

Full Length Research Paper

## Hepatitis C virus pathogenesis: Serum IL-33 level indicates liver damage

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Hepatitis C virus (HCV) is one of the major causes of chronic liver disease in the world. Majority of infected individuals are unable to clear the virus and as consequence of infection, cirrhosis and hepatocellular carcinoma can be developed in later stages. Immune system cells secrete cytokines which play a crucial role in intercellular communication system responsible for immune response. Interleukine-33 (IL-33) is a new member of the IL-1 family. It plays a key role in Th2-cells differentiation, activating mast cells and promotes dendritic cells development in bone marrow culture. IL-33 Pro-inflammatory properties are detrimental in inflammatory bowel disease (IBD), allergic contact dermatitis and other several experimental models of inflammation. In this study we examined the concentrations of serum IL-33 in patients with chronic hepatitis C (CHC) and healthy controls (C) to evaluate the potential role of IL-33 in the pathogenic process of CHC. We determined the concentrations of serum IL-33 and its correlation to the HCV virus activity and the degree of liver fibrosis. We indicated the liver viability by measuring liver enzymes and serum zinc levels. We found that IL-33 response is correlated positively to the increase of liver enzymes and degree of liver fibrosis which indicate its important role in the pathogenesis of CHC and its association with the severity of liver injury in CHC patients.

**Key words:** Hepatitis C virus, interleukine-33, liver damage.

### INTRODUCTION

Hepatitis C virus (HCV), a positive-strand RNA hepatotropic virus, is one of the major causes of chronic liver disease in the world (Alberti et al., 1999; Lauer and Walker, 2001). More importantly, 70–80% of acutely infected individuals unable to clear the virus eventually develop persistent chronic infection (Rehermann and Nascimbeni, 2005; Jacobson et al., 2010). In later stages of infection

cirrhosis and hepatocellular carcinoma can be developed (Crockett and Keeffe, 2005). The virus, hepatocytes and the host immune system interaction is the cause of chronic inflammation (Ciborowski and Gendelman, 2006) which result in immune system suppression as a consequence of hepatocyte injuries (Martini et al., 2005).

Immune system cells secrete cytokines which are small

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**Abbreviations:** HCV, Hepatitis C virus; IL-33, Interleukine-33; CHC, chronic hepatitis C; C, healthy controls; ALT, Alanine transaminase enzyme; AST, Aspartate aminotransferase; Th, T-helper cells.

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**Table 1.** Clinical features and Demographic characteristics of cases.

Parameter	CHC	C
Number	87	15
Age (years)	40 (22-60)	42 (30-55)
Sex (M/F)	45/ 42	9/6
Viraemia	6.27	NA
(Log copies /ml)	(3.11-6.7)	
Anti-HCV	Positive	Negative
AST(IU/L)	49.5 (11-123)	33.8 (18-65)
ALT(IU/L)	84.2 (21-301)	40 (25-57)

Normal Values (NA): ALT  $\leq$  50IU/L; AST  $\leq$  40 IU/L. Data were expressed as median and range

soluble proteins and play a crucial role in intercellular communication system responsible for immune response. These proteins act by binding to specific cell receptors that may either induce or inhibit cytokine production. Cytokines play a major role in both viral clearance or tissue damage during viral infection, (Steinke and Borish, 2006). Adaptive immune response to HCV is controlled by CD4<sup>+</sup> T cells that provide help in activation of both humoral and cellular responses. They can secrete Th1-cytokines and also may release Th2-cytokines, which limit Th1 cytokine-mediated response and prefer the development of humoral response (Moser and Murphy, 2000; Day Lauer et al., 2002).

Interleukine-33 is the most recently discovered member of the IL-1 family (Schmitz et al., 2005). IL-33 is synthesized as a 30000 molecular weight precursor protein and exhibits structural similarity to IL-18. The IL-33 receptor has recently been discovered, it has a structure analogous to other IL-1 cytokine receptors (Chackerian et al., 2007). IL-33 Plays a key role in Th2-cells differentiation and activation of mast cells which will lead to production of Th2 cytokine and Th2 response as well as mucosal and pulmonary Th2 inflammation (Schmitz et al., 2005). IL-33 promotes dendritic cells development in bone marrow culture (Mayuzumi et al., 2009). IL-33 proinflammatory properties are detrimental in several experimental models of inflammation, IL-33 plays a dichotomous role in inflammatory bowel disease (IBD), However, IL-33 enhances inflammatory responses, it promotes bowel epithelial integrity (Pastorelli et al., 2013). IL-33 Is Induced by Tumor Necrosis Factor alpha and Interferon gamma in Keratinocytes and participate in allergic contact dermatitis (Taniguchi et al., 2013).

It was found that the levels of serum IL-33 were elevated in autoimmune diseases as Systematic lupus erythematosus and Rheumatoid Arthritis patients (Alves-Filho et al., 2010). IL-33 is becoming a central molecule in allergic asthma that addresses various cascades of both arms of immune response that lead to inflammation

in the lung (Eiwegger and Akdis, 2011). Moreover, IL-33 has been found to be an important factor of the pathogenesis of some viral infections as HIV infection, dengue virus infection and HCV infection (Becerra et al., 2008; Miyagaki et al., 2011; Wang et al., 2012).

In this study we examined the concentrations of IL-33 and zinc levels in the serum as indicator of the viability of the liver in patients with chronic hepatitis C (CHC) and healthy controls (C) to evaluate the potential role of IL-33 in the pathogenic process of CHC. Moreover, we determined the concentrations of serum IL-33 and its correlation to the HCV virus activity and the degree of liver fibrosis. We found that IL-33 response is correlated positively to the increase of liver enzymes and degree of liver fibrosis which indicate its important role in the pathogenesis of CHC and its association with the severity of liver injury in CHC patients.

## MATERIALS AND METHODS

### Patients

A total of 87 patients (age median 40 years old) with chronic hepatitis C (CHC) were recruited at the outpatient service of El-Ahrar Hospital in Zagazig, Egypt. Individuals with positive anti-HCV antibodies and serum and serum HCV RNA for at least six months are diagnosed as CHC (Sherman, 2007; Sherman, Goodman et al., 2007). Another 15 age (age median 42 years old), gender and ethnic matched healthy controls (C) were recruited, and they had no historical liver disease and no evidence of HBV, HCV and HDV infection.

Individuals with HBV, HDV or HIV infections, autoimmune hepatitis or metabolic liver disease who received treatment were excluded and all patients denied drug use. Their clinical characteristics are shown in Table 1. Peripheral blood samples were collected from individual and their sera were prepared and then stored at -80°C till used.

### Measurement of IL-33 by ELISA

The concentrations of the serum IL-33 in individual patients and health controls were determined by Enzyme Linked Immunosorbent Assay (ELISA) using Human IL-33 ELISA Kit, according to manufacturers' instruction (Boster biological technology, Wuhan, China). Briefly, individual sera at 1:2 dilutions were subjected to ELISA analysis and the IL-33 serum concentrations were calculated according to the standard curve established using the recombinant IL-33. The detection range of the ELISA kit was 15.6-1000 pg/ml.

### Serologic analysis of hepatitis

The concentrations of serum antibodies against HCV were detected by ELISA kit (Abbott Laboratories, Abbott Park, USA). The levels of serum ALT and AST were detected using a Biochemistry Automatic Analyzer (Roche Diagnostics, Branchburg, USA). The amounts of serum HCV RNA were measured by quantitative PCR assay using a luciferase quantization detection kit, following the protocols (Roche Amplicor, Basel, Switzerland). The detection limit of viral RNA was 300 copies/mL.

**Table 2.** Metavir Scoring System for Chronic Hepatitis C

Activity score (A)	Fibrosis score (F)
A0 = no activity	F0 = no fibrosis
A1 = mild activity	F1 = portal fibrosis without septa
A2 = moderate activity	F2 = portal fibrosis with few septa
A3 = severe activity	F3 = numerous septa without cirrhosis
	F4 = cirrhosis

#### Measurement of zinc levels by colorimetric method

The concentration of Zinc in serum was determined by colorimetric method (Biodiagnostic, Cairo, Egypt). Briefly, zinc was chelated by zincon (2-carboxy-2'-hydroxy-5-sulfoformazyl-benzene) in the provided reagent at alkaline pH, the formation of this complex is measured at wavelength of 610 nm. Each individual sample was tested in triplicate and the zinc serum concentrations were calculated according to the standard curve established using the provided standard zinc.

#### Pathological examination of the CHC patients

Kindly, the data of pathological examination of patients were provided by the Department of Pathology, El-Ahrar Hospital, Zagzig, Egypt. Briefly, Liver tissue fragments were processed according to standard histology protocol, they were fixed in 10% formalin, embedded in paraffin, sectioned at 5 microns and stained hematoxylin-eosine and Van Gieson. The Knodell scoring systems used Histologic Activity Index (HAI) and modified by Ishak quantifies the necroinflammatory activity between 0 and 18 and fibrosis from 0 to 6. The Metavir score is described especially for patients with chronic hepatitis C, grading represents the activity level or amount of inflammatory infiltrate, and is rated from 0 to 3, and stage represents the amount of fibrosis, rated from 0 to 4. In this study we used Metavir scoring system to evaluate the degree of liver fibrosis (Table 2) (Mannan et al., 2014).

#### Statistical analysis

The ELISA determination of Zinc levels and IL-33 were repeated in triplicates and the data are presented as median and range unless specified. The differences between the groups were analyzed by t-test using the Graphpad Prism 5 software. The relationship between variables was evaluated using the Pearson rank correlation test. A two-tailed P value <0.05 was considered statistically significant.

## RESULTS

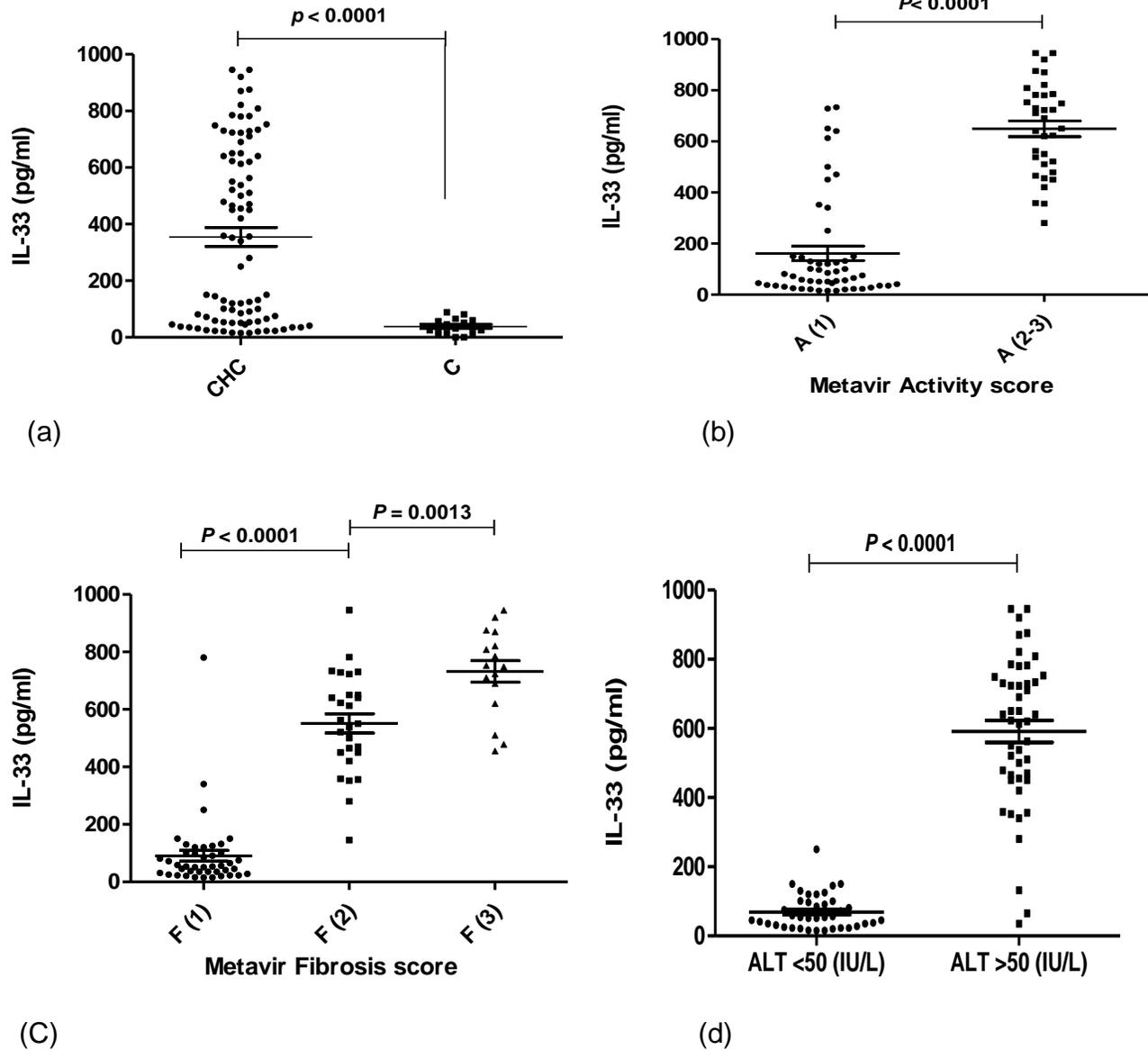
In order to determine the role of IL-33 in the pathogenic process of CHC and its correlation to health of liver, we also used liver enzymes Alanine transaminase (ALT) and Aspartate transaminase (AST) and zinc level in the serum to evaluate the liver functionality beside pathological examination of liver tissues. A total of 87 patients with CHC and 15 healthy controls were sequentially recruited. It is expected that there was no significant difference in the distribution of age and gender among these groups of subjects, but the concentrations of serum

ALT and AST in patients with CHC were significantly higher than those in the controls (Table 1). Also, the high levels of virus RNA were detected in CHC patients. In addition, the anti-HCV antibody was detected in CHC patients and not in controls.

The concentrations of serum IL-33 in individual CHC patients and C, and the levels of ALT and AST in serum were determined by ELISA and automatic enzymatic assays, respectively. Liver tissue fragments were processed according to standard histology protocol, and the Metavir score is used especially to describe the activity and fibrosis levels in patients with chronic hepatitis C. The potential association of the levels of serum IL-33, ALT, and AST was analyzed using the Spearman rank correlation test (Figure 1).

Analysis of serum IL-33 indicated that the concentrations in patients with CHC were significantly higher than those in controls ( $P < 0.0001$ , Figure 1(a)). Furthermore, IL-33 serum levels in patients with CHC showing activity levels (A 2-3) according to Metavir scoring system were significantly higher than CHC patients showing activity level (A 1) ( $P < 0.0001$ , Figure 1 (b)). In addition, IL-33 serum levels in CHC patients with fibrosis score (F 3) according to Metavir scoring system were significantly higher than CHC patients with fibrosis score (F2) and (F1) ( $P = 0.0013$  and  $P < 0.0001$  respectively, Figure 1 (c)). Moreover, patients with CHC revealed that the concentrations of serum IL-33 in CHC patients with higher levels of serum ALT (>50 units/L) or AST (>40 units/L) were significantly higher than those in CHC patients with normal levels of ALT (<50units/L) or AST (<40units/L), respectively ( $P < 0.0001$ ,  $P < 0.0001$ , respectively, Figures 1(d) and 1(e)). The concentrations of serum IL-33 in CHC patients were positively correlated with the levels of serum ALT and AST ( $r = 0.8$ ,  $P < 0.0001$ ;  $r = 0.82$ ,  $P < 0.0001$ , respectively, Figures 1(f) and 1(g)).

In addition to measuring serum liver enzymes (ALT and AST) we measured zinc levels in serum as an additional parameter for the liver viability. The concentrations of zinc in serum in individual CHC patients and C in serum were determined by colorimetric method, Zinc Normal values are 70-150  $\mu\text{g/dl}$  in healthy persons. Liver tissue fragments were processed according to standard histology protocol, and the Metavir score is used especially to describe the activity and fibrosis levels in patients with



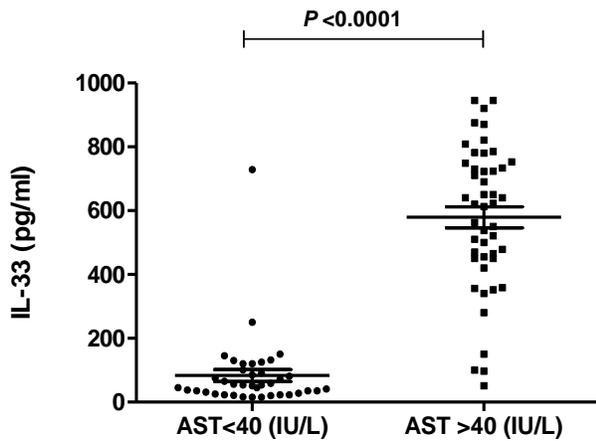
**Figure 1.** The serum IL-33 levels. Data are presented as the mean values of individual participants from three separate experiments. The horizontal lines refer to the median values of different groups. (a) The basal levels of IL-33 in serum of CHC and C; (b, c) the levels of serum IL-33 of CHC patients with different activity and fibrosis scores respectively; (d, e) the levels of serum IL-33 in those with different levels of serum ALT and AST respectively; (f and g) the correlation between the levels of serum IL-33 and ALT or AST respectively. C: healthy controls and CHC patients with Chronic Hepatitis C.

chronic hepatitis C (Figure 2).

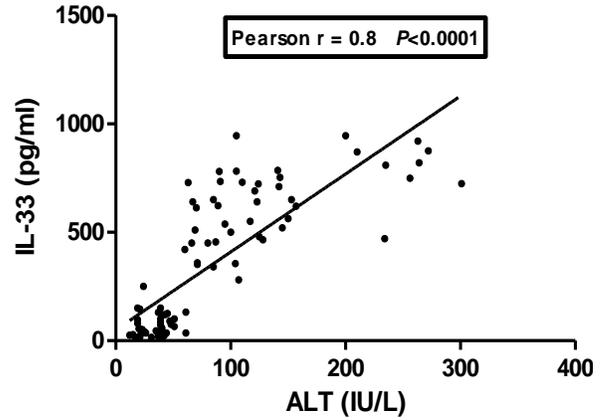
Analysis of serum zinc levels showed that the concentrations in patients with CHC were significantly lower than those in controls ( $P < 0.0001$ , Figure 2(a)).

Furthermore, zinc depletion was observed in higher stages of fibrosis, zinc levels in serum were significantly lower in CHC patients with fibrosis stage (F3) according to Metavir scoring system than those with (F2) and (F1) ( $P = 0.0004$ ,  $P < 0.0001$  respectively, Figure 2(b)).

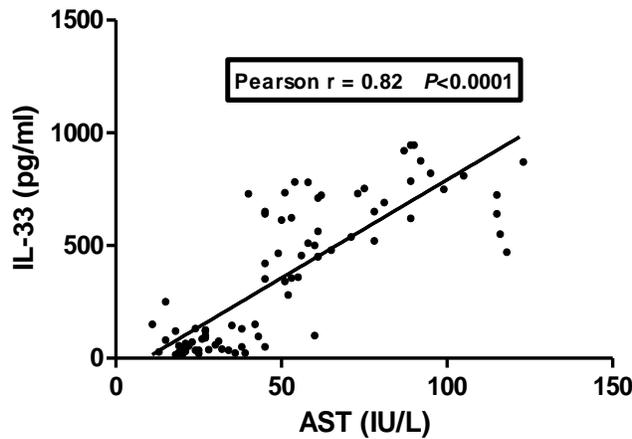
The concentrations of zinc and IL-33 in serum were determined by colorimetric method and ELISA respectively. Liver tissue fragments were processed according to standard histology protocol, and the Metavir score is used especially to describe the activity and fibrosis levels in patients with chronic hepatitis C. The potential association of the levels of serum zinc, IL-33 and viral load was analyzed using the Spearman rank correlation test (Figure 3).



(e)



(f)



(g)

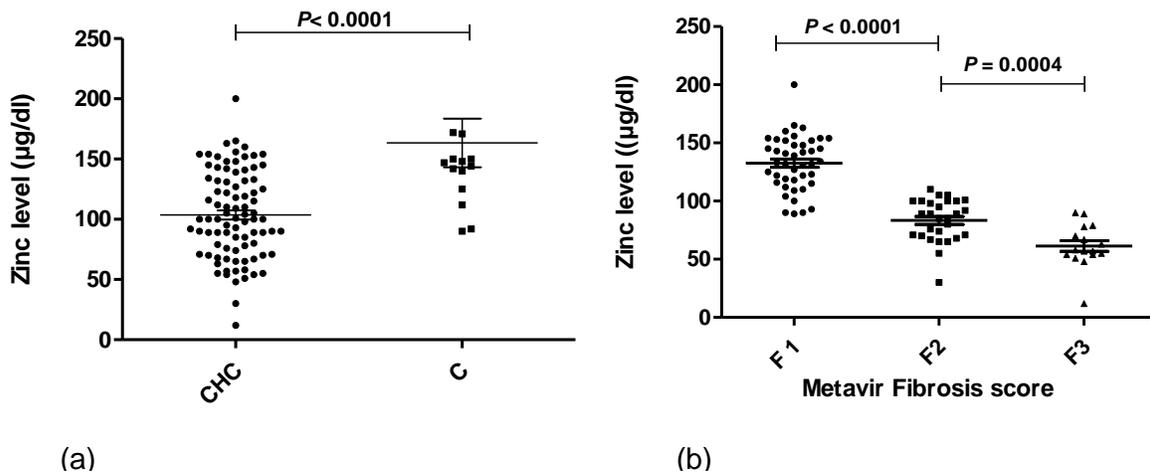
**Figure 1 Contd.** The serum IL-33 levels. Data are presented as the mean values of individual participants from three separate experiments. The horizontal lines refer to the median values of different groups. (a) The basal levels of IL-33 in serum of CHC and C; (b, c) the levels of serum IL-33 of CHC patients with different activity and fibrosis scores respectively; (d, e) the levels of serum IL-33 in those with different levels of serum ALT and AST respectively; (f and g) the correlation between the levels of serum IL-33 and ALT or AST respectively. C: healthy controls and CHC patients with Chronic Hepatitis C.

The viral load was measured by quantitative PCR assay using a luciferase quantization detection kit and amounts of serum HCV RNA were detected. The CHC patients with higher serum HCV RNA showed significantly higher level of activity or amount of inflammatory infiltrate (A 2-3) according to Metavir scoring system than those CHC patients with lower activity levels (A 1) ( $P < 0.05$ , Figure 3 (a)). The concentrations of serum IL-33 in CHC patients were positively correlated with the levels of serum HCV RNA ( $r = 0.76$ ,  $P < 0.0001$ ), while the zinc concentration were negatively correlated to HCV RNA ( $r = -0.67$ ,  $P < 0.0001$ ,

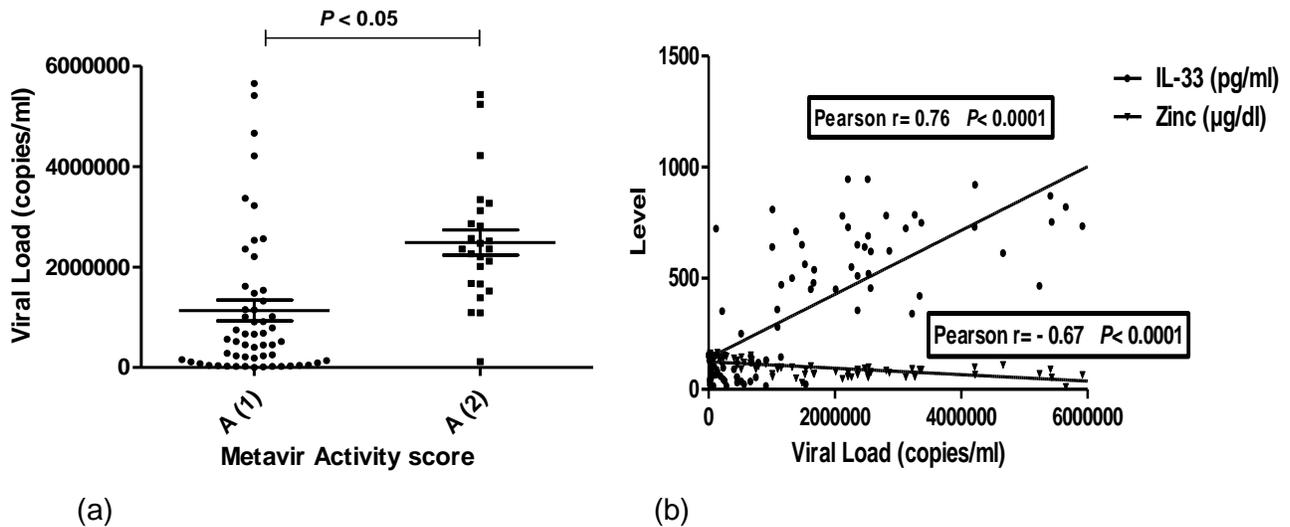
Figures 3(b)). Apparently, IL-33 is a pathogenic factor and plays role in chronic hepatitis C. IL-33 is associated with the damage of the liver in CHC patients and is correlated positively to viral load of HCV and liver enzymes ALT and AST.

**DISCUSSION**

Hepatitis C virus is considered a major cause of liver cirrhosis (Lauer and Walker, 2001). CHC patients are subjected routinely to liver functions testing which include



**Figure 2.** The Zinc levels in the serum. Data are presented as the mean values of individual participants from three separate experiments. The horizontal lines refer to the median values of different groups. (a)The basal levels of zinc in serum of CHC and C; (b) the levels of serum zinc of CHC patients with different fibrosis scores. C: healthy controls and CHC patients with Chronic Hepatitis C.



**Figure 3.** The Viral load. Data are presented as the mean values of individual participants from three separate experiments. The horizontal lines refer to the median values of different groups. (a)The levels of serum HCV RNA in CHC patients with different activity scores; (b) the correlation between the levels of serum IL-33, zinc and HCV RNA.

periodical monitoring of ALT and AST and pathological analysis of Liver biopsies. Zinc is essential trace element, distributed throughout the body of a healthy adult, including many organs (Fredricks et al., 1960; Herring, Leavell et al., 1960; Herring et al., 1960). In 1951, Vikbladh was the first to point out that the zinc content in serum was low in the case of various liver diseases, and zinc metabolism abnormality in liver disease (Vikbladh, 1951). It is well known that CHC patients show lower zinc

concentrations in blood as their pathologic condition worsens from chronic hepatitis, to compensated cirrhosis, to decompensated cirrhosis, to hepatocellular carcinoma. Lower serum zinc concentrations in chronic liver diseases and zinc depletion have been suggested to be a cause of liver fibrosis (Milman et al., 1986; Bode et al., 1988). Zinc level in the serum is used clinically to indicate the viability of the liver, and recently it was used to enhance the response interferon therapy for CHC patients (Ishikawa,

2012). To evaluate the degree of fibrosis, liver biopsies were aspirated and evaluated using Metavir scoring system which quantifies the necroinflammatory. The Metavir score was described especially for patients with CHC, the activity level or amount of inflammatory infiltrate were rated from 0 to 3, and fibrosis stages were rated from 0 to 4 (Mannan et al., 2014).

IL-33 is a member of IL-1 family cytokine, involved in various disease conditions (Barksby et al., 2007; Liew et al., 2010). IL-33 can activate the MAP kinase and NF- $\kappa$ B signal pathways and promote Th2 response and cytokine production via binding to the receptor complex composed of ST2 and IL-1RaP (Schmitz et al., 2005; Mok et al., 2010). It was observed that the levels of IL-33 were elevated in autoimmune diseases (Alves-Filho et al., 2010). IL-33 plays a significant role in IBD and lung inflammation (Eiwegger and Akdis, 2011; Pastorelli et al., 2013). Other studies showed that IL-33 is an important factor of the pathogenesis of some viral infections as HIV infection (Miyagaki et al., 2011) and dengue virus infection (Becerra et al., 2008). IL-33 serum levels were found associated with liver damage in patients with CHC (Wang et al., 2012). It contributes importantly in the pathogenic process of acute hepatitis induced by Con-A (Volarevic et al., 2012). IL-33 overexpression is associated with the development of HBV/HCV-related liver fibrosis (Marvie et al., 2010).

To investigate the role of IL-33 in HCV pathogenesis and its correlation to liver damage, we determined the levels of serum IL-33 in 87 CHC patients and 15 healthy controls. We found that the levels of serum IL-33 in CHC patients were significantly higher than in controls. Also, IL-33 levels were significantly elevated in patients with higher viral activity and higher fibrosis amount than those with lower activity levels and fibrosis stages. Compatible with these findings, the levels of serum IL-33 in CHC patients with abnormal concentrations of ALT or AST were significantly elevated than in those with normal levels of ALT and AST and the concentrations of serum IL-33 were correlated positively with the levels of serum ALT and AST in CHC patients. We determined the zinc levels in the serum of these population, zinc levels were significantly lower than those in controls. Moreover, zinc levels in serum were lower in CHC patients with higher stages of fibrosis than those with lower fibrosis amounts. In our tested population, we found that HCV RNA copies were correlated positively to the levels of IL-33 in serum while it was correlated negatively to the zinc levels in the serum.

The abnormal levels of ALT and AST, zinc levels and pathological examination of the liver tissues are indicatives of abnormal liver function and injuries, our data show that IL-33 may be an important pathogenic factor of the pathogenic process of CHC patients. Furthermore, we found that the levels of serum IL-33 were also correlated with pathogenic degrees of the liver

in CHC patients. More, interestingly, it has been shown that the treatment of CHC patients with IFN to inhibit the replication of HCV dramatically decreased the levels of serum IL-33 in CHC patients (Wang et al., 2012). From all the previous findings, we suggest that the levels of serum IL-33 may be used as a new biomarker for the diagnosis of liver damages in CHC patients. It is possible that higher levels of serum sST2, IL-33 receptors, are an early biomarker of liver injury, while higher levels of serum IL-33 may indicate the development and progression of liver fibrosis and damage (Marvie et al., 2010). It is considered that the pro-inflammatory cytokines, such as IFN- $\gamma$  and IL-6, are important factors for the clearance of infected HCV and for liver injury (Frese et al., 2002). It was found that the lower levels of serum IFN- $\gamma$  in CHC patients indicated continual viral replication and pathogenic progression (Bourgeois et al., 2009). The precise mechanism of IL-33 action is still not completely understood, but at least we can use IL-33 as cheap biomarker for HCV viral activity and fibrosis stage, but the other conditions which correlated with IL-33 elevation as IBD, Lung inflammation and allergic dermatitis should be rolled out.

In conclusion, our data indicate, that the concentration of serum IL-33 are significantly higher in CHC patients than controls and is significantly correlated with the levels of serum ALT and AST and degree of viral activity and amount of fibrosis. We suggest that IL-33 may be an important pathogenic factor of HCV-related liver injury in CHC patients. We also introduce IL-33 as a cheap biomarker can be used with considerations to evaluate the inflammation of the liver and the fibrosis stage in the CHC patients, however it is important to role out the other causes of inflammation.

### Conflict of interest

The authors confirm that there is no financial support from any company and confirm the absence of any interest to disclose.

### ACKNOWLEDGMENTS

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