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Some biochemical markers and expression of indoleamine-2, 3-dioxygenase in Egyptian patients with chronic Hepatitis C

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Abstract

Hepatitis C virus (HCV) is a main cause of chronic hepatitis and it may leads to cirrhosis, hepatic failure and hepatocellular carcinoma. Chronic HCV patients are subjected to treatment with ribavirin and interferon-α (IFN-α) but,which have achieved only limited success. The main aim of this study was to measure indoleamine-2, 3-dioxygenase (IDO) in chronic HCV patients which could explain the failure from therapy. Five ml of peripheral blood were collected from 30 patients with chronic HCV infection and 10 healthy control volunteers. Patients were categorized in to responders and non-responders according to viral titre upon IFN-α treatment. The levels of IDO were measured in the sera of the recruited subjects. Significant increases ($P<0.001$) in the concentration of IDO were observed in IFN-α non-responder patient when compared with responders and healthy control. Conclusion: Non-responsiveness of chronic HCV patients to IFNs based therapy associated with increases in suppressive mechanisms, opening a new avenue for targeting these molecules in HCV therapy.

Key words: Hepatitis C virus (HCV); Immune suppression; IFN-α; ribavirin; Indoleamine 2, 3- dioxygenase (IDO).

1 Introduction

Hepatitis C virus (HCV) infection is one of the major causes of liver diseases(Mohd Hanafiah et al., 2013; Afdhal et al., 2014). HCV estimated 170-200 million infected persons globally, while Egypt has the rising rate of HCV infection, ranging from 6 to 28%, with an average of approximately 13.8% in the general population(Mohamoud et al., 2013; Cuadros et al., 2014). Since, the discovery of the virus, the backbone drug used in all antiviral protocols was interferon-α (IFN- α), that attains viral clearance in 40% of the patients while 60% fail this therapy, which does not attack the virus directly but stimulates the immune system and activated it to become more efficient to eliminate the virus(Trembling et al., 2013; Vasudevan and Lubel 2015). Recently, it has been discovered drugs such as sovaldi, which act directly on the virus life cycle to prevent proliferation of the virus, by attacking enzymes necessary to manufacture the parts of the virus or prevent it from entering the liver cell or block the necessary proteins required for the formation of the virus (Dhingra et al., 2014).

Tryptophan has been considered as the main amino acid for proliferation and activation of T- cells, in particular various cells as macrophages and multiple malignant cells have been found to express high levels of Indoleamine 2, 3 dioxygenase (IDO) (Yu et al., 2013). IDO is a heme containing, immunosuppressive enzyme that catalyse tryptophan to kynurenine(Platten et al., 2012; Lepiller et al., 2015; Asghar et al., 2015). Interferon-γ (IFN-γ) cooperates with tumor necrosis factor (TNF), lipopolysaccharide (LPS) and interleukin-1 (IL- 1) to increase IDO expression [(Yeung et al., 2012; Asghar et al., 2015). Various types of tumors expressed high levels of IDO which in turn prevent local T-cell dependent anti-tumor immunity and as a result promote tumor growth (Yu et al., 2011). Furthermore, IDO may inhibit the immunity of T-cells by stimulating both differentiation and maturation of CD4+CD25+ regulatory T cells. As such IDO has been implicated in modulation of disorders cancer and autoimmune diseases (Yu et al., 2011). Recent studies showed a positive correlation between tumor-induced immunosuppression and IDO (Yu et al.,

2013). The suppressive effects of IDO is exerted via increase in suppressive metabolites at the tumor site as well decreasing the level of tryptophan (Yu et al., 2013).

Our study aimed to measure the expression of IDO in a population of Egyptian patients with chronic HCV in both responders and non-responders to IFN-α and ribavirin.

2 Materials and Methods

2.1 Subjects:

The research study was approved by the local ethics committee, Faculty of Medicine, Tanta University and informed consent was obtained from all patients before participation.The study was conducted among thirty patients with chronic HCV infection with a mean age of 44 ± 4.5.We also recruited healthy volunteers with a mean age of 39.5 ± 4.51.Patients were recruited from the Tropical Medicine & Infectious Diseases Department, Tanta University (Tanta, Egypt). Pegylated IFN-α (long acting interferon; Pegassys or Peg Intron) once every week for 48 weeks plus daily treatment with 800-1200mg ribavirinis considered the main treatment protocol which applies to the patients enrolled in this study.According to the clinical response, if no response occurs the treatment should stopped after 24 weeks from beginning the treatment course.

1.2. Inclusion and exclusion criteria:

The inclusion criteria of HCV patients before IFN- α treatment included the history of HCV infection and high levels of ALT. The exclusion criteria included co-infection or super infection with other hepatitis viruses.

1.3. Measurement of viral load:

Patients who were determined by positive for HCV by measuring HCV RNA level in their plasma using the transcription-mediated amplification quantification or real time PCR HCV quantification kits(Comanor et al., 2001)

1.4. Detection of plasma human IDO:

IDO concentrations in plasma were quantified by sandwich ELISA technique using (Ray Biotech) human IDO ELISA kit. All reagents, samples and standardswere prepared according to the recommended by the manufacture. Then, (100µl) standard and samplewere added to each well and incubated for 2 hours at 37°C. After that, (100 µl) prepared detection reagent A was aspirated, added and incubated for 1 hour at 37°C. Then,prepared detection reagent A was aspirated and washed 3 times. After that, (100 µl) prepared detection reagent Bwas added and incubated for 1 hour at 37°C. Then,prepared detection reagent B was aspirated and washed 5 times. After that, (90 µl) substrate solutionwas added and incubated for 15-25 minutes at 37°C. Finally, (50 µl) stop solution was added. Then, read immediately at 450nm.

1.5. Biochemical analysis:

Liver functions were assessed by measured serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, serum albumin were purchased from (Diamond)**(Shaker et al., 2010)**.

1.6. Statistical analysis:

According to the viral titer the CHC patients were divided into groups; responders and non-responders. The clinical data were collected along the study and analyzed for each patient, each value was calculated as the mean ± SD. Results were analyzed using a paired Student's t-test. *$P \leq 0.05$ (P values below 0.05 were considered significant); significant differences were performed with a one way analysis of variance (ANOVA). Linear regression test was used to detect whether there is a correlation between the two quantitative variables.

3 Results

IDO expression in chronic HCV patients:

Significant increases in the levels of IDO were found in IFN-α non- responderCHC patients when comperd to responder patients ($P< 0.001$) and healthy voulenteers ($P< 0.01$); 41.6 ± 3.1, 31.8 ± 3.1 and 19.6 ± 0.5, respectively as shown in **Table 1** and **Figure 1**.

Complete blood count (CBC) analysis in chronic HCV patients:

In **Table 2** significant decrease ($P< 0.05$) in the total numbers of white blood cellswere found in IFN-α responders and non- responder CHC patients when compared with the controls; 5.7× 10 3 ± 0.5× 10 3, 5.9× 10 3 ± 0.3× 10 3 and 7.4× 10 3 ± 0.4× 103, respectively as shown in **Figure 2A**. Also, significant decrease ($P< 0.05$) in the percentage of granulocytes were found in IFN-α responders and non- respondersCHC patients as compared with healthy controls; 46.6± 3.2, 47.7± 3.4 and 60.8± 2.8, respectively as shown in **Figure 2D**. In contrast, the increases in the percentage of monocytes and lymphocyteswere measuredin IFN-α responders and non-responderCHC patients when compared with healthy controls; 5.8 ± 0.2, 5.0± 0.3 and 4.2± 0.5, respectively for monocytes and 40.3± 3.9, 39.9± 2.7 and 29.6± 2.0, respectively for lymphocytes as shown in **Figures 2 (C, B)**.

Liver functions in chronic HCV patients:

In **Table 3**the increases in ALT and AST activitieswere foundin IFN-α responders and non-responderCHC patients when compared with healthy controls of note there were no significant differences (31.4± 6.2, 29.9± 4.8 and 25.1± 2.1) and (37.2± 4.5, 33.2± 4.9 and 27.1± 1.1), respectively as shown in **Figure 3 (A, B)**. However, total bilirubin levels were significantly increased ($P< 0.05$) in IFN-α non-responderCHC patients as compared to control volunteers; 0.9± 0.1 vs. 0.7± 0.1, respectively as shown in **Figure 3C**. In addition, albumin levels were decreased in IFN-α responder and non-responder CHC patients when compared with healthy controls; 3.4± 0.2, 3.5± 0.2 and 3.9 ±0.1, respectively as shown in **Figure 3D**.

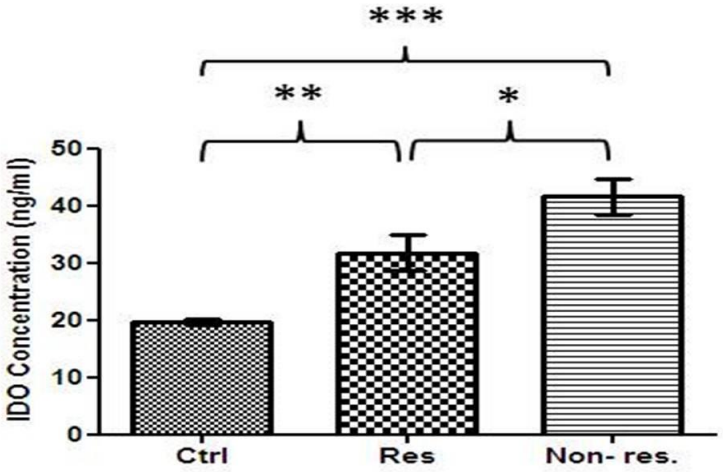

Figure 1

Figure 1: IDO Concentration in chronic HCV patients treated with IFN-α and ribavirin. Blood were collected from (n= 15) patients with chronic HCV respond to IFN-α and ribavirin treatment, (n= 15) patients with chronic HCV didn't respond as well as (n=10) samples for healthy control volunteers. * means $P< 0.05$: compared between responder and non-responder, ** means $P< 0.01$ and *** means $P< 0.001$: compared with control.

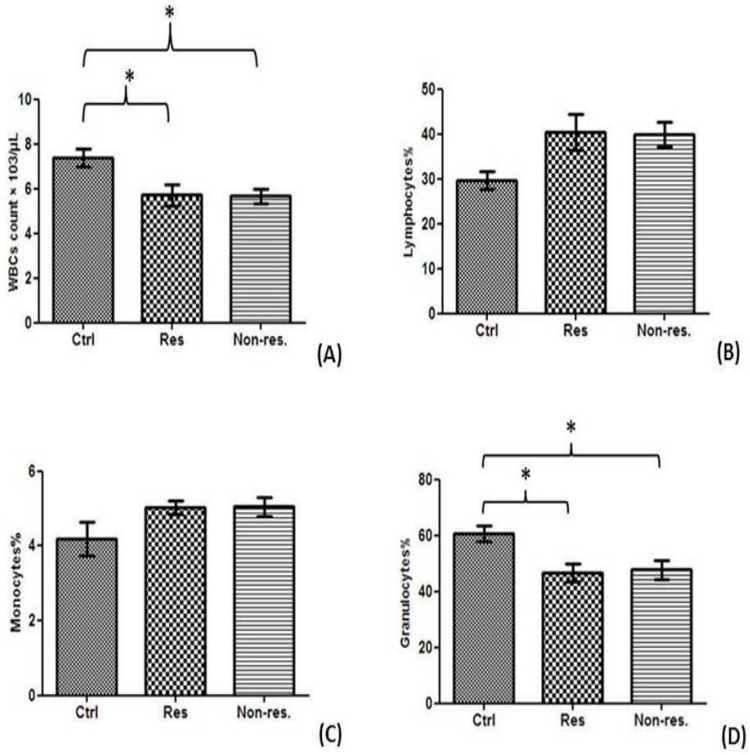

Figure 2

Figure 2:(A) representative average count of total WBCs, **(B)** percentages of lymphocytes, **(C)** percentages of Monocytes and **(D)** percentages of Granulocytes where blood were collected from healthy donors (n=10), patients with chronic HCV respond to IFN-α and ribavirin treatment (n= 15), and patients with chronic HCV didn't respond to treatment (n=15). * means $P< 0.05$: compared with control.

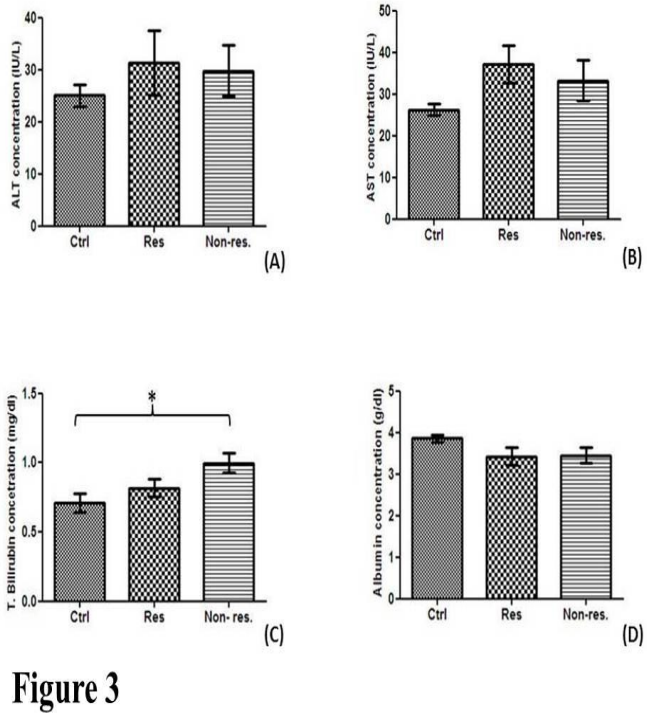

Figure 3

Figure 3: Liver function parameters in CHC patients treated with IFN-α and ribavirin. **(A)** ALT levels, **(B)** AST levels, **(C)** Total bilirubin levels, **(D)** Albumin levels. Blood were collected from healthy donors (n=10), patients with CHC patientsrespond to IFN-α and ribavirin treatment (n= 15), and CHC patientsdidn't respond to treatment (n=15). * means $P< 0.05$: compared with control

Table 1: Comparison between CHC patient groups; responder and non-responder versus control group as regard to IDO concentration

IDO Concentration (ng/ ml)	CHC patients		Healthy control
	Responders	Non-Responders	
Range	46.19 to 23.29	55.07 to 29.60	17.76 to 21.16
Mean ±SD	31.75 ± 3.122	41.55 ± 3.056	19.60 ± 0.458
P value	*P*<0.01	*P*<0.001	

Table 1

Table 2: Comparison between CHC patientsgroups; IFN-α responder and non- responder versus control group as regard to WBCs count, lymphocytes%, monocytes% and granulocyte%

WBCs count (×10³/μL)	CHC patients		Healthy control
	Responders	Non-Responders	
Range	3.321 to 6.792	2.781 to 5.523	3.678 to 9.510
Mean ±SD	5.712 ± 0.4558	5.663 ± 0.3241	7.390 ± 0.4045
P value	*P*<0.05	*P*<0.05	
Lymphocytes %	CHC patients		Healthy control
	Responders	Non-Responders	
Range	17.2 to 52.2	27.0 to 58.6	20 to 48
Mean ±SD	40.35 ± 3.946	39.91 ± 2.677	29.60 ± 2.007
P value	*P*>0.05	*P*>0.05	
Monocytes %	CHC patients		Healthy control
	Responders	Non-Responders	
Range	3.9 to 4.6	3.3 to 5.3	2 to 10
Mean ±SD	5.039 ± 0.1851	5.049 ± 0.2602	4.190 ± 0.4537
P value	*P*>0.05	*P*>0.05	
Granulocytes %	CHC patients		Healthy control
	Responders	Non-Responders	
Range	24.80 to 40.20	23.5 to 39.7	46 to 80
Mean ±SD	46.57 ± 3.238	47.71 ± 3.397	60.80 ± 2.768
P value	*P*<0.05	*P*<0.05	

Table 2

Table 3: Comparison between CHC patients groups; IFN-α responder and non- responder versus control group as regard to ALT (U/L), AST (U/L), total bilirubin concentration (mg/dl) and albumin concentration (g/dl).

ALT (U/L)	CHC patients		Healthy control
	Responders	Non-Responders	
Range	24 to 82	27 to 79	16 to 38
Mean ±SD	31.37 ± 6.179	29.81 ± 4.836	25.10 ± 2.111
P value	*p*>0.05	*p*>0.05	
AST (U/L)			
Range	18.7 to 76	22 to 70	19 to 33
Mean ±SD	37.17 ± 4.488	33.19 ± 4.870	27.04 ± 1.036
P value	*p*>0.05	*p*>0.05	
Total bilirubin (mg/dl)			
Range	0.52 to 1.0	0.43 to 1.33	0.2 to 1.2
Mean ±SD	0.818 ± 0.0667	0.998 ± 0.0691	0.710 ± 0.0706
P value	*p*>0.05	*P*<0.05	
Albumin level (g/dl)			
Range	3 to 3.8	3.1 to 4.2	3.5 to 5.5
Mean ±SD	3.427 ± 0.207	3.453 ± 0.194	3.860 ± 0.08655
P value	*p*>0.05	*p*>0.05	

Table 3

4 Discussion

To shed a light on some of the mechanisms associated with the failure of chronic HCV patients to IFN-based therapy, we measured the expression of IDO, as well the total numbers of blood leukocytes and granulocytes in both responder and non-responder chronic HCV patients who were treated with interferon and ribavirin. Overall, we found increases in IDO in patients regardless the viral response to IFN/ribavirin therapy. Interestingly, the non-responders showed higher IDO when compared to responders. Taken together, these data indicate to the presence of immunosuppressive mechanisms in HCV patients in general and in non-responders in particular. These data are of significant importance to the therapeutic approaches of HCV since it opens a new avenue to utilize or design drugs that can target these molecules as adjuvant therapy with the conventional therapy of HCV.

IDO is an immune regulatory enzyme which plays a critical role in different viral infection, human malignancies and autoimmune diseases. It enhances the immunosuppressive environment which in turn can lead to liver cirrhosis these findings are in agreement with(Barjon et al., 2015; Mehraj and Routy 2015; Asghar et al., 2015). As such, the increases in the levels of IDO in HCV patients in general than in controls and in IFN-α non-responders patients than responders would explain, at least in part the failure of chronic HCV patients to respond to IFN-α.

Previous studies have shown that overexpression of IDO enhance the expansion and function of Treg and suppress T-cell proliferation via tryptophan depletion at the onset of the viral infection(Mellor and Munn 2004; Hill et al., 2007; Sharma et al., 2007; Perrella et al., 2009).

Our findings are in line with in vivo pre-clinical studies that showed a transient increase in the level of hepatic IDO in the acute HCV infection in chimpanzees that eliminate HCV infection spontaneously (Iwamoto et al., 2009). Moreover, high levels of IDO was detected in HCC and were significantly associated with the frequency of liver metastases(Ishio et al., 2004; Pan et al., 2008; Asghar et al., 2015). In addition, this observation was confirmed by brandacher et al(Brandacher et al. 2006)who suggest that high levels of IDO expression participate to the metastasis of colon and endometrial cancer.

(Lepiller et al. 2015) suggest that at the commencement of HCV infection, the high level of IDO may play a role in the innate antiviral immune response by retarding the replication of HCV.

Finally, we found a significant decrease in total white blood cells and granulocytes percentage while we found increased levels of monocytes and lymphocytes percentage in IFN-α responder and non-responder HCV patients regardless their response to the treatment during the treatment course. This decrease may be due to chronic HCV patients who subjected to treatment with interferon and ribavirin developed leukopenia and neutropenia, because of the inhibitory effect of IFN-α on erythropoietin and the bone marrow, these results are consistent with other(Olariu et al., 2010; Striki et al., 2014; Jadoon et al., 2015).

This study demonstrated that an increase in both ALT and AST levels in IFN-α responder and non-responder patients. The infection with HCV leads to attack of the liver then make damaged to liver cells which considered one of the main causes of elevated liver enzymes, these results are in accordance with(Sarwar and Tarique 2010; Khalil and Hassan 2012). Our results indicated that there was a slight decrease in albumin levels in IFN-α responder and non-responder which is due to HCV infection and liver failure which in turn leads to decrease the levels of albumin in the blood which leads to edema and ascites since, the liver considered the only source for producing albumin in the body, these consistent with other(Umar and DiBaise 2010; Lee 2012). In addition, there was a significant increase in total bilirubin in IFN-α non- responder patients when compared with IFN-α responders and healthy control. Furthermore, the up regulation of bilirubin levels in blood may be due the liver is not functioning correctly and may lead to scarring of the liver or cirrhosis, these results are in accordance with(Sarwar and Tarique 2010; Khalil and Hassan 2012). Our findings suggested that the expression of IDO and some biochemical markers that have immune suppressive function can be reversed and enhance responsive of HCV patients to interferon- α and ribavirin.

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