, and no fibrosis was defined as F0, mild fibrosis as F1, moderate fibrosis as F2, severe fibrosis as F3, and cirrhosis as F4. Significant fibrosis was also defined as F2-4. Hepatic inflammatory activity was also scored[27].

Statistical analysis

Statistical analysis was performed using the statistical package for social studies (SPSS) version 16 for windows. Patient baseline characteristics and results were descriptively sum-marized and reported as mean ± standard deviation (SD) or number (percentage) of patients with a condition. Comparisons between groups were made using Student's t test or the Mann-Whitney U test for continuous variables. P-values less than 0.05 were considered significant. The diagnostic performance of serum biomarkers for significant fibrosis and cirrhosis prediction was measured according to sensitivity, specificity, PPV and NPV parameters. They were expressed as percentage. The diagnostic value of the method was assessed by calculating the area under the curve ROC (AUROC) and their corresponding 95% confidence intervals (CI).

RESULTS

One hundred and thirty patients with CHC were included in this study. All patients were white and 76.9 % were male (n=100), with a mean age of 39±8.7 years. The significant fibrosis was found in 81% of CHC patients using the METAVIR system. Ninety (69.2%) patients had significant fibrosis (F2-4) and fifty four (41.5%) had cirrhosis (F4).The main characteristics of patients according to the fibrosis scores are shown in Table 1 and Table 2.

The mean length of liver biopsy core (LBC) was 1.48 ± 0.42 cm. The mean length of LBC was 1.3 ± 0.4 cm in A0-A1 (n=77; 59.23%), 1.43 ± 0.55 cm in A2-A3 (n=35; 26.92%) and 1. 41±0.45 cm in A4 (n=18; 13.85%). There was no statistical significance between length of LBC and grade of inflammation (p= 0.12). Mean length of LBC was 1.38 ± 0.58 cm, 1.51± 0.61 cm, 1.38 ± 0.38 cm and 1.58 ± 0.41 cm in fibrosis stages F0-F1 (n=40; 30.7%), F2 (n=26; 20%), F3 (n=10;7.7%) and F4 (n=54; 41.5%) respectively. Mean LBC was significantly longer (1.58 ± 0.45 cm) in stage F4 fibrosis than stage F0-F1 (1.28 ±0.39 cm, p= 0.01) as shown in Table 2.

Overall, the mean number of portal tracts in liver biopsy core was 11 ± 3.9. The mean number of portal tracts were 8.7 ± 4.8 in A0-A1 (n=77; 59.23%), 10.9 ± 4.2 in A2-A3 (n=35; 26.92%) and 13.6 ± 5.3 in A4 (n=18; 13.85%, Table 2) with high statistical significance ($p < 0.001$). The mean number of portal tracts were 7.9 ± 3.2 in F0-F1 (n=40; 30.7%), 11.5 ± 3.7 in F2 (n=26; 20%), 11.8 ± 2.8 in F3 (n=10; 7.7%) and 13.9 ± 4.7 in F4 (n=54; 41.5%), again with high statistical significance ($p < 0.001$) as shown in Table 2.

Mean AST, ALT, platelet count, Hyaluronic acid (HA), AFP, TNF-α, HA-to platelet ratio index (HAPRI); AFP-to platelet ratio index (AFPPRI), and TNF-to platelet ratio index (TNFPRI) in patients with no-mild fibrosis (F0-1) vs significant fibrosis (F2-4) and with no cirrhosis (F0-3) vs cirrhosis (F4) are shown in Table 3. The data obtained showed significant decrease in platelet count and increase (0.001) in AST, ALT, Hyaluronic acid (HA), AFP, TNF-α, HAPRI, AFPPR, and TNFPRI in patients with cirrhosis compared to other stages of liver fibrosis.

Table 1. Demographic, laboratory and histological characteristics of 130 patients with chronic hepatitis C. and Control Subjects

	All CHC Patients N, mean ± SD	Controls N, mean ± SD	P*
No.	130	70	
Age (Year) ++	39±8.7	38.6±7.4	0.0001**
Sex (Male/Female) ++	100/30 (76.9/23.1) %	50/20	0.016*
BMI (kg/m2) ++	23.3 ± 3.2	24.7 ± 4.9	0.52
AST (IU/ml) +	63.56 ± 46.8	22.3 ± 6.3	0.001**
ALT (IU/ml) +	79.65 ±58.51	28.3 ± 5.6	0.001**
Platelets (109/L) ++	219.8 ± 66.5	196 ± 24.4	0.001**
HCV Genotypes 1	0 (0%)		
4	56 (43.08 %)		
2,4	27 (20.77 %)		
2,3	25 (19.23 %)		
3,4	22 (16.92 %)		
Stage of Fibrosis, (METAVIR) n (%)	105/130 (81%)		
F0	25 (19.2)		
F1	15 (11.5)		
F2	26 (20)		
F3	10 (7.7)		
F4	54 (41.5)		
Mean length of liver biopsy core (LBC +SD)	1.48 ± 0.42 cm.		
mean number of portal tracts (NoP+SD)	11 ± 3.9		
NecroinflammationA0-A1	77(59.23)		
A2-A3	35 (26.92)		
A4	18 (13.85)		

* p for controls vs all HCV patients; ++Student t test; +Mann Whitney U test; * p<0.05; **p<0.01; SD: Standard deviation; HAPRI: HA-to platelet ratio index; AFPPRI: AFP-to platelet ratio index; TNFPRI: TNF-to platelet ratio index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase;TNF-α; Tumor necrosis factor-Alpha; AFP: alpha-fetoprotein.

Table 2. Distribution of length of liver biopsy core (LBC) and number of portal tracts (NoP) with grades of inflammation and stages of fibrosis

Grade of inflammation:	Mean length of LBC +SD	p	Mean number of portal tractsNoP+SD	p
A0-A1(n=77) +	1.3 ± 0.4 cm	0.12	8.7 ± 4.8	0.001
A2-A3(n=35) +	1.43 ± 0.55 cm	0.12	10.9 ± 4.2	0.001
A4 (n=18) +	1.41 ± 0.45 cm	0.12	13.6 ± 5.3	0.001
Stage of fibrosis:				
F0-F1+	1.38 ± 0.58 cm		7.9 ± 3.2	
F2+	1.51 ± 0.61 cm	0.03	11.5 ± 3.7	0.001
F3+	1.38 ± 0.38 cm	0.04	11.8 ± 2.8	0.001
F4+	1.58 ± 0.41 cm	0.01	13.9 ± 4.7	0.001

++Student t test; +Mann Whitney U test; * p<0.05; **p<0.01.

Based on genotype analysis of Egyptian patients infected with HCV, the most frequently detected genotype was 4 (43.1%). Patients with mixed HCV genotype 2&4 (20.8%), 2&3 (19.23%), and 3&4 (16.9%) were also identified. The frequency distribution of different genotypes is given in Table 1.

Our patient's data showed significant differences in genotype distribution in relation to serum markers and liver fibrosis. The most prevalent

Table 3. Comparison of variables associated with the presence of significant fibrosis and cirrhosis

	Significant fibrosis		p	Cirrhosis		p
	F0-F1	F2-F4 Mean ± SD		F0-F3	F4 Mean ± SD	
AST (IU/ml)+	69.7 ± 14.6	80.5±22.0	0.001**	74.8 ± 16.4	110.5 ±27.8	0.001**
ALT (IU/ml)+	96.3 ± 26.9	118.4±17.6	0.001**	105.3±28.5	134.3±27.14	0.001**
Platelets (109/L)++	231.8±29.2	217.3±12.5	0.001**	206.3±19.5	151.4±84.5	0.001**
Hyaluronic acid (ng/ml)+	43.6 ± 12.5	129.0 ±27.8	0.001**	164.0 ±34.3	198.0 ± 42.5	0.001**
AFP (ng/ml)+	14.7± 3.0	16.5 ± 3.9	0.001**	17.6 ±4.3	25.8 ± 5.2	0.001**
TNF-α (pg/ml)+	14.5± 2.4	22.2 ± 5.6	0.001**	25.4 ±5.7	35.8 ±7.6	0.001**
HAPRI++	0.76±0.45	1.83±1.46	0.001**	0.79±0.53	2.32±1.63	0.001**
AFPPRI++	1.48±0.79	3.28±2.38	0.001**	1.65±0.93	4.22±2.58	0.001**
TNFPRI++	0.86±0.09	1.73 ± 0.18	0.001**	0.93±0.09	2.42±0.25	0.001**
Population, n (%)	40 (30.7)	90 (69.2)		76 (58.5)	54(41.5)	

++Student t test; +Mann Whitney U test; * p<0.05; **p<0.01; SD: Standard deviation; HAPRI: HA-to platelet ratio index; AFPPRI: AFP-to platelet ratio index; TNFPRI: TNF-to platelet ratio index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase;
TNF-α; Tumor necrosis factor-Alpha; AFP: alpha-fetoprotein.

Table 4. Genotype-specific representation according to serum markers and liver fibrosis

Characteristics	Genotypes				
	4 (n=56)	2&4 (n=27)	2&3 (n=25)	3&4 (n=22)	P ¥
AST (IU/ml)+	85.9±12.9	75.0 ± 15.2	69.5 ± 14.3	56.8±11.23	0.001**
ALT (IU/ml)+	145.5 ± 26.03	125.7 ± 16.5	104.9 ± 23.9	94.8 ± 25.6	0.001**
Hyaluronic acid (ng/ml)+	165.9 ±15.8	142.0 ± 32.6	138.3 ± 29.1	121.3 ±37.9	0.001**
AFP (ng/ml)+	19.6 ±4.4	16.5 ± 4.7	15.7 ±2.5	13.5± 2.1	0.001**
TNF-α (pg/ml)+	28.3 ± 5.9	19.4 ± 4.5	17.3 ± 5.7	15.1 ± 3.8	0.001**
Duration of HCV (years)+	6.5 ± 1.7	4.72 ± 1.6	3.8 ± 1.5	2.72 ± 1.36	0.001**
Stage of Fibrosis, (n)F0	3	5	7	12	0.001**
F1	9	8	5	3	
F2	7	3	2	2	
F3	10	4	5	3	
F4	27	7	6	2	
Population, n F0-F1	12	13	12	15	0.001**
F2-F4	44	14	13	7	
F0-F3	29	20	19	20	
F4	27	7	6	2	

¥p for genotype 4 vs other HCV genotypes; ++Student t test; +Mann Whitney U test; * p<0.05; **p<0.01; SD: Standard deviation; HAPRI: HA-to platelet ratio index; AFPPRI: AFP-to platelet ratio index; TNFPRI: TNF-to platelet ratio index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TNF-α; Tumor necrosis factor-Alpha; AFP: alpha-fetoprotein

HCV genotype in our study was: genotype 4 (n =56; 43.1) which showed a significant increase ($p < 0.001$) in the levels of serum biomarkers and stages of liver fibrosis compared to other genotypes as shown in Table 4.

Prediction of Significant Fibrosis

We analyzed the data comparing the different biomarkers to hepatic fibrosis using ROC. The results confirmed that HAPRI; AFPPRI, and TNFPRI were predictive of level of hepatic fibrosis. The AUROC curves of the HAPR index, AFPPRI and TNFPRI to predict significant fibrosis (F2-4) were 0.73, 0.879 and 0.79, respectively (Table 5).

For patients with a HAPR of score ≤ 25, 35 of 52 did not have significant fibrosis, and for those with a HAPR score > 48, 37 of 45 had significant fibrosis. A HAPR score ≤ 25 excluded significant fibrosis in 67.3% (35/52) (NPV) of patients, with a sensitivity of 84.34%, and a HAPR score >48 predicted significant fibrosis in 82.2 % (37/45) (PPV) of patients, with a specificity of 91.04 % in 74.6 % (97/130) of patients (Table 6).

For patients with an AFPPRI of score ≤0.19, 40 of 50 did not have significant fibrosis, and for those with an AFPPRI of >37, 30 of 45 had significant fibrosis. An AFPPRI ≤0.19 excluded

Table 5. AUROC of fibrosis tests in the prediction of significant fibrosis and cirrhosis

	Significant fibrosis (F0-1 vs F2-4)		Cirrhosis (F0-3 vs F4)	
Test	Area	95% CI	Area	95% CI
HAPRI	0.73*	0.70 - 0.85	0.83 **	0.77 -0.91
AFPPRI	0.879*	0.82 - 0.940	0.912 *	0.82 -0.932
TNFPRI	0.79**	0.70 - 0.85	0.92*	0.82 - 0.940

P for TNFPRI vs HAPRI or AFPRI in case of significant fibrosis and p for HAPRI vs TNFPRI or AFPRI in case of cirrhosis;* $p<0.05$; **$p<0.01$; CI: Confidence intervals; HAPRI: HA-to platelet ratio index; AFPPRI: AFP-to platelet ratio index; TNFPRI: TNF-to platelet ratio index.

Table 6. Diagnostic accuracy of tests in the prediction of significant fibrosis (F2-4)

	Total (n)	Fibrosis		Sen % 95% CI	Spe% 95% CI	PPV% 95% CI	NPV% 95% CI
		F0-F1 (n=40) (30.8 %)	F2-F4 (n=90) (69.2)				
HAPRI							
≤25	52	35	15	84.34 (54.4 - 96)	44.8 (25 -75.3)	65.42 (35.4 -85)	69.8(54.4 - 96.0)
> 25	79	29	50				
≤48	85	62	23				
> 48	45	8	37	43.4 (35.4 - 84.8)	91.04 (86–100)	85.7(63.6 -92.8)	56.5(53- 84.1)
AFPPRI							
≤19	50	40	10	97.5(69.8 - 99.8)	66 (54.4 - 96.0)	78.4 (72.6 - 98)	69.8 (58.6 - 96)
>19	80	64	16				
≤37	85	65	20				
> 37	45	15	30	92.3 (72.6 - 97.8)	94.04 (87 – 100)	85.71 (81 - 100)	56.5 (46.2 – 95)
TNFPRI							
≤ 0.16	60	45	15	86.7(81 – 100)	93.7(87 – 100)	90.7(73 - 97.8)	58.6 (51.7 - 96)
> 0.16	70	38	32				
≤45	68	15	53				
> 45	62	18	44	93.98 (86.8 – 100)	75.3 (72.6 – 97)	63.93(44.2 - 85)	82.14 (79.4 – 100)

significant fibrosis in 80.0 % (40/50) (NPV) of patients, with a sensitivity of 97.5%, and an AFPPRI I >37 predicted significant fibrosis in 66.6 % (30/45) (PPV) of patients, with a specificity of 94.04 % in 73.1 % (95/130) of patients (Table 6).

For patients with a TNFPRI of score ≤ 0.16, 45 of 60 did not have significant fibrosis, and for those with a TNFPRI of ≥45, 44 of 62 had significant fibrosis. A TNFPRI ≥0.16 excluded significant fibrosis in 75.0 % (45/60) (NPV) of patients, with a sensitivity of 86.7 %, and a TNFPRI ≤45 predicted significant fibrosis in 71.0 % (44/62) (PPV) of patients, with a specificity of 75.3% in 93.8 % (122/130) of patients (Table 6).

Prediction of Cirrhosis

The AUROC curves of the HAPR index, AFPPRI and TNFPRI to predict cirrhosis (F2-4) were 0.83, 0.912 and 0.92, respectively (Table 5).

For patients with a HAPR of score ≤ 75, 50 of 60 did not have cirrhosis, and for those with a HAPR score > 100, 38 of 48 had cirrhosis. A HAPR score ≤ 75 excluded cirrhosis in 83.3% (50/60) (NPV) of patients, with a sensitivity of 90.5 %, and a HAPR score >100 predicted cirrhosis in 79.2 % (38/48) (PPV) of patients, with a specificity of 94.6 % in 83.1 % (108/130) of patients (Table 7).

For patients with an AFPPRI of score ≤0.19, 18 of 30 did not have cirrhosis, and for those with an AFPPRI of >37, 36 of 50 had cirrhosis. An AFPPRI ≤ 0.19 excluded cirrhosis in 60.0 % (18/30) (NPV) of patients, with a sensitivity of 92.4%, and an AFPPRI I >37 predicted cirrhosis in 72.0 % (36/50) (PPV) of patients, with a specificity of 90.6 % in 61.5 % (80/130) of patients (Table 7).

For patients with a TNFPRI of score ≤0.16, 27 of 30 did not have cirrhosis, and for those with a TNFPRI of ≥45, 22 of 27 had cirrhosis. A TNFPRI ≤0.16 excluded cirrhosis in 90.0 % (27/30) (NPV) of patients, with a sensitivity of 98.04 %, and a TNFPRI ≤ 45 predicted cirrhosis in 81.5 % (22/27) (PPV) of patients, with a specificity of 90.7% in 43.8 % (57/130) of patients (Table 7).

Table 7. Diagnostic accuracy of fibrosis tests in the prediction of cirrhosis (F4)

	Total (n)	Fibrosis		Sen % 95% CI	Spe% 95% CI	PPV% 95% CI	NPV% 95% CI
		F0-F1 (n=40) (30.8 %)	F2-F4 (n=90) (69.2)				
HAPRI							
≤75	60	50	10	90.5 (86 – 100)	80.9(73 - 98)	65.42(64 – 93)	85.11(81 - 100)
> 75	70	25	45				
≤100	82	68	14				
> 100	48	10	38	89.7 (87 – 100)	95 (86 – 100)	82(81 - 100)	77(72.6 - 97.8)
AFPPRI							
≤19	30	18	12	92.4(81 - 100)	67.3(64 - 93)	62.8 (61.1 – 91)	91.3(86.8 – 100)
>19	100	70	30				
≤37	80	68	12				
> 37	50	14	36	82.5(63.6 - 93)	91 (82- 100)	75.8(71.5 – 100)	78.9(75.3 – 100)
TNFPRI							
≤0.16	30	27	3	98.(88.1 – 100)	57.3(51 – 96)	46.7(43 - 94.5)	96.5(86.8 – 100)
> 0.16	100	75	25				
≤45	103	68	35				
> 45	27	5	22	80.3(73 - 97.8)	91(87 – 100)	79.1(73 - 97.8)	85.5 (81 - 100)

DISCUSSION

Assessment of the degree of hepatic fibrosis is essential in deciding on antiviral therapy for chronic HCV infection[28-30]. Although liver biopsy remains the gold standard method for the assessment of hepatic fibrosis, it has some limitations[31-34].

Noninvasive methods to measure severity of liver injury are clinically important in Egypt where advanced liver disease from HCV is common and access to liver biopsy is limited[35, 36]. Many of

the reports evaluating biomarkers for detecting hepatic fibrosis have used scoring systems encompassing combinations of results from several blood tests and demographic data[37-39]. Most of the indexes proposed in these studies would not be practical in Egypt and other developing countries because of cost and unavailability of some tests.

For this reason we evaluated a few blood tests routinely performed on patients with chronic HCV in Egypt in addition to the levels of alpha-fetoprotein (AFP),tumor necrosis factor- alpha (TNF-α), and hyaluronic acid (HA) using commercially available tests for measuring hepatic fibrosis in patients with different HCV genotypes. The diagnostic performance of the HA-to platelet ratio index (HAPRI) index, AFP-to platelet ratio index (AFPPRI) and TNF-to platelet ratio index (TNFPRI) to predict significant fibrosis and cirrhosis were also evaluated in our CHC patient. In the present study, the significant fibrosis was found in 81% of CHC patients (105/130) using the METAVIR system. Ninety (69.2%) patients had significant fibrosis (F2-F4), 54 (41.5%) had cirrhosis (F4), and 40 (30.7) (F0- F1) had Mild or no fibrosis. It was found that HCV infection has a rapid course of disease progression in chronic hepatitis C patients[40-44].

In our study, the area under the ROC curves of the HAPRI for predict-ing significant fibrosis and cirrhosis were 0.73 (0.70-0.85) and 0.83 (0.77-0.91), respectively. Based on the high and low predictive cut-off values of HAPRI score (≤ 25 and > 48), the diagnostic accuracy of significant fibrosis in accordance to liver biopsy was reported in 74.6 % (97/130) of CHC patients. This matched with other studies which reports the increase of serum hyaluronic acid with the progression of liver fibrosis or cirrhosis in chronic HCV patients, and that HA alone has a very good diagnostic accuracy for the non-invasive assessment of fibrosis and cirrhosis[14, 45-51].

For prediction of cirrhosis, the best determined diagnostic cut-off values of HAPRI score was (≤ 75, > 100). Together, using HAPRI cut-off values (≤75 and >100), the diagnostic accuracy of significant cirrhosis in accordance to liver biopsy was reported in 83.1 % (108/130) of CHC patients.

The diagnostic accuracy of significant cirrhosis in our Egyptian patients infected with different HCV genotypes shows a close correlation to the average of Oberti *et al*. [52] who evaluated HA as the best performed marker having a diagnostic accuracy of 86% for detecting cirrhosis in subpopulations having viral or combined viral and alcoholic etiologies. Furthermore, HIV co-infection did not reduce the value of noninvasive biomarkers to detect and measure fibrosis in HCV infected patients[53-54]. Also, Wong et al. showed that HA had 85% sensitivity and 88% specificity for predicting stage 4 and 5 fibrosis[53]. However, our study yielded a slightly higher PPV (79.2%) than those of Patel *et al.*[50] who reported a 75% PPV and NPV in differentiating moderate/severe fibrosis from no/mild fibrosis in CHC patients.

This may be related to the higher proportion of patients with significant fibrosis, since diagnostic performance of non-invasive tests varies according to the prevalence of significant fibrosis[23, 54-56].

Currently, AFP is widely used as a serum marker for diagnosing HCC, especially in patients with chronic liver disease[57]. However, Elevated serum AFP levels have also been observed in patients with CHC, with a prevalence ranging from 10% to 42% [58].

In our study, the AUROCs of the AFPPR index for predict-ing significant fibrosis and cirrhosis were 0.879 (0.82 - 0.94) and 0.912 (0.82 - 0.932), respectively. Together, using AFPPRI cut-off values (≤ 19 and > 37), the diagnostic accuracy of significant fibrosis and cirrhosis in accordance to liver biopsy was reported in 73.1 % (95/130) and 61.5 % (80/130) of CHC patients with a specificity of 90.04 % and 90.06 % respectively. In similar studies by Hu *et al*.,[62] and Bayati *et al*.,[63], the AFP level was used to predict liver fibrosis with a sensitivity of 22.8–35% and a specificity of 94.5–98.6% in CHC patients with advanced fibrosis. Some studies showed different elevated serum AFP levels (10-30ng/ml) in CHC patients with cirrhosis. The prevalence of elevated AFP levels ranged between 10% and 43% [58, 61, 62]. The high prevalence may have been associated with the severity of liver fibrosis or cirrhosis. Recent studies have reported that liver fibrosis (stage F4) is associated with hepatic progenitor cell activation which increases AFP production in patients with advanced fibrosis[63-65].

In the past, strong correlation has been observed between the degree of liver inflaammation and serum levels of TNF- α in HCV patients, thus indicating its possibility as a marker of liver fibrosis[65].

In the present study, the AUROCs of the TNFPR index for predict-ing significant fibrosis and cirrhosis were 0.79 (0.70 - 0.85) and 0.92 (0.82 - 0.94), respectively. Together, using TNFPR cut-off values (≤ 16 and > 45), the diagnostic accuracy of significant fibrosis and cirrhosis in accordance to liver biopsy was reported in 93.85 % (122/130) and 43.85 % (57/130) of CHC patients with a specificity of 75.3 % and 90.7 % respectively. In patients with CHC, TNF-alpha levels along with other demographic and laboratory tests were significantly associated with the severity of liver fibrosis (F3-F4), and that TNF-alpha was a putative candidate involved in the fibrosis mechanism[66-67]. Also, our data matched with Morais *et al*.,[67] who were able to suggest TNF-alpha and alkaline phosphatase (AP) as non-invasive biological markers to evaluate fibrosis and necroinflammatory activity in CHC patients.

In our population, using our own diagnostic cut-off values of HAPRI, AFPPRI, and TNFPRI significant fibrosis could be classified correctly according to liver biopsy in74.6%, 73.1%, and 75.3% with specificity 91.04%, 90.04%, and 93.85% respectively. Also, cirrhosis was predicted correctly according to liver biopsy in 83.1%, 61.5%, and 43.85% with specificity 94.1%, 90.1%, and 90.7% respectively. This may be due to the higher proportion of patients with significant fibrosis, since diagnostic performance of noninvasive tests varies according to the prevalence of significant fibrosis[29, 55, 56]. However, using TNFPR index in the absence of cirrhosis was predicted better according to liver biopsy in 90.0 % of CHC patients with sensitivity 98.04 % compared to HAPRI and AFPPRI of our study and Forns index in Guzelbulut *et al.*'s study[72].

Our patient's data showed differences in HCV genotype distribution. Genotypes 4 showed higher distribution (n=56, 43.08 %), followed by mixed HCV genotypes 2, 4 (n=27, 20.77 %), genotype2, 3 (n=25, 19.23 %) and genotype3, 4 (n=22, 16.92 %). Genotype 1 was absent from patients under study. Accumulated data showed that there are two main patterns for the distribution of HCV genotypes in the Middle East; in the first pattern, genotype 4 is prevalent in most of the Arab countries and in the second pattern, genotype 1a or 1b predominates in the non-Arab countries[4]. Mixed HCV genotype infection may be related to a high mutation rate of HCV-RNA and that the infection rate is extremely variable for different regions and for the same group of patients. Also, it was observed that the different genotypes are relevant to epidemiology, vaccine development, and clinical management of chronic HCV infection[70- 73].

The correlation among HCV genotypes with serum markers and their association with disease severity and sensitivity to interferon treatment remains controversial till date[74].

Evaluating the correlation between different clinical markers with genotypes, our results showed that a combination of five clinical markers ALT, AST, HA, AFP and TNF-α level can have high positive predictive values for diagnosis of different HCV genotypes. We observed elevated ALT and AST levels in all genotypes compared to normal range but in patients infected with genotype 4 values were quite higher. These results could lead to the association of genotype 4 with increased risk of cirrhosis[75-77]. Also, higher serum levels of HA, AFP and TNF-α were reported in patients with HCV genotype 4 compared to other mixed genotypes. These results confirmed with other studies[23, 56, 78-81], which reported the increment of HA, AFP and TNF-α biomarkers in CHC patients. In our study, patients with genotype4 and mixed HCV genotypes 2, 4 showed higher significant fibrosis and cirrhosis stages compared to other mixed HCV genotypes. This may be related to many extra hepatic complications such as, decline of Hb level which may lead to autoimmune hemolytic anemia (AIHA) in CHC patients with different HCV genotype 4 [82-86]. Hence, HCV genotypes may play a key role along with other demographic parameters in predicting severity of liver disease in chronic HCV infection. However, a multivariate analysis is required to further validate the results of our study.

In conclusion, we have shown that in comparison with liver biopsy, HAPRI, AFPPRI, and TNFPRI could identify significant fibrosis and cirrhosis at a high degree of accuracy. Significant fibrosis and cirrhosis could be correctly predicted

in 74.6%, 73.1%, 75.3% for fibrosis and 83.1%, 61.5%, 43.85% for cirrhosis respectively. Also, significant variable response of HCV genotypes with serum markers and severity of disease were reported. This may play a role in predicting severity of liver disease and possibility of treatment response in chronic HCV infection.

Abbreviations

HCV: Hepatitis C virus; PPV: positive predicted value; NPV: negative predicted value; AUC: area under the curve; ROC: receiver operating characteristic; HA: Hyaluronic Acid; AFP; Alpha Fetoprotein; TNF-œ: Tumor Necrosis Factor Alpha.

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