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Evaluation of serum fibrotic markers; CTGF, IL-17and TGF-β1 versus liver biopsy for detection of hepatic fibrosis in Egyptian patients with chronic hepatitis C

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ABSTRACT

Objectives: Hepatitis C virus (HCV) infection in Egypt is the most serious health problem, where 10%–15% of the population is infected. The severity of the disease and diagnostic decision-making is evaluated by liver biopsy; an invasive technique with many drawbacks. Recently, attention has been directed to non-invasive, accurate alternatives using serum biochemical markers. Thus, CTGF, IL-17and TGF-β1 were assessed versus liver biopsy in relation to hepatic inflammatory and fibrotic status in chronic HCV patients.

Design and methods: 58 chronic HCV patients and 30 normal healthy controls were enrolled in the study. Serum samples were collected for detection of CTGF, IL-17and TGF-β1 levels using ELISA. Liver biopsies were obtained from some patients for revaluation of the expression levels of the studied biomarkers genes by quantitative Real Time PCR and for histopathological assessment of grades of inflammation and stages of fibrosis.

Results: CTGF, IL-17and TGF-β1serum levels were significantly higher in HCV patients versus healthy controls, and decreased significantly in response to antiviral therapy. There were significant differences in their serum levels and the relative expression levels of their genes in relation to hepatic fibrotic stages and inflammatory grades. Significant Correlation existed between the Serum levels of measured biomarkers and the relative expression levels of their genes in the corresponding liver tissue; verifying the hepatic source of serum biomarkers.

Conclusion: CTGF, IL-17and TGF-β1are serum promising biomarkers, being non-invasive, specific, sensitive and accurate method for assessment of liver fibrosis and inflammation in Chronic HCV infection. They could be alternatives to invasive liver biopsy.

1. Introduction

Hepatitis C virus (HCV) infection, is a major health problem, with an estimated prevalence of > 170 million people infected worldwide, (Lauer and Walker, 2001).To evaluate the severity of the liver disease, liver biopsy still remains the golden standard to date (Gebo et al., 2002). However, liver biopsy is associated with many problems that could limit its applicability as a diagnostic procedure (Rousselet et al., 2005). In addition, liver biopsy is an invasive and painful procedure, with rare but potentially life-threatening complications (Bravo et al., 2001). Thus, many patients with chronic HCV infection are reluctant to undergo liver biopsy and are discouraged from starting therapy for that reason (Castéra et al., 2009).

Connective tissue growth factor (CTGF) has received special focus with respect to its fibrotic actions. It mediates expression of some fibrotic markers during HCV infection. Some studies have indicated the presence of an association between CTGF and stage of fibrosis in chronic HCV infected patients (Kovalenko et al., 2009). And also demonstrated that increased CTGF expression underscore the importance of hepatocytes in producing CTGF during HCV infection (Tong et al., 2009; Nagaraja et al., 2012).

Several studies have reported that the frequency of IL-17 cells is increased in the portal areas of livers from chronic HCV infected patients (Harada et al., 2009). HCV antigen-specific Th17 cells were also induced in their peripheral blood (Rowan et al., 2008). However, the role of IL-17 in HCV infection still has not been well investigated. It is plausible to speculate that IL-17 could have an important role in stimulating liver inflammation during HCV infection, as the condition in HBV infection (Lafdil et al., 2009).

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Transforming growth factor (TGF)-β1 is known to be the best characterized profibrogenic cytokine. It increases the production of many extracellular matrix proteins together with their receptors; moreover, it inhibits the matrix degrading proteolytic enzymes synthesis (Marek et al., 2005). It is known that TGF-β1 is a strong immunosuppressive cytokine. Thus, high levels of TGF-β1 in patients with Chronic HCV could suppress specific immune reactions including cytotoxic T cell function which in turn will contribute to the development of chronicity of the disease. It also may induce apoptosis of liver cells (Kirmaz et al., 2004).

Increased levels of TGF-β1, which is detected in patients with cirrhosis, could be attributed to the chronic viral hepatitis background. The strong correlation of TGF-β1 levels with fibrosis reveals an important role for TGF-β1 in the pathogenesis of fibrosis in HCV patients. It has been shown before that Hepatitis C virus core antigen upregulates TGF-β1 expression (Taniguchi et al., 2004).

The present study was carried out on patients having chronic hepatitis C virus infection. The three biochemical markers CTGF, IL-17 and TGF-β1 were measured to evaluate both their serum levels and the relative expression of their genes in liver tissue, to find out if they could be used as a reliable testing for the assessment of liver inflammatory and fibrotic conditions replacing liver biopsy and to evaluate their serum levels in response to therapy.

2. Subjects and methods

The present study was carried out at Medical Biochemistry Department, Faculty of Medicine, Ain Shams University, Egypt. It was performed in accordance with Declaration of Helsinki, and was approved by the Research Ethics Committee of Ain Shams University, Cairo, Egypt. An informed consent was obtained from all participants.

The study included 88subjects. They were stratified into 3 groups: The first group included 58 patients with chronic hepatitis C virus infection. They were recruited from the HCV Clinic, Tropical Medicine Department, Ain Shams University Hospital, Cairo, Egypt, and diagnosed by positive anti-HCV antibodies and positive HCV viral load test (Quantitative PCR for HCV RNA).The second group consisted of 30 individuals without any hepatic diseases served as the normal control group. Patients and normal individuals were matched for the demographic variables: age, gender, BMI (body mass index). Finally, the third group represented the follow up group; which consisted of 32 patients selected from the chronic HCV infected patients included in the study. They received combination therapy (pegylated interferon and ribavirin). Follow up was performed after 4 weeks, 12 weeks and 24 weeks of therapy.

As regards the histopathological data referring to the stages of fibrosis and grades of inflammation, it was according to Metavir scoring system. On that base two more classifications were designed for HCV patients. *Classification according to presence of inflammation*: A0-A1: HCV patient with no to mild inflammation (n = 27), A2-A3: HCV patients with moderate to severe inflammation (n = 31). *Classification according to presence of fibrosis*: F0-F1: HCV patients with no to mild fibrosis (portal fibrosis without septa) (n = 6), F2: HCV patients with moderate fibrosis (some septa) (n = 38), F3-F4: HCV patients with severe fibrosis (numerous septa) to cirrhotic liver (n = 14). Full clinical data were obtained from all patients enrolled in the study.

Inclusion criteria were as follows: 18–60 years age, proven HCV infection by HCV Ab and HCV RNA detection. Exclusion criteria were as follows: co-infection with hepatitis B virus, other causes of liver disease, decompensated liver disease, diabetes mellitus, arthritis or any collagen disease, chest disease namely sarcoidosis and suppurative lung disease, Liver transplantation, anticoagulant treatment and patients who had received specific antiviral therapy prior to enrollment in the study.

All patients were subjected to full history taking and thorough clinical examination. The Full laboratory tests data (CBC, ALT, AST, S. Bilirubin, INR, albumin and viral load) and the report of abdominal ultrasonographic examination were collected from the patients' files.

Serum samples (5 ml of blood) were collected from all subjects, samples from HCV patient were drawn before antiviral therapy and at 4, 12 and 24 weeks from starting treatment, and then stored at − 80 °C for later detection of serum fibrosis markers CTGF, IL-17and TGF-β1 using ELISA kits according to the manufacturer's instructions (DRG®. CTGF, DRG Instruments GmbH, Germany Division of DRG International, Marburg Quantikine® Human IL-17, Immunoassay R & D systems, inc, USA and. TGF-β1 M ELISA Kit - Life Technologies – Invitrogen) respectively.

Liver biopsies were obtained from chronic HCV patients and were divided into two samples: one was examined by a pathologist for histological examination and quantification of liver fibrosis and inflammation. Second liver biopsy samples were stored at − 80 °C for later evaluation of the relative RNA expression levels of serum fibrosis markers CTGF, IL-17and TGF-β1 by quantitative Real Time PCR.

RNA extraction from liver biopsies was done using the RNeasy Mini Kit QIAGEN; (Catalog no.74104). Reverse transcription of the extracted RNA into cDNA was performed using the Sensiscript Reverse Transcriptase for Two-tube RT-PCR Kit QIAGEN (Catalog no. 205211). The quantitative real-time PCR was done using QuantiTect® SYBR® Green PCR kit, QIAGEN (Catalog no. 204141).

Primers of CTGF were as follows (Liu et al., 2007): sense: 5′-GCCTGTTCCAAGACCTGT-3′ and antisense: 5′-GGATGCACTTTTTGCCCTTCTTA-3′, Primers of IL-17 (Loverrea et al., 2011) were as follows: sense 5′-GAATCTCCACCGCAATGAGG-3′ and antisense 5'CCCACGGACACCAGTATCTT-3′ and Primers for TGF-β1 (Nagaraja et al., 2012) were as follows sense: 5′-ACCTGAACCCGTGTTGCTCT-3′ and antisense: 5′-CTAAGGCGAAA GCCCTCAAT.

The prepared real time PCR mixtures were placed into the Rotor-Gene Q MDx (QIAGEN Hilden, Germany) to be amplified and quantified according to the following program: Initial PCR activation at 95 °C for 15 min followed by 45 cycles, each with three stages: denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s.

The hepatic relative expression level (fold change) for fibrosis markers CTGF, IL-17and TGF-β1 was calculated by the comparative cycle threshold $2^{-(\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001).The raw data were normalized to a housekeeping gene (B-actin) as the invariant control for the samples, and compared with a reference sample.

2.1. Statistical analysis

Statistical analysis was done using the statistical package for social sciences (SPSS software version 20, Chicago, Illinois) on a personal computer. The following analyses were performed: Student *t*-test (*t*-test) and one way Anova were used for assessment of the statistical significance of differences between sample mean values of quantitative data between two groups and more than two groups' respectively. The nonparametric Mann-Whitney and Kruskal-Wallis tests were used for statistical comparison of the variables between the various groups. The positivity rates were compared by Chi-square test. Statistical significance was set at a value of $P < 0.05$.Receiver Operating Characteristics (ROC) curve was used to discriminate positive from negative results. It was constructed by calculating the true positive fraction (sensitivity percent) and the false positive fraction (100-specificity) of markers at several cutoff points. Pearson correlation coefficient (*r*) measured the relationship between the variables.

3. Results

3.1. CTGF, IL-17 and TGF-β1 serum levels in different study groups

CTGF serum levels were found to be significantly higher in chronic HCV patients than the healthy control individuals with a *P*-value of (0.008). As regards the relation with the stages of fibrosis, there was a

Table 1
Serum levels of CTGF,IL-17and TGF-β in different study groups.*

Classification according to		IL-17 (pg/ml)					CTGF (ng/ml)					TGF-β1 (pg/ml)				
		Mean ± SD	Median	Range	*t* or *f*	*P*-value	Mean ± SD	Median	Range	*t* or *f*	*P*-value	Mean ± SD	Median	Range	*t* or *f*	*P*-value
Health status	Patients (n = 58)	62.6 ± 15.2	60.5	36.8–125	$t = -2.515$	0.014*	37.1 ± 9.3	36.4	22.9–59.4	$t = -2.718$	0.008*	113.3 ± 46.9	100.4	49–189.43	$t = 12.050$	*0.0001
	Healthy control individuals (n = 30)	57.3 ± 3.8	57.1	50.9–68.4			32.1 ± 7.7	31.2	22.0–57.5			32.6 ± 15.1	27.2	15.8–84.0		
Inflammatory status of the liver	A0-A1(n = 27)	57.7 ± 13.8	52.1	36.8–87.8	$t = -2.373$	0.021*	36.5 ± 7.5	36.8	22.0–48.4	$t = 0.532$	0.597	96.3 ± 36.0	87.0	49.0–181.0	$t = 2.834$	0. 006
	A2-A3(n = 31)	66.9 ± 15.4	62.7	48.2–125			37.8 ± 10.7	37.1	22.0–59.4			128.6 ± 50.1	155.4	49.0–189.4		
Fibrotic status of the liver	F0-F1(n = 6)	55.3 ± 11.8	53.8	36.8–64.4	$f = 2.048$	0.139	26.3 ± 5.1	25.5	22.0–36.1	$f = 8.712$	0.001**	62.7 ± 11 0.5	60.2	49–84	$f = 4.508$	0.015
	F2(n = 38)	62.6 ± 13.2	60.5	44.1–88.4			36.8 ± 7.1	35.4	23.8–48.4			118.2 ± 42.9	104.0	57.37–189.4		
	F3-F4(n = 14)	67.1 ± 20	61.0	48.2–125			43.0 ± 11.4	40.05	23.0–59.4			122.6 ± 53.8	141.2	49–189.2		

t = independent-samples *t*-test.
f = one way ANOVA.
* $P < 0.05$: is significant.
** $P < 0.01$: is highly significant, but $P > 0.05$: is not significant.

significant difference with a *P*-value of (0.001) between F0-F1, F2 and F3-F4 stages of fibrosis. CTGF serum levels seemed to be non-significant giving a *P*-value of (0.597) in relation to grades of inflammation (Table 1).

As regards IL-17 serum levels, it was also found to be significantly higher in chronic HCV patients than in healthy control individuals with a *P*-value of (0.014). Furthermore there was a significant difference in its levels between A0-A1 and A2-A3 grades of inflammation with a *P*-value of (0.021), while, it seemed to be non-significant between the different stages of fibrosis as the *P*-value was (0.139), Table1.

Results of serum levels of TGF-β1, also revealed a higher significant difference between chronic HCV patients and healthy control individuals with a *P*-value of (0.0001). Moreover, a significant difference between patients with different grades of inflammation (A0-A1 and A2-A3) with a *P*-value of (0. 006), and different stages of fibrosis as well, with a *P*-value of (0.015) between F0-F1, F2 and F3-F4, Table1.

Correlation of CTGF, IL-17 and TGF-β1 serum levels with the laboratory data gathered from the patient's files (WBC, Hb, Platelets, INR, Albumin, Bilirubin, AST, ALT, and Viral Load) was examined. It revealed no correlation except for a strong positive correlation between serum level of CTGF and AST with a *P*-value of (0.003) and a positive correlation between TGF-β1 serum levels and bilirubin with a *P*-value of (0.008), Supplementary Table 1. As regards the correlation with the clinicopathological parameters (history of Bilharzial infection, BMI (body mass index), fever, nausea, headache, diarrhea, jaundice and epigastric pain), results revealed that CTGF serum levels were statistically significant with a *P*-value of (0.039) between patients with history of bilharziasis or those without, as well as TGF- ß1 serum levels with a *P*-value of (0.049). While, IL-17 serum levels seemed to be non-significant between patients either having history of bilharziasis or not. Additionally, a significant correlation was detected between IL-17 and jaundice ($P = 0.009$) Supplementary Table 2.

ROC curve analysis for CTGF, IL-17 and TGF-β1 serum levels in patients versus healthy controls was performed to calculate the best discriminating cut off values, which gave the highest sensitivities and specificities. They were 34.53 ng/ml for CTGF, 60.31 pg/ml for IL-17, and 48.3 pg/ml for TGF-β1, as shown in Fig. 1 and Table 2.

3.2. Results of the follow up of HCV patients after 4, 12 and 24 weeks of treatment in relation to the starting point (before therapy)

CTGF, serum levels decreased significantly only after 24 weeks of

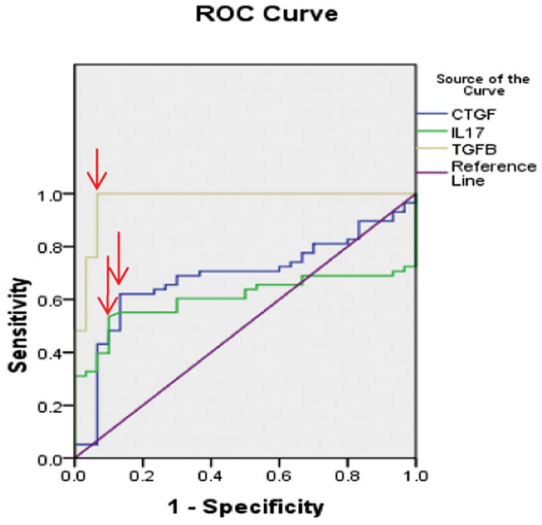

Fig. 1. ROC curves for CTGF, IL-17 and TGF-β1 serum level, Area under the curves were, 0.686/*P*-value 0.004, 0.605/*P*-value 0.106 and 0.975/*P*-value 0.000 respectively. The best serum cutoff values were 60.31 pg/ml for IL-17, 34.53 ng/ml for CTGF and 48.33 pg/ml for TGF-β1.

Table 2
Performance characteristics of CTGF, IL-17 and TGF-β1

Parameter	Sensitivity	Specificity	NPV	PPV	Accuracy
IL-17	53.4%	90%	91.1%	50%	65.9%
CTGF	62%	86.6%	90%	54%	70%
TGF-β	100%	90%	100%	96.6%	89.8%

therapy. As regards IL-17 serum levels, it declined significantly after 4 weeks of treatment. TGF-β1 serum levels declined significantly after 12 weeks. Concerning the viral load, it declined significantly after 4 weeks, all in relation to their levels, before starting treatment (Table 3).

A strong Correlation was detected between the serum levels of the three studied serum biomarkers in HCV Patients through the course of treatment, with P values < 0.05 (Table 4).

3.3. Results of quantitative real time PCR; evaluating the CTGF, IL-17 and TGF-β1 relative expression levels (fold change) in different chronic HCV infected patients subgroups of the study

CTGF relative expression levels were found to be significant with a P-value of (0.015) between F0-F1, F2 and F3-F4 stages of fibrosis. On the other hand, CTGF relative expression levels were insignificant with a P-value of (0.058) with all grades of inflammation (Table 5, Fig. 2).

On the other hand, IL-17 relative expression levels were found to have a significant difference in relation to the grades of liver inflammation; between A0-A1 and A2-A3 with a P-value of (0.002). It was also found to be significant regarding its relation to the stages of liver fibrosis; between F0-F1, F2 and F3-F4 with a P-value of (0.040) (Table 5, Fig. 2).

As regards TGF-β1 relative expression levels were found to have a significant difference in relation to the grades of liver inflammation; between A0-A1 and A2-A3 with a P-value of (0.005). It was also found to be significant regarding its relation to the stages of liver fibrosis; between F0-F1, F2 and F3-F4 with a P-value of (0.003) (Table 5, Fig. 2).

Table 4
Correlations between serum levels of studied biochemical markers in HCV patients during the course of treatment.

	CTGF	IL17	TGF-β1
IL17	$r = 0.186$ $P = 0.036$	–	–
TGF-β	$r = 0.206$ $P = 0.019$	$r = 0.371$ $P = 0.0001$	–
Viral load (PCR)	$r = 0.226$ $P = 0.010$	$r = 0.243$ $P = 0.006$	$r = 0.251$ $P = 0.004$

r = Pearson correlation (parametric). $P < 0.05$: is significant.

3.4. Correlation between serum levels and relative expression levels of IL-17, CTGF and TGF-ß1 in liver tissue

Regarding the Correlation between Serum levels of the 26 serum samples and relative expression levels of CTGF, IL-17 and TGF-ß1 of the corresponding liver tissue samples; there was a statistically significant correlation, with a P-value of (0.001), (0.014) and (0.048) respectively (Table 6).

4. Discussion

Worldwide, HCV is considered one of the major causes of chronic liver diseases, which include inflammation, fibrosis and cirrhosis. Furthermore, HCV leads to increased morbidity and mortality in hepatocellular carcinoma (Liu et al., 2007). HCV is currently the most substantial public health problem in Egypt.

Although liver biopsy is an invasive procedure and produces complications, it is still the gold standard for grading the degree of necro-inflammation and staging the level of liver fibrosis in chronic HCV infected patients (El-Attar et al., 2010). The drawbacks of liver biopsy have revealed the urgent need to identify alternative ways to assess liver inflammation and fibrosis by noninvasive methods. Among the noninvasive methods, three serum biomarkers CTGF, IL-17 and TGF-ß1 were chosen to be assessed, aiming to evaluate their efficiency in replacing liver biopsy.

Furthermore, to confirm the hepatic source for these serum biomarkers, the relative expression levels of their genes in paired liver tissues obtained from some HCV patients representing different stages of inflammation and fibrosis were estimated. Correlation was detected

Table 3
Different biochemical parameters used for following up HCV patients during the course of treatment and their significance.

Parameter	Mean ± SD	Minimum	Maximum	f	P	Median	Mean rank	X^2	P
IL17(pg/ml) before therapy	61.0 ± 17.13	36.8	125	4.31	*0.006	58.16	86.66	13.98	**0.003
4 weeks	57.5 ± 14.9[a]	34.3	110.4			54.65	72.06		
12 weeks	50.7 ± 12.0[a]	33.3	80			47.80	53.61		
24 weeks	50.4 ± 12.03[a,b]	33.1	82.8			47.20	51,67		
CTGF (ng/ml) before therapy	36.95 ± 7.7	25.8	51.4	5.193	*0.002	35.40	76.8	13.970	**0.003
4 weeks	35.7 ± 7.8	17.6	49.3			34.18	71.9		
12 weeks	34.2 ± 7.9	16.8	48.2			33.56	64.5		
24 weeks	29.7 ± 7.49[a,b,c]	13.5	49.2			29.71	44.6		
TGF β (pg/ml) before therapy	106.76 ± 36.75	57.3	189.2	23.32	*0.000	99.76	93.0	54.561	**0.000
4 weeks	92.16 ± 40.41	26.5	179.0			87.17	79.4		
12 weeks	66.97 ± 40.40[a,b]	22.9	166.5			58.01	56.1		
24 weeks	38.97 ± 16.38[a,b,c]	14.0	79.12			35.84	29.3		
PCR (viral load) before therapy	836,037.2 ± 887,816.4	50,000	3.60E + 006	21.212	*0.000	390,500	103.7	64.186	**0.000
4 weeks	206,596.9 ± 230,166.5[a]	0	782,000			87,500	70.7		
12 weeks	43,101.1 ± 112,567.2[a]	0	520,000			0.000	43.2		
24 weeks	39,233.9 ± 564,325.0[a]	0	409,000			0.000	40.2		

f = one way ANOVA.
X^2 = Kruskal-Wallis Test (Chi-Square value).
* $P < 0.05$: is significant.
** $P < 0.01$: is highly significant, but $P > 0.05$: is not significant.
[a] In relation to the patients before treatment.
[b] In relation to treatment for 4 weeks.
[c] In relation to treatment for 12 weeks.

Table 5
Significance of CTGF, IL-17 and TGF-β1 relative expression levels in relation to inflammatory and fibrotic status of the liver.

Classification according to		IL-17				CTGF				TGF-β1			
		Median	Mean rank	[a]X^2or U	P-value	Median	Mean rank	[a]X^2or U	P-value	Median	Mean rank	[a]X^2or U	P-value
Inflammatory status of the liver	A0-A1 (n = 16)	0.100	9.81	U = 21	0.002*	0.037	11.25	U = 44	0.058	1.6200	10.25	U = 28.0	0.005*
	A2-A3 (n = 10)	22.200	19.40			46.347	17.10			249.509	18.70		
Fibrotic statusof the liver	F0-F1 (n = 3)	0.050	10.00	[a]X^2 = 6.44	0.040*	0.000	5.67	[a]X^2 = 8.442	0.015*	0.0009	6.33	[a]X^2 = 11.36	0.003*
	F2 (n = 18)	0.380	11.94			0.180	12.67			2.5964	12.00		
	F3-F4 (n = 5)	30.907	21.20			63.687	21.20			258.71	24.00		

X^2 = Kruskal-Wallis Test (Chi-Square value). U = Mann-Whitney U Test.
* $P < 0.05$: is significant but $P > 0.05$: is not significant.

between both the serum and the hepatic relative gene expression levels (which was one of the most salient outcomes of this study) indicated that the serum biomarkers are of hepatic source and they represent a true reflection of their liver mRNA level.

To the best of our knowledge, this study was the first to evaluate the serum levels of CTGF, IL-17 and TGF-ß1 in HCV in correlation to their hepatic genes relative expression, altogether in the relation to the inflammatory and fibrotic status of the liver, moreover with the effect of positive history of bilharzial infection.

Data reported in past years have provided convincing evidence that CTGF was a critical factor in developing hepatic fibrosis (Tong et al., 2009).

In the present study, the role of CTGF in HCV-induced liver fibrosis was investigated Its serum concentrations in HCV patients were significantly higher compared with the control group; this was proved with many other literatures as Kovalenko et al., 2009.

The relation of the serum levels of CTGF with stages of liver fibrosis and the grades of liver inflammation was also studied; they showed that CTGF levels were significantly elevated in advanced stages of fibrosis as evidenced also in many previous studies (Nagaraja et al., 2012; Manivannan et al., 2011). Weng et al., 2007, reported that CTGF production was triggered by transforming growth factor during hepatic fibrosis and was highly expressed in fibrotic tissues, inducing collagen synthesis.

On the other hand, there was no significant difference between the mild, moderate and severe grades of inflammation in relation to CTGF serum levels. This was in agreement with Piao et al., 2012, who found that the levels of CTGF were not correlated with the degree of inflammation. However this previous study was conducted on HBV patients not HCV. To the best of our knowledge, No previous studies examined the relation between CTGF in sera of HCV patients and inflammation grades to compare with, but from our results we assume that CTGF is not related to grades of inflammation.

When comparing the relative expression of CTGF gene of some of the HCV patients representing the same different inflammatory and fibrotic status, it gave us the same statistically results as those results of serum CTGF levels previously discussed. The results were significant to stages of fibrosis, but not with the grades of inflammation.

Table 6
Correlation between Serum levels and relative expression levels of CTGF, IL-17 and TGF-β1.

Biomarkers	IL-17 relative expression levels	CTGF relative expression levels	TGF-β1 relative expression levels
IL-17 serum levels	r = 0.475 P = 0.014*	–	–
CTGF serum levels	–	r = 0.603 P = 0.001*	–
TGF-β1 serum levels	–	–	r = 0.391 P = 0.048*

r = Pearson Correlation (parametric).
* $P < 0.05$: is significant.

Only few previous studies explored the relation between CTGF and bilharzial infection in chronic HCV patients. These studies showed that a high serum CTGF level was a useful biomarker for assessment of liver fibrosis and is a candidate marker for human schistosomiasis as well (Kovalenko et al., 2009). These previous studies are in line with our findings which revealed that serum CTGF levels were higher in HCV patients with bilharziasis than HCV patients with negative shistosomiasis (Manivannan et al., 2011).

Previous studies suggested that HBV virus infection itself does not cause liver injury directly. It is the host immune responses which are triggered by the invading viruses are considered to be blamable for the liver injury. Many studies have focused on virus specific T cells which are believed to be implicated in the liver damage under HBV infection (Rehermann and Nascimbeni, 2005).T-helper type 17 (Th17) cells are responsible for the inflammatory conditions which is always associated with liver fibrosis (Lemmers et al., 2009). IL-17 is the cytokine which is secreted by CD4 + Th17 cells, and is considered to be pro-inflammatory and pro-fibrotic (Miossec et al., 2009). Therefore; this study was performed to explore the role of IL-17 in HCV infected patients.

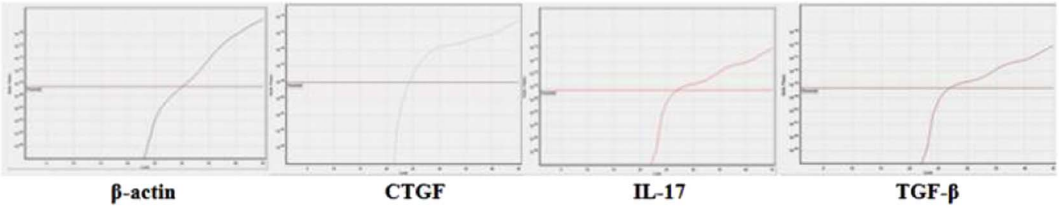

Fig. 2. Real time PCR amplification curves of β-actin, CTGF, IL-17 and TGF-β1.Y axis = Rn (Rox normalization) and the X axis = the number of the cycles, the threshold cycle (Ct) is the intersection between an amplification curve and a threshold line.

Serum IL-17 levels were significantly higher in chronic HCV patients when compared to controls and was in agreement with other literatures, (Fathy et al., 2011; Ghazy et al., 2012).The correlation between IL-17 serum levels and the grades of inflammation of the liver tissue biopsies of these patients was studied. A significant difference between the two groups of inflammation was detected, which confirmed that IL-17 played an important role in regulation of inflammation and this was in concordance with other studies (Tan et al., 2013).

When correlating the serum levels of IL-17 with the different stages of fibrosis, no significant difference was detected. Though one could assume that if Th17 is implicated in chronic HCV inflammation, the hallmark cytokine would be correlated with hepatic fibrosis, and it would be a potential hepatic fibrosis biomarker in cases of chronic HCV infection. Nevertheless, a study conducted on a large cohort of patients with varying degrees of fibrosis; was unable to find any significant association between fibrotic scores and serum IL-17 in chronic HCV patients (Lemmers et al., 2009).This was with an agreement of another study which found even an inverse correlation of liver fibrosis stage with HCV-specific IL-17 (Li et al., 2012).

As regards the effect of shistosomiasis, comparing the levels of IL-17 in the serum of HCV patients with previous bilharzial infection with those who had never been infected with shistisomiasis before, there was no statistically significant difference between the two groups. This is most likely because IL-17A is chiefly regarded as a pro-inflammatory cytokine and has been found to be related to numerous inflammatory diseases (Iwakura et al., 2008). Despite being reported to have a role in the Schistosoma induced pathological events in murine studies, (Rutitzky et al., 2008), a previous study showed the presence of less systemic IL-17A levels in S. haematobium infected people; compared to uninfected ones (Milner et al., 2010).

When comparing the hepatic relative expression of IL-17 gene, there was a detectable significant difference with the grades of inflammation as previously discussed with serum IL-17 levels. Surprisingly there was a statistically significant difference when comparing the expression level of IL-17 with the stages of fibrosis. This could be contributed to the small sample size examined in this study, though there were previous studies that support this finding (Tan et al., 2013; Hassan et al., 2014). They suggested that IL-17A was contributed to chronic hepatitis-induced liver fibrosis through two independent pathways: first, IL-17 stimulates Kupffer cells to express numerous inflammatory cytokines IL-6, IL-1β, and TNF-α, as well as the major fibrogenic cytokine TGF-β1; which in turn, induce activation of the Hepatic Stellate Cells (HSC) into myofibroblasts. Second, IL-17 directly induce HSCs to express collagen Type I thus, promoting their activation into fibrogenic myofibroblasts via the Stat3 signaling pathway. Moreover, it has been reported elsewhere that intrahepatic IL-17 expression was strongly correlated with the serum indices of hepatic fibrosis, which is considered a vital pathological process in the profression of liver cirrhosis (Woltman et al., 2000).

Transforming growth factor beta-1 (TGF-β1) is another key cytokine that is positively implicated in the development of liver inflammation and fibrosis. It participates in many important events leading to liver fibrosis, such as HSC activation, hepatocyte apoptosis, ECM production and expression of other profibrogenic intermediaries (Nagaraja et al., 2012; Kirmaz et al., 2004).

TGF-β1 is strongly related to the other two serum biomarkers studied here, as CTGF is often co-expressed with TGF-β1 in various fibrotic conditions (Nagaraja et al., 2012), TGF-β1 is considered a key upstream mediator of CTGF production. Moreover, TGF-β1 has s unique capacity to direct T cell lineage commitment to pro-inflammatory Th17 (Wahl, 2007).

In this study, we have investigated TGF-β1 serum concentrations and its hepatic expression levels in HCV patients in relation to degrees of liver inflammation and fibrosis. Serum levels were significantly higher in HCV patients compared with the control group; and were significantly elevated in relation to stages of fibrosis and grades of inflammation as evidenced also in many previous studies as Kamal et al., 2006.

Presser et al., 2013, revealed the molecular mechanism of TGF-β gene expression in reaction to HCV infection. They demonstrated that HCV-induced transcription factors AP-1, Sp1, NF-kB and STAT-3 were involved in TGF-β gene expression. They also demonstrated that HCV-induced TGF-β1 gene expression was mediated via the activation of certain cellular kinases such as p38 MAPK, Src, JNK, and MEK1/2. Furthermore, they determined the role of TGF-β1 in human (HSCs) activation and invasion. Moreover, Chusri et al., 2016 reported that TGF-β1, play salient roles in liver fibrogenesis through the generation of different reactive oxygen species (ROS).

Liver fibrosis is not an end point of therapy; Previous studies provided an excellent evidence in both human liver disease and animal models that hepatic fibrosis is potentially reversible (Ramachandran and Iredale, 2012). Iredale et al., 1998, reported that a well-established hepatic fibrosis can resolve to near normal liver architecture within 4–6 weeks. Analysis of histological changes occurring during fibrosis resolution reveals a rapid loss of those activated hepatic myofibroblasts, which are the principal scar-producing cells in the fibrotic liver, by apoptosis (Friedman, 2008).

In the present study, a significant decrease in CTGF, IL-17 and TGF-β1 in the patients responding to combination therapy after 12 weeks and 24 weeks of therapy was detected. It was correlated with the decline in the viral load as well, indicating that serial measurements of markers of fibrosis CTGF, IL-17 and TGF-β1 could predict regression of fibrosis throughout the course of treatment and reflect the inflammatory and fibrotic status of the liver.

In conclusion, the obtained results highlight the potential clinically useful value of a panel of cytokines and fibrosis markers that could help in determining the rate at which hepatic fibrosis is progressing or regressing, thus, replace or guide the use of liver biopsy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2017.05.003.

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