

## Expression of Toll Like Receptors 3 & 7 in Peripheral Blood from Patients with Chronic Hepatitis C Virus Infection and Their Correlation with Interferon- $\alpha$

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Toll-like receptor 3 (TLR3) and Toll-like receptor 7 (TLR7) are pathogen- recognition receptors that are expressed on innate immune cells. They recognize viral RNA which induces their activation with subsequent increase in IFN- $\alpha$  transcription. It has been postulated that HCV may cause down regulation of these receptors as one of immune evading mechanisms that participate in viral persistence. The aim of this work is to investigate the expression of Toll-like receptors 3 (TLR3) and 7 (TLR7) in peripheral blood from patients with chronic hepatitis C infection and correlate their expression to the peripheral blood expression of interferon- $\alpha$  (IFN- $\alpha$ ), viral load and histopathology of the liver. IFN- $\alpha$ , TLR3 and TLR7 expression in peripheral blood from patients with chronic hepatitis C infection (n= 30) and from healthy controls (n= 20) were measured by real time polymerase chain reaction. Viral load and Liver biopsy were done for all patients. The results showed lower expression of TLR3 and TLR7 in patients than controls, and levels of expression correlated positively with IFN- $\alpha$  expression. No correlation was found between TLR3 and TLR7 and viral load or histopathological staging and grading of the liver tissue. In conclusion, HCV may induce down regulation of TLRs (TLR3 and TLR7) expression on innate immune cells with subsequent decrease in INF-  $\alpha$  production suggesting that new therapies that aim to increase the expression level of TLRs may help in treatment of HCV infection.

**T**oll-like receptors (TLRs) are membrane-bound receptors that recognize pathogen-associated molecular patterns and signal through adaptor molecules, myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain containing adaptor protein, Toll/IL-1 receptor domain containing adaptor inducing interferon- $\beta$  (TRIF), and TRIF-related adaptor molecule to activate transcription factors, nuclear factor (NF)- $\kappa$ B, activator protein 1, and interferon regulatory factors (IRFs) leading to the initiation of innate immunity. This system promptly initiates host defenses against invading microorganisms (Mihai & van der Meer, 2011).

Among the mammalian TLRs, TLR3 and TLR7 are related to recognition of RNA. TLR3 is expressed on endosomal membranes

(and the plasma membranes of some cells) and thus senses double stranded dsRNA of viral origin and is expressed preferentially in dendritic cells (Wang *et al.*, 2009). Once engaged, TLR3 triggers the activation of Interferon-regulatory factor 3 (IRF-3), a transcription factor playing a critical role in the induction of type I interferon and NF- $\kappa$ B through signaling processes that require the protein TRIF (Leea *et al.*, 2012). The type I IFN further up-regulates TLR3 in an autocrine/ paracrine manner, a phenomenon linked to its anti-viral gene defense action (Agarwal *et al.*, 2011).

TLR7 is sensor for viral, single-stranded ssRNA. Toll like receptor 7 appears to be preferentially expressed by plasmacytoid dendritic cells and B lymphocytes. This TLR also trigger IRF-7 mediated type I IFN

production upon activation, but unlike TLR3, the induction of IFN by TLR7 is coupled to the adaptor protein MyD88 and not to TRIF (Chen *et al.*, 2009).

Recent studies have shown that TLR3 also plays important roles in the pathophysiology of a variety of liver diseases, which may attribute to the wide expression of TLR3 on all types of liver cells including hepatocytes, stellate cells, sinusoidal endothelial cells, Kupffer cells, biliary epithelial cells as well as immune cells such as NK cells, and liver lymphocytes (Seki & Brenner, 2008).

TLR3 and TLR7 are generally believed to play an important role in the viral hepatitis infection. The antiviral effects of signaling on viral hepatitis infection are likely mediated via stimulating the production of type I IFN that subsequently inhibits hepatitis C virus (HCV) replication (Yin & Gao, 2010).

However, several lines of evidence suggest that TLRs signaling is suppressed during viral hepatitis infection, which may contribute to the escape of hepatitis virus from the surveillance of innate immunity and lead to the chronic infection. A greater understanding of the specific cellular source of TLR signals and TLR pathway changes in different stages of viral infection may assist in the design of appropriate therapeutic interventions that target these receptors in patients with chronic viral hepatitis infection (Seki & Brenner, 2008).

Type I IFN induces the maturation of dendritic cells (DCs) by increasing both the expression of co-stimulatory molecules such as CD80, CD86, and CD40 and antigen presentation via major histocompatibility complex class I in addition to classical endogenous antigen presentation; it also facilitates the cross-presentation of viral antigens. A cumulative report has shown that DC activation via TLR signaling is a prerequisite for the subsequent induction of

vigorous T-cell responses (deLuca *et al.*, 2011).

HCV infects many millions of people worldwide. It causes chronic liver inflammation and fibrosis; resulting in cirrhosis and hepatocellular carcinoma. HCV is classified within the family *Flaviviridae*, HCV is a small, enveloped RNA virus that possesses a positive-sense, single-stranded RNA genome. Some HCV-derived products, including HCV-RNA and several HCV proteins trigger host defense. Immune responses, including cell-mediated immunity and type I IFNs, are vital in controlling and clearing HCV infection (Chang *et al.*, 2010). HCV evades the host immune system to sustain a chronic infection. HCV interferes with signals of the TLR3-TRIF –IRF-pathway. NS3/4A hepatitis C protein induces degradation of TRIF as TRIF shares amino acid homology (Ser-Thr-Pro-Cys-Ser) with the HCV polyprotein at the NS4B/5A site of cleavage by NS3/4A. Also HCV interferes with signals of the TLR7 as NS5A interacted with MyD88 through the IFN sensitivity-determining region (ISDR) (Chang *et al.*, 2010).

The recommended standard therapy for HCV is a once-weekly injection of peg-interferon alpha 2 (IFN- $\alpha$ 2) plus daily oral ribavirin. The sustained viral response is approximately 50% and the therapy is associated with significant side effects including flu-like symptoms, depression, and injection site reactions. In addition, some patients develop neutralizing antibodies to recombinant IFN, which may contribute to treatment failures. There is, therefore, a need to discover new drugs with improved efficacy and reduced side effects.

Whereas current IFN therapy involves the administration of a single IFN- $\alpha$  subtype (2a or 2b), TLR activation induces a range of different IFN subtypes. For example, the TLR7 agonist imiquimod has been shown to

induce IFN- $\alpha$ 1, - $\alpha$ 2, - $\alpha$ 5, - $\alpha$ 6, and - $\alpha$ 8. This could offer improved antiviral efficacy, since different IFN subtypes have been shown to have different antiviral potencies against HCV, and some subtypes have synergistic activities in combination with others. It is also possible that different subtypes could have differences in side effects (Puig *et al.*, 2012).

The aim of this work is to investigate the expression of TLR3 and TLR7 in peripheral blood from patients with chronic hepatitis C infection and correlate their levels with that of IFN- $\alpha$  expression, viral load and histopathology of the liver.

## Patients and Methods

### Study participants

The study included 30 chronic hepatitis C patients, enrolled from Tropical Medicine out-patients clinics, who were candidates for interferon therapy. None of patients had received antiviral treatment before involvement into the study. Chronic hepatitis C patients were diagnosed by elevated both Anti-HCV antibodies (ELISA second generation, Ortho Diagnostic System, Raritan, NJ, USA) and liver ALT and AST for more than 6 months and confirmed by real time quantitative PCR. 20 healthy subjects were enrolled as a control group.

Patients who had co-existing liver diseases, decompensated liver disease, hepatic focal lesion, contraindication for liver biopsy and patients with inadequate liver biopsy were excluded from the study.

Informed written consent was obtained from all participants before enrollment in the study. The study protocol was approved by Ethical Committee of Faculty of Medicine, Ain Shams University.

### Sample Preparation

Fasting Blood samples were taken from both study group and control group. Part of the sample was added to a tube containing trisodium citrate (final concentration 1mg/mL) for prothrombin time (PT) determination and the other part was used to separate sera for further lab investigations. For that purpose the sera was preserved at  $-20^{\circ}\text{C}$ .

### RNA extraction and reverse transcription

For RNA extraction, peripheral blood was collected in EDTA contain tubes, from both patients and controls.

MagNA Pure LC RNA Isolation Kit (Roche Diagnostics) was used for manual extraction. In brief, samples were lysed in a buffer containing a chaotropic salt and RNase inactivator. Nucleic acids were bound to the surface of glass magnetic particles. After a DNase digestion step, unbound substances were removed by several washing steps. The purified RNA was separated from the MGPs in the wells of the Elution Cartridge at  $70^{\circ}\text{C}$  with a low-salt buffer. cDNA was synthesized with use of the First Strand cDNA Synthesis Kit (Roche Diagnostics) containing Transcriptor Reverse Transcriptase, random hexamer primers and an anchored-oligo(dT)<sub>18</sub> primer and ThermoStable Protector RNase Inhibitor according to manufacturer's instructions.

### Relative Quantitative Real-time RT-PCR

Real time RT-PCR was carried out using Light Cycler® 480 DNA SYBR Green I Master (Roche Applied Science) with addition of primers and template according to manufactures instructions. Human glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as housekeeping gene. For TLR 3 the forward primer was: TGGTTGGGCCACCTAGAAGTA and reverse primer: TCTCCATTCCTGGCCTGTG. For TLR 7 the Forward primer was: TTTACCTGGATGGAAACCAGCTA and Reverse primer: TCAAGGCTGAGAAGCTGTAAGCTA. IFN $\alpha$  forward primer was: TGC TTT ACT GAT GGT CCT GGT and reverse primer: TCA TGT CTG TCC ATC AGA CAG. The GAPDH-forward primer was ATG GCT ATG ATG GAG GTC CAG and GAPDH-reverse was TTG TCC TGC ATC TGC TTC AGC (Hornung *et al.*, 2002).

To control for specificity of the amplification products, a melting curve analysis was performed. No amplification of nonspecific products was observed. . Quantitative real-time RT-PCR curves were analyzed by LightCycler 4 software (Roche Diagnostics). The expression of the target gene concentration was expressed as a ratio of target to the control gene in the same sample, rather than an absolute value.

Assessment of HCV load was done with Taqman MasterMix (Applied Biosystems, Foster City, CA.)

Percutaneous liver biopsy was performed for all enrolled patients under ultrasound guidance using 16 gauge needles. Specimens of at least 2.5 cm in length, including a minimum of 12 portal tracts were considered reliable for adequate grading and staging using modified Knodell's score (Ishak *et al.*, 1995). Reading of liver biopsies was done by a single pathologist who was blind to the clinical data.

### Statistical Analysis

Data was collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 13; Inc., Chicago. IL). Quantitative data was shown as mean, SD. Student t-test was used to compare mean and SD of 2 sets of quantitative normally distributed data, while Mann Whitney test was used when this data is not normally distributed. Pearson's correlation was used to study correlation between two variables having normally distributed data. Box and scatter plots have been plotted when appropriate. P-value was considered statistically significant when it is less than 0.05.

### Results

The study was conducted on 30 patients (21 male and 9 female) suffering from chronic HCV infection and 20 control subjects (15 males and 5 female) with mean age  $33.52 \pm 9.85$  and BMI  $26.35 \pm 10.64$ . Cases and controls were age and sex matched. All patients were Child A category according to Child Pugh classification. Demographic and laboratory data of the patients are summarized in Table (1).

Histopathology of liver biopsies showed that 7 patients (23.33%) had fibrosis stage 1/6, 5 patients (16.67%) had fibrosis stage 2/6, 4 patients (13.33%) had fibrosis stage 3/6, 12 patients (40%) had fibrosis stage 4/6 and 2 patients (6.67%) had fibrosis stage 5/6.

Regarding activity index, 10 patients (33.33%) had activity score 4/18, 3 patients (10%) had activity score 6/18, 5 patients (16.67%) had activity score 7/18, 4 patients

(13.33%) had activity score 11/18 and 8 patients (26.67%) had activity score 12/18.

The analysis of the RT-PCR data showed that levels of the two receptors TLR3 and TLR7 and the level of IFN  $\alpha$  are significantly lower in patients with chronic hepatitis C in comparison to control subjects (Table 2, figure 1).

Table 1. Demographic and laboratory data of the chronic HCV patients.

Parameter	Patients (n=30)
Age (year)	36.600 $\pm$ 8.792
BMI	25.88 $\pm$ 42.866
*AST (IU/ML)	68.300 $\pm$ 35.440
*ALT (IU/ML)	67.900 $\pm$ 28.663
*ALP (IU/ML)	142.90 $\pm$ 59.083
Albumin (mg/dl)	3.980 $\pm$ 0.5367
Fasting Glucose (mg/dl)	107.20 $\pm$ 12.564
Creatinine (mg/dl)	0.9120 $\pm$ 0.1380
Bilirubin (mg/dl)	0.9690 $\pm$ 0.4433
WBCS / mm <sup>3</sup>	6124 $\pm$ 2029
*Hb (mg/dl)	15.029 $\pm$ 1.815
Platelets /mm <sup>3</sup>	198.30 $\pm$ 66.116
PT time /second	13.010 $\pm$ 0.8938
Viral load /mm <sup>3</sup>	64890 $\pm$ 64933

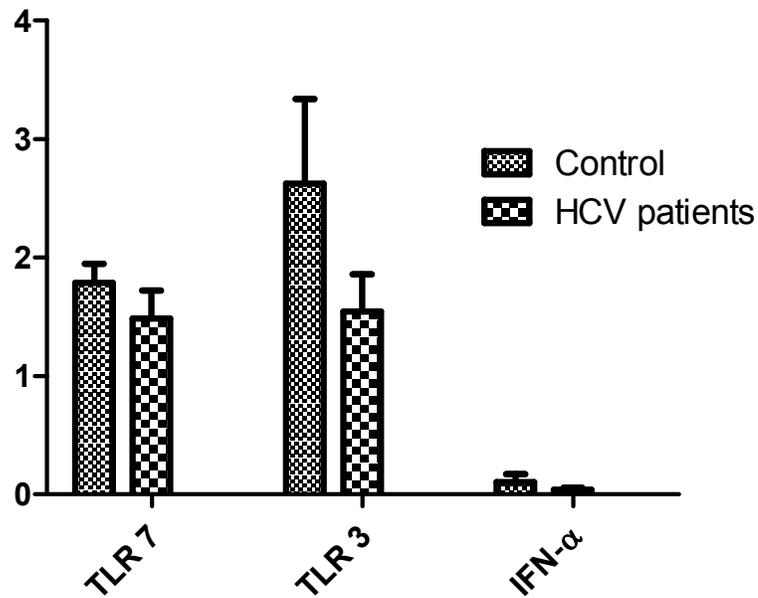
\*ALT (alanine aminotransferase), \*AST (aspartate aminotransferase), \*ALP (alkaline phosphatase), \*Hb (hemoglobin)

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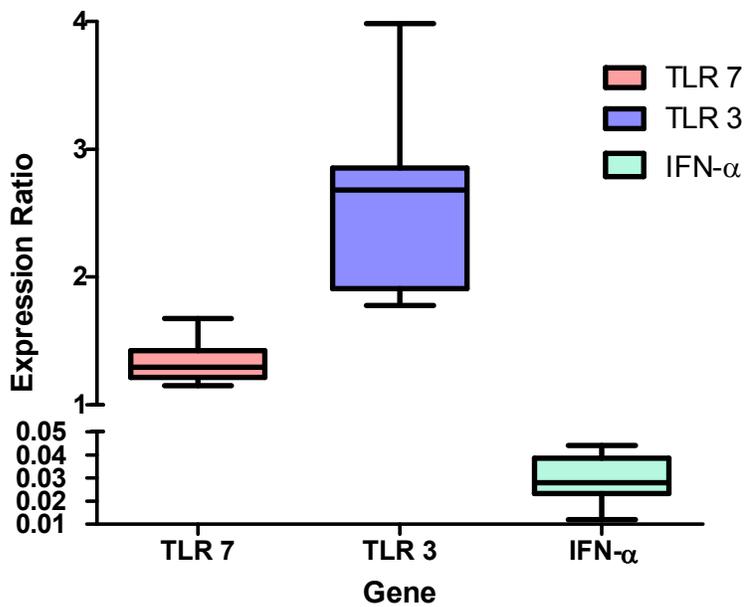
Table 2. Comparison between peripheral blood expression of TLR3, TLR7 and IFN- $\alpha$  in chronic HCV patients and controls.

Parameter	patients (n=30) Mean $\pm$ SD	Controls (n=20) Mean $\pm$ SD	P Value
TLR 7	1.322 $\pm$ 0.1330	1.531 $\pm$ 0.1621	0.0008
TLR 3	1.390 $\pm$ 0.08907	1.546 $\pm$ 0.3118	0.0437
IFN- $\alpha$	0.02925 $\pm$ 0.01004	0.03970 $\pm$ 0.01540	0.0329

P<0.05 is significant.



**Figure 1.** Comparison between peripheral blood expression of TLR3, TLR7 and IFN-α in chronic HCV patients and controls.



**Figure 2.** Relative expression of TLR-3, TLR 7 and IFN-α genes by RT-PCR data. Boxes represent the range or the middle 50% observation. Whiskers represent the minimum and maximum observation.

Univariate analysis showed that the level of both TLR 3 and TLR 7 correlate significantly with IFN- $\alpha$  ( $r = 0.6450$ ,  $P = 0.0021$  and  $r = 0.8427$ ,  $P < 0.0001$  respectively (figure 3, 4)

with no correlation to hepatic histopathological changes or HCV viral load (table 3).

Table 3. Correlation between TLR 3, TLR 7 and laboratory and histopathological parameters findings in the chronic HCV patients.

		AST (IU/ML)	ALT (IU/ML)	HCV PCR	Albumin (mg/dl)	Bilirubin (mg/dl)	fibrosis	Activity index	IFN- $\alpha$
TLR3	r	0.0033	0.2731	0.664	0.3842	0.0529	0.0319	0.3169	0.6450
	P value	NS	NS	NS	NS	NS	NS	NS	0.0021
TLR7	r	0.2245	0.0884	0.1983	0.0181	0.099	0.2708	0.2616	0.8427
	P value	NS	NS	NS	NS	NS	NS	NS	< 0.0001

$P > 0.05$  is not significant (NS).

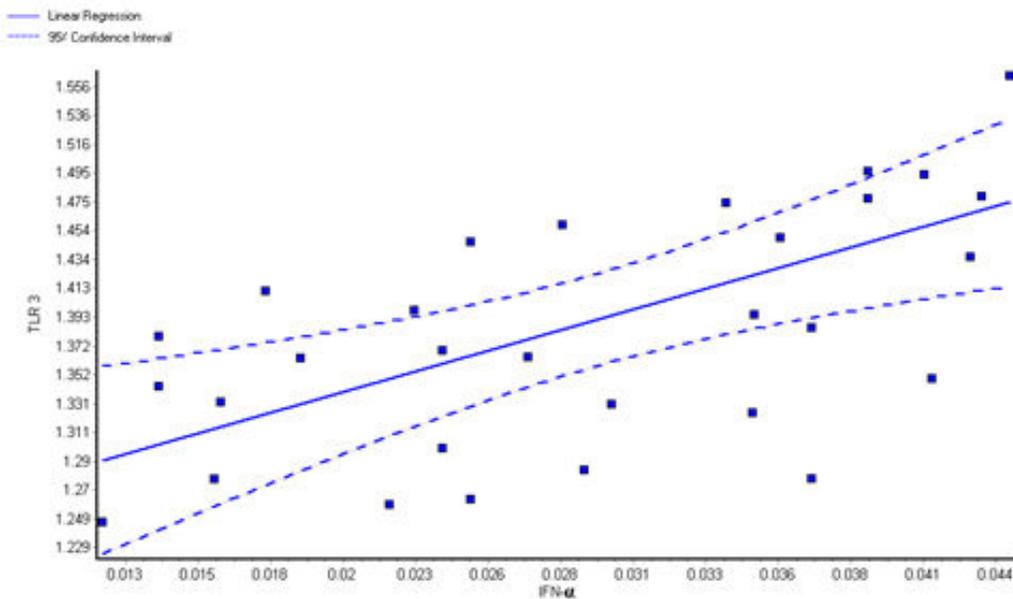
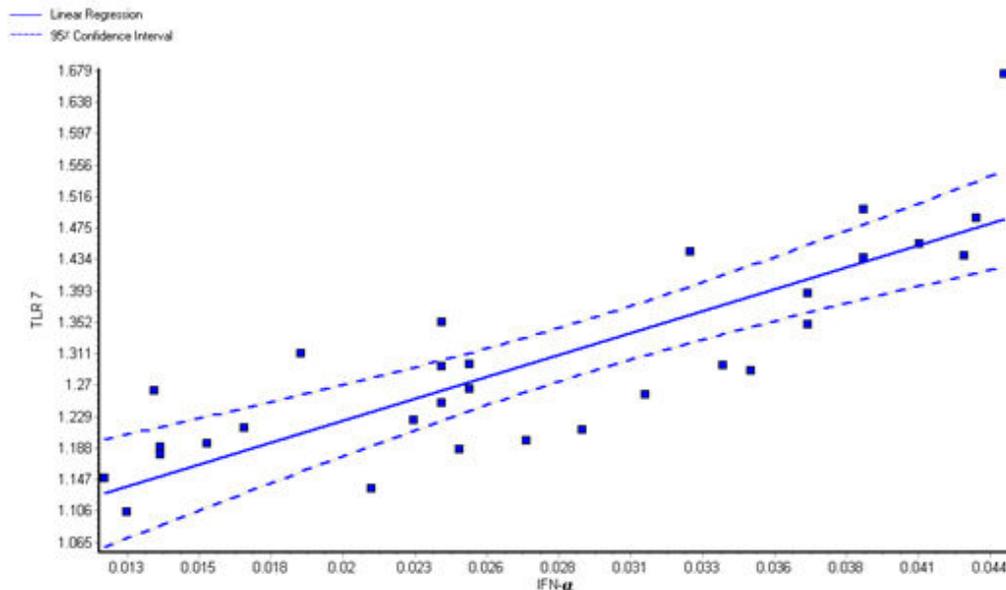


Figure 2. Correlation between levels of IFN-  $\alpha$  and TLR 3 in the chronic HCV patients.



**Figure 3.** Correlation between levels of IFN-  $\alpha$  and TLR 7 in the chronic HCV patients.

## Discussion

Immune evasion by HCV has been documented in several different host cell types, and it has been suggested to play a key role in viral persistence and development of chronic infection (Jason *et al.*, 2012). HCV employs a novel mechanism for immune evasion by specifically targeting TLR expression, mRNA stability and function (Chang *et al.*, 2010).

The capacities of TLR3 signaling pathways to restrict virus replication are consistent with the convergence downstream at the level of kinases responsible for the activation of IRF-3 and NF- $\kappa$ B (Qu *et al.*, 2011). DsRNA engagement by TLR3 results in the activation of IRF-3-dependent type I IFN responses, as well as in NF- $\kappa$ B-dependent proinflammatory cytokine production. Both types of IFN, as well as a number of interferon stimulated genes (ISGs), possess antiviral activity against HCV *in vitro* (Jiang *et al.*, 2008) making it likely that the antiviral state induced by TLR3 signaling is dependent on the activation of

IRF-3. However, the proinflammatory cytokine interleukin-1 $\beta$  also inhibits the replication of HCV RNA (Torres-Pedraza *et al.*, 2010).

The disruption of TLR3 signaling pathways may favor HCV infection by impairing the establishment of an antiviral state within infected cells. However, disruption of TLR3 signaling may play an additional role in promoting viral persistence at the level of the host. Both the magnitude and the breadth of the adaptive T-cell response are critical determinants in the control and clearance of HCV infection (Agrawal *et al.*, 2012). TLR3 is known to promote the cross-priming of T lymphocytes to viral antigens and has recently been shown to be essential for type II IFN responses to coxsackie virus infection (Negishi *et al.*, 2008). Although much remains to be learned about how early innate immune responses shape the subsequent development of adaptive immunity to HCV, viral disruption of the TLR3-TRIF axis is likely to figure

prominently among the HCV-host interactions that determine the outcome of infection (Rebecca *et al.*, 2012)

Activation of TLR7 depends on the adaptor molecule MyD88 that stimulates IFN regulatory factor-3 (IRF-3) and IRF-7. These IRF molecules are phosphorylated in the cytosol and are translocated to the nucleus upon activation. DNA-dependent protein kinase (DNA-PK) has also been shown recently to phosphorylate and activate IRF-3. The IRFs can both foster type I IFN production and enhance the antiviral activity of these cytokines (Huang *et al.*, 2009). Activity of IRF-3 and IRF-7 expression is inhibited in HCV replicon cells. Thus, pharmacologic agents that activate IRFs in hepatocytes may exert direct anti-HCV effects, as well as potentiate IFN action (Colonna, 2007).

This study was designed to assess TLR3 and TLR7 expression in patients with chronic hepatitis C virus infection and its correlation with IFN- $\alpha$  expression, viral load and histopathology of the liver.

In the present study, the expression of TLR3, TLR7 and IFN- $\alpha$  were significantly lower in patients with chronic hepatitis C in comparison to control subjects. This is in agreement with many authors, who found that the expression of TLR3 and TLR7 receptors are down-regulated in patients with chronic HCV infection (Atencia *et al.*, 2007; Chang *et al.*, 2010; Chung *et al.*, 2011). Also, Katsounas *et al.* (2012) found a negative regulation of the MyD88-dependent toll-like receptor (TLR) pathway and decreased expression of TLR3 in patients with chronic HCV. Several strategies have been developed to evade the innate immune signaling by HCV. Three signaling pathways have been demonstrated to be interfered: TLR3-TRIF-TBK1-IRF-3 pathway, TLR-MyD88 pathway, and RIG-I/MDA5-IPS-1 pathway (Seki & Brenner, 2008). The NS3/4A serine protease

of HCV mediated cleavage of TRIF, and then reduced its abundance and inhibited TLR3 signaling pathway, which made HCV escape attack from the innate immune system (Li *et al.*, 2005). HCV core protein blocked TLR3-mediated secretion of IFN- $\alpha/\beta$  and TNF-related apoptosis-inducing ligand (TRAIL) in KCs in the liver, which affect viral recognition and contribute to the inflammation in the liver (Chang *et al.*, 2007; Tu *et al.*, 2010).

On the other hand, Dolganiuc *et al.* (2004) concluded a wide up-regulation of almost every TLR including TLR3 and TLR7 in monocytes and lymphocytes of patients with chronic HCV infection. However, this difference could be explained by the difference in patients selection and severity of the disease.

The statistical regression analysis of the quantitative RT PCR data and clinical data showed no correlation between the expression levels of the studied receptors and other factors as viral load, liver functions, degree of hepatic fibrosis or activity. However, we found that the measured levels of TLR3 and TLR7 correlated significantly with those of IFN- $\alpha$ . This goes with the result of Atencia *et al.* (2007) who reported that the expression levels of TLR3 and TLR7 correlate strongly with the expression level of IFN- $\alpha$ , suggesting that this down-regulation appears to be related with the infection, as the same analysis performed on patients with liver cirrhosis not related with viral infections (mainly of alcoholic origin) did not show significant differences when compared with healthy controls.

In conclusion, TLR3 and TLR 7 are down regulated in chronic HCV infection. They were correlated to interferon- $\alpha$  level. Further studies to detect the possible role of TLRs as a predictor of response to antiviral therapy are needed. Also, the possibility of targeting these

receptors to enhance the immune response and clear the infection needs further studies.

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