ASSOCIATION OF PRETREATMENT SERUM INTERFERON γ INDUCIBLE PROTEIN 10 (IP-10) LEVELS WITH SUSTAINED VIROLOGICAL RESPONSE TO THE STANDERD THERAPY IN PATIENTS WITH CHRONIC HEPATITIS C

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ABSTRACT:

Hepatitis C virus (HCV) is a major cause of chronic liver disease. HCV is currently the most significant public health problem in Egypt. Related studies have found elevated IP-10/CXCL10 levels to be associated with increased liver damage. Forty samples were collected before the start of treatment (at zero point) in the period from January 2009 to July 2009, while the other 40 samples were collected from the same patients after 24 weeks from the beginning of treatment with interferon and ribavirin (standard therapy of chronic hepatitis C) in the period from August 2009 to February 2010. RNA extraction of hepatitis C and amplification by real time PCR for all samples were performed. Determination of IP10 levels in all samples was done. Higher level of the mean IP-10 (CXCL 10) in group B (non-responders) than group A (responders) was noticed. But there was no statistically significant difference between the 2 groups regarding the mean pretreatment IP-10 (CXCL 10) (P value = 0.12). No or weak negative association between IP- 10 (CXCL 10) at 24 week and response to treatment at 72 week was found so the high level of pretreatment IP-10(CXCL 10) at 24 week may not be associated with absence of sustained virologic response SVR.

KEY WORDS: Hepatitis C Interferon γ IP-10

INTRODUCTION:

Egypt has been widely regarded as having an epidemic with the highest recorded prevalence of HCV in the world (Alter, 2007). Egypt Demographic and Health Survey (EDHS) in 2009 estimated an overall anti-HCV antibody prevalence of 14.7% and the number of Egyptians estimated to be chronically infected was 9.8% (El-Zanaty F, 2009).

Generally when an individual is exposed to HCV, his body produces antibodies to the virus. These antibodies remain in the HCV-infected individual throughout his or her life, even if the virus is eliminated from the body naturally or following medical treatment. The diagnostic tests for HCV are divided into two categories; serological assays which detect the antibodies to HCV in the serum or plasma and molecular assays that detect, quantify or characterize HCV RNA in an infected patient (Yang, 2004).

Chronic HCV infection is curable, and cure is the goal of antiviral therapy. The current recommended therapy for chronic hepatitis C is a combination of peginterferon and ribavirin given for 24 or 48 weeks, depending on the viral genotype (Hoofnagle and Seeff, 2006, Dienstag and McHutchison, 2006).

Successful treatment is characterized by a sustained virological

response (SVR), defined by undetectable HCV RNA in a sensitive assay (detection limit ≤ 50 international units (IU)/ml) 6 months after the end of therapy. Recent large-scale follow-up studies have shown no relapse or recurrence after 4–6 years in more than 99% of patients who have an SVR (McHutchison et al., 2008, Swain et al., 2010).

Previous studies have reported increased serum and intrahepatic levels of the interferon (IFN)–γ–inducible protein (IP-10/CXCL10) in HCV genotype 1–infected individuals (Mihm et al., 2003, Butera et al., 2005). Related studies have found elevated IP-10/CXCL10 levels to be associated with increased liver damage and it has also been shown that serum IP-10/CXCL10 concentrations were higher in non-responders to HCV therapy than in those who achieve a sustained virological response (Romero et al., 2006, Lagging et al., 2006). In several independent studies, elevated serum/plasma levels of CXCL10 predict the failure of IFN-α–based HCV treatment (Butera et al., 2005, Diago et al., 2006).

In this study we aimed to measure the level of IP 10 in the serum of the patients with chronic hepatitis C before treatment with interferon 24 weeks after treatment, and then correlate the results with the response to treatment.

MATERIALS AND METHODS:

This is a retrospective study carried on samples that were collected in a previous study which is: Beneficial Health Effects of Probiotic Supplement to Hepatitis C Infected Patients.

80 serum samples were collected from 40 patients having chronic HCV infection at The Hepatology and Tropical Medicine Research Institute, Cairo, Egypt.

40 samples were collected before the start of treatment (at zero point) in the period from January 2009 to July 2009, while the other 40 samples were collected from the same patients after 24 weeks from the beginning of treatment in the period from August 2009 to February 2010.

Samples included in this study from patients who had underwent treatment with interferon and ribavirin (standard therapy of chronic hepatitis C) for 48 weeks, from patients who came back at 24 week to confirm virological response also from patients who came back at 72 week to confirm sustained virological response or relapse. RNA extraction of hepatitis C for all samples was performed and amplification by real time PCR. Genotyping was done by Restriction Fragment length Polymorphism (RFLP). Determinations of IP10 levels in all samples were done by EIA. Samples from patients not completing the full course of treatment, not returning at 24 week to confirm virological response or not returning after 72 week to confirm sustained virological response or relapse were excluded from the study.

Detection of hepatitis C by real time PCR

Peripheral venous blood was obtained from each subject and RNA extraction was done by Qiacube instrument (Qiagen, Germany) and then amplification was done by real time PCR. PCR products were obtained using 25 uL reactions (16.5 **master** mix and 8.5 RNA). The amplification conditions were performed as follows: 95^{o}C for 10 min, followed by 40 cycles of 15 seconds at 95^{o}C, for 60 seconds at 60 ^{o}C and for 90 seconds at 72^{o}C, and ending with a single 10 min

extension step at 72°C (Applied Biosystem, U.S.A).

Genotyping of HCV by (RFLP) procedure:

From the nested PCR products of 237 bp, 10µl were digested by restriction endonuclease enzymes for 2 hours at 37°C by both MvaI/HinfI in buffer H and RsaI/HaeIII in buffer L (Boehringer Manheim, Germany) (McOmish et al., 1994, Constantine et al., 1995) in a total volume of 20µl. All enzymes were used at 7.5units/ reaction. Electrophoresis was done in a 4 % Metaphore gel in 0.5X TBE buffer (FMC Bio products, USA).

Determination of IP 10 level:

The determination of IP 10 level was done by EIA technique using RayBio® Human IP-10 ELISA Kit, RayBiotech, Inc., December 2011. The RayBio®

Human IP-10 EIA kit is an in vitro enzyme-linked immune-sorbent assay for the quantitative measurement of human IP-10 in serum. This assay employs an antibody specific for human IP-10 coated on a 96-well plate. Standards and samples were pipetted into the wells and IP-10 present in a sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human IP-10 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells and color developed in proportion to the amount of IP-10 bound. The Stop Solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

Statistical analysis:

Statistical analysis was done using SPSS 17.0 software for Windows. Since all tested variables were continuous (scale), data were compared between the two groups using Chi-Square and Mann-Whitney U-test and correlation with Spearman's coefficient r test.

RESULTS:

Patients were divided into 2 groups according to the SVR. Group A included 35 patients with sustained viral response (SVR) and group B included 5 patients (with no SVR including relapsers and non- responders). Both groups A and B were randomly selected. Group A included 25 males (71.4%) and 10 females (28.6%), and age ranged from 21 to 58 years. The mean age was 38.25±11.47 SD. Group B included 2 males (40%) and 3 females (60%), and age ranged from 37 to 47 years. The mean age was 42.5±+4.36 SD.

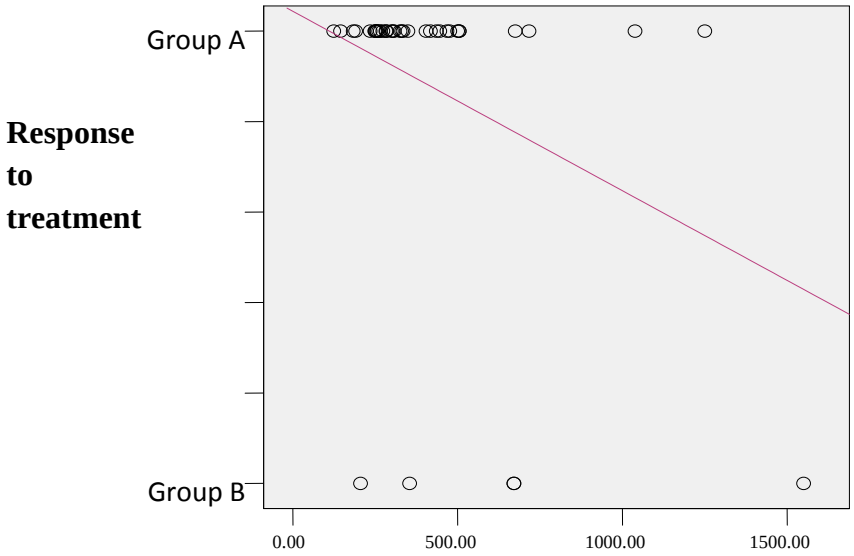
There was no statistically significant difference between the 2 groups regarding the mean age (P value =0.39 NS). In Group A, viral load ranged from 2600 to 18,000,000 IU/ml with a mean of 2,658,017±4,947,208.8 SD. In Group B, viral load ranged from 640,000 to 11,000,000 IU/ml with a mean of 3,808,000±5875572.9 SD. There was no statistically significant difference between the 2 groups regarding the mean Pretreatment viral load (P value =0.12).

In group A, There were 2 patients with genotype 1 (6%), 2 patients with genotype 3 (6%), 29 patients with genotype 4 (88%), and 2 patients with undetected genotype. (Genotype has not been detected due to lack of documentation). In group B, all the patients had genotype 4 (100%).

In group A, the mean IP-10 (CXCL 10) was 364.1±234 SD, while in group B, the mean IP-10 (CXCL 10) was 507±193.6 SD.

Higher level of the mean IP-10 (CXCL 10) in group B (non responder) than group A (responders) was noticed. But there was no statistically significant difference between the 2 groups regarding the mean Pretreatment IP-10 (CXCL 10) (P value =0.12).

Correlation between IP-10 (CXCL 10) at 24 week and response to treatment: No or weak negative association between IP- 10 (CXCL 10) at 24 week and response to treatment at 72 week was found so the high level of pretreatment IP-10(CXCL 10) at 24 week may not be associated with absence of SVR (Fig 1).

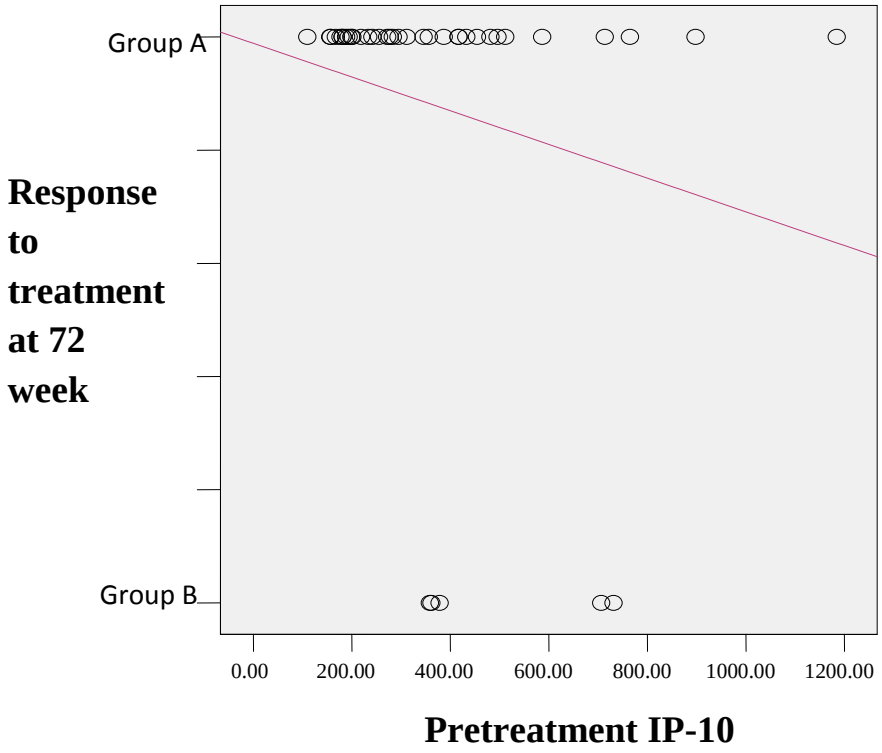


r= -0.23, p= 0.15

Grades of r: 0.00 to 0.24 (weak or no association), 0.25 to 0.49 (fair association), 0.50 to 0.74 (moderate association), 0.75+ (strong association).

Figure (1) the correlation between response to treatment and IP-10 at 24 week.

Correlation between pretreatment IP-10 (CXCL 10) and response to treatment at 72 week: A fair negative association between pretreatment IP-10 (CXCL 10) and response to treatment at 72 week was found as the high level of pretreatment IP-10(CXCL 10) was associated with no SVR (Fig 2).

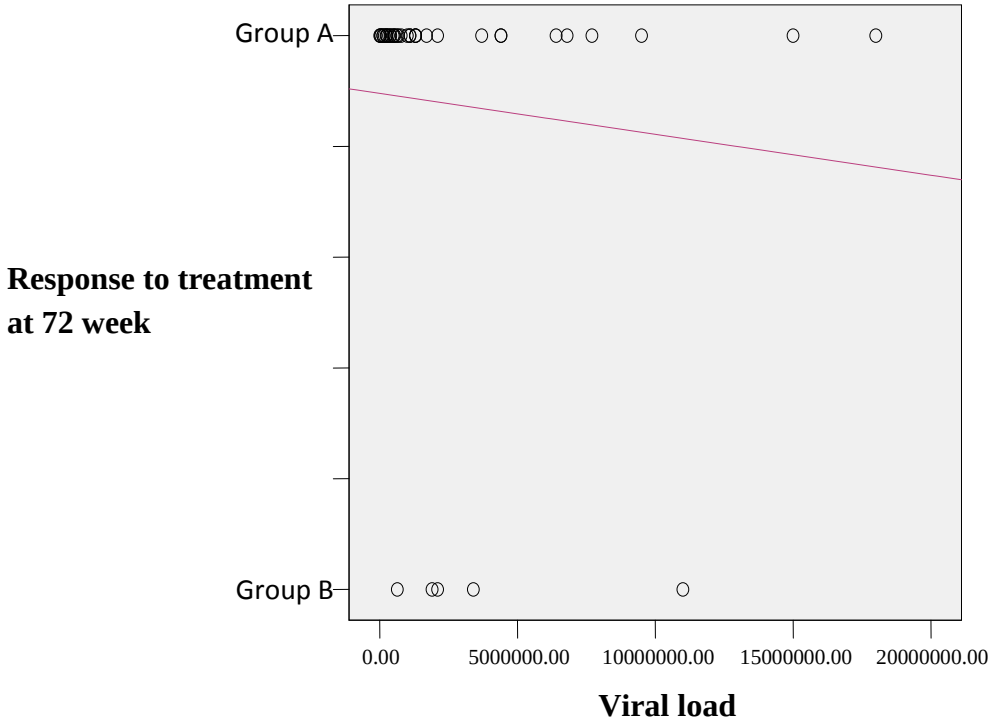


r= -0.27, p= 0.09

Grades of r: 0.00 to 0.24 (weak or no association), 0.25 to 0.49 (fair association), 0.50 to 0.74 (moderate association), 0.75+ (strong association).

Figure (2): the correlation between pretreatment IP-10 (CXCL 10) and response to treatment at 72 week.

Correlation between viral load and response to treatment at 72 week: A fair negative association between pretreatment viral load and response to treatment at 72 week was found as the low viremia is associated with SVR (Fig 3).



r= -0.25, p= 0.12

Grades of r: 0.00 to 0.24 (weak or no association), 0.25 to 0.49 (fair association), 0.50 to 0.74 (moderate association), 0.75+ (strong association).

Figure (3): the correlation between pretreatment viral load and response to treatment at 72 week.

DISCUSSION:

Chronic infection with hepatitis C virus (HCV) is a major public health problem, with nearly 170 million infe-cted individuals' worldwide (Shepard et al., 2005). Chronic HCV infection leads to a wide spectrum of liver diseases, ranging from mild chronic hepatitis to end stage cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). For more than a decade, Egypt has been widely regarded as having an epidemic with the highest recorded prevalence of HCV in the world (Alter, 2007). However, this treatment is effective in fewer than 50% of patients infected with HCV genotype1 or 4 (Casrouge et al., 2011). The current recommended therapy for chronic hepatitis C is a combination of pegylated IFN-α2 and ribavirin given for 24 or 48 weeks, depending on the viral genotype (Hoofnagle and Seeff, 2006). Studies identified the chemokine IP-10 (CXCL10) as an important negative prognostic biomarker. Given that CXCL10 mediates chemo-attraction of activated lymphocytes, it is counterin-tuitive that this chemokine correlates with therapeutic non responsiveness (Casrouge et al., 2011).

We conducted a retrospective randomized study on 40 chronically-infected HCV patients that were being treated with pegylated IFN-α2 plus ribavirin. The patients were divided into 2 groups: group A included 35 patients showing SVR (if the serum viral load was undetectable 24 weeks after the completion of treatment) and group B showing no SVR (patients having a relapse if the serum viral load was undetectable at end of treatment but detectable 24 weeks after the completion of treatment or non responders (NR) if HCV RNA was detectable in serum at the end of treatment and 24 weeks after the completion of treatment).

Pretreatment plasma levels of IP-10(CXCL10) and IP-10(CXCL10) levels at 24 week were measured. The virological response was assessed at 24 weeks after the end of treatment, i.e. at 72 weeks.

In the present study, the majority of patients were genotype 4 (89.4%) while (5.3%) were genotype 1 and (5.3%) were genotype 3. Other scholars found different genotype distribution which is possibly due to the place of the study. In a study carried by Diago and his colleagues (2006), 137 patients with chronic hepatitis C infection treated with interferon-α plus ribavirin were investigated. Genotyping of patients' samples showed that 103 patients were genotype 1 while 34 patients were non-1 genotype (Diago et al., 2006).

We found no statistically significant difference between the two groups regarding mean age and pretreatment viral load but there was statistically difference between the two groups regarding mean gender.

We noticed a higher level of the mean IP-10 (CXCL 10) (507±193.6 SD) in group B (non-responder) than group A (364.1±234 SD) (responders), also there was fair negative correlation between pretreatment IP-10(CXCL 10) level and the response to treatment which was more or less statistically non significant (P= 0.09). Also there was a non significant correlation between IP-10(CXCL 10) level at 24 week and the response to treatment (P=0.15). We found a fair negative association between pretreatment viral load and response to treatment at 72 week but it was statistically non significant (P=0.15).

Apolinario and coworkers found a significant association between baseline serum IP-10 levels and the type of therapeutic response, IP-10 (CXCL10) levels being significantly higher in patients with no SVR (381 ± 138 pg/ml) than in those who obtained an SVR (245 ± 154 pg/ml) (P = 0.01). This relationship was independent of the type of peginterferon or ribavirin used (Apolinario et al., 2004).

In 2005, Butera and coworkers studied 29 patients with HCV infection.11 patients (38%) achieved SVR while 18 (62%) showed no SVR. IP-10 (CXCL10) levels measured 7 days pretreatment were lower in patients with SVR than in those with no SVR (p=0.018) (Butera et al., 2005). Similar results were obtained by Lagging and coworkers who found that pretreatment IP-10 levels predict RVR and SVR in patients infected with HCV genotype 1(Lagging et al., 2006).

Darling and colleagues (2011) also studied 272 patients with chronic hepatitis C treated with peginterferon

and ribavirin. There were 157 sustained responders and 115 non responders. Mean IP-10 was lower in sustained responders compared with non responders (437 ± 31 vs 704 ± 44 pg/mL, P <0.001) (Darling et al., 2011).

We found that all previous results come in agreement with ours which confirm that the pretreatment IP-10 serum level is associated with the SVR in chronic hepatitis C patients.

Our results came in some agreement with the results of Moura and coworkers (2011) who measured pretreatment plasma levels of IP-10 (CXCL10) among 41 patients with chronic hepatitis C infection treated with interferon-α plus ribavirin. The mean of the pretreatment plasma levels of IP-10 (CXCL10) in patients with SVR was 289.9 (157.7-512.9) and in those with no SVR was 142.7 (86.7-206.6). Analysis of the association between pretreatment levels of IP-10 (CXCL10) and virological response showed that elevated IP-10 (CXCL10) levels were associated with a lack of SVR (p = 0.045) (Moura et al., 2011).

A study carried by Romero and colleagues reported that strong association was observed between low IP-10 levels and SVR (P=0.01) which is also in agreement with our results (Romero et al., 2006).

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