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RESEARCH ARTICLE

Serum Interferon –gamma inducible protein – 10: a possible player in progression of Hepatitis B Virus related chronic liver diseases.

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Abstract

Objective: To assess relation of serum Interferon gamma inducible protein - 10 (IP-10) to clinical, biochemical and histopathological characteristics in different clinical stages of HBV.

Methods: The 116 HBsAg positive patients (24; carriers, 81; chronic hepatitis and 11; cirrhosis) were tested for HBV Quantitative PCR and genotyping and IP-10 serum levels.

Results; By ROC curve, optimal IP-10 level for potential prediction of cirrhosis development within chronic hepatitis patients was 343.5 ng/ mL The area under curve was 0.862 (95% CI :, P:<0.001). According to this level, patients were sub-classified into 2 groups. Most of patients in the above cutoff value (>343.5 pg/ml) group (n=32) were genotype D (84.4%), revealing significant higher viral load (P:<0.001), ALT (P: 0.002), and more severe histopathologic inflammatory and fibrotic (P: <0.0001 for each) patterns. Also, these patients were more prone to progress to liver cirrhosis [RR= 2.8, 95% CI (1.7 -4.7)]. Spearman's correlation revealed significant positive correlations between serum IP-10 levels and patient's age (P: 0.009), ALT (P: 0.005), viral load, and stages of fibrosis and inflammation (P: <0.0001 for each). By linear regression analysis, ALT, viral load, hepatic inflammation and IP-10 were independent predictors of hepatic fibrosis (P: 0.02, 0.007, <0.0001, <0.0001 respectively). The multivariate analysis showed IP-10 as an independent predictor for both hepatic inflammation and fibrosis (P: <0.0001 for each).

Conclusions; The observed strong association between IP-10 levels and HBV viral loads points to IP-10 role in recruitment of immune cells to infected liver and induction of cellular immunity against HBV infections.

INTRODUCTION

Hepatitis B viral infection affects approximately240 million people and is responsible for over 1 million deaths per year (WHO 2012, Ganem& Prince 2004, Lok& McMahon 2007). Egypt is considered a region of intermediate prevalence for HBV infection with a reported endemicity of 4.5% (Ismail et al., 2014)

HBV is non-cytopathic and its effect is mainly immune-mediated (Wieland &Chisari2005, Dunn et al., 2007, Liu et al., 2012, Kimkong et al., 2013)˙The immune status varies with different clinical stages and may affect disease progression leading to cirrhosis and hepatocellular carcinoma(Jiang et al., 2002, Martin et al., 2009).

In liver diseases, progression from healthy tissue to cirrhosis is mediated by a chronic inflammatory reaction within the parenchyma that leads to the excess deposition of extracellular matrix proteins(Friedman 2008). This inflammatory reaction is the main predictor of disease progression. The recruitment of immune cells into the damaged liver is orchestrated by chemokines (Holt et al., 2008). The predominant chemokine receptor expressed by infiltrating lymphocytes is CXCR 3that binds the chemokines CXCL 9, CXCL 10 (Interferon-gamma inducible protein 10 kD (IP-10)) and CXCL 11 (Lasagni et al., 2003).

IP-10 is widely involved in enhancement of Th1 immune response, homing of immune cells, apoptosis, cell growth and angiostatic effects(Liu et al., 2011, Xu et al., 2013). IP-10 is closely correlated with hepatic inflammation and fibrosis in HCV (Zeremski et al., 2011). However, its role in HBV infection is not clear**.**

The current study was designed to assess the relation of serum IFN-γ inducible IP-10 to clinical, biochemical and histopathological characteristics in different stages of HBV mediated hepatic injury.

Materials and Methods:

1. Study populations:

In a cross-sectional case control study, one hundred and sixteen treatment-naiveHBsAg positive patients attended or referred to the Tropical Medicine and/or Internal Medicine Departments, Mansoura University Hospitals, Egypt, between 2011 and 2014 were included in the study.

Based on clinical presentations, past history, imaging data, liver histopathology, Child-Pugh (CP) scores (Durand & Valla 2005)antiHBc IgM/IgG status and as per AASLD practices guidelines (Lok& McMahon 2009),patients were grouped into; inactive carrier, chronic hepatitis, and cirrhosis groups; that matched with twenty five apparently healthy volunteers. The clinical and the histopathologic assessment of the studied population were performed at the time of blood sampling.

All patients gave their informed consents. The study protocol was approved by the constitutional ethical committee.

Exclusion criteria

Patients co-infected with other viral hepatitis,pregnant females, patients < 18 years old, having a concomitant disease, decompensated liver cirrhosis or alcoholic were not eligible for the present study.

2. Laboratory Investigations(all procedures were done according to the manufacturer's instructions***):***

Samples:

Three venous blood serum samples: 1st one for biochemical and, viral serological markers;2ndone for HBV quantitative PCR and genotyping and3rdfor IP-10 measurement (latter two stored at -80°C till time of use). All patients and control persons were subjected to:

A. Routine laboratory tests

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum total and indirect bilirubin, serum albumin, and creatinine by Hitachi 911Analyzer (Roche Diagnostics, Branchburg, NJ) were done for all subjects.

B. Serological detection of virological markers

All sera were tested for hepatitis markers (HBsAg, HBeAg, anti-HBe, anti-HBc total/IgM, anti-HCV, anti-HDV and anti-HIV antibodies)by the ChemiluminiscentMicroparticle Immunoassay method (Abbott ARCHITECT® Assay (Architect i2000SR, Abbott Diagnostics; Abbott Laboratories, Chicago, IL, USA).

C. Molecular diagnosis of HBV

DNA extraction:

Extraction was done from 200 µL serum samples (stored at -80°C)by the QIAamp DNA extraction mini kit (QIAGEN, Germany)

***Quantitative PCR of HBV-DNA**

HBV DNA was assessed by sensitive quantitative PCR the artus HBV TM PCR Kit. It is based on the amplification and simultaneous detection of a 134 bp region of the HBV genome using real-time PCR. The kit provides 5 HBV quantitation standards. Use of the standards enables accurate quantitation of viral load. The artus HBV TM PCR Kit covers a linear range from 8.8 IU/ml to at least 5.6 x 10^9 IU/ml. The samples with an HBV DNA level that exceeded 5.6 x 10^9 IU/mL required a 1:999 dilution. The amplification was performed on Stratagene Mx3000P PCR system (La Jolla, CA, USA). The threshold cycle (Ct) is the cycle at which a significant increase in fluorescence occurs.

***HBV Genotyping Method:**

Genotyping system based on nested PCR, using type specific primers for determination of six genotypes A through F of HBV according to previous method described by Naito et al (2001). The nested PCR primers were designed on the basis of the conserved nature of the nucleotide sequences in regions of the pre-S1 through S

genes.Two nested PCRs were performed in different mixture for each sample. (mix A) applied for identification of genotypes A, B, C and (mix B) for genotypes D, E, F.

D. Measurement of IP-10 in serum:

Human IP-10 was quantified using Quantikine (R&D Systems Minneapolis, MN, USA)—a solid-phase ELISA. Every titer was measured twice by a Robonik Eliza reader analysis instrument (India) and the final average OD was calculated. Results were expressed in pg/ml. The sensitivity of the kits was 1.67pg/mL and the mean inter- and intra-assay precision of the kit were respectively 3.6% and 6.7%. IP-10 concentrations in serum samples were calculated using the standard curves in the kits.

3. ***Liver biopsy:***

Liver tissues were fixed in 10% formalin, paraffin-embedded, and serially sectioned. Each biopsy was staged for fibrosis (score: 0–6) and graded for a necro-inflammatory score (0–18) in accordance with the method ofIshak et al. (1995).

Statistical Analysis:

All statistical analyses were performed using the IBM SPSS program for Windows (version 21). Continuous variables are expressed as mean ± standard deviations or medians (range), and were analysed by the Mann–Whitney U-test and Kruskal-Wallis test. Categorical variables are expressed as the number of cases (percentage). Frequencies were compared using the Fisher's exact test. Diagnostic performance was determined by constructing a ''receiver-operating characteristic'' (ROC) curve and calculating the area under the ROC (AUROC) curve. From these curves, sensitivities, specificities and the best cut-off valueswere established. Spearman correlation was used to estimate the relation between variables. General linear regression analysis was done. Serum IP-10 was used as dependent factor, and all the significantly correlated variables as independent factors. Other models were explored in which hepatic inflammation and hepatic fibrosis were the dependent factors, and serum IP-10 was independent variable. Significance was defined as*P< 0.05, **P < 0.01.

Results:

General characteristics of the studied population (Tables 1)

The 3 studied patients groups; inactive carriers (n=24), CH (n=81) and LC (n=11) were with a mean age (in years) 40.7±8, 40.9± 10.2 and 47.1 ±5.7 respectively. Patient groups were matched with control group (n=25) with a mean age 44.1±6.1 years. Male gender was the predominant in the studied participants. All the patients were negative for HDV, HIV and HCV antibodies. The CH subset of patients showed the highest viral load, AST and ALT levels in comparison to other groups, this difference was statistically significant (**P< 0.0001 for each).

Meanwhile the minimal inflammatorypattern was the prevalent among carrier group (79.2%) and CH subsets (45.7%), all LC patients demonstrated either moderate (45.5%) or severe (54.5%) inflammatory pattern (**P<0.0001). Similarly, on one hand, none of the carrier patients revealed fibrosis, and on the other hand 100% of LC patients had grade 5-6 fibrosis. The CH group demonstrated (65.4%) grade 1 fibrosis and (34.6%) grade 2-4 fibrosis (**P<0.0001). Genotype D was the prevalent among studied patients comprising (**57.4%**), followed by genotype C (**19.9%)**.

Serum IP-10:

The median (range) of serum IP-10 [478 (293-917)] was the highest in LC group of patients when compared to that of controls [82.5 (49-170)], carriers [80 (30-489)] and CH group [236 (41-965)] (**P<0.0001) (**Figure 1**).

Receiver-operating characteristic'' ROC analysis was conducted to identify the optimal IP-10 levels for prediction of development of LC within the entire cohort of hepatitis B patients. From these curves, sensitivities, specificities and the best cut-off values were established. IP-10 best cut-off values were 343.5 ng/ mL, with a sensitivity of 90.9% and a specificity of 74.1%. The area under the curve was 0.862 (95% CI =0.779-0.945, **p<0.001)(**Figure 2)**

According to IP-10 cutoff level **(343.5 pg/ml)** patients were sub-classified into 2 groups (**Table 2**). Most of the patients in the above cutoff value (**>343.5pg/ml)** group (n=32) were genotype D (84.4%), revealing statistically significant higher viral load (**P<0.001), ALT (0.002), and more severe histopathologic inflammatory (**P<0.0001) and fibrotic (**P<0.0001) pattern.

Patients with IP-10 >343.5 pg/ml were more likely to have liver cirrhosis [RR= 2.8, 95% CI: 1.7 -4.7]**.**

Spearman's correlation between serum IP-10 levels and biochemical and histopathological characteristics of studied patients **(Table 3)** revealed a statistically significant positive correlation between serum IP-10 levels and each of the patient's age (rho= 0.3, **P= 0.009), ALT (rho= 0.3, **P= 0.005), viral load (rho= 0.4, **P<0.0001), stage of fibrosis (rho= 0.5, **P<0.0001), stage of inflammation (rho= 0.6, **P<0.0001).

General linear regression analysis

General linear regression analysis models were explored in which hepatic fibrosis was the dependent factor and serum IP-10 and other clinical biochemical characters as independent variables.

ALT (R^2= 0.1,*P= 0.02), viral load (R^2= 0.1, **P= 0.007), hepatic inflammation (R^2= 0.6, **P<0.0001), and IP-10 (R^2= 0.3, **P<0.0001) were independent predictors of hepatic fibrosis in chronic hepatitis group (**Table 4).** Further analysis models were explored, after adjustment for age, genotype and ALT levels, in which IP-10 was an independent predictor for hepatic inflammation (R^2= 0.44, **P<0.0001), and hepatic fibrosis (R^2= 0.42, **P<0.0001)**(Figure3).**

Table (1) Baseline Characteristics of study population

		Control (n=25)	Carrier (n=24)	CH (n=81)	LC (n=11)	*P*
Age (year) (mean±SD)		44.1±6.1	40.7±8	40.9± 10.2	47.1 ±5.7	ns[2]
Gender, M/F		17/8	17/7	61/20	6/5	ns[1]
HBV genotype						
D		-	17 (70.8%)	55 (67.9%)	9 (81.8%)	ns[1]
Non D		-	7 (29.2%)	26 (32.1%)	2 (18.2%)	
Clinical data						
Fatigue *n(%)*		-	2 (8.3%)	48 (59.3%)	11(100%)	<0.0001[1]
Anorexia *n(%)*		-	1(4.2)	13 (16.1%)	7 (63.6)	0.0003[1]
arthralgia *n(%)*		-	3 (12.5%)	18 (22.2%)	4 (36.4%)	ns[1]
Jaundice*n(%)*		-	0 (0%)	25 (30.9%)	2 (18.2%)	0.002[1]
Hepatomegaly*n(%)*		-	0 (0%)	23 (28.4%)	0 (0%)	0.0007[1]
Splenomegaly*n(%)*		-	0 (0%)	6 (7.4%)	9 (81.8%)	<0.0001 [1]
Laboratory data						
HBV-DNA titer, log IU/mL			2.5 (0.6-3.3)	4.4 (1.6-5.2)	3.1 (2.6-4.97)	<0.0001[2]
ALT, IU/L			21.5 (15-28)	169 (17- 631)	47 (14-537)	<0.0001[2]
AST, IU/L			22.5 (17-35)	143 (21-631)	63 (17-369)	<0.0001[2]
Pathologic findings						
Grade of inflammation *median(range)*			2 (0-6)	5 (0-14)	13 (11-15)	<0.0001[2]
Inflammation n(%)	Minimal	-	19 (79.2%)	37(45.7%)	0 (0%)	
	Mild	-	5 (20.8%)	18 (22.2%)	0 (0%)	
	Moderate	-	0 (0%)	24(29.7%)	5 (45.5%)	
	Severe	-	0 (0%)	2 (2.4%)	6 (54.5%)	
Stage of fibrosis, *median(range)*		-	1 (0-2)	2 (0-5)	5 (5-6)	<0.0001[2]
Fibrosis n(%)	no/mild (0-1),	-	24 (100%)	53 (65.4%)	0 (0%)	
	moderate/severe (2-4)	-	0 (0%)	28 (34.6%)	0 (0%)	
	Cirrhosis (5-6)	-	0 (0%)	0 (0%	11 (100%)	

[1]= fisher's exact for a 2×3 contingency table
[2]=Kruskal- Wallis test
CH: chronic hepatitis, LC: liver cirrhosis
M/F= male / female
HBV: Hepatitis B virus
ALT; alaninie transferase, AST; aspartate transferase
ns= non-significant
P≤ 0.05 is considered significant,

Table (2) HBV Patients characteristics according to interferone protein -10 (IP-10) cut off level.

IP-10		**≤343.5 (n=84)**	**>343.5 (n=32)**	***P***
Age (year) (mean±SD)		43.5±8.8	45.6±7.31	ns[4]
Gender, M/F		60/24	24/8	ns [3]
Patient category				
Carrier (n=24)		23(95.8%)	1(4.2%)	<0.0001[1]
Chronic hepatitis(n=81)		60(74.1%)	21(25.9%)	
Liver cirrhosis(n=11)		1(9.1%)	10(90.9%)	
HBV genotype				
D		54 (64.3%)	27 (84.4%)	0.04[3]
Non D		30 (35.7%)	5(15.6%)	
Laboratory data				
HBV-DNA titer, log IU/mL		3.15 (0.6-5.18)	4.4 (0.98-6.4)	<0.001[4]
ALT, IU/L		**98.5** (15- 631)	238(21-476)	0.002[4]
AST, IU/L		99.5(14-698)	223(19-695)	0.002[4]
Pathologic findings				
Grade of inflammation *(Median, range)*		4(0-15)	11 (1-15)	<0.0001[4]
Inflammation n(%)	**Minimal**	49(58.3%)	7 (21.9%)	
	Mild	23(27.4%)	0 (0%)	
	Moderate	11(13.1%)	18 (56.2%)	
	Severe	1(1.2%)	7 (21.9%)	
Stage of fibrosis *(Median, range)*		1(0-5)	4 (0-6)	<0.0001[4]
Fibrosis n(%)	**Mild**	70(83.3%)	7 (21.9%)	
	Moderate	14 (16.7%)	14 (43.8%)	
	Cirrhosis	0 (0%)	11 (34.4%)	

[1]= fisher's exact for a 2×3 contingency table
[3]= fisher's exact for a 2×2 contingency table
[4]= Mann-Whitney U test
M/F= male / female
HBV: Hepatitis B virus
ALT; alaninie transferase, AST; aspartate transferase
IP-10: Interferon gamma inducible protein 10
ns= non-significant
P≤ 0.05 is considered significant,

Table (3) Spearman correlation between interferone protein -10 and laboratory and pathological parameters.

Parameters	IP-10	
	rho	P
Age	0.3	0.009
ALT U/L	0.2	ns
AST U/L	0.3	0.005
HBV Viral load log IU/mL	0.4	<0.0001
Stage of inflammation	0.6	<0.0001
Stage of fibrosis	0.5	<0.0001

HBV: Hepatitis B virus
ALT; alaninie transferase, AST; aspartate transferase
IP-10: Interferon gamma inducible protein 10
ns= non-significant
P≤ 0.05 is considered significant.

Table (4): Predictors of disease progression in chronic hepatitis B (CHB) patients.

Parameters	Liver fibrosis	
	R^2	P
Age/years	0.03	ns
Gender (M/F)	0.04	ns
ALT U/L	0.02	ns
AST U/L	0.1	0.02
HBV genotype	0.02	ns
HBV Viral load (log) IU/mL	0.1	0.007
Inflammation	0.6	<0.0001
IP-10 pg/ml	0.3	<0.0001

ALT; alaninie transferase, AST; aspartate transferase
HBV: Hepatitis B virus
IP-10: Interferon gamma inducible protein 10
P≤ 0.05 is considered significant

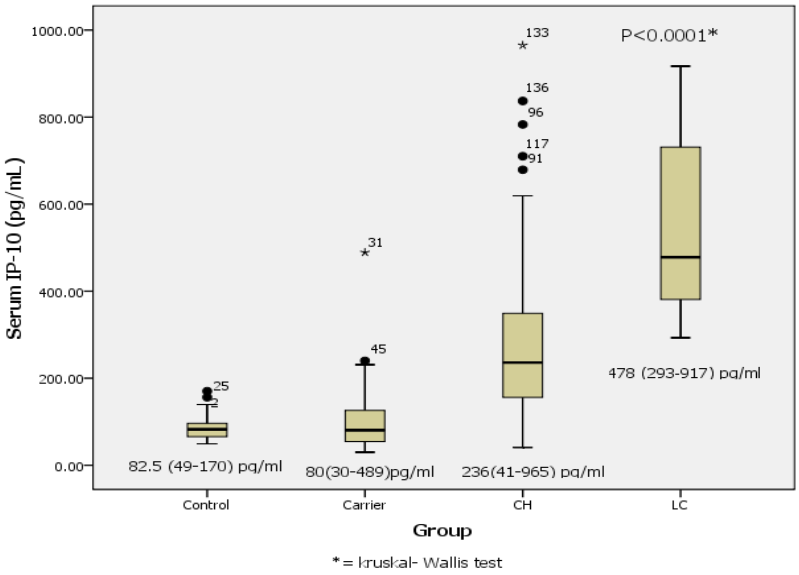

Figure (1) Serum interferone protein -10 levels among studied groups (presented data median and range) CH: chronic hepatitis, LC: liver cirrhosis

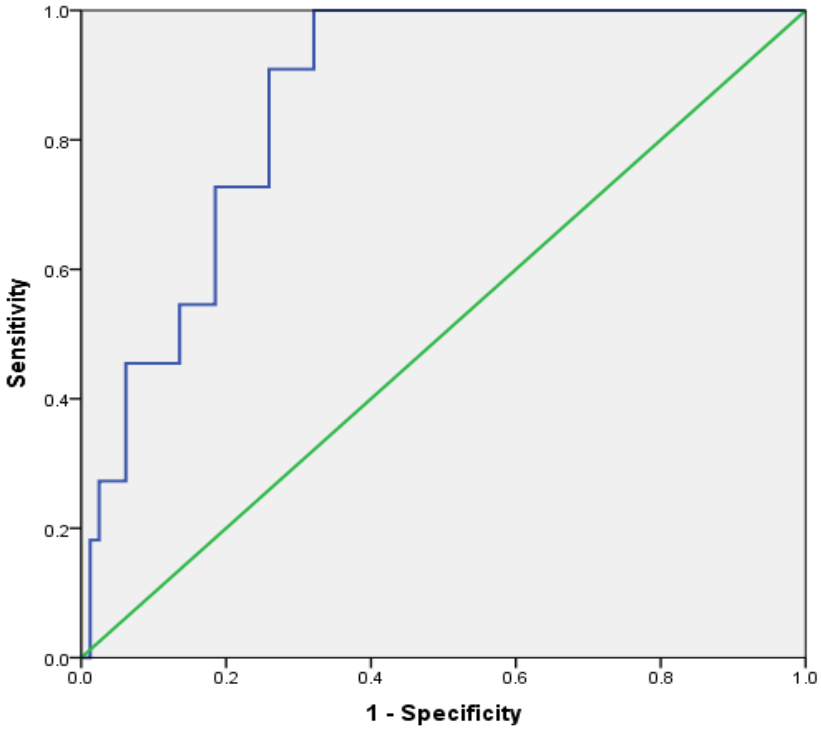

Figure (2) A Receiver Operating Curve (ROC) analysis of interferone protein -10 levels for the prediction of development of liver cirrhosis (LC) patients within chronic hepatitis (CH) patients subjects. Diagonal line is the reference line

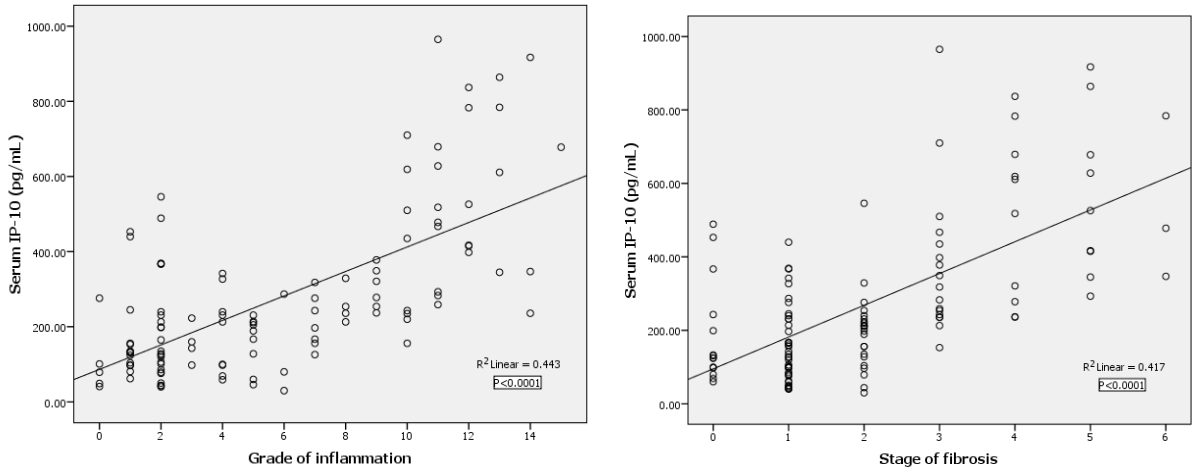

Figure (3) Serum interferone protein -10 is an independent predictor of hepatic inflammation and fibrosis

Discussion

HBV-associated liver damage is considered to be the consequence of a protracted cytolytic immune response generated against infected hepatocytes (Robek et al., 2004, Rossi &Zlotnik2000). Chemo-attraction activity of IP-10 was observed on monocytes, macrophages, T cells, NK cells, and dendritic cells(Dufour et al., 2002).

The major pathway for viral clearance in liver has been ascribed to the non-cytopathic antiviral mechanism. Thisnon-cytopathic viral clearance occurs by inhibition of replication and expression of HBV genome through the intrahepatic secretion of IFN-γ(Robek et al., 2004, Wieland &Chisari2005). IP-10 is closely correlated with hepatic inflammation and fibrosis in hepatitis C infection (HCV) (You et al., 2011). However, this relationship with CHB has not been fully identified.Based on this hypothesis, we tried to assess the relation of serum IFN-γ inducible IP-10 to clinical, biochemical and histopathological characteristics in different clinical stages of HBV mediated hepatic injury

IP-10 attracts monocytes, lymphocytes and NK cells(Hassanshahi et al., 2008) to liver tissues through interaction with its receptor CXCR3 expressed on target cells, so that many hepatocytes are destroyed and release quantities of ALT into the blood, which can not only achieve an effective antiviral response but also lead to tissue damage, thus contributing to progressive liver injury (Wanget al., 2010). In agreement with this observation, ALT levels, the hepatic necroinflammation and serum IP-10 levels in the current study demonstrated a crescendo starting by the inactive carrier group, passing through the chronic hepatitis patients and ending by the liver cirrhosis patients. This might represent a crescendo of immune response that culminates in liver cirrhosis. Similar findings were reported by Wang and co-workers 2014. In their study, IP-10 expression gradually increased from asymptomatic carriers to patients with CHB and was highest in patients with active on chronic liver failure. Serum IP-10 levels were positively correlated with the hepatic Inflammation activity score and ALT level. This is further supported by the finding in the current study that the patient group having the above cut off values had a higher ALT level and a more aggressive hepatic inflammatory and fibrotic pattern.

However, Mohamadnejad et al. 2006 reported that serum ALT alone can be used as a reliable marker for liver inflammation but liver fibrosis could not be predicted by serum ALT(Martinot-Peignouxet al., 2002).Additionally, patients with HBeAg negative CHB have wide fluctuations in serum ALT (Manesiset al., 2002) and they may have advanced liver fibrosis despite normal serum ALT.

The conflicting results about the correlation between the hepatic fibrosis and the concentration of serum ALT might be due to several potential causes such as lack of statistical power, heterogenecity of the groups of patients studied, different indices used to measure hepatic fibrosis and variable assays used for ALT measurement.

On the other hand, ALT may not be a crucial factor in determining the disease activity. It is believed that HBV infection is such a complex disease, that measurement of one biochemical factor is unlikely to reflect the whole disease process(Mohamadnejad et al. 2006).

In depth reading of the strong association between the viral load and the IP-10 serum levels observed in the present study supports the role of IP-10 in the recruitment of immune cells to the infected liver and induction of cellular immunity against viral infections. Therefore, studied patientswith IP-10 levels >343.5 pg/ml had a

significantly higher viral load.In a previous Study by Zhou and coworkers 2012, they demonstrated that hepatitis B virus protein X (HBx) increases IP-10 expression in a dose-dependent manner. Furthermore, they showed that HBx induces activation of NF-κβ, leading to up-regulation of IP-10 expression. As a consequence, up-regulation of IP-10 may mediate the migration of peripheral blood leukocytes thus causing immune pathological injury of liver.

More interestingly, IP-10 was independently related to hepatic fibrosis in the current study. Thus the IP-10 might be closely related to the progression of fibrosis in Chronic HBV- mediated disease. Higher concentration of serum IP-10 may recruit more CD4+ T cells to the liver, inducing more vigorous immune reactions. This was contradictory to the findings of (Shravanthi& Mukherjee 2012)thatreduction in IP-10 expression was significant in patients of all disease categories than controls which were most evident in cirrhosis group. This contradiction can be explained on the basis that they assessed the expression pattern of IP-10 genes in peripheral blood mononuclear cells (PBMCs) of HBV infected patients, rather than serum level. Meanwhile the source and the higher concentration of IP-10 might be in the liver itself.

Moreover, Wang and associates2014 reported that IP-10 was mainly derived from hepatocytes around portal areas and necro-inflammatory regions, as well as from interstitial cells in perisinusoidal spaces. They also found that the serum and intrahepatic IP-10 levels were higher in patients group. In an earlier study by Chuang etal 2005,plasma IP-10 levels in Primary biliary cirrhosis (PBC) was increased significantly compared to controls and appeared to increase with disease progression. By immunohistochemistry, IP-10 was evident in the portal areas in PBC. This more evidenced by the finding that patients, in the current study, with IP-10 level >343.5 pg/ml were more prone **to progress to liver cirrhosis [RR= 2.8,** 95% CI **(1.7 -4.7)].**

In conclusion, we demonstrated that a high serum IP-10 level was an independent predictor of disease progression in CHB infection. Further studies are needed to determine whether IP-10 may be used to monitor the natural course and progression of HBV-associated liver disease, and also may be regarded as a new potential therapeutic target.

Conflict of interest: We declare that we have no conflict of interest.

Funding; Self Support

Ethics;

This study was approved by Ethical Committee of Mansoura Faculty of Medicine, Mansoura University

Authors Contributions

All the authors shared study design, data collection, and literature research and writing the initial manuscript. NS was responsible for performing the biochemical analyses, and revised the final version of the manuscript. DS recruited patients, assembled data, and she was responsible for the statistical analysis of the clinical results the final version of the manuscript. SZ coordinated patient recruitment and clinical data collection and revised the final version of the manuscript. AA coordinated patient recruitment and clinical data collection. MEH shared in performing the biochemical analyses and revised the final version of the manuscript.

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