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RESEARCH ARTICLE

ASSESSMENT OF TISSUE INHIBITOR METALLOPROTEASES-1 (TIMP-1) AS A BIOMARKER IN HCV PATIENTS

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ABSTRACT

Egypt contains the highest prevalence of hepatitis C virus (HCV) infection in the world. The goal of this study was to elucidate where tissue inhibitor metalloproteases-1 (TIMP-1) acts as a diagnostic biomarker of HCV infection and studying the effect of the treatment of interferon for different intervals on TIMP-1 levels with studying the relation of TIMP-1 with different liver markers. This study was carried out on 65 blood samples divided into six groups. TIMP-1 and different liver markers were assessed in all groups. There was significant increase in mRNA expression of TIMP-1 levels, all liver enzymes and bilirubin in HCV patients. Albumin, total protein and A/G ratio showed significant decrease. AFP showed insignificant increase in all groups. Treatment with interferon for 24 weeks caused good regression in TIMP-1 levels to nearly normal ranges which indicate the improvement effect of interferon on HCV patients. TIMP-1 had significant correlations with different liver markers. TIMP-1 can be considered as a diagnostic biomarker for HCV infection which can monitor the liver status.

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INTRODUCTION

Hepatitis C virus (HCV) is a common chronic viral infection documented worldwide (Soriano *et al.*, 2006). The World Health Organization has declared hepatitis C a global health problem, with approximately 3% of the world's population (roughly 170-200 million people) infected with HCV. Egypt contains the highest prevalence of hepatitis C in the world (Mohamed, 2004). The percentage of Egyptians with HCV is 14.7%. This is ten times greater than any other country in the world. The prevalence of HCV in Western countries is less than 2%. The drivers of the HCV epidemic in Egypt are not well understood, but the mass parenteral antischistosomal therapy (PAT) campaigns in the second half of the 20th century are believed to be the determinant of the high prevalence (Cuadros *et al.*, 2014). There are at least 6 major genotypes or genetic strains of HCV, each comprising multiple subtypes, have been identified worldwide (Zein and Persing, 1996). Substantial regional differences appear to exist in the distribution of HCV genotypes. HCV genotype 4 appears to be prevalent in North Africa and the Middle East, particularly in Egypt (Abdulkarim *et al.*, 1998).

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The presence of numerous subtypes of each HCV genotype in some regions of the world, such as Africa and Southeast Asia, may suggest that HCV has been endemic for a long time. Conversely, the limited diversity of subtypes observed in the United States and Europe could be related to the recent introduction of these viruses from areas of endemic infection (Smith and Simmonds, 1997). The gold standard to treat patients with chronic HCV infection is interferon (IFN) with ribavirin (Palumbo, 2011). Under physiological conditions, IFN is a key cytokine produced by virtually all cells in the mammalian organism in response to a variety of bacterial and viral stimuli.

In response to viral infection, IFN produced by the infected target cells stimulated NK-cells and T-cells induce a number of cellular genes and exert a multitude of immune stimulatory effects of innate and adaptive immunity involved in inhibition of viral replication (Pestka, 1997). Ribavirin is an antiviral drug taken orally that alters the body's immune response to viruses. In the treatment of hepatitis C, it has been shown to be very effective in combination with interferon rather than as a treatment on its own. Combination therapy is the standard form of treatment for hepatitis C. It consists of interferon and ribavirin. Treatment consists of a 24 week course of interferon

injections, self-administered once a week, plus ribavirin capsules daily taken (Ward, 2005). The matrix metalloproteinases are inhibited by specific endogenous tissue inhibitors of metalloproteases (TIMPs), which comprise a family of four protease inhibitors: TIMP1, TIMP2, TIMP3 and TIMP4 (Brew *et al.*, 2000). These endogenous inhibitors control the activity of matrix metalloproteases (MMP) and other metalloproteases (Brew and Nagase, 2010). In this respect, TIMP-1 is an important regulator of extracellular matrix turnover. TIMP-1 is a widely expressed and secreted protein that plays a critical role in tissue remodeling via inhibiting members of a large family of MMPs (Chirco *et al.*, 2006). TIMP-1 has been suggested to be a serum marker for liver fibrosis, and the expression is induced during liver injury (Nobili *et al.*, 2009). In addition, TIMP-1 also plays an important role in promoting liver fibrosis (Parsons *et al*., 2004), but inhibiting liver regeneration (Gieling *et al.*, 2008). The profibrogenic effects of TIMP-1 are thought to be mediated via preventing collagen degradation through inhibition of MMPs and protecting against activated hepatic stellate cell (HSC) death (Mohammed *et al.*, 2005). It is believed that activated HSCs and Kupffer cells are the major sources for TIMP-1 production during liver injury (Roeb *et al.*, 1994). Early studies also showed TIMP-1 mRNA and protein expressions are up-regulated by inflammatory cytokines. The aim of this study was to elucidate where TIMP-1 may act as a biomarker for prognostic or diagnostic of HCV infection with studying the effect of interferon (INF) treatment for different intervals on TIMP-1 levels and studying the relation of TIMP-1 with different liver function markers.

MATERIALS AND METHODS

Subjects

This study was carried out on 65 blood samples. Blood samples of 15 healthy volunteers were considered as the negative control (GI). Fifty HCV patient samples divided according to the treatment into 5 groups (10 for each). The second group (GII) included HCV patient samples who didn't receive any treatment. The third (GIII), fourth (GIV) and fifth (GV) groups were the samples of HCV patients who treated with INF (800 units once/week) and ribavirin (two tablets 200 mg, three times daily) for only 4, 16 weeks and for 24 weeks (the end of treatment course), respectively. The last group (GVI) was the samples of HCV patients who treated only with Cyncholine plus as a liver support by a dose of two capsules (each capsule contains 215 mg) three times per day. Cyncholine plus was obtained from Arab Company for Pharmaceuticals and Medicinal plants MEPACO-Egypt (Enshas El-Raml, Sharkeiya, Egypt).

Each capsule of Cyncholine plus contains Cynara dry extract (100 mg) + Mentha dry extract (25 mg) + Heavy magnesium oxide (40 mg) + Silymarin (50 mg). HCV patient samples were admitted from the National Institute of Endemic Disease and Liver, Ministry of health, Egypt, Ministry of medicine, from June 2014 to January 2015. Full medical history was taken with special attention to any associated medical problems. All individuals were subjected to full clinical examination and didn't show clinical symptoms or signs of any other problems except hepatitis. The study protocol was revised and approved ethically by the ministry of medicine, Egypt.

Blood samples

Four milliliters of venous blood were collected from all subjects and were divided into two parts. The first part included about 3.5 ml of total blood which was left to clot at 37 ºC for 10 minutes, and then centrifuged at 4000 rpm for 10 minutes. Serum was then separated and divided into several aliquots and stored at -20°C for using in biochemical parameters determination. The second part included 0.5 ml of whole blood samples separated in EDTA-tubes and stored in a refrigerator at 4°C for few hours or approximately one day, then it used in the total RNA extraction. These samples used as soon as possible to give the highest yield of extracted RNA.

Liver markers tests

Liver enzymes as GGT (EC 2.3.2.2), AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were spectrophotometrically determined according to the methods of Szasz *et al.*, 1969; Bergmeyer *et al.*, 1986; 1985, respectively, using the commercial kits of Biodiagnositic Co. Whereas, total protein was estimated using the biuret reaction according to Doumas *et al* (1981) and albumin was determined in the presence of Bromcresolgreen in acidic medium according to Gendler (1989). Serum total bilrubin was estimated using the commercially available kits (Vitro-Scient Co.) depending on the reaction between bilirubin and diazotisedsulfanilic acid. The colored complex was spectrophotometrically measured according to the Jendrassik-Grof method (Jendrassik and Grof, 1938). Quantitative determination of AFP was carried out on the principle of Enzyme Immunoassay method. Serum alpha fetoprotein (AFP) was estimated using the commercially available kits (BioCheck Chemical Co., UK). The Alpha fetoprotein AFP ELISA test is depended on the principle of a solid phase enzyme-linked immunosorbent assay. The concentration of AFP is directly proportional to the color intensity of the test sample (Engall *et al.*, 1980).

TIMP-1 assessment

Total RNA isolation was estimated using the commercially available kits (Thermoscientific Gene JET RNA Co., #k0731). Samples were lysed and homogenized in Lysis Buffer, which contains guanidine thiocyanate, a Chaotropic salt capable of protecting RNA from endogenous RNases (Chomczynski and Sacchi, 1987). The lysate is then mixed with ethanol and loaded on a purification column. The chaotropic salt and ethanol cause RNA to bind to the silica membrane while the lysate is spun through the column (Boom *et al.*, 1990). The purity of the RNA was estimated by the ratio of the absorbance at 260/280 ($A_{260/280}$). The RNA was stored at -80 °C until use. Reverted reverse Transcriptase of cDNA was estimated using the commercially available kits (Thermoscientific revertAid, First strand Synthesis Kit, #k1621). cDNA calibrators were prepared by PCR amplification run to saturation (35 PCR cycles; each consisting of 5 min at 95 °C for predenaturation, 30 s at 95 °C for denaturing, 30 s at 56 °C for annealing, 4 min at 72 °C for extension and 10 min at 72 °C for final extension

after the last cycle) using the kit of ThermoScientific Co. (#K1081) with the appropriate primer of TIMP-1 (the Forward primer 5′dTACAGTTCCTCTACGCCCCA3′ and the Reverse primer 5′dACATCCCCCAAGCTCCCTAT3′). The samples showed a unique band in agarose electrophoresis. The relative mRNA gene expression of TIMP-1 was determined using SYBR Green by the real-time thermal cycler (Rotor-Gene Q 5-plex). Relative mRNA levels were calculated by means of $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT$ = difference of crossing points of test samples and respective control samples as extracted from amplification curves by the Rotor-Gene Q series software 2.0.3).

Statistical Analysis

Statistical analysis was performed by Graphpad prism 6 software (Graphpad, San Diego, CA). Data are expressed as means±SD (n=10 in each group except in the control group n=15). Student t test was performed to compare values from 2 groups. ANOVA (1-factor analysis of variance) was used to compare values obtained from three or more groups, followed by Tukey's post hoc test. Statistical significance was taken at the $P < 0.05$ level.

with INF + ribavirin for 16th& 24th weeks showed good improvement in liver aminotransferases. Patients who treated with only Cyncholine plus capsules as a liver support showed statically significant increase in aminotransferases levels in comparison with control group (Table 1).

Serum total protein and albumin in HCV patients (GII) showed statically significant decrease. HCV patients who treated with INF + ribavirin for 4th week, 16th and 24th weeks showed insignificant decrease with values were very close to normal concentrations. However, treatment with only Cyncholine plus capsules didn't show good regression in both total protein and albumin. Serum globulin concentration showed insignificant changes in all groups when compared to the control group. A/G ratio was very high statically significant decrease in HCV patients (GII) and HCV patients treated with only Cyncholine plus capsules as a liver support (GVI).

There were insignificant changes in all HCV patients treated with interferon + ribavirin for different intervals, when compared to the healthy volunteers (GI) (Table 2).

Table 1. Assessment of different liver enzymes in serum of all groups

Group (treatment)	GGT (µkat/L)	AST	ALT
GI (-ve control)	0.217±0.029	0.618±0.026	0.434± 0.016
GII (HCV)	0.294±0.013[a]	0.929± 0.032[a]	1.18± 0.019[a]
GIII (4week,INF+ribavirin)	0.287±0.013[a]	0.718± 0.025[a,b]	0.531± 0.023[a,b]
GIV (16week,INF+ribavirin)	0.237±0.028	0.595± 0.025[b]	0.498± 0.027[b]
GV (24week,INF+ribavirin)	0.223±0.018	0.601±0.033[b]	0.450± 0.008[b]
GVI (HCV+cyncholine)	0.256±0.025	0.656± 0.009[a,b]	0.517±0.202[a,b]

Data are expressed as mean±SD. The symbols (a) and (b) indicate a significant changes ($p<0.05$) in comparison with GI (healthy volunteers, negative control) and GII (HCV patients, positive control), respectively.

Table 2. Total protein, Albumin, Globulin and A/G ratio assessment in serum of different groups

Group (treatment)	Total Protein (g/L)	Albumin (g/L)	Globulin (g/L)	A/G ratio
GI (-ve control)	73.3± 5.7	38.3±5.2	35.0± 1.1	1.10±0.10
GII (HCV)	56.3±3.2[a]	23.0±1.7[a]	33.3±1.5	0.69±0.05[a]
GIII(4week,INF+ribavirin)	63.3±5.8	38.4±5.7[a]	34.9±2.5	0.81±0.17
GIV(16week,INF+ribavirin)	69.8±1.0[b]	35.0±4.0[b]	35.0±1.7	1.00±0.15
GV(24week, INF+ribavirin)	72.2±7.0[b]	36.1±8.1[b]	35.0±1.7	1.02±0.20
GVI (HCV+cyncholine)	59.3±3.0[a,b]	26.0±3.6[a]	33.3±1.5	0.78±0.15[a]

Data are expressed as mean±SD. The symbols (a) and (b) indicate a significant changes ($p<0.05$) in comparison with GI (healthy volunteers, negative control) and GII (HCV patients, positive control), respectively.

Table 3. Total Bilirubin and AFP assessment in serum of different groups

Group (treatment)	Total bilirubin (µmol/L)	AFP (µg/L)
GI (-ve control)	9.57±0.992	0.63±1.53
GII (HCV)	15.91±2.61[a]	0.73±0.05
GIII (4weeks,INF+ribavirin)	14.71±1.86[a]	0.53±0.05[b]
GIV (16weeks,INF+ribavirin)	14.19±2.61[a]	0.46±0.06[b]
GV (24weeks, INF+ribavirin)	11.97±1.71	0.47±0.11[b]
GVI (HCV+cyncholine)	19.32±5.2	1.00±0.26

Data are expressed as mean±SD. The symbols (a) and (b) indicate a significant changes ($p<0.05$) in comparison with GI (healthy volunteers, negative control) and GII (HCV patients, positive control), respectively

RESULTS

Aminotransferases (ALT and AST) and GGT enzyme activities showed significant elevation in HCV patients without treatment. Patients treated with INF + ribavirin for 4th weeks showed also significant elevation, while Patients treatment

Total bilirubin levels significantly increased in HCV patients (GII) in comparison with the healthy individuals. HCV patients treated with interferon + ribavirin for four weeks and HCV patients who treated with only Cyncholine plus also showed significant increase. One the other hand, INF + ribavirin

treatment for 16th and 24thweeks showed good improvement. Bilirubin concentrations of groups IV and V were nearly normal levels. Serum AFP levels showed insignificant increase in all studied groups in comparison to the normal levels obtained from the control group (Table 3). TIMP-1 appeared at 470 bp in all samples and showed a unique band in agarose electrophoresis (Figure 1).

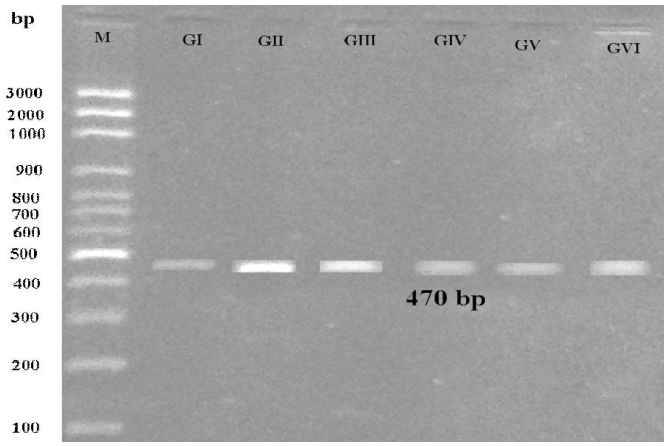

Figure 1. Analysis of PCR products by ethidium bromide stained agarose gel electrophoresis. Gel band pattern of PCR product (TIMP-1 appeared at 470 bp) of serum of different groups. Where, M = marker of DNA (100-3000 bp, lane 1). GI=negative control group (lane 2), GII=HCV group (lane 3), GIII=4w (INF+ribavirin) (lane 4), GIV=16w (INF+ribavirin) (lane 5), GV=16w (INF+ribavirin) (lane 6) and GVI= HCV+cyncholine (lane 7)

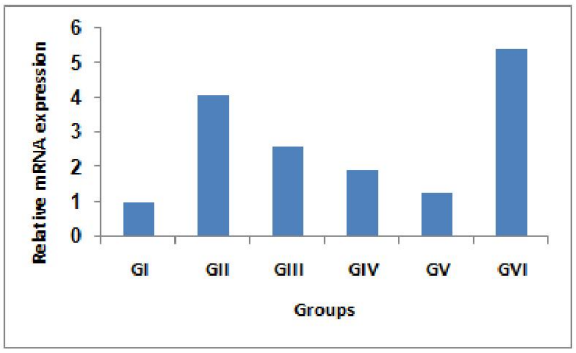

Figure 2. mRNA expression of TIMP-1 gene in different groups relative to the calibrator. Where GI=negative control group, GII=HCV group, GIII=4w (INF+ribavirin), GIV=16w (INF+ribavirin), GV=16w (INF+ribavirin) and GVI= HCV+Cyncholine

The mRNA expression of TIMP-1 gene increased in HCV patients in relative to the control group. Treatment with interferon + ribavirin showed TIMP-1 mRNA expression return back to its nearly normal levels, especially after 24th week treatment. However, HCV patients treated with only Cyncholine plus as a liver support showed mRNA expression of TIMP-1 gene appeared high statically significant in comparison to the healthy control (Figure 2). TIMP-1 had significant positive correlation with serum AST, ALT, GGT, total bilirubin, AFP and A/G ratio in HCV patients. TIMP-1 had significant negative correlation with serum total Protein, albumin in the HCV patients. On the other hand, TIMP-1 had no statically significant positive correlation with serum globulin (Table 4).

Table 4. Correlation analysis betweenTIMP-1 andthe other liver parameters in HCV patients (results are expressed as correlation coefficients)

Parameter	TIMP-1	
	r	*p*
AST	0.068	0.0001
ALT	0.549	0.0001
GGT	0.373	0.0015
Total protein	-0.433	0.005
Albumin	-0.550	0.053
Globulin	0.089	0.528
A/G ratio	0.590	0.002
Total bilirubin	0.118	0.0164
AFP	0.0004	0.0344

AST, Asparatate aminotransferase; ALT, Alanine aminotransferases; GGT, Gamma glutamyltransferase; A/G ratio, Albumin/globulin ratio; AFP, Alpha fetoprotein.

DISCUSSION

Hepatitis is inflammation of the liver, the irritation or swelling of liver cells from any cause. It most commonly caused by a viral infection. Liver enzymes especially transaminases (AST and ALT) are useful biomarkers of liver injury in a patient with some degree of intact liver function (Johnston, 1999). From the data obtained in the results, both ALT and AST enzymes showed significant increase in HCV patients (GII) reached to about 2.7 and 1.5 folds for ALT and AST, respectively. Patients treated with INF for four weeks (GIII) also showed significant increase. Patients treated with INF + ribavirin for 16th and 24th weeks (GIV and GV) showed nearly normal values of ALT and AST; this can be considered as indicator for good recovery. These results are in agreement with that of Diehal *et al.* (1984) who stated that "A significant number of patients with HCV infection had elevated serum ALT and AST levels". Among HCV patients, higher GGT activity has been associated with more severe liver disease in a number of cross-sectional studies (Paolicchi *et al.*, 2005).

GGT is also a component of many scores that were constructed for noninvasive evaluation of fibrosis stage (Hessien *et al.*, 2010). The GGT activities obtained in this study showed significant increased in HCV patients who did not receive any treatment (GII) and in HCV patients who treated with INF + ribavirn for only 4 weeks (GIII). GGT activities in the two other intervals of INF + ribavirn treatment (16th and 4thweeks, represented as GIV and GV) were very close to normal. Patients who treated with only Cyncholine plus (GVI) didn't show any regression in all liver enzyme activities in comparison to the healthy control. These results are in agreement with that of Silva *et al.* (2004) who stated that "A significant number of patients with chronic HCV infection had elevated serum GGT levels. Other study suggested that GGT is an independent predictor of both virological response and clinical outcomes among HCV patients. GGT activity was found to predict INF treatment response and liver disease outcomes in a large cohort of HCV patients and advanced fibrotic disease (Everhart and Wright, 2013).

Bilirubin assessment is one of the most common liver function tests and used as a sensitive indicator of hepatic dysfunction (Raymond and Galambos, 1971). Our results demonstrated that

total bilirubin levels significantly increased in HCV patients (GII) in comparison with healthy individuals (GI). The HCV patients treated with INF + ribavirin for four weeks and HCV patients who treated with Cyncholine plus (GIII and GVI, respectively) also showed significant increase. The two other intervals of INF + ribavirin treatment (16^{th} and 24^{th} weeks, represented in GIV and GV)_ showed nearly normal bilirubin levels. Total protein and albumin concentrations showed statically significant decrease in patients suffering from HCV without receiving any treatment (GII) and in HCV patients treated with only Cyncholine plus capsules (GVI) in comparison to the healthy normal group (GI). Combination therapy of INF + ribavirin for different intervals showed insignificant decrease, especially after 24 weeks (GV).

Albumin is synthesized in the liver, and low serum albumin may be indicative of liver failure or diseases such as cirrhosis or chronic hepatitis (Anderson and Douglas, 2000). This can be considered as good improvement of liver and its synthetic functions capacity recovery. These data are in agreement with many previous studies which reported decreased serum albumin concentrations in chronic activity hepatitis and liver cirrhosis in general and especially in high anti-HCV (+) patients (Osman *et al.*, 2007). The albumin/globulin (A/G) ratio is a very sensitive liver function biomarker because whether in some cases, total protein is normal, elevated, or low; a decrease in the A/G ratio often indicates the presence of impaired liver function.

The normal range of A/G ratio is about 1 (McClatchey and Kenneth, 2002). The data obtained in this study revealed great fall in A/G ratio to about 63 % of its normal ratio in HCV patients who did not receive any treatment. Treatment with INF + ribavirin showed good regression of liver represented as elevation in A/G ratio, especially after 16^{th} and 24^{th} week treatment (GIV and GV, respectively) which showed ratio nearly equal to the normal. However, treatment with only cyncholine plus (GVI) didn't show any improvement; A/G ratio was fallen to about 70% of the normal value. These results are in agreement with a study of Cucu *et al* (2015) which reported the decrease in the (A) fraction and the A/ G ratio in patients with chronic hepatitis.

AFP is the most abundant plasma protein found in the human fetus. AFP level may be elevated in patients with chronic liver disease such as hepatitis or cirrhosis, or patients with drug or alcohol abuse (van-der Veek *et al.*, 2011). Patients with acute and chronic liver disease have also shown increased values (Arrieta *et al.*, 2007). Our data revealed that serum AFP levels increased by insignificant way in all studied groups in comparison to the negative control group. These results were in agreement with a previous study of Abdel-Baki and Zaki (2014). Other study suggested that among patients with advanced chronic hepatitis C, serum AFP values are frequently elevated, even in the absence of HCC (Di Bisceglie *et al.*, 2005). On the other hand, another study stated that AFP is often measured in subjects with chronic hepatitis C for diagnosing hepatocellular carcinoma. However, its prevalence and clinical significance remain inconclusive in subjects without hepatocellular carcinoma (Chen *et al.*, 2008).

mRNA expression of TIMP-1 gene is induced in the liver during liver injury. It is generally believed that activated hepatic stellate cells (HSCs) and Kupffer cells are the major source of TIMP-1 production as strong TIMP-1 immunostaining was detected in activated HSCs and Kupffer cells (Jeong *et al*., 2006). Our results obtained in this study showed up-regulation of mRNA expression of TIMP-1 gene in all HCV patients. HCV infection caused increase of TIMP-1 levels by incredible way in untreated HCV patients. HCV patients (GII) showed TIMP-1 mRNA expression increased than the healthy volunteers by about 4 folds.

Treatment with INF + ribavirin showed TIMP-1 mRNA expression return back to its nearly normal levels, especially after 24^{th} week treatment (GV). However, HCV patients treated with only cyncholine plus as a liver support (GVI) showed mRNA expression of TIMP-1 gene higher than HCV patients who didn't receive any treatment (GII); these patients had TIMP-1 mRNA expression reached to about 5.4 folds in comparison to the healthy control (GI).These results are in agreement with early studies proved that TIMP-1 mRNA and protein expression are up-regulated in HCV patients (Fontana *et al*., 2008). By using the statistical analysis of the obtained data, it is found that there were significant positive correlations between TIMP-1 and serum AST, ALT, GGT, total bilirubin, AFP and A/G ratio. On the other hand, TIMP-1had a significant negative correlation with total protein and albumin. However, TIMP-1 had insignificant correlation with globulin.

Conclusion

From the obtained results in this study it's found that, mRNA expression of TIMP-1 gene levels were elevated in HCV patient; this could be happened as liver disease progress. Interferon treatment showed improvement in TIMP-1 levels to nearly normal ranges which indicate the treatment effect of interferon on HCV infections. Tissue inhibitor metalloprotease-1 (TIMP-1) can be considered as a diagnostic and prognostic biomarker for HCV infection which can monitor the liver status.

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