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Interleukin 17 levels used as diagnostic marker for following up hepatitis C virus treatment

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ABSTRACT

Background: Hepatitis C virus (HCV) causes significant morbidity and mortality worldwide. The infection induces up- regulation of cytokine and chemokine's commonly linked to the development of cellular and pro-inflammatory antiviral responses. The current standard in hepatitis C treatment consists of combination regimens of pegylated interferon-alpha plus ribavirin. ***Aim:*** This study was carried to follow up and monitoring the level of IL-17 in Hepatitis C infected patient before and after 120 days of treatment. ***Materials & Methods:*** Our study involved 120 HCV infected patients & 20 healthy persons; divided into three groups. Group I: IL-17 levels were measured in all subjects before and after 120 days of treatment. Also, Routine liver function tests were performed to all subjects included (aminotransferase (AST; alanine aminotransferase (ALT); Albumin; Total bilirubin; Direct Bilirubin, and alpha fetoprotein.

Results: our results demonstrated that; the level of IL-17 was increased in hepatic infected patients than the healthy group and also increased after 120 days of treatment, there is significance increased in other parameters as AST , ALT, AFP, while significance decrease in the level of Albumin. AUC (area under curve) value represents the combined effects of both sensitivity and specificity of Il-17 before and after treatments, as the sensitivity and specificity of it was 100% and 100%; respectively before treatment, was 70% and 100%; respectively after treatment. ***Conclusion:*** IL-17 was a sensitive & specific marker in the following-up and monitoring HCV patients treated with interferon and ribavirin.

Introduction

Hepatitis C virus (HCV) is highly morbid and mortal agents to nearly 3% of the World population infected by this virus (1). HCV is a leading cause of end-stage liver disease, and is

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the most common indication for liver transplantation. HCV nearly always recurs in liver-transplanted patients, and 10 to 25% of them develop cirrhosis within five to 10 years (2). The current standard in hepatitis C treatment consists of combination regimens of pegylated interferon-alpha (Peg-INF-alpha) with ribavirin (RBV). Such treatment regimens are quite successful in patients with HCV genotypes 2 and 3 infections, but they are much less effective in patients with genotypes 1 and 4 infections (3). The combination of Peg-INF-alpha with RBV therapy substantially improves the efficacy of HCV treatment by targeting several steps of viral replication and/or cellular pathways (4). However, the exact mechanism of action of these drugs is not yet well understood, neither is their impact on the host's immune response.Interleukin-17 (IL-17) is a prototype member of a new cytokine family with six species identified to date. IL-17 is secreted mainly by activated CD4+ and CD8+ T lymphocytes, while its receptor is distributed ubiquitously,IL-17 has been classified as a pro inflammatory cytokine because of its ability to induce the expression of many mediators of inflammation, most strikingly those that are involved in the proliferation, maturation and chemotaxis of neutrophils [(5)]. Increased levels of IL-17 have been associated with several conditions, including airway inflammation, rheumatoid arthritis, intraperitoneal abscesses and adhesions, inflammatory bowel disease, allograft rejection, psoriasis, cancer and multiple sclerosis. This review provides an overview of IL-17 activities, concentrating on viral hepatitis [(6)]. Pegylated interferons have been investigated by several investigators to treat acute hepatitis C. In 2005, Santantonio published on a cohort of 16 Italian patients with acute HCV treated with PEG-IFNa-2b for 24 weeks. Sustained clearance of HCV-RNA was observed in 15 patients (94%). Treatment was initiated 12 weeks after the clinical onset of hepatitis. The proportion of individuals infected with HCV genotypes 2 or 3 was high (63%).

The fairly good tolerability and the high sustained response rate to PEG-IFNa-2b treatment of acute hepatitis C was confirmed by the German Hep-Net Acute HCV-II study [(7)]. Possible sources of infections were medical procedures, IV drug abuse, and sexual exposure accounting for about three-fifths of cases. Sixty-six percent of patients were infected with HCV genotype 1 and maximum ALT levels before treatment ranged between 24 and 3,399 U/L (median 599). The median time from the most likely date of infection to start of therapy was 76 days and the time from the onset of symptoms to the start of therapy ranged between 5 and 131 days with a median of 27 days. Thus, therapy was started 1–2 months earlier than in most of the other recent studies on acute hepatitis C where treatment was usually delayed until 3 months after the patient first presented [(8)].

SUBJECTS, MATERIAL & METHODS

Materials: All chemicals used in these experiments were provided from Sigma Chemical Co. of high quality and purity.

Subjects: 140 subjects were included in this study, 105(75%) were males (mean age: 37 ± 9 years) and 35(25%) were females (mean age: 32 ±9 years) collected from the Hepatology section of the "ELAHRAR HOSPITAL", Zagazig, Egypt.

Approval of the study protocol, for both the scientific and the ethical aspects, was obtained from the Scientific Committee for Clinical Research of the participating hospital. Subjects were classified to three groups, Group (I) "healthy control group": consisted of 20 persons (13) males and (7) females (age range: 30–60 years), healthy not infected with hepatitis c virus (n = 20) served as negative control group. Group (II): comprised sixty persons with hepatitis C infection without any treatment. Group (III): sixty persons with hepatitis C infection undergoing 120 day of interferon & ribivarin treatment course (Peg-INF-alpha (1.5 µg/kg/week) plus RBV (1,000-1,200 mg/day according to weight) [(9)].

Samples: A blood sample was collected into tube before the beginning of the treatment. A second blood sample was obtained 120 days after treatment initiation. Serum was obtained after appropriate centrifugation and was immediately frozen at - 70°C until quantification of the immune mediator.

Methods

Viral load: HCV viral load was determined from serum using the COBAS® TaqMan HCV with the COBAS® AmpliPrep instrument (Roche®) [10]. The viral RNA load was measured before beginning of the treatment and after 120 days after treatment initiation.

Biochemical assays:

Liver Function Tests: liver functions (alanine aminotransferase (ALT) [11], Aspartate aminotransferase (AST) [12], total Bilirubin and Direct bilirubin [13], Albumin [14], and Alfa fetoprotein (AFP) [15]) were assayed.

Interleukin 17: IL-17 was measured by Elisa technique according to the method described by Toy. *et al.* [16]. ELISA Assay for IL-17 Detection in Serum the Human IL-17A (homodimer) Enzyme-linked immunosorbent assays (ELISA). The assay was performed according to the manufacturer's instructions. In brief, capture antibody was incubated overnight at 4_C. Wells were then washed and incubated for 1 h at room temperature in Assay Diluent. Serum samples were thawed overnight at 4_C then brought to room temperature. Next, 100 ll of standard or serum sample was added to each well, and incubated at 4_C overnight. Detection antibody was incubated for 90 min at room temperature. Wells were washed and Avidin-HRP was added and incubated at room temperature for 50 min. Wells were again washed, and 100 ll of substrate solution was added to each well and incubated at room temperature for 30 min. Then, 50 ll of stop solution (2 N sulfuric acid) was added to each well prior to analysis using Spectra MAX 250 instrument (Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro software (Molecular Devices) at 450 nm with subtraction of 570 nm wavelength.

Statistical Analysis: All results were analyzed by SPSS software (version 14). Data were expressed as mean ± SD. The student's t test was used for statistical analysis of differences between each two groups. Comparison of mean values of studied variables among different groups was done using ANOVA test. Pearson's correlation coefficient was used to quantify the relationship between the studied parameters. P<0.01 was considered to be significant [17].

Results

Table 1: summarized the laboratory data for patients before treatment compered to negative control. The mean activities of ALT, AST& ALP were found to be 30.2±8.5 U/l; 30.5±8.7 U/l; respectively in healthy group, while these activities were significantly increased to 67.9±56.8 U/l, 54.3±47.8 U/l, respectively; in infected HCV patients. Also; Total Bilirubin, Direct Bilirubin, AFP were found to be 0.95±0.16 mg/dl, 0.17±0.1 mg/dl, 4.2±1.5 U/ml; respectively in healthy group while these levels were increased in HCV patients (group before treatment) to 0.85±0.63 mg/dl, 0.20±0.26 mg/dl, 6.8±7.9 U/ml; respectively. While, the mean level of albumin in patients before treatment showed non-significant reduction (4.3±0.56 g/dl) compared to healthy group (4.8±0.41g/dl). The mean levels of IL-17 in healthy group were 51.2±12.3 (pg/ml) while these levels were significantly increased in the group before treatment to 183.4±96.3 (pg/ml); (p< 0.0001).

Table 2: Summarize the laboratory data for patients after treatment and control. The mean activities of ALT, AST were decreased to 57.2±42.7 IU/l, 48.8±34.7 IU/l; respectively compared to their activities before treatment. Also Total Bilirubin, Direct Bilirubin, AFP levels were non-significantly decreased to 0.82±0.60 mg/dl, 0.19±0.1 mg/dl, 5.3±4.5 U/ml; respectively compared to their levels before treatment. While, after treatment, albumin levels recorded a slightly increase to 4.6±0.45 compared to its concentration before treatment. On the other hand, Il-17 levels were demonstrated a significant reduction to 143.1±48.5 (pg/ml) compared to its levels before treatment (p value; 0.005).

ROC of IL-17 for differentiated between healthy individuals and HCV patients before and after treatment*:* the levels of Il-17 were higher than normal values in all serum samples of HCV-infected patients

(before treatment) with sensitivity (100 %), and with specificity (100%) as area under the ROC curve (AUC) =1.0 ($p< 0.0001$), Fig. (1). While after treatment, its sensitivity (70 %), and with specificity (100%) as area under the ROC curve (AUC) =0.97 ($p< 0.0001$), Fig.(2). So, the efficiency of Il-17 for discriminating HCV-patients after treatment from those before treatment was 73%. The area under ROC curve of Il-17 for discriminating patients after treatment from those before treatment was 0.76 ($P <0.0001$) fig. (3).

DISCUSSION:

Hepatitis C virus (HCV) is one of the most important etiologic agents of postransfusional hepatitis and a common cause of chronic hepatitis, cirrhosis and hepatocarcinoma. In the early phase of acute infection, HCV continues to replicate in the liver, overcoming innate and acquired immunity and therefore usually transforms into a chronic infection. Among 60 - 80% people infected with HCV develop chronic hepatitis that leads to end-stage liver disease in about 20% and hepatocellular carcinoma in 10– 15% [(17)]. Both effector subsets and regulatory Lymphocytes are found in the liver of chronic HCV patients, and the balance of the cells recruited will determine the severity of the liver disease. The naïve CD4 T cell is a multipotential precursor with defined antigen specificity, but substantial plasticity distinguishes effector or regulatory lineages. The most important lineages are T helper (Th) Th1, Th2, Th17 and Th9 effector cells and anti-inflammatory regulatory T cells (Tregs). While the underlying mechanisms for HCV disease pathogenesis are not fully understood, a role for Th17 immunity has been proposed based on the finding of Th17 cells in other hepatic viral diseases. The elevation of many parameters as IL-17, ALT, AST, Bilirubin, AFP in patient due to the fibrosis and cirrhosis of the liver of chronic patient In both chronic Hepatitis B (HBV) and Hepatitis C Virus (HCV) infections, reports indicate a close correlation between virus-induced liver inflammation, infiltration and activation of Th17 cells and the amount of liver damage caused by the antiviral immune response For instance, a close correlation between liver infiltrating as well as circulating Th17 cells and the amount of liver damage has been shown in chronically infected Hepatitis B patients. A shift from Th1 to Th17 seems to be potentially disadvantageous for the patient in terms of antiviral defense and liver disease progression, since stronger Th17 responses are associated with higher viral plasma load, increased levels of serum transaminases, and enhanced activation of blood monocytes as well as liver macrophages. Antigen-specific responses of virus-specific Th17 cells have been described for both HBV and HCV, leading to similar pathophysiological Changes in both infections [(18)].

For HBV, HBcAg especially has been shown to be one of the key Th17- inducing antigens, leading to an IL-17R-induced activation of macrophages and monocytes followed by upregulation of CD86, B7H1, B7DC, and CD83 and also cytokines such as IL-1β, IL-6, TNFα, IL-23p19 and IL-12p35. HCV-RNA can be detectable in serum within 3–7 days after exposure. HCV-RNA levels rise rapidly during the first weeks followed by a rise of serum aminotransferases 2–8 weeks after exposure. The elevation of serum alanine aminotransferase (ALT) indicating hepatic injury, inflammation, and necrosis commonly may reach levels greater than 10 times the upper limit of normal. Unfortunately, the serological development of acute HCV infection is accompanied by clinical symptoms only in a minority of cases. Jaundice occurs in only 20–30% of patients, mostly between 2 and 12 weeks after infection [(19)]. More commonly, nonspecific symptoms, such as fatigue, low-grade fever, myalgia, nausea, vomiting, or itching, are clinical correlates of the infection leading to high rates of unrecognized cases in the acute phase of the disease. It is quite well established that patients are more likely to recover spontaneously if they are symptomatic. Thus, patients with more severe hepatitis may require less stringent therapies and the natural course of the infection can be monitored for some time before treatment is initiated. Importantly, in none of the studies conducted

to date was HCV genotype clearly associated with treatment outcome. This is significantly different than what is observed in patients with chronic HCV infection.

Interleukin (IL)-17-producing T-helper (Th) 17 cells have been reported to trigger tissue inflammation and damage and there is accumulating evidence that Th17 cells are important contributors to hepatic inflammation and liver cirrhosis [(20)]. IL-17 is produced by monocytes/DCs through recognition of viral pathogen-associated molecular pattern (PAMP)

such as Toll-like receptor (TLR)3 ligands [(21)]. In addition to the ability of HCV to trigger the TLR3 pathway [(22)]. The increased number of Th17 cells appears to be associated with the severity of liver inflammation in chronic HCV patients As one of the crucial factors for Th17 differentiation, thymic stromal lymphopoietin (TSLP), a member of the common c-chain cytokine, is capable of activating (conditioning) DCs, thereby stimulating naive T cells to differentiate into Th2 cells.12 In addition, DCs treated with both TSLP and poly (I: C) activate naive T cells and differentiate into Th2 and Th17 cells [(23)]. Thus, TSLP-activated DCs, which are known to be strong inducers of Th2 responses, can simultaneously induce Th17 cells under certain pathological conditions.

AFP is a fetal protein that is not normally present in the serum of adults and is commonly used as a tumor marker for hepatocellular carcinoma (HCC). However, serum AFP is also elevated during pregnancy and in chronic hepatitis patients [(24)]. In this study, a considerable number of type C chronic hepatitis and compensated cirrhosis patients demonstrated persistently elevated AFP levels in the absence of HCC. In addition, the AFP level decreased significantly after IFN treatment. Changes in alanine aminotransferase (ALT) after treatment. Paired *t*-test was used. $*P < 0.05$ was regarded as significant. Furthermore, the AFP decrement was universally observed regardless of treatment response to IFN therapy. Transient AFP elevation has been observed after a rise in transaminase in acute hepatitis and fulminant hepatitis [(25)]. This type of AFP elevation is explained as a result of hepatocyte regeneration accompanied by necroinflammatory change.

AFP production is supposed to regulate the transcription level of hepatocytes [(26)]. Among HCV-infected patients, the HCV-coding core protein is regarded to be one of the proteins responsible for hepatocarcinogenesis, up-regulating several molecules resulting in activation of the cell cycle and cell proliferation at the transcriptional level in hepatocytes [(27)]. The HCV-coding core protein may also up regulate AFP production at the transcriptional level. In contrast, IFN is considered to down-regulate cell cycle progression at the transcriptional level and induce apoptosis via the IFN receptor-mediated JAK-STAT signaling pathway [(28)]. This competing action of IFN against HCV-related protein may be a direct anticancer mechanism that inhibits HCC. Many reports have cited elevated AFP baselines as an independent HCC risk factor [(29)] along with age, gender, liver histology stage, and ethnicity in HCV-infected patients.

Conclusion:

In was concluded that, IL-17 may be used as diagnostic marker for following and monitoring patient perform HCV treatment as it is high specific, sensitive & sheep method.

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Table 1: Laboratory data of healthy individuals and patients before treatment

Variables	Mean ± S.D		P value [b]
	Healthy (n= 20)	Before treatment (n = 60)	
AST (U/ml) [a]	30.5±8.7	54.3±47.8	0.006
ALT (U/ml) [a]	30.2±8.5	67.9±56.8	0.023
Total Bilirubin (mg/dl)[a]	0.95±0.16	0.85±0.63	0.531
Direct Bilirubin (mg/dl)[a]	0.17±0.1	0.20±0.26	0.590
Albumin (g/L) [a]	4.8±0.4	4.3±0.56	0.002
AFP (U/ml) [a]	4.2±1.5	6.8±7.9	0.148
IL-17 (pg/ml) [a]	51.2±12.3	183.1±96.3	< 0.0001

[a] References values: Aspartate aminotransferase (AST) up to 40 U/L; alanine aminotransferase (ALT) up to 45 U/L; Albumin 3.8-5.4 g/L; Total bilirubin: up to 1 mg/dl; Direct Bilirubin up to 0.25 mg/dl and alpha fetoprotein up to 10 U/ ml; [b]p > 0.05 is considered non-significant; P <0.05 is considered significant.

Table 2: Laboratory data of healthy individuals and patients after treatment

Variables	Mean ± S.D		P value [b]
	Before treatment (n = 60)	After treatment (n = 60)	
AST (U/ml) [a]	54.3±47.8	48.8±34.7	0.472
ALT (U/ml) [a]	67.9±56.8	57.2±42.6	0.241
Total Bilirubin	0.85±0.64	0.82±0.60	0.373

(mg/dl)[a]			
Direct Bilirubin (mg/dl)[a]	0.20±0.26	0.19±0.10	0.811
Albumin (g/L) [a]	4.3±0.56	4.6±0.45	
AFP (U/ml) [a]	6.8±7.8	5.3±4.5	0.192
HCV-RNA (U/ml)	727,189±134,8230	375,416± 731,834	0.153
IL-17 (pg/ml) [a]	183.1±96.3	143.4±48.5	0.005

[a] References values: Aspartate aminotransferase (AST) up to 40 U/L; alanine aminotransferase (ALT) up to 45 U/L; Albumin 3.8-5.4 g/L; Total bilirubin: up to 1 mg/dl; Direct Bilirubin up to 0.25 mg/dl and alpha fetoprotein up to 10 U/ ml; [b] p > 0.05 is considered non-significant; P <0.05 is considered significant.

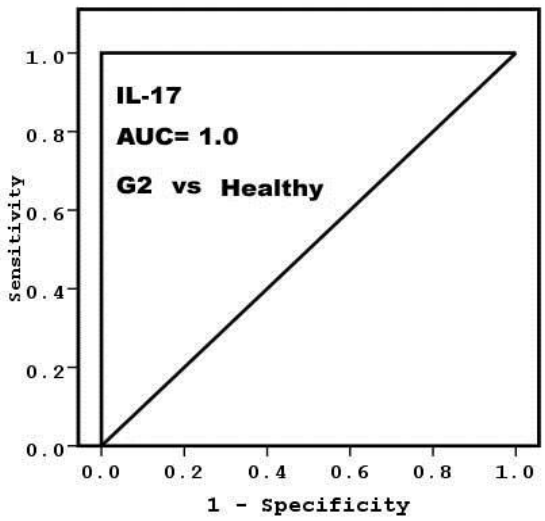

Figure (1): ROC of IL-17 for differentiated between healthy individuals and HCV patients before treatment.

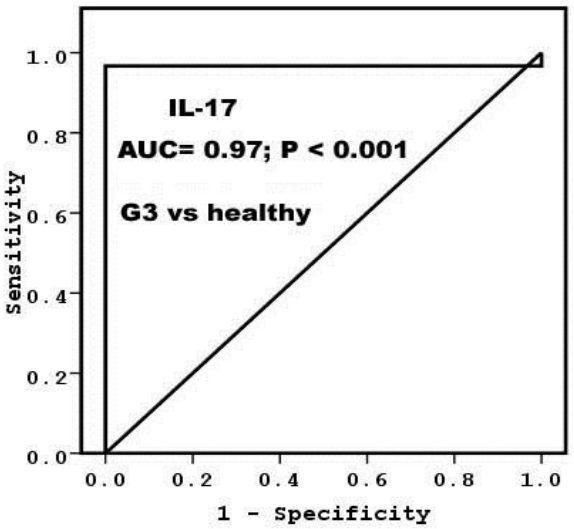

Figure (2): ROC of IL-17 for differentiated between healthy individuals and HCV patients after treatment.

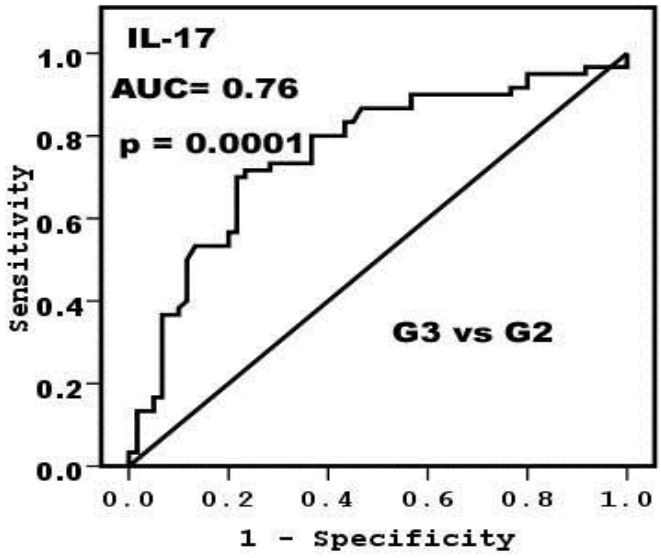

Figure (3): ROC of IL-17 for differentiated between HCV patients before treatment and HCV patients after treatment. The true positive rate (sensitivity) is plotted as a function of the false rate (1−specificity). Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. AUC (area under curve) value represents the combined effects of both sensitivity and specificity.