Original article

Impact of Sofosbuvir and Daclatasvir therapy on the expression levels of inflammasomes in chronic hepatitis C infected patients

Aya Fergany 1, Helal F. Hetta 2, Khaled Hassanein 2, Mohammed A. Medhat 3, Gaber El-Saber Batiha 4, Asmaa S. Shaltout * 2

1- Microbiology and Immunology Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.
2- Medical Microbiology and Immunology Department, Assiut, Egypt.
3- Tropical Medicine and Gastroenterology Department, Assiut University Hospitals, Assiut University, Assiut, Egypt.
4- Department of Pharmacology and Therapeutics, Faculty of Veterinary Medicine, Damanhour University, Damanhour, 22511, Egypt.

ABSTRACT

Background and Aim: Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis. Inflammasomes are multi-protein complexes that sense specific microbial molecules and trigger signaling cascades, leading to caspase1 activation and generation of pro-inflammatory cytokines, including IL-1β and IL-18. We aimed to investigate the expression levels of NLRP3, AIM2 and IFI16 inflammasome genes and serum levels of IL-18 in chronic HCV infected patients before treatment and after SVR12. Methods: The study included 30 chronic HCV infected patients and 30 healthy controls. The expression levels of inflammasome genes were evaluated by Quantitative real-time PCR (qPCR) and serum levels of IL-18 were evaluated by ELISA at baseline and after SVR12 with three months regimen of Sofosbuvir and Daclatasvir. Results: At baseline, the expression level of NLRP3, AIM2 and IFI16 inflammasome genes were higher in comparison to controls (p = 0.018, 0.000 and 0.155 respectively). In addition, the level of serum IL-18 was upregulated in comparison to controls (p = 0.000). After treatment, there was a statically significant decrease in the expression level of NLRP3, AIM2 and IFI16 inflammasome genes (p < 0.0001 for all). Also, there was a statically significant down regulation in the level of serum IL-18 (p = 0.000). Conclusion: Direct acting antiviral (DAA) therapy not only cause viral eradication but also has an immunological restitution effect as it decreases the expression level of NLRP3, AIM2 and IFI16 inflammasome genes and serum level of IL-18. This down regulation may decrease the risk of HCC development in chronic HCV infected patients.

Introduction

Hepatitis C virus (HCV) infection is a worldwide public health problem. HCV prevalence had an estimated 2.8% increase over the last decade, with more than 185 million infections (3% of the world's population) [1]. The majority of patients is unable to clear the virus and develops viral persistence despite the ongoing innate and adaptive immune response [2-5]. In most acutely infected patients, HCV is able to escape the immune response and establish persistent infection which increases the risk of progressive liver fibrosis and HCC [6].

The innate immune system is considered as the first line of defense mechanisms against different invading organisms. HCV eradication or persistent infection is linked to the outcome of the interactions between HCV and the different innate immune cells [7-9]. HCV infection activates the
innate immune response through different mechanisms. This activation results in the increased expression of many genes and proteins associated with inflammation and finally triggers liver fibrosis [10-12]. Recognition of the virus by innate sensors as TLRs leads to the production of type I interferons and the assembly of inflammasome complexes [13].

Inflammasomes are intracellular innate immune sensors, including: NLRP3, AIM2 and IFI16 [14]. They are multi-protein cytosolic complexes that integrate several pathogens triggered signaling cascades, ultimately leading to caspase-1 activation and secretion of pro-inflammatory cytokines including IL-18 and IL-1β [15]. Although, multiple types of inflammasomes were reported to be involved in HCV, others are still unknown and need further investigations [16].

Inflammasome derived IL-1β and IL-18 are central regulators of hepatic inflammation [17,18]. Inflammasome activation has been the only mechanism described to convert pro-IL-18 to mature IL-18 [14]. Thus, higher levels of IL-18 are considered as an indication of inflammasome activation [15]. IL-18 has been implicated in HCV pathogenesis and correlated with liver injury during HCV infection [14-21]. The continuous stimulation of innate immunity in response to HCV may lead to the progression of liver disease. So, many studies focused on finding a treatment which that not only causes eradication of HCV virus but also, can reconstitute immune parameters - as inflammasomes - to decrease the incidence of liver progression diseases [11].

The discovery of direct acting antiviral agents (DAAs) considered as a revolution in the treatment of patients with chronic HCV infection [22,23]. The combination of Sofosbuvir plus Daclatasvir is related with a high degree of sustained viral response (SVR) in chronic HCV patients infected with genotype 1 or 4 who are as summed to be difficult to be treated [22].

Some recent reports have revealed that the immune dysfunction and altered cytokine in chronic HCV patients treated with DAAs still persist despite the absolute eradication of HCV [24,25]. On the other hand, other studies reported rapid down regulation of the innate immune parameters in chronic HCV patients treated with DAAs treatment compared to patients treated with IFN [11,24].

Therefore, we aimed to analyze the expression level of NLRP3, AIM2, and IFI16 inflammasome genes and IL-18 serum level in chronic HCV patients before treatment and after SVR12 and to correlate these levels with viral load before treatment.

**Materials and Methods**

**Study type, settings, and duration:**
The present study was a cohort study conducted from February 2019 to February 2020 at Al-Rajhi Liver hospital, Assiut University Hospital, Egypt. Assiut Medical School Ethical Review Board reviewed and approved the study protocol; approval number was (17100798). Clinical Trials code was (NCT04244383). All of the attendees signed an informed consent form before sample collection. Assuming type I statistical error in the range of 0.05 significance level and type II statistical error in the range of 20%, we aiming to obtain statistical power of 80%. Based on previous studies [19], 30 subjects per group were estimated.

**Study definitions and patients’ selection:**
Patients attending the AL-Rajhi Hospital were invited to participate in the study. Eligible participants were assigned to two groups. Group 1 consisted of chronic HCV patients who were treatment naïve. Group 2 consisted of apparently healthy control group who were recruited from the blood donation banking department after exclusion of hepatitis infection.

Chronic HCV patients were defined when HCV-Ab and HCV-RNA-PCR were positive. HCV viral load was determined before treatment and after 12 weeks from the end of treatment (SVR12) by using Artus1 HCV-RG RT-PCR Kit (cat#4518265, QIAGEN, Germany) according to the manufacturer’s protocol. Quantitative real time-PCR was performed on 7500 Fast real-time PCR Thermal cycler (Applied Biosystems, CA, USA).

All patients were subjected to full medical history, including the history of previous treatment for HCV and any features of decompensated cirrhosis. Baseline laboratory studies were done, including: complete blood count, AST, ALT, serum bilirubin, Prothrombin time (PT), serum creatinine, and pregnancy test for females at childbearing age. Patients with HCC, autoimmune disease, liver cirrhosis, pregnant women and patients who refuse to participate in the study were excluded. Patients eligible for study received Sofosbuvir 400 mg and Daclatasvir 60 mg daily for 12 weeks. Patients who did not complete the treatment course were
excluded. The volunteer controls were matched for age and sex.

**Sample taking:**
Nine milliliters of venous blood were collected from each patient before treatment and 4 ml from controls by clean venipuncture. The sample taken before treatment was into 5ml for viral load estimation, CBC, liver function tests, kidney function test investigation (patients only), 2 ml on EDTA tube for PBMCs isolation, and the remaining 2 ml on blank tube for ELISA.

Six milliliters of venous blood were collected from each subject at 12 weeks after SVR12, then divided into 2 ml for viral load estimation, 2 ml on EDTA tube for PBMCs isolation, and 2 ml on a blank tube for ELISA.

**Isolation of PBMCs:**
Isolation of PBMCs was done from the whole blood of selected patients before treatment and after SVR12 and from controls using Ficoll Histopaque (Biowest, Germany) according to the manufacturer's instructions.

**RNA extraction, reverse transcription, and quantitative real-time PCR:**
The total RNA of PBMCs was extracted with TRIzol (Invitrogen, Applied Biosystems, USA). Reverse transcription was conducted using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific - Applied Biosystems, USA). The primers used in this experiment were as follows: NLRP3 (forward) 5¢-GATCTTCGGTGGCATACA-3¢ and (reverse) 5¢-GGGATTCGAAACACGTGCAAT-3¢; AIM2 (forward) 5¢-CTGCAGCTGATGAGACCATTCGTA-3¢ and (reverse) 5¢-GGTGCAGCACGTTGCTTTG-3¢; IFI16 (forward) 5¢-GAAGTGCCAGCGTAACTCCTAA-3¢ and (reverse) 5¢-TGGTGACGACCTTGCTTTG-3¢; and GAPDH (forward) 5¢-GCACCGTCAAGGCTGAGAAC-3¢ and (reverse) 5¢-TGGTGAAGACGCCAGTGGA-3¢ [20]. The quantitative real time PCR (qPCR) was performed using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific - Applied Biosystems, USA) with ROX solution provided in Applied Biosystems Step One Plus Real-Time PCR (Applied Biosystems, USA). The relative gene expression was normalized to the level of GAPDH transcript and relative quantification was performed using the 2−ΔΔCT method.

**Enzyme-linked immune-sorbent assays (ELISA):**
IL-18 serum level in chronic HCV patients before and after SVR12 and controls were determined by ELISA kits (SinoGeneclon, China) according to the manufacturer's instructions.

**Statistical analysis:**
The statistical analysis was performed with statistical package for social science using IBM SPSS Statistics version 20 (SPSS Inc., Chicago, IL, USA). All variables tested before evaluation for normally. 2 groups of dependent continuous variables were evaluated using Mann-Whitney U test. Difference in median between the same group was calculated using Wilcoxon signed ranks test. Spearman’s correlation was used to correlate the studied parameters. p<0.05 was considered significant.

**Results**

**Demographic data:**
Thirty chronic HCV patients and equal number of healthy controls were included in this study. The age of the patients ranged from 20 -79 (median = 51) years. The study included 19 (63%) males and 11 (37 %) females. There was no significant difference in age and gender between the enrolled groups. Biochemical measurements of the participant groups are summarized in table (1).

**Gene expression interpretations:**
There was a statistically significant increase in gene expression of NLRP3 and AIM2 inflammasomes in chronic HCV patients before treatment in comparison to controls with p-value = (0.018 and 0.000) respectively. Conversely, there was no significant increase in gene expression of IFI16 inflammasome in chronic HCV patients before treatment in comparison to controls with p-value (0.155) as shown in figure (1). Also, the gene expression level of NLRP3, AIM2, and IFI16 inflammasomes in chronic HCV patients after SVR12 were significantly down-regulated than before treatment with (p <0.0001) for all genes as shown in figure (2). In addition, there was no significant correlation between gene expression of NLRP3, AIM2, or IFI16 inflammasomes and viral load before treatment in chronic HCV patients (r = -0.342, -0.023, -0.08) respectively (p= 0.06, 0.9, 0.65).
IL-18 serum levels in patient before and after SVR12:
IL-18 serum level was significantly up regulated in chronic HCV patients before treatment than healthy controls with \( p \)-value = 0.000 as shown in figure (3). Also, IL-18 serum level was significantly decreased after SVR12 than before treatment with \( p \)-value = 0.000 as shown in figure (4). In addition, there was no significant correlation between viral load and IL-18 serum level before treatment in chronic HCV patients.

<p>| Table 1. laboratory characteristics of chronic HCV patients and control group. |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chronic HCV patients (30)</th>
<th>Healthy control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-RNA copy number expressed in median (rang)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral load before treatment (UL/ml)</td>
<td>971500 (64.000 - 3997519)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Complete blood count expressed in median (rang)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dL) Normal rang (12.2-18.1)</td>
<td>15.15 (11.7 - 17)</td>
<td>15.55 (13.5-17.2)</td>
</tr>
<tr>
<td>RBCs (x10^6/ul) Normal rang (4.04-6.13)</td>
<td>5.27 (4.15 - 6.38)</td>
<td>5.13 (4.09-6)</td>
</tr>
<tr>
<td>Platelets (x10^3/ul) Normal rang (142-424)</td>
<td>208 (119 - 419)</td>
<td>322.5 (198-420)</td>
</tr>
<tr>
<td>WBCs (x10^3/ul) Normal rang (4.6-10.2)</td>
<td>6.64 (3.2 – 13.4)</td>
<td>7.8 (5-10)</td>
</tr>
<tr>
<td>Lymphocytes (x10^3/ul) Absolute normal rang (0.6-3.4)</td>
<td>2.9 (1.5-5.2)</td>
<td>1.95 (1-2.8)</td>
</tr>
<tr>
<td>Monocytes (x10^3/ul) Absolute normal rang (&lt;0.9)</td>
<td>0.5 (.09-4.1)</td>
<td>0.6 (0.3-0.77)</td>
</tr>
<tr>
<td>Liver function tests expressed in median (rang)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (U/L) Normal rang (&lt; 41)</td>
<td>49.5 (9 - 162)</td>
<td>28 (22-34)</td>
</tr>
<tr>
<td>AST (U/L) Normal rang (&lt; 38)</td>
<td>35 (10 - 142)</td>
<td>28 (22-33)</td>
</tr>
<tr>
<td>Bilirubin-T (umol/L) Normal rang (3.4-17.1)</td>
<td>7.9 (2.4 - 23)</td>
<td>11.45 (5.5-15.4)</td>
</tr>
<tr>
<td>Albumin (g/L) Normal rang (34-48)</td>
<td>44 (30 - 50)</td>
<td>39.5 (35-47)</td>
</tr>
<tr>
<td>PT (%) Normal rang (70-120)</td>
<td>105.8 (9.7 - 128.8)</td>
<td>91.5 (72-112)</td>
</tr>
</tbody>
</table>
**Figure 1.** Showing the effect of HCV infection on the expression of genes of NLRP3, AIM2, and IFI16 inflammasomes in chronic HCV patient compared with healthy controls. Data expressed as median.

**Figure 2.** Showing the expression of genes of NLRP3, AIM2, and IFI16 inflammasomes before treatment with DAAs and after SVR12 in chronic HCV patients. Data expressed as median.

**Figure 3.** Showing IL-18 serum level in chronic HCV patients before treatment in comparison to healthy controls. Data expressed as median.
**Figure 4.** Showing comparison between IL-18 serum level before treatment and after SVR12 in chronic HCV patients. Data expressed as median.

**Discussion**

To the best of our knowledge, we reported for the first time the down regulation in gene expression level of NLRP3, AIM2, IFI16 inflammasomes in chronic HCV patients after treatment (SVR12) with three months regimen DAAs (Sofosbuvir/Daclatasvir) therapy. We found that the treatment with DAAs (Sofosbuvir/Daclatasvir) not only eradicate the virus completely, but also has immunological restitution effect on both transcription and post-transcription level.

The expression level of NLRP3 inflammasome gene was up regulated in chronic HCV patients before treatment in comparison to controls. This result consistent with previous studies which documented that NLRP3 inflammasome participated in the antiviral innate immune response against HCV and contributed to liver inflammation through the release of inflammatory cytokines [17,26-28].

In addition, AIM2 inflammasome gene expression was up regulated in chronic HCV patients before treatment in comparison to controls. This result indicates the involvement of AIM2 inflammasome in the immune attack against HCV.

Also, IFI16 inflammasome gene expression was up regulated in chronic HCV patients before treatment in comparison to controls. This result suggested the involvement of IFI16 inflammasome in immune attack against HCV as previous studies reported that, IFI16 inflammasome was engaged with other inflammasomes in many diseases including hepatic diseases [29-31].

To investigate whether the treatment with DAAs can achieve restitution of the up regulated inflammasomes, we analyzed their expression levels in the same patients after SVR12.

The results showed that, expression level of NLRP3 inflammasome gene decreased significantly after SVR12 than before treatment. This result is very promising in relation to liver inflammation state as NLRP3 was strongly suggested to be involved in liver pathogenesis [26,27].

To the best of our knowledge, it was reported for the first time the decrease in expression level of AIM2 inflammasome gene significantly after SVR12 than before treatment with p-value = 0.000. In a study was done by Han and others, they found that AIM2 gene expression was proved to be correlated with the inflammation state in HBV patients [32]. So, we suppose this decline may be a predictor of inflammation state of liver after viral eradication.

In addition, we reported for the first time the decrease in expression level of IFI16 inflammasome gene significantly after SVR12 than before treatment with p-value = 0.000. This result is supported by a study was done by Pang and others on chronic HBV, they found that IFI16 was closely related to the degree of inflammation in CHB [33].

The viral load did not show any significant correlation with the expression level of NLRP3, AIM2, or IFI16 inflammasome genes before treatment. So, it was suggested that, amount of the virus does not have an effect on the increased level of the inflammasome genes before treatment. Hence, any amount of the virus will be able to induce infection and activation of the immune system in the same way.
In fact, these results are promising in relation to liver disease progression situation, as the successful treatment of HCV is determined by the ability to completely eradicate the virus and rapidly decline liver inflammation [11,25]. Assessment of inflammasomes expression after SVR12 can be used as a marker for liver inflammation and determine who is liable for HCV complications and needs strict follow up. In addition a recent study was done by Wu and others, they focused on NLRP3 inhibitors as a novel therapy for chronic HCV that have the ability to suppress development of chronic liver disease [27].

IL-18 and IL-1β are important factors contributing to the development of chronic hepatocellular inflammation [11,21,34,35]. Considering that, inflammasome activation is the only described mechanism to convert pro-IL-18 to mature IL-18 [15,19]. So, we focused in our study on IL-18 concentration being a marker of liver inflammation that induced mainly from inflammasomes assembly.

In the study, IL-18 serum level was significantly increased in chronic HCV patients before treatment in comparison to controls. This result is consistent with results from many studies that confirmed the elevation of IL-18 concentration in HCV patients [19,21,25].

In addition, the results showed that, IL-18 serum level decreased significantly after SVR12 than before treatment. This result proved the immune regulating effect of the DAAs (Sofosbuvir/Daclatasvir). This result was consistent with many studies which reported the normalization of IL-18 serum level after viral eradication [15,36].

A study was made on chronic HBV patients showed that, there was no significant increase in serum levels of IL-18 in CHB despite the increased expression level of AIM2 and IFI16 inflammasomes [30]. This discrepancy might be due to the difference in the type of virus, the mechanism of evasion of each virus, how immunity deal with them and different antiviral drugs.

No significant correlation was found between viral load and IL-18 serum level before treatment. This result proved our pervious result that, any amount of the virus can stimulate the immunity nearly at the same way. This result is consistent with pervious results from study done by Chattergoon and others, they showed that there was no correlation between HCV RNA and IL-18 level despite the wide fluctuation of the viral amount [36].

In addition, IL-18 serum level did not show significant correlation with liver enzymes: ALT or AST before treatment. Likewise, results from previous study was done by Chattergoon and others showed that, there was no correlation between the increased level of IL-18 and ALT [36].

Importantly, these finding enrich our understanding of HCV interaction with innate immune response and subsequent effect on liver pathogenesis and help in finding a marker for liver inflammation or disease severity and finding novel therapeutic targets able to reduce hepatic inflammation.

Conclusion

HCV infection induces the activation of many inflammatory parameters including NLRP3, AIM2, and IFI16 inflammasomes. The up regulation of these inflammasomes will finally trigger the increase in the secretion of IL-18. According to the decrease in expression levels of inflammasome genes and serum IL-18 level after SVR12 we concluded that, the combination of Sofosbuvir plus Daclatasvir not only cause eradication of HCV virus but also have the ability to restitute immune parameters as inflammasomes.

Acknowledgments: None.

Funding: We thank Assiut Faculty of Medicine grant office for funding this research.

Conflicts of Interest: None.

References


3-Hetta HF, Mekky MA, Khalil NK, Mohamed WA, El-Feky MA, Ahmed SH, et al. Extra-


21-Veenhuis, R.T., et al., Systemic Elevation of Proinflammatory Interleukin-18 in Human Immunodeficiency Virus (HIV)/Hepatitis C Virus (HCV) Coinfection Versus HIV or HCV


