Hepatic expression of STAT1, SOCS3 & PIAS1 in HCV Patients and their Role in Response to therapy

Manal A. Eid, MD, Manal A. Tawfeek, MD, Hesham A. El-Serogy, MD, Sherif A. El-Saadany, MD, Wael F. Farrag, MD, Medhat A. Ghazy, MD, & Hoda A. Salem, MD.

ABSTRACT

Objective: To explore some of the mechanisms of HCV resistance to IFN, we investigated the expression of signal transducer and activator of transcription (STAT1) and its negative regulators protein inhibitor of activated STATs (PIAS1) and suppressor of cytokine signaling (SOCS3) in liver tissues of both IFN responders and non-responders chronic HCV patients.

Methods: 60 patients divided in to group 1a: 38 treatment responder Chronic HCV, group 1b: 22 treatment non-responder chronic HCV patients & control group: 6 subjects. Liver biopsies were taken from them and examined for histological scoring & Western blot analysis of STAT1, SOCS3, and PIAS1 expression.

Results: STAT1 expression was significantly increased in liver tissue from group 1 compared to group 2 ($P = 0.001$), while a non-significant difference was observed between group 1a & group 1b ($P = 0.747$). Phosphorylated STAT1 protein was expressed at a significantly higher level in liver tissue of group 1a compared to group 1b ($P = 0.001$). Western blotting of PIAS1 and SOCS3 protein expression in liver tissues from group 1 & 2 revealed significantly increased expression in group 1 compared to group 2 ($P = 0.001$). In addition comparing liver tissue from group 1a & group 1b showed that PIAS1 and SOCS3 protein expression was significantly higher in liver of group 1b.

Conclusion: Assay for phosphorylated STAT1 and/or its negative regulators, PIAS1 and SOCS3 proteins expression could be a good predictor of response to therapy and these could be used as biomarkers that can be easily detected by western blotting or immune-staining during standard histopathological liver biopsy analysis.

From the Departments of Clinical Pathology (Eid, Tawfeek, El-Serogy), Department of Tropical Medicine (El-Saadany), Department of Internal Medicine (Farrag, Ghazy) Faculty of Medicine, Tanta University and the Department of Clinical Pharmacy Misr University for science & Technology, Egypt

Address correspondence and reprint request to: Dr. Manal A. Tawfeek, Taibah University faculty of Medicine Tel.: 0508141044. E-mail: manalhammoda1965@yahoo.com

Hepatitis C virus (HCV) infection has been recognized as a major public health problem worldwide. HCV infection is a significant cause of chronic liver disease, with frequent progression to cirrhosis and an elevated risk for the development of hepatocellular carcinoma. It infects more than 170 million people worldwide (3% of the world population). Egypt has one of the highest prevalence rates of HCV infection in the world. About 13% of the Egyptians are positive for HCV antibodies and about 9.8% are HCV RNA positive. This means that almost 10% of the total population are infected and are infectious to other people.

Among the six major HCV genotypes found worldwide, genotype 4 is the most predominant in Egypt, with 4a as the dominant subtype. Yet there is no effective vaccine available, and the current treatment is the combination of interferon alpha (IFN-α) and a nucleotide analog, ribavirin. Unfortunately, Patients infected with HCV genotype 4 show poor responses to IFN-α therapy compared with genotypes 2 and 3, but a similar response to those infected with type 1b HCV. Genotype 4 HCV RNA clearance has been recorded in only 22.2%. The mechanisms of IFN antiviral action as well as the mechanisms of viral IFN resistance are not well defined. Thus a major
challenge to the HCV community is to uncover such mechanisms and improve therapy for IFN non-responders\(^2\).

IFN is one of the most potent innate antiviral cytokines. Its activity against viruses is believed to depend on both activation of many IFN stimulatory genes (ISGs) and on modulation of the immune system. IFN intracellular signaling is mainly through the Janus kinase (Jak) family, Jak Stat (signal transducer and activator of transcription) signaling pathway\(^2\). STATs are a family of cellular proteins that mediate transduction of extracellular signals from growth factors and cytokines such as IFNs and IL-6 and regulate transcription directly\(^6\). The interaction of these cytokines with specific cell surface receptors triggers activation of Stats through its phosphorylation by the receptor associated Jak kinases. Once phosphorylated Stats homo- or heterodimerise and translocate to the nucleus, where it regulates the transcription of Stat responsive genes\(^7\). It is well documented that activation of STAT1, STAT2 and STAT3 are essential for the antiviral effect of IFNs\(^8,9\).

Negative regulators of STATs include suppressor of cytokine signaling (SOCS) family members and protein inhibitor of activated STATs (PIAS). SOCS1 and SOCS3 prevent phosphorylation of STATs by binding to receptor associated Jak kinases. PIAS1 and PIAS3 inhibit binding of STAT1 and STAT3 respectively to the promoter of target genes thus preventing gene transcription\(^10,11\).

As STATs and their regulators are essentials to set the antiviral mechanisms within the HCV infected cells, the present work aims to analyze the expression of STAT1 and its negative regulators SOCS3 and PIAS1 in liver cells, and to define the relation between their expression, if any, and the response to interferon therapy.

**Methods.** 112 patients were enrolled in the study; only 60 HCV-RNA positive patients (36 men, 24 women) with age ranging from 26 to 45 years fulfilled the criteria for this study & continued the follow up period. These patients were followed-up at out-patient clinics of Tropical Medicine & Internal Medicine Departments, Tanta University Hospitals for 18 months during the period from February 2008 to August 2009 to assess treatment responders & non-responders. A written informed consent was taken from studied patients. The study was approved by the ethical committee of our institution and the recruited subjects gave their informed consent for the genetic analysis. Normal liver samples were obtained from 6 patients (serving as control) at laparotomy performed for cholecystectomy in 5 cases and hiatal hernia in one case. The control subjects had not received anti-inflammatory therapy previous to the operation and in all cases histological examination of the liver biopsies revealed normal liver architecture.

Diagnosis of patients was based on positivity for anti-HCV antibodies and positivity for HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR) and histological evidence of
chronic hepatitis. Alcohol consumption and other causes of liver disease were excluded. None of the patients had received antiviral therapy before sample collection.

**Treatment and Criteria of response to therapy:**
All patients fulfilled the criteria of Interferon therapy, received pegylated interferon (PEG-IFN alpha 2a) (40 Kd; PEGASYS, F. Hoffmann-La Roche, Basel, Switzerland) at a dose of 180 mg once weekly plus ribavirin (RBV) given at doses of 1000 mg/day if body weight was <75 kg and of 1200 mg/day if >75 kg. The duration of treatment was 48 weeks. As recommended in HCV infected patients, virological stopping rules were applied: thus patients who did not achieve a reduction of >2 log10 in serum HCV-RNA at week 12 of therapy were considered to represent treatment failures and discontinued HCV therapy prematurely (12,13). Likewise, patients with detectable serum HCV-RNA at week 24 were also considered to represent treatment failures and stopped HCV treatment. Sustained virological response (SVR) was defined as an undetectable HCV-RNA 6 months after the end of treatment. Viral loads were repeated at 12 wk to assess those who achieved an early virological response (EVR), at the end of treatment to assess the end of treatment virological response (ETVR), and six months later to confirm an SVR (14). Follow up of patients was done during treatment and for 6 months after treatment to detect responders and non-responders. Follow up of these patients during treatment & 6 months after treatment revealed 38 patients as responders & 22 as non responders.

The subjects were divided into:

- **Group 1:** 60 chronic HCV patients who were subdivided into two groups according to treatment response.
  - **Group 1a:** 38 treatment responder chronic HCV patients.
  - **Group 1b:** 22 treatment non-responder chronic HCV patients.
- **Group 2** (control group): 6 subjects with normal liver biopsy taken during laparotomy operation.

All patients of the study were subjected to the following:

- Detailed history taking & full clinical examination.
- **Laboratory investigation** including: complete blood count, blood urea and s. creatinine, liver function tests, viral markers for HCV and HBV using commercial ELISA Kits (Abbott GmbH, Delkenheim, Germany), fasting & postprandial blood glucose level, ANA, TSH, a-fetoprotein, HIV & Cytomegalo-virus.
- Quantitative estimation of HCV RNA (15).
- Body mass index
- Fundus examination
- Electrocardiography
- Abdominal ultrasonography.
- **Liver biopsy examination:** Liver biopsies from all patients were reviewed for the purpose of this study. The liver biopsies were divided into 2 pieces, one was used for histological examination and the other was used for protein extraction.

**Histological assessment:** Paraffin-embedded sections of specimens were stained with hematoxyline and eosine (H&E), masson trichrome, and periodic acid-shiff after diastasde digestion. All specimens were evaluated using Ishak modified histological activity index (HAI) (3). Inflammation scoring ranged 0 to 18 and included assessment of periportal hepatitis, confluent necrosis, focal necrosis, and portal inflammation. Fibrosis scoring ranged 0 to 6, distinguishing mild architectural changes (1–2) from fibrosis (3–4) and cirrhosis (5–6).

**Protein extraction from liver tissue:** Liver tissue was homogenized in 300 µl of NP-40 lysis buffer (50 mM Tris-Hcl [pH 8.0], 150 mM NaCl, 1% NP-40, containing 1X protease inhibitor cocktail from Roche Diagnostic Corporation, Indianapolis, IN, USA) for 1 hour at 4°C. Cell debris was removed by centrifugation at 12,000 r.p.m. for 15 minutes. Supernatant was collected and Protein concentration was determined by Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA) (7).

**Western blot analysis of STAT1, SOCS3, and PIAS1 expression:** Equal amounts of liver protein extract (80µg) were fractionated on NuPAGE 10% Bis-Tris gels (Novex precast mini gel, Invitrogen, Carisbad, CA) and transferred to
polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Membranes were incubated with anti-STAT1 (Epitomics, Inc. CA, USA), anti-STAT1 phospho (ps727) (Epitomics, Inc. CA, USA), anti-SOCS3 (Cell Signaling tec. USA), and anti-PIAS1 primary antibodies (Abcam, USA). Binding of the primary antibodies was detected by incubating the membranes with the corresponding secondary antibody. The blots were then developed using enhanced chemiluminescence system according to the manufacturer recommendation (ECL system, Amersham, Arlington Heights, IL), and signals were visualized by exposure to X-ray film (X-Omat films, Kodak Company). To ensure equal loading of the protein, the membranes were stripped in stripping buffer (1 M Tris, 20% SDS, and 0.8% β-mercaptoethanol) at 55°C for 20 minutes, incubated with anti-actin (Sigma Chemical Co., Mo, USA) primary antibody. The actin signals were developed by sequential incubation of the membranes with the corresponding secondary antibody and ECL system followed by exposure to X-ray film. Signals were scanned using Alpha Imager 3.2 software (IS-1000 Digital Imaging System, USA) and normalized to that of actin

Statistical analysis: Analysis of the data was performed by using the computer program SPSS version 16. Unpaired student t-test was used to test significance between two groups in quantitative data. Comparison of non-parametric quantitative data in two different groups using their mean rank was performed by Mann-Whitney (Z).

Results. 112 patients were enrolled in the study; only 60 patients (34 men, 26 women) with ages ranging from 26 to 45 years were included in the study. These patients were subdivided into 38 HCV treatment responders (group 1a) & 22 HCV treatment non-responders (group 1b), in addition to 6 patients as controls.

The clinical characters of the patients and controls are summarized in table 1. Serum levels of AST & ALT were significantly higher in group 1 compared to group 2 (P<0.05), while no difference was observed between group 1 & group 2 as regards age, body mass index, albumin & total serum bilirubin (P>0.05) (table 1).

Comparison between group 1a & group 1b showed significant difference as regard serum ALT (P<0.05), while no difference was observed between group 1a & group 1b regarding age, body mass index, AST, serum albumin, total bilirubin, viral load & histological finding (P>0.05) (table 2).

To examine the activation state of JAK-STAT signaling pathway in chronic HCV patients and analyze its effect on their response to antiviral therapy, STAT1 protein expression was
examined in pretreatment liver tissue by western blotting. STAT1 expression was significantly increased in liver tissue from group 1 compared to group 2 ($P = 0.001$). However no difference was observed between group 1a & group 1b ($P = 0.747$). The present work showed a positive correlation between STAT1 protein expression & viral load in IFN responder (group 1a) ($r=0.735$ & $P > 0.001$) and non-responder patients (group 1b) ($r = 0.735$ & $P > 0.001$). No correlation was found between STAT1 expression and serum level of measured liver enzymes.

Since the active forms of STAT proteins which are involved in target gene transcription are the phosphorylated ones, we tested the expression of phosphorylated STAT1 using specific anti-STAT1 phospho antibody which detects STAT1 phosphorylation at serine residue 727. Phosphorylated STAT1 protein was expressed at a significantly higher level in liver tissue of group 1a compared to group 1b ($P=0.001$) (tables 2), with no correlation between phosphorylated STAT1 expression & viral load in group 1a ($r=0.249$ & $P=0.263$) & group 1b ($r=-0.155$ & $P=0.353$).

Western blotting of PIAS1 and SOCS3 protein expression in liver tissues from group 1 & 2 revealed significantly increased expression in group 1 compared to group 2 ($P=0.001$). Comparing liver tissue from group 1a & group 1b showed that PIAS1 and SOCS3 protein expression was significantly higher in liver of group 1b ($P=0.001$).

PIAS1 showed no correlation with measured liver enzymes or viral load in group 1a ($r=0.375$ & group 1b ($r=-0.225$ & $P=0.175$). Also, SOCS3 showed no correlation with measured liver enzymes or viral load in group 1a ($r=-0.138$ & $P=0.539$) & in group 1b ($r=-0.222$ & $P=0.181$).

Table 1- Characteristics of subjects enrolled in the study (group 1&2)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group 1 HCV group N=60</th>
<th>Group 2 Controls (6)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>38.9±11.8</td>
<td>39.4±13.5</td>
<td>0.922</td>
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<tr>
<td>Sex (M/F)</td>
<td>36/24</td>
<td>4/2</td>
<td>-</td>
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<td>BMI (Kg/m2)</td>
<td>23.1±4.87</td>
<td>22.7±4.15</td>
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<td>AST (u/l)</td>
<td>52.55±11.41</td>
<td>24.17±5.84</td>
<td>0.001*</td>
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<tr>
<td>ALT (u/l)</td>
<td>61.22±14.74</td>
<td>26.17±4.16</td>
<td>0.001*</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.9±0.3</td>
<td>4.2±0.2</td>
<td>0.020</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>1.2±0.4</td>
<td>0.9±0.1</td>
<td>0.074</td>
</tr>
<tr>
<td>Viral load (X10^5)</td>
<td>6.69±6.81</td>
<td>------</td>
<td>------</td>
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<tr>
<td>STAT1</td>
<td>407.9±72.48</td>
<td>20.83±4.44</td>
<td>0.001*</td>
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<tr>
<td>STAT1-phos</td>
<td>161.3±110.1</td>
<td>17.33±4.36</td>
<td>0.002*</td>
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<tr>
<td>PIAS1</td>
<td>503.4±306.1</td>
<td>26.67±7.99</td>
<td>0.001*</td>
</tr>
<tr>
<td>SOCS3</td>
<td>244.0±176.2</td>
<td>32.17±12.19</td>
<td>0.005*</td>
</tr>
</tbody>
</table>

*Significant values ($P < 0.05$)
Table 2 - Characteristics of HCV patients (group 1a & group 1b)

<table>
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<tr>
<th>Characteristics</th>
<th>HCV group N=60</th>
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</thead>
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<tr>
<td></td>
<td>Group1a</td>
<td>Group1b</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>Responders (N=38)</td>
<td>non-responders (N=22)</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>38.6 ±0.8</td>
<td>39.3 ±0.9</td>
<td>0.573</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>23/15</td>
<td>13/9</td>
<td></td>
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<tr>
<td>BMI (Kg/m2)</td>
<td>22.8±11.35</td>
<td>23.3±5.11</td>
<td>0.846</td>
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<td>AST (u/l)</td>
<td>50.91±11.15</td>
<td>53.5±11.6</td>
<td>0.401</td>
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<tr>
<td>ALT (u/l)</td>
<td>55.05±13.07</td>
<td>64.79±14.62</td>
<td>0.012*</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.9±1.3</td>
<td>3.8±0.6</td>
<td>0.735</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>1.1±0.1</td>
<td>1.2±0.4</td>
<td>0.146</td>
</tr>
<tr>
<td>Viral load (X10⁵)</td>
<td>7.37±8.06</td>
<td>6.3±6.06</td>
<td>0.562</td>
</tr>
<tr>
<td>Histological finding</td>
<td></td>
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<tr>
<td>• activity score</td>
<td>4.0±1.81</td>
<td>4.1±2.86</td>
<td>0.869</td>
</tr>
<tr>
<td>• fibrosis score</td>
<td>1.8±0.77</td>
<td>2.2±0.8</td>
<td>0.061</td>
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<tr>
<td>STAT1</td>
<td>411.9±77.5</td>
<td>405.5±40.37</td>
<td>0.747</td>
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<td>STAT1-phos</td>
<td>28±80.8</td>
<td>91.9±46.46</td>
<td>0.001*</td>
</tr>
<tr>
<td>PIAS1</td>
<td>142.0±52.41</td>
<td>712.7±158.8</td>
<td>0.001*</td>
</tr>
<tr>
<td>SOCS3</td>
<td>45.8±16.43</td>
<td>358.7±111.8</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*Significant values (P < 0.05)

Fig. 4 - Correlation between STAT1 & viral load in treatment responder (group 1a).

Fig. 5 - Correlation between STAT1 & viral load in treatment non-responder (group 1b).
Discussion. Approximately half of chronic HCV patients do not respond to current interferon/ribavirin combination therapy. The response rates of therapy vary depending on viral and host factors. However, factors determining the responsiveness are not well understood. With the exception of some clinical and biochemical factors, degree of inflammation and degree of fibrosis, there are no other markers which may serve as valid predictors of response to therapy.

So, assessments of biomarkers that predict the response to IFN in HCV patients are necessary. In this work we analyzed the status of STAT1, the key regulator of IFN signaling, and its negative regulator PIAS1 and SOCS3 proteins expression in the liver tissue of pretreatment chronic HCV patients using western blotting technique.

STATs proteins are part of the signaling pathway of interferon. Dysregulation of STATs expression is involved in cell resistance to viral infection. Several studies showed that activation of STATs pathways are predictive of treatment outcome in chronic HCV infection. The present work showed increased STAT1 protein expression in HCV infected liver tissue relative to control tissue, with a positive correlation with the viral load but no difference was observed between IFN responder and non-responders. In consistence with our findings, Bautista et al., and Kim et al., demonstrated overexpression of STATs proteins, STAT1, STAT2, and STAT5, in the HCV infected liver relative to healthy donors with no difference between IFN responders and non-responders, but rather increasing level of STATs proteins paralleled the degree of liver injury. In another study, STAT1 mRNA expression correlated positively with higher inflammatory score in chronic HCV infected liver tissue. In contrast, a previous study reported decreased STAT1 expression in cells transfected with HCV genomic constructs. The discrepancy is mostly because this study was performed under conditions of forced expression of HCV proteins in the cells without accompanying viral replication using an in vitro assay.

Interestingly, the activated form of STAT1, phosphorylated STAT1, showed a significant over expression in IFN responder patients with a nearly blocked expression in non-responders. These observations are in agreement with the antiviral activity of STAT proteins. Supporting this finding, Larrea et al., using HCV replicon system, reported that HCV replication blocks activation of STAT1, STAT2, and STAT3 after activation with IFN-α. This indicates interference of HCV with IFN-α induced Jak-STAT signaling cascade. Helbig et al., confirmed this finding and reported that IFN-α mediated STAT activation is blocked in hepatic cell lines containing HCV genomic replicon. In study to identify the hepatitis C viral proteins that block STAT signaling, NS3/4a protein has been shown to inhibit phosphorylation of STAT1 at the serine residue 727. These studies indicated that HCV proteins play an important role in the escape of HCV from interferon system.

The observation that STAT1 phosphorylation is blocked in therapy non-responder patients led us to investigate the STAT1 activation inhibitors. Among the suppressors of Jak-Stat signaling pathway are SOCS3 and PIAS1 proteins. SOCS3, a member of SOCS family, has been shown to inhibit INF-mediated antiviral activities and IFN signaling. The members of PIAS are special inhibitors of STATs that inhibit STAT-mediated gene activation by blocking the DNA binding activity of STATs. PIAS1 is an inhibitor of STAT1.

To determine the role of PIAS1 and SOCS3 in blocking the signaling pathway of interferon and resisting therapy in chronic HCV infection, we analyzed the expression of STAT1 inhibitors, PIAS1 and SOCS3 in liver tissue of pretreatment HCV patients. The study showed over expression of both PIAS1 and SOCS3 in HCV patients resisting IFN therapy with no correlation with the viral load or serum level of liver enzymes. In consistence with this observation Zhu et al., Kim et al., and Miyaaki et al., reported that interferon
resistant HCV infected hepatic cells exhibited enhanced SOCS3 expression and blocking SOCS3 partially restores interferon sensitivity. Moreover, post treatment relapse was associated with increased expression of SOCS3 and PIAS3\(^{20}\). They concluded that SOCS3 expression in the liver prior to IFN therapy might be a useful predictor of HCV clearance by interferon. In another study, intense PIAS1 immunostaining was observed in IFN resistant HCV infected hepatic cells compared to sensitive cells\(^{19}\).

In summary, the study showed blocking of activation of STAT1 protein and significant abundance of STAT1 inhibitors, SOCS3 and PIAS1 proteins in liver tissue of chronic HCV patients resistant to interferon/ribavirin therapy. These observations indicate that alteration of Jak/Stat signaling either by blocking activation of STAT proteins or up-regulation of STAT negative regulators, SOCS3 and PIAS1, represent a potential mechanism by which HCV resists interferon therapy and could be used as pretreatment predictors of HCV response to therapy.

References


