Study of toll-like receptor 7 expression and interferon α in Egyptian patients with chronic hepatitis C infection and hepatocellular carcinoma

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HCC;
IFN-α;
Innate immunity;
Viral hepatitis;
Viral load

Abstract Background: Hepatitis C virus is considered to be one of the most important devastating causes of chronic hepatitis, cirrhosis, and hepatic cellular carcinoma. Toll-like receptor 7 (TLR7) is a pathogen-recognition receptor that is expressed on innate immune cells. It recognizes viral RNA which induces its activation with a subsequent increase in IFN-α 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 the work: Was to investigate the expression of TLR7 in peripheral blood of patients with chronic HCV infections and patients with HCC, comparing it with normal individuals, and correlating it with both serum levels of IFN-α and viral load.

Results: The results of this study showed a significant decrease in TLR7 expression in patients with chronic HCV and no detection at all in patients with HCC, in addition a significant negative correlation was observed between levels of TLR7 expression and interferon α when compared to viral load.

Conclusion: Down regulation of TLR7 expression in HCV and HCC patients may contribute to the decrease of IFN-α and increase in viral load. These results raise the possibility that by targeting TLR7 with high affinity pharmacological stimulants may be able to control HCV infection by induction of IFN-α and direct activation of antiviral mechanisms in hepatocytes. Additionally, they provide insight about the potential use of TLR7 as a new set of molecular markers for prognosis and outcomes of chronic HCV infection and HCC.

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1. Introduction

Hepatitis C virus infection has become a global problem necessitating a wide range of control and preventive measures. It is a
member of the Flaviviridae family, which is a small enveloped positive sense single stranded RNA virus [1]. Approximately 130–150 million people globally have chronic hepatitis C infection, and from 350,000 to 500,000 people die from hepatitis C-related liver diseases each year [2].

HCV is immunogenic and triggers immune responses in most healthy adults. Intrinsic components of the virus presumably activate the innate immune system that effectively limits viral replication. One of the earliest responses to HCV is the production of type I-IFNs, critical cytokines that establish an antiviral state and bridge the innate and adaptive immune systems [3].

Toll-like receptors (TLRs), which belong to a family of pathogen recognition receptors, are an essential part of the innate immune response that detects conserved pathogen-associated molecular patterns (PAMPs) of bacteria, parasites, fungi, protozoa components, and viruses. [4] They are expressed by a variety of immune and non-immune cells, such as B lymphocytes, T lymphocytes, antigen-presenting cells, and fibroblastic synoviocytes [5].

TLR7 is mainly expressed in the endosome-lysosome membrane of plasmacytoid dendritic cells (pDCs), B lymphocytes, hepatic natural killer cells, and virally infected hepatocytes. In these cells, endosomal proteases digest HCV particles, uncoating and releasing viral ssRNA which is recognized by TLR7 [6].

TLR7 is interesting in regard to HCV-infection, because its engagement leads to production of increased levels of interferon-α [7]. Stimulation of TLR7 induces recruitment of myeloid differentiation factor 88 (MyD88) which in turn recruits members of the interleukin-1 (IL-1) receptor-associated kinase 4 (IRAK4) family that associates temporarily with a member of the TNF receptor-associated factor (TRAF6) family [8]. Both IRAK4 and TRAF6 activate Interferon regulatory factor 7 (IRF7) that stimulates expression of type I IFN [9]. Moreover, pDCs possess a dsRNA-independent pathway for recognizing HCV and produce high levels of type I-IFN after virus infection [10]. Since TLR expression in human pDCs is limited to TLR7 and TLR9, TLR7-mediated pDC recognition of HCV ssRNA may be an important antiviral pathway [11]. Meanwhile, TLR7 stimulation in virally infected hepatocytes may inhibit HCV replication through direct activation of antiviral genes independent of interferon regulation factors. Accordingly, TLR7 signaling is directly critical for the efficient control of HCV infection, not only by IFN induction, but also through IFN-independent mechanisms. However, 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 [12].

HCV employs a novel mechanism for immune evasion by specifically targeting TLR7 expression, mRNA stability and function [13].

Combination therapy with polyethylene glycol modified IFN-α and ribavirin suppresses HCV replication in 40–80% of patients. However, severe side effects are associated with this treatment leading to poor patient compliance and sustained virological response rate (SVR) for HCV-G4—the predominant genotype in Egypt—does not exceed 60%. Moreover, the treatment of HCV-G4 non-responders, thalassemic patients, patients on hemodialysis and patients with HCV-G4 recurrence after liver transplantation still represents a significant therapeutic challenge [14].

For these reasons, it is crucial to develop alternative therapies. Because TLR7 agonists can impede HCV infection both via type I IFN and independently of IFN, they may be considered as an alternative treatment of chronic HCV infection, especially in IFN-α-resistant patients.

Disease progression and complications of chronic hepatitis, including the development of cirrhosis and HCC, generally result from CTL cytotoxicity and the recruitment of inflammatory cells to lesions in the liver [15].

The action of IFNγ, secreted by activated immune cells which either reside in the liver or are recruited to the liver in response to inflammation or injury, extends beyond immune modulation to include regulation of hepatocyte apoptosis and cell cycle progression during liver disease [16, 17]. IFNγ might play a crucial role in limiting HBV and HCV pathogenesis during acute infection. This is supported by the observation that IFNγ suppresses HCV replication in the HuH-7 human hepatoma cell line [18] and inhibits HBV replication in transgenic mice [19].

Inflammation-induced carcinogenesis may involve abnormal responses of the innate host immune system to different kinds of antigens [20]. Down-regulation of TLR7 in hepatocytes has been proposed to be the exclusive mechanism accounting for persistent hepatitis virus infection and hepatocyte transformation [13].

The aim of this study was to investigate the expression of TLR7 in peripheral blood of patients with chronic HCV infections and patients with HCC, comparing it with normal individuals, and correlating it with both serum level of IFN-α and viral load.

2. Patients and methods

The study included three groups:

Group I: included 40 chronic HCV patients with positive HCV antibodies for more than 6 months detected by the EIA technique and positive HCV RNA detected by RT-PCR.

Group II: included 20 patients having hepatocellular carcinoma on top of chronic HCV infection. Diagnosis is based on focal lesion in the liver detected by radiological diagnosis and elevated α fetoprotein.

Patients were enrolled from the Tropical Medicine Department (inpatients and outpatients).

Group III: included 20 healthy subjects with no past history of liver disease as a control group.

All patients were subjected to:

- full history taking,
- thorough clinical examination,
- laboratory investigations including: complete blood picture, liver function tests: serum ALT, AST, albumin, and bilirubin level,
- abdominal ultrasonography.

Patients who had positive HBs Ag, and those with alcoholic liver disease were excluded from the study. Informed written consent was obtained from all participants before enrollment in the study. The study protocol was approved by Ethics Committee of Faculty of Medicine, Ain Shams University, and has been carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for experiments involving humans.
2.1. Sample collection and preparation for RT-PCR and ELISA

Four milliliters (ml) of venous blood was collected from each person under complete aseptic conditions and divided as follows:

- Two milliliters was put into Ethylene Damien Tetra Acetic Acid (EDTA) containing sterile tube; for estimation of TLR7 mRNA in peripheral mononuclear cells using the PCR technique [9].
- The remaining 2 ml was put into another sterile tube, left to clot at room temperature, then centrifuged at 3000 rpm/min for 10 min.

Serum was separated and stored in aliquots at −20 °C till used for measurement of serum IFN-α by ELISA according to Zhang et al. [3].

RNA extraction: RNA was extracted from PBMCs using QIAamp RNA Blood Mini Kits supplied by (QIAGEN) Clinic Lab.

Principle: during the QIAamp procedure for purification of RNA from blood, erythrocytes were selectively lysed and leukocytes were recovered by centrifugation. Leukocytes then were lysed using highly denaturing conditions that immediately inactivated RNases, allowing the isolation of intact RNA. After homogenization of the lysate by a brief centrifugation through a QIA shredder spin column, ethanol was added to adjust binding conditions and the sample was applied to the QIAamp spin column. RNA was bound to the silica membrane during a brief centrifugation step. Contaminants were washed away and total RNA was eluted in RNase-free water for direct use. Amplification by RT-PCR was done using Quantitect Reverse Transcription kit for cDNA synthesis with integrated removal of genomic DNA contamination for gene expression analysis. Detection of amplification product was done by SYBR Green-based real-time RT-PCR using Quantitect SYBR Green PCR kit For TLR7 the Forward primer was: TTTACCTGGATGGAAAACCAGCTA and Reverse primer: TCAAGGCTGAAGAGCTGTAAGCTA and The GAPDH-forward primer was AGAAGCTGTAAGCTA and Reverse primer: TCAAGGCTGTAAGCTA and Reverse primer: TCAAGGCTGTAAGCTA. The amplified product (IFN-α) was detected by SYBR Green and collected data were revised and introduced to a PC using Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Data were presented and suitable analysis was done according to the type of data obtained for each parameter. P value was considered statistically significant when it is < 0.5 and highly significant when it is < 0.001.

Laboratory data are summarized in Table 1. The analysis of RT-PCR and ELISA data showed a significant decrease in PMNCs TLR7 mRNA and serum IFN-α levels in HCV patients compared to controls (Table 2).

In the HCV group, TLR mRNA levels showed a highly significant positive correlation with serum IFN-α levels (Fig. 1), and a highly significant negative correlation with viral load (Fig. 2), while there was a highly significant negative correlation between serum IFN-α levels and viral load (Fig. 3).

Regarding the 20 patients in the HCC group, no TLR7 mRNA was detected, while serum IFN-α level was highly significantly lower than the control group (Table 3).

Regarding liver function tests, there was a highly significant increase in ALT, AST and bilirubin in both patients groups compared to controls, with a highly significant decrease in albumin in the HCV group compared to both HCC and control groups. On the other hand, there was a highly significant decrease in RBCs and platelet counts in both patient groups compared to the control group, with a highly significant decrease in WBCs count and Hb level in the HCV group compared to HCC and control groups.

4. Discussion

Hepatitis C virus is considered to be one of the most important devastating causes of chronic hepatitis, cirrhosis, and hepatic cellular carcinoma [22]. In viral infection, TLR stimulation presents a double-edged sword: it is indeed essential for triggering innate and adaptive immunity against pathogens, but TLR over-activation is known to contribute to the pathogenesis of chronic inflammatory and infectious diseases. The strength and duration of TLR activation is thus tightly controlled by multiple negative regulatory mechanisms. Research in TLR biology has mainly focused on identification of ligands and signaling pathways triggered by ligand-TLR interaction [23].

TLR7, a sensor for viral ssRNA, plays a significant role in anti HCV immunity responses by inducing type I IFNs and other IFN independent antiviral mechanisms.
However, HCV has strategies to avoid activation of antiviral pathways by TLRs and their ligands. HCV selectively impairs innate immunity pathways that limit HCV replication such as type I IFNs, while at the same time generating a chronic inflammatory state that causes persistent liver injury [24].

In this study, there was a highly significant decrease in TLR7 mRNA expression in PBMCs among HCV patients compared to the control group. These results are in agreement with many previous studies. Atencia and his colleagues [9] found a significant down regulation in TLR3 and TLR7 mRNA levels in chronic HCV infection with cirrhosis compared to healthy controls. They didn’t found this down regulation in patients with liver cirrhosis not related to viral infections (mainly of alcoholic origin). Furthermore, Taylor and co-workers [25], observed a reduced level of expression of TLR7 in HCV patients with poor response to interferon therapy. Moreover, Kang et al. [26] found that the incubation of PBMCs with HCV core proteins triggers the expression of TLR2 and suppresses that of TLR4&TLR7. A more recent study by Mohammed et al. [27] also demonstrated lower

### Table 1  Laboratory data in studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Control (10)</th>
<th>HCV (40)</th>
<th>HCC (20)</th>
<th>P</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (IU/ML)</td>
<td>26.55 ± 8.28</td>
<td>46.43 ± 15.68</td>
<td>48.45 ± 8.33</td>
<td>.001</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>AST (IU/ML)</td>
<td>24.00 ± 6.48</td>
<td>57.42 ± 22.36</td>
<td>50.75 ± 16.01</td>
<td>.001</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>T. bilirubin (mg/dl)</td>
<td>.43 ± .24</td>
<td>1.28 ± .58</td>
<td>1.58 ± .60</td>
<td>.001</td>
<td>HS</td>
<td></td>
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<tr>
<td>D. bilirubin (mg/dl)</td>
<td>.06 ± .07</td>
<td>.28 ± .11</td>
<td>.24 ± .10</td>
<td>.001</td>
<td>HS</td>
<td></td>
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<tr>
<td>Albumin (mg/dl)</td>
<td>3.49 ± .52</td>
<td>2.76 ± .57</td>
<td>3.21 ± .66</td>
<td>.001</td>
<td>HS</td>
<td></td>
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<tr>
<td>WBCs/mm³</td>
<td>8.88 ± 1.71</td>
<td>6.36 ± 1.49</td>
<td>8.40 ± 2.53</td>
<td>.001</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>RBCs/mm³</td>
<td>4.89 ± .39</td>
<td>4.00 ± .44</td>
<td>3.94 ± .58</td>
<td>.001</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>Hb (mg/dl)</td>
<td>12.60 ± 1.21</td>
<td>10.49 ± 1.45</td>
<td>12.16 ± 1.60</td>
<td>.001</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>Platelets/mm³</td>
<td>305.2 ± 54.12</td>
<td>140.60 ± 48.29</td>
<td>157.60 ± 55.96</td>
<td>.001</td>
<td>HS</td>
<td></td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; WBCs, white blood cells; RBCs, red blood cells.

### Table 2  Comparison between HCV and control groups as regards TLR7 mRNA and serum IFN-α levels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV (20)</td>
<td>Controls (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR7</td>
<td>1.05 ± 0.55</td>
<td>1.27 ± 0.37</td>
<td>0.37 S</td>
</tr>
<tr>
<td>IFN-α pg/ml</td>
<td>7.69 ± 7.65</td>
<td>9.12 ± 8.57</td>
<td>.041 S</td>
</tr>
</tbody>
</table>

In this study, there was a highly significant decrease in TLR7 mRNA expression in PBMCs among HCV patients compared to the control group. These results are in agreement with many previous studies. Atencia and his colleagues [9] found a significant down regulation in TLR3 and TLR7 mRNA levels in chronic HCV infection with cirrhosis compared to healthy controls. They didn’t found this down regulation in patients with liver cirrhosis not related to viral infections (mainly of alcoholic origin). Furthermore, Taylor and co-workers [25], observed a reduced level of expression of TLR7 in HCV patients with poor response to interferon therapy. Moreover, Kang et al. [26] found that the incubation of PBMCs with HCV core proteins triggers the expression of TLR2 and suppresses that of TLR4&TLR7. A more recent study by Mohammed et al. [27] also demonstrated lower

![Figure 1](image1.png)  Correlation between levels of TLR7mRNA and serum IFN-α in the HCV group.

![Figure 2](image2.png)  Correlation between TLR7mRNA and viral load in the HCV group.
expression of TLR7 mRNA in patients with chronic HCV infection as compared to controls. Chang [28] postulated that HCV down-regulates TLR7 by decreasing mRNA stability which could facilitate evasion of host immune surveillance.

Our study revealed a highly significant negative correlation between TLR7 mRNA level and viral load in HCV patients. Chang et al. [13] reported that reduction of HCV replication increases TLR7 mRNA and protein expression, while HCV infection produces instability of mRNA leading to low TLR7 RNA and protein expression and impairment of TLR7 signaling pathway and activation of IRF7. However, Mohammed and his co-workers [27] found no correlation between TLR7 expression and viral load or histopathological staging and grading of the liver tissue in chronic hepatitis C patients.

Regarding serum IFN-α level, we found a significant decrease in the HCV group compared to the control group. Ishii and koziel [29] demonstrated that expression of viral NS5A protein inhibits the signaling pathway of TLR2, TLR4, TLR7 and TLR9 by binding to the adaptor protein MyD88 and inhibiting the recruitment of IRAK4 leading to a decrease in MyD88-dependent signals responsible for IFN-α production. While plasma DCs—which can produce type I IFN 200–1000 folds more than other types of cells in blood representing the front line HCV innate antiviral immunity-use TLR7 for viral detection [30].

Abe et al. [31] postulated that shutting TLR7 signaling by HCV leads to suppression of maturation and differentiation of pDCs which are the main source of IFN-α in infected pts. Moreover Shina and Rehermann [32] suggested that HCV may specifically target plasma DCs, while Szabo and Mandrekar [33] found that TLR7, TLR8 and TLR9-induced IFN-α production is diminished in peripheral pDCs of infected HCV patients. In the current study, we found that serum IFN-α level was positively correlated with TLR expression in PBMCs and negatively correlated with viral load in HCV patients. The same results were obtained by, Mohammed et al. [27] and Atencia et al. [9] who suggested that one of the mechanisms leading to the chronicity of infection could be the acquisition of an “exhausted” state of this antiviral machinery at the late stages of infection.

Innate immunity plays a critical role in the development and progression of HCC. In particular, the activation of Toll-like receptor signaling results in the generation of immune responses that often result in the production of pro-inflammatory cytokines and chemokines, and could cause acute inflammation in the liver [34].

Regarding the HCC group in this study, no TLR7 mRNA was detected, while serum IFN-α level was significantly lower than the control group.

Similarly, Chang co-workers [13] detected a significant decrease of TLR7 expression in the presence of HCV infection both in vivo in human HCV-infected livers and in vitro in hepatoma cells. They found that HCV infection in hepatoma cells can impair expression and function of TLR7, while HCV clearance by interferon alpha treatment or self-restriction of viral replication through high passage number of culture cells, restored the decreased TLR7 expression.

Furthermore, Lin and his colleagues [23] found a significant downregulation in TLR7 expression in cancerous regions in all HCC liver tissues compared to adjacent normal hepatocytes. The expression levels of TLR7 were higher in adjacent normal liver tissue compared to tumor tissue from HBV-related, HCV-related, and non-HBV/HCV-related HCC. They hypothesized that IFNγ induced by hepatitis virus infection significantly decreases TLR7 promoter activity leading to downregulation of TLR7 gene expression resulting in immune escape and even immunological tolerance thereby facilitating its persistence within liver cells and modulating inflammatory signaling in hepatoma cells.

In conclusion, there is evidence that down regulation of TLR7 expression by HCV may contribute to the decrease of IFN-α and increase in viral load in chronic HCV and HCC patients.

These results raise the possibility that targeting TLR7 with high affinity pharmacological stimulants may be able to control HCV infection by induction of IFN-α and direct activation of antiviral mechanisms in hepatocytes. Furthermore, they provide insight about the potential use of TLR7 as a new set of molecular markers for prognosis and outcomes of chronic HCV infection and HCC. A larger scale clinical investigation should be undertaken to determine this novel therapeutic and prognostic approach.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>$P$ value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α pg/ml</td>
<td>HCC (20)</td>
<td>Controls (20)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>5.78 ± 4.2</td>
<td>9.12 ± 8.57</td>
<td>0.001</td>
<td>HS</td>
</tr>
</tbody>
</table>

![Figure 3](image-url) Correlation between viral load and serum IFN-α level in the HCV group.
Conflict of interest

All authors have no conflicts of interests and no financial disclosure.

References


