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Serological studies on circulating interferon gamma in patients infected with hepatitis C virus.

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Egypt has possibly the highest hepatitis C virus (HCV) prevalence worldwide. HCV is a leading cause of liver cancer and cirrhosis. The pathogenesis of chronic liver disease still poorly understood. Recently, experimental data have shown the critical role of pro-inflammatory cytokines like IFN-γ in the development of liver injury. *Methods:* Liver function tests such as, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and albumin were measured. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting to identify interferon gamma (IFN-γ) and Enzyme linked immunosorbent assay (ELISA) as a sensitive and specific method was used to evaluate the levels of IFN-γ in sera of chronic HCV patients (n = 110); and hepatocellular carcinoma (HCC) patients (n = 30) in addition, sera of 30 healthy individuals were used as negative controls. *Results:* hepatocellular carcinoma (HCC) patients had higher values of AST, ALT, and bilirubin but they had lower levels of serum albumin than those of chronic hepatitis C (CHC) patients or healthy individuals ($P < 0.0001$ for all comparisons). Sera of disease groups showed elevated IFN-γ levels compared with healthy ($p < 0.0001$). The differences were also remain statistically significant ($P<0.0001$) when CHC group was compared with HCC group. After 3 months of receiving daclatasvir and sofosbuvir combination treatment for non-cirrhotic and compensated cirrhotic patients and receiving daclatasvir, sofosbuvir and ribavirin combination treatment for decompensated cirrhotic patients, serum concentration of IFN-γ was found to be significantly reduced (from 46.1 ± 1.2 to 41.8 ± 1.1; $P=0.023$). *Conclusion:* The findings in the present study provided evidence that the surveillance of serum IFN-γ may be a non-invasive biomarker for the potential diagnosis of HCC.

Keywords: IFN-γ, liver, blood markers, diagnosis, HCC.

INTRODUCTION

The liver has a unique spectrum of functions. It regulates the amount of energy; stores, distributes, and disposes various nutrients; and synthesizes, transforms, and metabolizes many endogenous substrates and pollutants (Jeschke, 2009). Many different diseases may occur in the liver, including infections such as hepatitis, cirrhosis, cancers, and damage by medications or toxins (Pinter et al., 2016). Chronic liver diseases usually progress to cirrhosis (Sharma and John, 2017) which is not only the major risk factor for the development of hepatocellular carcinoma but also a limiting factor for anticancer therapy of liver and non-hepatic malignancies (Pinter et al., 2016). The two major risk factors of chronic liver diseases are chronic hepatitis B and hepatitis C infections (Patel et al., 2012). Current diagnostic methods, of ultrasound and α-fetoprotein, are expensive and lack sensitivity in tumor detection. Early diagnosis is integral to improved survival rates and there have been recent advances in

technology that have enabled early identification of the process of hepatic carcinogenesis *(*Patel et al., 2012*)*. Several cytokines may also have influence on hepatic carcinogenesis progression (Budhu and Wang, 2006). Interleukin 10 (IL-10) and transforming growth factor beta (TGF-b) are immune-suppressive cytokines and have been shown to inversely correlate with clinical outcome in patients with HCC (Karpenko et al., 2018). Moreover, many studies demonstrate that interferon-gamma (IFN-γ) supplementation can elicit tumor suppressive effects in models of HCC (Horras et al., 2011). It is believed that macrophage-derived cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 were the most important forces for liver regeneration after partial hepatectomy (Li and Hua, 2017). Interferon gamma inducible protein 10 (IP-10), also known as CXCL10, is secreted by several types of immune cells in response to IFNg. Pretreatment IP-10 levels may predict interferon-based treatment response in patients with chronic hepatitis B and C (Limothai et al., 2016), but its role in HCC has not been studied. In addition, the relation between interferon-gamma (IFN-γ) levels and the severity of liver diseases through fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) not been fully clarified. Thus, we aimed to characterize IFN-γ levels in liver-diseased patients before and after treatment.

MATERIALS AND METHODS

Study design and grouping

This study was conducted on 140 patients with HCV related chronic diseases classified into: 30 patients with HCC and 110 patients with CHC recruited from El Demerdash and Ain Shams University Hospitals, Cairo, Egypt, in addition to 30 healthy subjects enrolled as the control group. HBV co-infected patients or those who received previous antiviral treatment for HCV were excluded from the study. Diagnosis of HCC patients were assessed by computed tomography of the abdomen, abdominal ultrasonography and serum AFP > 200 ng/mL.

Collection of serum samples

Fresh venous blood samples collected, first part were collected and allowed to coagulate for thirty minutes and then centrifuged at 1,000×g for ten min. Then, the serum portion was aliquoted and stored until use at -20°C. until analysis and the second part in EDTA was collected and processed within 24 h for platelets count.

METHODS

Biochemical tests

Liver function tests alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and albumin. Liver function tests were measured on an automated biochemistry analyzer (*Hitachi 917;* Roche Diagnostics, Mannheim, Germany).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

Serum samples at 25µg/lane were separated by SDS-PAGE according to the method of Laemmli (Laemmli, 1970). The solution of proteins (antigens) to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non disulfide linked tertiary structures, and applies a negative charge to each antigen in proportion to its mass. Serum samples from chronic hepatitis C (CHC) patients, hepatocellular carcioma (HCC) and healthy individuals were analyzed by 12 % one-dimensional SDS-PAGE under reducing conditions. A mixture of reference proteins (Sigma Chemical Co., St. Louis, MO, USA) was run in parallel which includes Myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.0 kDa), and lysozyme (18.3 kDa). Then gel was stained with Coomassie blue. The Coomassie blue stained separated polypeptides have a wide range of molecular weights ranged from 215 KDa to 18.3 KDa.

Immunobloting technique Western blot

According to the method of Towbin (Towbin et al., 1979), SDS-PAGE separated samples were electro transferred onto nitrocellulose membrane (0.45-µm pore size; Sigma). Western blot was performed according to Attallah *et al* (Attallah et al., 2016) run conditions as NC filter was blocked in blocking buffer and was then rinsed in TBS and incubated with anti-gamma interferon IgG antibody with constant shaking overnight then washed in TBS three times, 10 min each. The NC filter was incubated with anti-mouse IgG alkaline phosphatase conjugate, for 2 hours with dilution of (1: 300) followed by washing in TBS as mentioned before. The target antigen for anti anti-gamma interferon IgG antibody was visualized by incubating the NC filter in substrate solution (BCIP/NBT) system. Then the reaction was stopped by distilled water.

Gel electroelution

IFN-γ bands were electroeluted separately as follow: In the unstained preparative gel, the target protein band was cut and electroeluted at 200 volts for 3 hr in a dialysis bag (Sigma). After dialysis against one liter of phosphate-buffered saline (PBS), pH 7.2 overnight at 4°C with constant stirring, the electroeluted protein band was concentrated using 50 mL polyethylene glycol for 1 hr. For further concentration the protein was precipitated using 40% trichloroacetic acid, final concentration (V/V) centrifuged at 6500 g for 15 min. The precipitate was washed twice using diethyl ether to remove the excess trichloroacetic acid. The excess diethylether was removed by gentle drying and the pellet was reconstituted in PBS (pH 7.2). The protein content of the purified IFN-γ was determined (Lowry et al., 1951) before the remainder was stored at -20°C until use.

Determination of IFN-γ

According to Attallah et al., (2016) Diluted serum sample (1:50), in coating buffer (pH 9.6), was tested (50-µl per well) for gamma interferon antigen. In brief, coated ELISA plate was sealed with an acetate plate sealer and incubated overnight at 2-8 ºC. After blocking of free binding sites, specific anti-gamma interferon antibody in PBS-T20 was added (50 µl per well) and incubated at 37 ºC for 2 h. After washing, 50 µl / well of anti-mouse IgG alkaline phosphatase conjugate diluted in 0.2% (w/v) BSA in PBS-T20, was added and incubated at 37 °C for 1 hr. The amount of coupled conjugate was determined by incubation with p-nitrophenyl phosphate substrate for 30 min at 37 °C. The reaction stopped and absorbance was read at 490 nm using ELISA reader (Σ960 Metretech, Germany).

Statistical analysis

The SPSS software package (version 24) and the GraphPad Prism package; v.6.0 (GraphPad Software, San Diego, CA) were used to analyze all statistical calculations. Continuous variables were expressed as the mean ± standard deviation (SD). Comparisons between different groups were analyzed by χ^2 test for categorical variables and nonparametric one-way ANOVA or Student *t*-test for continuous variables.

RESULTS

The Demographic clinical data of the studied groups

As shown in **Table 1**, in general, HCC patients tended to be older (50.2±11.6) than CHC (47.5±14.1) and healthy controls (48.51±4.9), without significant differences between all the participating groups (*P* =0.671). In addition, regarding gender differences, there were no significant differences between all the participating groups (*P* =0.834).

Biochemical laboratory data

As shown in Table 1, HCC patients had higher values of AST, ALT, and bilirubin but they had lower levels of serum albumin than those of CHC or healthy individuals (*P* < 0.0001 for all comparisons) except for ALT and serum total bilirubin significant difference was (*P* = 0.003 and 0.001; respectively).

Identification of INF-γ antigen

Selected serum samples from CHC and HCC patients as positive controls and healthy individuals as negative controls were analyzed by western blotting. Anti-INF-γ antigen monoclonal antibody was used as a primary antibody and an intense sharp band corresponding to IFN-γ target antigen was observed at 26 kDa in serum samples of HCV-infected patients. There was no reaction observed in healthy controls; Figure (1-A).

Quantitation of serum IFN-γ level

The mean ± standard error of mean (SEM) of serum IFN-γ level was (53.0±2.1 µg/ml) in HCC patients higher than in CHC patients (44.2±0.8 µg/ml) and healthy individuals (23.5 ±0.7 µg/ml) with extremely high significant difference (*P* <0.0001). The differences were also remain statistically significant (*P*<0.0001) when CHC group was compared with HCC group; Figure (1-B).

Level of INF-γ antigen in sera of CHC patients at different stages of liver fibrosis

Liver fibrosis was present in 110 CHC patients and was divided according to fibrosis-4 (FIB-4) index (Sterling et al., 2006; Kim et al., 2010) into (F1=25, F2-F3=55, and F4-F6=30). Moreover, INF-γ antigen level, mean ± SD, increase with progression of liver fibrosis where it was 38.6±1.2 µg/ml, 43.7±0.6 µg/ml 50.0±2.3 and µg/ml in F1, F2-F3, and F4-F6 respectively and there was significant difference (p < 0.0001) among serum samples of groups of patients**.**

Table 1: Baseline demographic data of participants

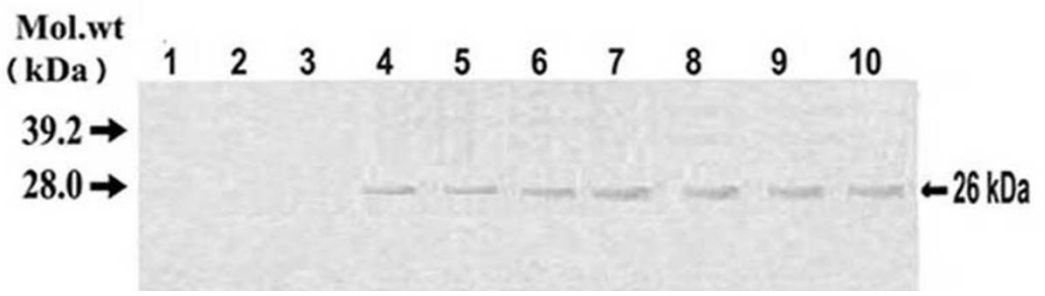
Variables[a]	Healthy	CHC[b]	HCC[c]	P value[d]
No	30	110	30	
Gender (male/female)	16/14	63/47	17/13	0.834
Age (years)	48.51±4.9	47.5±14.1	50.2±11.6	0.671
ALT (U/L)	28.0±5.2	67.4 ± 4.5*	72.7±3.4*	0.003
AST (U/L)	20.1±1.3	52.1±5.7*	75.3±1.6*#	0.0001
Total bilirubin (mg/dL)	0.7±0.1	1.5±0.2*	2.2±0.12*#	0.001
Direct bilirubin (mg/dL)	0.06±0.01	0.23±0.02*	1.10±0.05*#	0.0001
Indirect bilirubin (mg/dL)	0.71±0.03	0.67±0.42*	1.6±0.04*#	0.0001
Albumin (g/dL)	5.4±0.06	2.9±0.1*	1.8±0.09*#	0.0001

Data are presented as means ± S. Error.

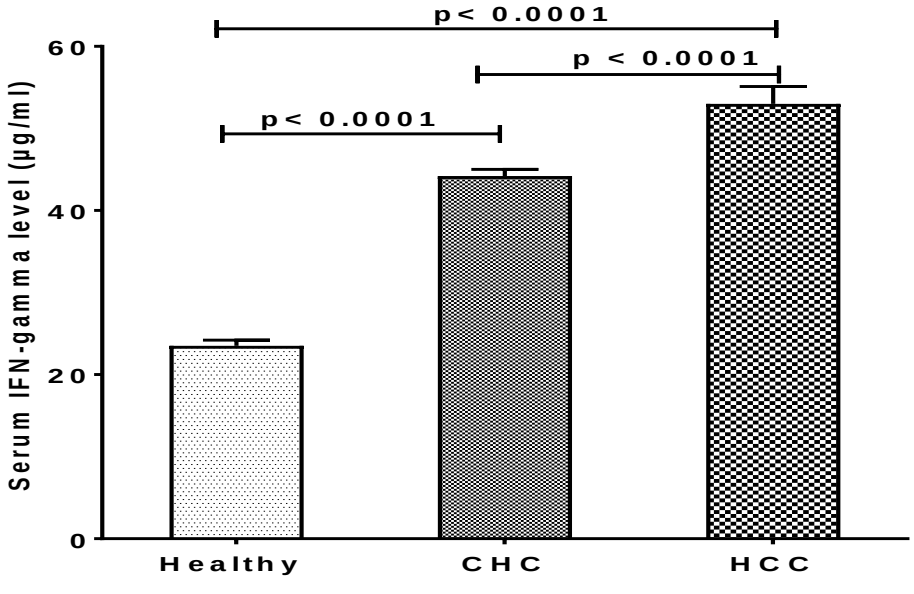
[a] Reference ranges: AST up to 40 U/L; ALT up to 45 U/L; total bilirubin: up to 1 mg/dl; and serum albumin 3.8-5.4 g/dL .[b]CHC: Chronic hepatitis C patients. [c]HCC: Hepatocellular Carcinoma.

*, **, *** = p < .05, p < 0.001, p < 0.0001; respectively significance difference versus Healthy group.

#, ##, ### = p < .05, p < 0.001, p < 0.0001; respectively significance difference versus CHC group.



1-A



1-B

Figure 1: (A) Western immunoblot analysis for detection of INF-γ antigen in serum samples from healthy individuals, CHC and HCC patients. Lanes 1-3: serum samples from healthy individuals; **Lanes 4-6:** serum samples from CHC patients; Lanes 7-10: serum samples from HCC patients. Lane (Mr): Molecular weight marker (sigma) was included myosin (215.0 kDa), phosphorylase B, (120.0 kDa), bovine serum albumin (BSA) (84.0 kDa), Ovalbumin (60.0kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.2 kDa), and lysozyme (18.3 kDa). (B) Quantitation of serum IFN-γ level antigen in serum samples from healthy individuals, CHC and HCC patients.

Biochemical response after receiving treatment

Out of 110 CHC patients, a Group of 70 CHC patients with available treatment information, in which 40 non cirrhotic patients who had received daclatasvir and sofosbuvir combination therapy and 30 cirrhotic patients divided into: 15 compensated patients received daclatasvir and sofosbuvir combination therapy and 15 decompensated patients received daclatasvir, sofosbuvir and ribavirin combination therapy for 3 months , were enrolled in this part of the study. In selected CHC patients, the post treatment liver function tests are listed in Table 2. After treatment, liver function parameters including ALT, AST, bilirubin and albumin all improved after antiviral therapy. ALT and AST levels normalized and showed an extremely significant decrease (*P*= 0.0003 and 0.0002; respectively). In addition, bilirubin level significantly improved after the antiviral therapy. Subsequently, albumin levels also significantly improved (baseline albumin 3.182 ± 0.04 g/dL *vs*. 3.65 ± 0.10 at treatment week 12; *P* < 0.0001).

Level of IFN-γ after 3 months treatment

After 3 months of receiving treatment for all the studied groups, serum concentration of IFN-γ was found to be significantly reduced (from 46. ±1.2 to 41.8 ± 1.1; *P*=0.023) as shown in Figure 2.

Table 2: Liver function tests for all studied groups before and after 3 months treatment

Variables	Before treatment	After treatment	*P* value*
ALT (U/L)	44.42 ± 3.80	29.04 ± 1.59	0.0003
AST (U/L)	50.29 ± 4.60	30.38 ± 2.29	0.0002
Total bilirubin (mg/dL)	0.88 ± 0.08	0.61 ± 0.03	0.003
Direct bilirubin (mg/dL)	0.26 ± 0.02	0.13 ± 0.01	0.0002
Indirect bilirubin (mg/dL)	0.66 ± 0.07	0.63 ± 0.02	0.667
Albumin (g/dL)	3.182 ± 0.04	3.65 ± 0.10	<0.0001

Data are presented as means ± S. Error.

[a]Reference ranges: AST up to 40 U/L; ALT up to 45 U/L; total bilirubin: up to 1 mg/dl; and serum albumin 3.8-5.4 g/dL .[b]CHC: Chronic hepatitis C patients. [c]HCC: Hepatocellular Carcinoma. [d]*P* value : $p > 0.05$ is non-significant; $p < 0.05$ is significant. **P* value: $p > 0.05$ is non-significant; $p < 0.05$ is significant.

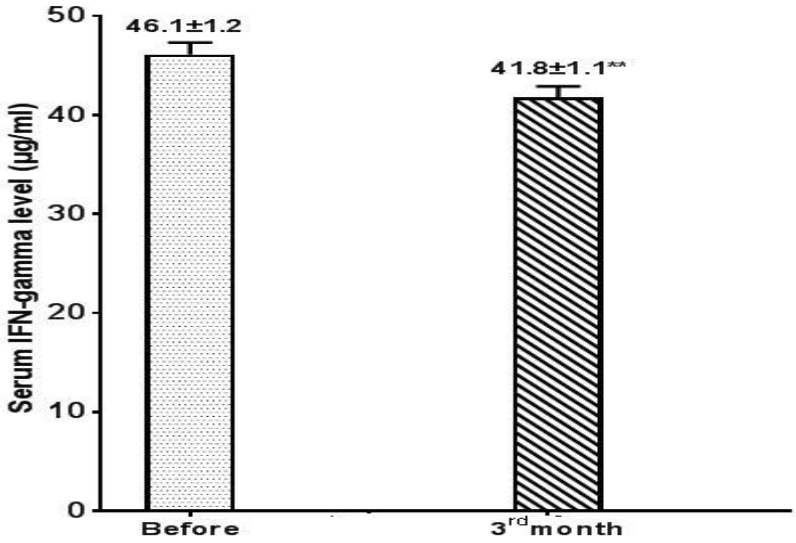


Figure 2: Level of IFN-γ after daclatasvir and sofosbuvir ± ribavirin treatment

DISCUSSION

The liver parenchyma is comprised of hepatocytes, which are fully differentiated, metabolically active cells. Under normal conditions, hepatocytes are mitotically quiescent, yet they can be induced to replicate following injury due to toxicant exposure, viral infection, or following surgical resection of a substantial portion of the liver (Michalopoulos, 2010). Host immunity may have important role in the prognosis of hepatocellular carcinoma (HCC) (Jemal et al., 2011). Interferon (IFN)-γ is a cytokine known for its immunomodulatory and anti-proliferative action. In the liver, IFN-γ can induce hepatocyte apoptosis or inhibit hepatocyte cell cycle progression (Horras et al., 2011). The aim of the present study was evaluation of IFN-γ in sera of patients with liver diseases using specific monoclonal antibody with immunochemical techniques. For this porous, the level of IFN-γ was quantified in serum samples of CHC and HCC patients using ELISA against sera collected from healthy individuals. In the present study, immunoblotting (Western blot) analysis, showed a molecular weight of 26 KDa for the single immunoreactive band in the sera of hepatic patient. Our finding agreed with (Jung, 2008; Attallah et al., 2016) when found that depending on its molecular form and extent of glycosylation, the literature values for the molecular weight of IFN-γ vary from 20 to 70 KDa.

A study *in vivo* showed that the level of IFN-γ increases from the 4th days post-infection (Karalyan et al., 2012); increased IFN-γ serum expression has been reported to promote antitumor activity, whereas sustained low-level expression of IFN-γ triggers tumorigenesis (He et al., 2005). *IFN-γ* has dual role as a tumor cell growth suppressor and apoptotic activity promoter, which has been described in human breast tumor cells (Ruiz-Ruiz et al., 2000). The relation between interferon-gamma (IFN-γ) levels and the severity of liver diseases through fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) not been fully clarified. Thus, we aimed to characterize IFN-γ levels in liver-diseased patients before and after treatment. In the present study, the levels of serum IFN-γ were increased in HCC patients than CHC patients and healthy controls as a function of the progression of liver disease. Previous study described an elevation in the transcript levels of various growth inhibitory inflammatory cytokines, such as transforming growth factor-β, interferon (IFN)-γ and interleukin (IL)-1 in the hepatic tissue (Singh et al., 2008). Attallah et al., (2016) found the increase in IFN-γ was associated with HCC ($P = 0.002$) compared with CHC patients. There was significant ($P < 0.0001$) association between IFN-γ levels and the fibrosis stages and activity. During liver injury and inflammation, hepatocytes increase expression of the transmembrane IFN-γ receptor (Valente et al., 1992), which presumably increases their sensitivity to IFN-γ stimulation (Lai et al., 2009). In the liver, IFN-γ also activates the IFN-γ receptor expressed on nonparenchymal cells, which include resident macrophages called Kupffer cells, the activation of which is important for mediating both innate and adaptive immune responses **(**Crispe, 2009).

In the present study, After 3 months of daclatasvir and sofosbuvir combination treatment and daclatasvir, sofosbuvir and ribavirin combination treatment, serum concentration of IFN-γ was found to be significantly reduced (from 46.1 ± 1.2 to 41.8 ± 1.1; P=0.023).

Several pretreatment factors are predictive of SVR in patients treated with IFN-α, including young age, a short duration of illness, mild activity on liver biopsy, the absence of cirrhosis, and low pretreatment serum level of HCV RNA (Fukutomi et al., 2000). However, the molecular basis underlying the failure of antiviral therapy in HCV is not fully understood; several studies related the risk of treatment failure to multiple factors, including both viral and host factors (Pawlotsky, 2003; Gao et al., 2004). Host immune factors have predictive value in influencing SVR. It has been reported that increased serum levels of cytokines (such as tumour necrosis factor- α (Larrea et al., 1996), interleukin (IL)-1b (Kishihara et al., 1996), IL-10 (Cramp et al., 2000), and chemokines such as IL-8 (Polyak et al., 2001; Mihm et al., 2004) correlated with a poor response to antiviral therapy in HCV patients. Also, elevated intrahepatic and serum levels of a T-cell specific chemokine termed interferon-γ inducible protein10 (IP-10) were detected in HCV infected patients, and serum IP-10 concentrations were higher in non-responders (NR) than in responders to antiviral therapy (Diago et al., 2006; Omran et al., 2014).

Great efforts have gone into understanding the reasons for pegylated interferon plus rebavirin treatment failure in chronic HCV infection. This is an important issue, since the standard treatment is physically demanding and costly (Asselah et al., 2010) and also still a propotion of patients did not achieve SVR after treatment with new direct antiviral drugs.

Early identification of non-responder patients is of particular importance as this might influence the decision for treatment prolongation (Reiberger et al., 2008). Our results may support the hypothesis that pretreatment may affect gamma interferon gene expression level in PBMCs and its protein could be used to predict treatment outcome. As serum interferon gamma may be higher in PBMCs of non-responders when compared to responders (Attia et al., 2013) but this waits further confirmatory study.

CONCLUSION

In conclusion, the steady increase of IFN-γ indicates that it may be involved in the pathogenesis of liver damage and the surveillance of serum IFN-γ may be used as a non-invasive biomarker for the potential diagnosis of HCC.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

The present study was the Ph.D. thesis of Al Najdi R. Al Najdi R collected the samples, performed the analysis and draft the manuscript. Barakat AB, Shoman SAH and Attallah AM were supervisors of the study and participated in its design and coordination, helped to draft the manuscript, interpreted the data, revised and approved the final revision.

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