Overexpression of Cyclooxygenase-2 and Transforming Growth Factor-Beta 1 is an Independent Predictor of Poor Virological Response to Interferon Therapy in Chronic HCV Genotype 4 Patients

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ABSTRACT

Background/Aims: COX-2 and TGF-β1 are overexpressed in hepatitis C virus (HCV) infection and are related to hepatitis pathogenesis and hepatic fibrosis. The current study investigated the relationship between pretreatment COX-2 and TGF-β1 hepatic expression in HCV genotype 4 and the virological response to interferon therapy. **Patients and Methods:** Liver biopsies of 55 patients with HCV infection genotype 4 were selected together with 10 liver biopsies as control. The patients' clinicopathological data were collected. Immunohistochemistry was done using anti-COX-2 and anti-TGF-β1 antibodies. Statistical tests were used to determine the association between both COX-2 and TGF-β1 expression in relation to clinicopathological parameters and response to interferon therapy. **Results:** COX-2 was upregulated especially in nonresponders and was an independent predictor of poor virological response. However, COX-2 showed no association with other clinicopathological features. TGF-β1 was upregulated and associated with nonresponders, histological activity, and fibrosis stage. There was no association between TGF-β1 and other clinicopathological features. There was an association between COX-2 and TGF-β1 immunoexpression. **Conclusion:** Overexpression of COX-2 and TGF-β1 is an independent predictor for poor outcome of interferon and ribavirin therapy and these might be useful markers for the response to treatment. Both molecules are associated together; however, their role during hepatitis treatment has to be clarified.

Key Words: Chronic hepatitis, cyclooxygenase-2, hepatitis C virus genotype 4, immunohistochemistry, transforming growth factor-beta1, virological response

Internationally, there is a range of 130-170 million infected with hepatitis C virus (HCV).[1] Worldwide, 3-4 million people are newly infected each year, with the predominant prevalence of infection with genotype 1, followed by genotypes 2 and 3. Specific geographical distribution is characteristic for genotypes 4,5, and 6. Chronicity develops in more than 80% of acutely infected persons and a considerable percentage die of HCV-related illnesses.[2] Egypt has the largest percentage of HCV infected people worldwide. Among different regions and demographic areas, a range of 6%-40% of the population have anti-HCV antibodies, and 10% have chronic HCV infection.[3,4] HCV transmission is ongoing in Egypt, and incidence rate is estimated to be 165,000 new infections per year.[5] Genotype 4 is very high among Egyptian HCV patients. HCV is a leading cause of hepatocellular carcinoma (HCC) and chronic liver disease in Egypt.[6,7] HCV genotype 4 represents about 55% of all Egyptian HCV patients.[8]

Cyclooxygenase-1 (COX-1) and COX-2 are enzymes that convert arachidonic acid into prostaglandins and thromboxanes. COX-1 is constitutively expressed in various tissues and plays important roles in homeostasis. However, COX-2 is involved in inflammation, angiogenesis, antiapoptosis, and cancers.[9-11] Increased COX-2

immunoexpression in liver tissues from patients with HCV,[12] and in HCV-positive hepatocellular carcinoma[13] has been reported. The HCV core protein was able to upregulate COX-2 expression in hepatocyte-derived cells, providing a potential mechanism for hepatic fibrosis during chronic HCV infection.[14] Interferon (IFN) treatment has been shown to decrease COX-2 expression in the liver in HCV infection.[12]

Transforming growth factor-beta (TGF-β) is a member of the multifunctional cytokines. The TGF-β superfamily consists of three main isoforms, namely, TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is a dimeric protein that acts either as a hormone or locally as a regulator of proliferation, differentiation, extracellular matrix production, and cell death.[15] It has a role in cell growth control, cell adhesion and motility, alteration of cellular phenotype, production and degradation of ECM protein, and apoptosis of hepatic cell lines.[16] The main sources of TGF-β1 in the liver are activated stellate cells and Kupffer cells.[17] In HCV infection, TGF-β1 is upregulated in the liver and may be involved in the pathogenesis of chronic liver disease.[18] In HCV infection, TGF-β1 role in the pathogenesis of hepatic fibrosis may be the most important[19] and is a prognostic marker of hepatic damage.[20]

Understanding the molecular mechanisms that are involved in the pathogenesis of HCV-liver damage is crucial in improving the current nonsatisfying therapeutic strategy. COX-2 and TGF-β1 are investigated in liver cirrhosis and hepatocellular carcinoma. COX-2 expression in early stages of HCV infection expression plays a role in liver pathology. However, in HCV infection COX-2 and TGF-β1 immunoexpression has not yet been extensively investigated. There is little data about the effect of hepatic COX-2 and TGF-β1 immunoexpression on the virological response. Hence the current study is dedicated to demonstrate the relationship between the expression of COX-2 and TGF-β1 in hepatic tissue of HCV genotype 4 patients before starting INF-α-based therapy and virological response to treatment.

PATIENTS AND METHODS

Patients
The current study involved liver biopsies and clinicopathological data of 55 chronic HCV patients. The patients' data were retrieved from the archives of the departments of Internal Medicine and Pathology, Faculty of Medicine, Minia University, Egypt. Data are presented in Table 1. Patients had anti-HCV antibodies detected by enzyme-linked immunosorbent assay and confirmed by detection of HCV-RNA by quantitative PCR. HCV genotype was determined by a second-generation line probe assay and patients with genotype 4 were selected. There were 26 patients categorized with low viral load

and 28 with high viral load. All patients had pre-treatment liver biopsy as a final confirmation of hepatitis along with grading and staging. Exclusion criteria included HBsAg or HIV seropositivity and other chronic liver disease. Patients were known to receive a combined pegylated IFN-α2a and ribavirin therapy. The virological response was reported as (1) early responders (ER): Undetectable or ≥ 2 log reduction of HCV-RNA 3 months after initiation of therapy, (2) early nonresponders (ENR): HCV-RNA was more than 2 log at 3 months after initiation of therapy, (3) sustained responders (SR): HCV-RNA was undetectable at 6 months after stop of therapy, and (4) relapsers: HCV-RNA was detectable at 6 months after stop of therapy. Table 2 shows the incidence of virological response. There were a total of 56.3%

Table 1: Baseline characteristics of patients

Parameter	Value
Number (*n*)	55
Age range (years)	37-53
Sex	54:1
Male: female	
Alanine transaminase (ALT) (IU/L)	38.89 (2.847)
Aspartate transaminase (AST) (IU/L)	41.25 (2.505)
Bilirubin (mg/dL)	1.03 (0.0517)
Viral load	
Low	28 (50.9%)
High	27 (49.1%)
Grade of histological activity (Metavir system)	
A (1)	31 (56.4%)
A (2)	20 (36.4%)
A (3)	4 (7.2%)
Stage of fibrosis (Metavir system)	
F (1)	23 (41.8%)
F (2)	18 (32.7%)
F (3)	14 (25.5%)
F (4)	0 (0%)
Fatty change	
0	23 (41.8%)
1	13 (23.7%)
2	15 (27.3%)
3	4 (7.2%)

Values represent the number (*n*) (%), and mean (the SEM). SEM: Standard mean of error

Table 2: Virological response to combined interferon and ribavirin

Response	*n* (%)
Responders	
Early responders	46 (83.4)
Sustained responders	31 (56.3)
Non-responders	
Early nonresponders	9 (16.4)
Relapsers	15 (27.3)

with sustained virological response and 43.7% nonresponders. This work was completed according to the ethical rules of the Faculty of Medicine, Minia University, Egypt.

Histopathology

Paraffin blocks of liver biopsies of patients were retrieved from the archives of the Department of Pathology, Faculty of Medicine, Minia University. New sections were prepared and stained with hematoxylin and eosin stain and Masson trichrome stain and examined for grading and staging according to the Metavir System.[21] Grading of fatty change was done as follows: Grade 0: <5% of hepatocytes are affected; grade 1: 5%-33% of hepatocytes are affected, grade 2: >33%-66% of hepatocytes are affected; and grade 3: >66% of hepatocytes are affected.[22] In addition, 10 liver biopsies diagnosed as no pathological changes were retrieved belonging to age-matched individuals with normal clinical examination, normal liver chemistry, negative hepatitis markers and HIV, and normal abdominal ultrasonography. Pathological findings of patients are reported in Table 1.

Immunohistochemistry

Sections were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich® Germany) diluted in acetone. Sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked using DAKO® peroxidase blocking reagent for 10 min. Slides were pre-treated in microwave oven in citrate buffer (pH 6). Subsequently, slides were incubated with mouse monoclonal anti-COX-2 antibody (Thermo Fisher Scientific Anatomical Pathology (CA, USA), dilution 1:100) and mouse monoclonal anti-TGF-β1 antibody (AbD Serotec (UK), dilution 20 µg/mL). Econo Tek horse radish peroxidase (HRP) goat anti-polyvalent ready to use detection system was used (ScyTek Laboratories Inc., UT, USA). Econo Tek biotinylated anti-polyvalent was applied for 30 min at room temperature. Then, Econo Tek HRP was applied for 30 min at room temperature and finally diaminobenzidine (DAB) was applied for 10 min. Slides were counterstained and mounted. Appropriate washing between all steps was performed. Positive control tissue for each antibody was used and negative control slides were included. Assessment of immunostaining was performed semi-quantitatively. Positively stained hepatic cells for COX-2 and TGF-β1 were counted at ×200 in each case. The mean values were calculated and expressed as percentage to total hepatic cells. The fractions of percentage of positive hepatic cells for COX-2 and TGF-β1 were divided as follows: (1) 0%-25%, (2) 26%-50%, and (3) 50%-100%. The staining intensity was expressed as follows: 0, none; 1, weak; 2, intermediate; and 3, strong. A 6-scale scoring system was used to categorize COX-2 and TGF-β1 immunoexpression that combines the staining intensity and extent. For the statistical analysis, COX-2 and TGF-β1 immunostaining scores 1-3 were considered as low expression and immunostaining scores 4-6 were considered as high expression.

Statistical analysis

Data are presented as the mean ± the standard error of mean (SEM). Differences between two groups of patients for one variable were tested by using the Mann–Whitney test. To test the association between three groups of patients for one independent variable the Kruskal–Wallis test was used. Pearson Chi-square test was used to determine the association between two independent categorical data. Binary logistic regression analysis was used to predict the virological response in relation to immunoexpression of COX-2 and TGF-β1. Estimated odds ratio [exponential (B)], 95% confidence interval (CI) for exp (B), and significance were denoted for each analysis. Statistical procedures were performed using SPSS® Release 16.0. Statistical significance was determined at P value of ≤ 0.05 and tests were two sided.

RESULTS

Correlations between pathological findings, laboratory findings, and virological response

There was a significantly higher level of alanine transaminase (ALT) in patients with fatty change score 3 ($P = 0.021$). On the other hand, there was no significant difference between the levels of aspartate transaminase (AST) and bilirubin in relation to scores of fatty change ($P = 0.074$ and $P = 0.424$, respectively). High-grade histological activity (A3) has higher levels of ALT and AST ($P = 0.05$). However, there was no association with bilirubin levels ($P = 0.398$). The stage of fibrosis, viral load, and virological response showed no association with the level of ALT, AST, or bilirubin. The clinicopathological features such as fatty change, grade of histological activity, stage of fibrosis, and viral load were not able to predict the virological response [Table 5].

COX-2 immunostaining and its relationship with virological response and clinicopathological features

COX-2 immunostaining was observed in the cytoplasm of hepatocytes. The highest incidence of staining was near portal tracts and in hepatocytes in interface hepatocytes near lobular inflammation [Figure 1]. Overall, there were more cases with high COX-2 immunoexpression than low expression [Table 3]. There was no COX-2

Table 3: Distribution of COX-2 and TGF-β1 immunostaining

Score of expression	COX-2		TGF-β1	
	Control (%)	Patients (%)	Control (%)	Patients (%)
Low expression	10 (100)	25 (44.5)	10 (100)	26 (47.3)
High expression	0 (0)	30 (54.5)	0 (0)	29 (52.7)

immunostaining in control biopsies. Expression of COX-2 was significantly higher in nonresponder patients than in responders (P = 0.002) [Table 4]. There was no statistical difference between high and low COX-2 expression in relation to ALT (P = 0.918), AST (P = 0.593), bilirubin (P = 0.328), fatty change (P = 0.920), grade of histological activity (P = 0.734), stage of fibrosis (P = 0.604), and viral load (P = 0.320). Logistic regression analysis showed that high hepatic COX-2 immunoexpression was an independent predictor of poor virological response (P < 0.001) [Table 5]. COX-2 immunostaining is significantly associated with TGF-β1 immunostaining (P < 0.001).

TGF-β1 immunostaining and its relationship with virological response and clinicopathological features

TGF-β1 showed a granular diffuse staining in hepatocyte cytoplasm of patients [Figure 2]. Control biopsies revealed faint cytoplasmic TGF-β1 immunostaining. In hepatitis patients, high TGF-β1 immunostaining was observed in more cases than low expression, whereas all control biopsies revealed a very low expression. TGF-β1 immunoexpression was significantly higher in nonresponder patients than in responders (P = 0.001) [Table 4]. Also, there was higher TGF-β1 immunoexpression with higher grade of histological activity (P < 0.001), and stage of fibrosis (P = 0.001). There was no statistical difference between high and low TGF-β1 expression in relation to ALT (P = 0.589), AST (P = 0.617), bilirubin (P = 0.738), fatty change (P = 0.335), and viral load (P = 0.508). Logistic regression analysis showed that high hepatic TGF-β1 immunoexpression was an independent predictor of poor virological response (P = 0.024) [Table 5].

DISCUSSION

The current study demonstrates for the the first time the relationship between COX-2 and TGF-β1 immunoexpression in HCV genotype 4 and their relationship to response to
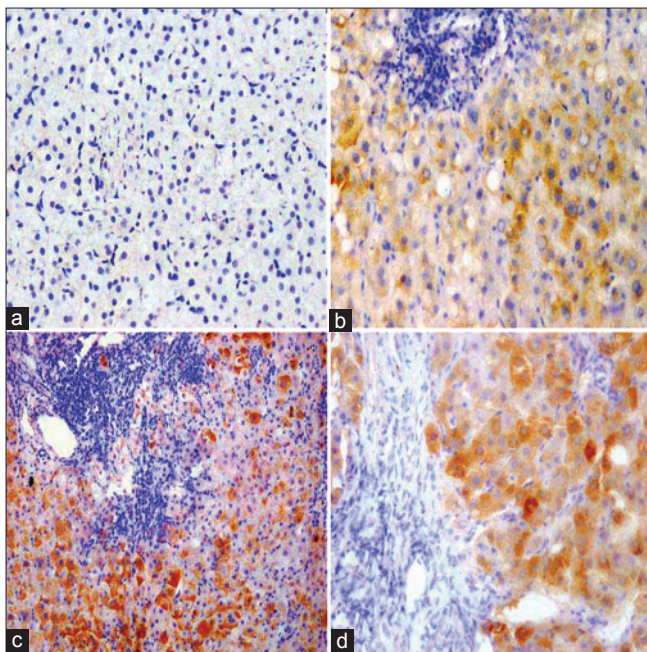

Figure 1: Immunostaining of COX-2 (a) Control liver tissue, which is completely negative; (b) Low-grade histological activity shows a moderate staining in lobular hepatocytes. (c) High-grade histological activity shows an intense staining in periportal hepatocyte. (d) Stage 3 fibrosis shows extensive and strong staining in the vicinity of portal tract. Magnification used was ×200.
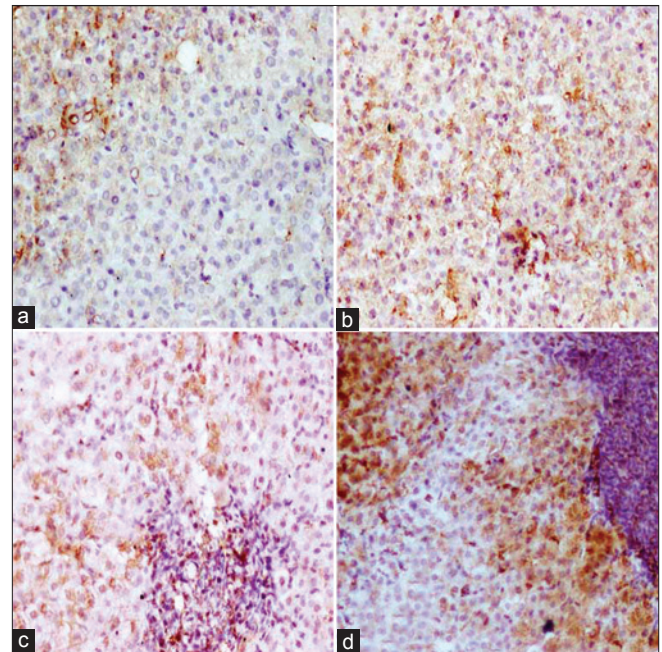

Figure 2: Immunostaining of TGF (a) Control liver tissue shows low expression. (b) Low-grade histological activity shows a moderate granular cytoplasmic staining in lobular hepatocytes. (c) Low-grade histological activity shows an intense staining in periportal hepatocyte. (d) Grade 3 histological activity shows extensive and strong staining in the periportal region. Magnification used was ×200.

Table 4: Distribution of COX-2 and TGF-β1 immunostaining in relation to virological response

Score of expression	COX-2		TGF-β1	
	Responders	Nonresponders	Responders	Nonresponders
Low expression	25/27 (92.6%)	0/28 (0%)	19/27 (70.4%)	7/28 (28%)
High expression	2/27 (7.4%)	28/28 (100%)	8/27 (29.6%)	21/28 (75%)
P value	0.002		0.001	

Table 5: Regression analysis for virological response

Variable	Exp (B)	95% CI for exp (B)	*P* value
Viral load	1.442	0.499-4.171	0.499
Histological activity	0.256	0.023-2.851	0.161
Fibrosis	0.636	0.155-2.613	0.531
Fatty change	1.537	0.501-4.717	0.453
COX-2 immunostaining	0.147	0.043-0.504	<0.001
TGF-β1 immunostaining	0.368	0.155-0.876	0.024

combined therapy with pegylated IFN-α2a plus ribavirin. There is a genotype-specific interaction between the key players of HCV infection pathogenesis.[23] COX-2 has a potential role in HCV-RNA replication.[13] The current study demonstrated that COX-2 was upregulated in a subset of chronic HCV genotype 4. This finding is similar to previous reports from other HCV genotypes.[12,24,25] COX-2 inhibition was claimed to provide a mechanism-based chemopreventive approach to hepatitis.[26] In the current study, COX-2 immunoexpression was higher in nonresponders than in sustained responders. Similar findings had been previously reported.[12,27] The present study had shown for the first time that COX-2 upregulation is an independent predictor of poor virological response. Manning *et al.* reported a reduction of liver Cox-2 level after initiation of INF therapy,[12] however, they found that pre-treatment and post-treatment hepatic COX-2 expression were not related to treatment outcome. In their study, the sample size was very small and they only used the intensity of staining to score COX-2. However, in our study we used a combination of the extent and intensity of staining.

Data from the present study could not establish a positive link between liver expression of COX-2 and increased inflammatory activity and fibrotic stage. This finding is contrary to previous reports, which correlated COX-2 overexpression with high inflammatory grades, advanced stages of fibrosis, and cirrhosis.[14,24,25,28-32] This conflict may be related to the difference in genotype of HCV, sample size, or the method of scoring of COX-2. However, there was no association with COX-2 immunoexpression and ALT level, which is similar to a previous finding.[12] On the contrary, serum ALT correlated with COX-2 levels.[27,33] So, COX-2 is involved in the hepatic inflammatory process; however, it may not be a rate-limiting factor for inflammatory activity and stage of fibrosis.

In the current study, TGF-β1 is upregulated in HCV infection. Similar finding has been previously reported.[20,24,34,35] Upregulation of TGF-β1 expression by HCV core is considered a mechanism by which the virus induces cellular proliferation, fibrosis, and predisposes to malignancy.[36] It is well known that TGF-β1 is a very potent profibrogenic factor[37] and it seems to be the most important factor in the pathogenesis of fibrosis in HCV infection.[19] In the current

study, there was a higher level of TGF-β1 immunoexpression in advanced stage of fibrosis than in lower stage. This is consistent with several reports correlating the level of TGF-β1 (both in serum and in hepatic tissue) with the progression of fibrosis in HCV infection.[19,20,38] In the current study, there was no association with TGF-β1 and serum ALT level and viral load. These results are supported by similar findings.[35,38,39] In the present study, fatty change and ALT levels were not associated with TGF-β1 immunoexpression. However, there was an association between high TGF-β1 expression and high inflammatory grade, which was reported earlier by Bedossa *et al.*[39] ALT is known to reflect hepatocellular damage, which is scored histologically by inflammation and necrosis. TGF-β1 in our study is associated with higher histological grade of inflammation but not with high ALT level. This notion may need further investigation on a large scale study to verify that conflict.

In this study, the relationship of TGF-β1 to the therapeutic outcome in patients' population was investigated. TGF-β1 tissue expression was higher in nonresponders than in responders. Several studies reported that TGF-β1 expression is decreased in HCV infection after treatment with IFN-α both in responders and nonresponders.[19,39-42] However, others have shown that TGF-β1 expression only declines after therapy in responders and not in nonresponders, and Marek *et al.*, found an increase in TGF-β1 levels in nonresponders after treatment.[40,43] The difference in post-treatment levels in responders and nonresponders may be due to pre-treatment high TGF-β1 as in our study. Overall, therapeutic outcome in HCV hepatitis patients is associated with significant changes in TGF-β1, which may have a prognostic significance regarding treatment efficacy.[19] Our results from logistic regression analysis have shown that high TGF-β1 tissue level is an independent predictor for nonresponders. Although we did not examine post-treatment tissue levels of TGF-β1, our novel finding is supported by the studies, which found that the post-treatment TGF-β1 level is high in nonresponders.

In the present study, a positive association between COX-2 and TGF-β1 immunoexpression was found. This was reported previously.[24] In our study both factors were found to be independent predictors of poor virological response, which suggests a synergistic effect in the aetiopathogenesis of HCV infection.

Limitations of our study include a relatively small number of patients and lack of biopsy during treatment and after treatment.

In conclusion, the current study has shown upregulation of COX-2 and TGF-β1 in HCV infection, which is associated with poor virological response. COX-2 and TGF-β1 can be considered useful predictive markers for the response to INF

therapy in HCV infection. The exact role that COX-2 and TGF-β1 play during hepatitis treatment has to be clarified by *in vitro* and *in vivo* studies to reveal intracellular events associated with response to treatment.

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