Potential Genetic Markers for Prediction of Treatment Response in HCV Affected Children

SAHAR A. SHARAF, M.D.*; IMAN MANDOUR, M.D.*; HANAA M. EL-KARAKSY, M.D.**
RANIA DARWISH, M.D.*; NORMEEN H. RADY, M.D.* and FATMA EL-MOUGY, M.D.*
The Departments of Clinical & Chemical Pathology* and Pediatrics**, Faculty of Medicine, Cairo University, Egypt

Abstract

Background: Egypt has high prevalence of hepatitis C virus (HCV) infection in children who are younger than 10 years. Interleukin 10 (IL-10) is an anti-inflammatory cytokine which helps to dampen inflammation that could be harmful to the host, consequently limiting potential tissue damage. High production of IL-10 facilitates viral evasion by down regulating protective inflammatory response and opposing the response to antiviral treatment.

Aim of Work: To assess the prevalence of the 2 SNPs -1082 G/A and –592 C/A in IL-10 promotor region and if they affect* the response to antiviral therapy in children and young adults with HCV infection.

Patients and Methods: Seventy three HCV patients underwent quantitation of HCV-RNA viral load by polymerase chain reaction (PCR) meanwhile liver function testing were followed-up for 72 weeks after the start of HCV therapy. The IL-10 genotyping was assayed by real time PCR.

Results: No significant association was found between polymorphisms in IL-10 gene (–1082G/A and –592 C/A) and the response to HCV therapy in children and young adults with HCV infection.

Conclusion: There is no association between response to therapy for HCV in our group of children and IL-10 polymorphisms (–1082G/A and –592C/A), however using logistic regression analysis, IL10 gene SNPs –592 A>C and basal viral load showed that they are independent factors predicting response to interferon therapy in chronic hepatitis C patients.

Key Words: HCV – Interleukin 10 – RT-PCR – SNP -1082 G/A – SNP -592 C/A.

Introduction

HEPATITIS C Virus infection (HCV) is a major etiologic agent of transfusion-associated hepatitis.

Regulatory mechanisms which control production of IL-10 include genetic polymorphisms especially in the promoter region [9,10]. IL-10 is encoded by IL-10 gene on chromosome 1q 31-32 [11,12]. The IL-10 promoter area is polymorphic containing three frequent point mutations –1082 (G/A), –819 (C/T), and –592 (C/A) [13], that are suggested to be associated with differential IL-10 levels in humans [14].
This suggests that heterogeneity in the promoter region of IL-10 gene may have a role in the response of chronic hepatitis C to antiviral therapy. It was hypothesized that genetically predisposed patients to high IL-10 production are poor responders to IFN-α and hence, may benefit from other treatment modalities designed to enhance Th1 response [15].

The present study aimed at detecting if the polymorphisms in the IL-10 promoter gene could affect the response to antiviral therapy. The single nucleotide polymorphisms −1082 G/A and −592 C/A in the promoter region of IL-10 gene were assayed in the studied group and their effect on response to HCV therapy was assessed.

**Patients and Methods**

The current study was approved by the Ethical Committee of Faculty of Medicine, Cairo University. It was done over the years 2010 to 2014 on 73 children chronically infected with HCV who were attending the Pediatric Hepatology Unit in Cairo University Pediatric Hospital. All children were treated for chronic HCV by pegylated interferon (Peg-IFN) α 2b (1.5 μg/kg weekly subcutaneously) and ribavirin (15mg/kg/day).

The objective of the study was clearly explained to all the patients participating in the study and parents of patients signed an informed consent before participating in the study.

Diagnosis was based on the serological, virological and histological testing. Basal, pre-treatment and post-treatment histopathological examination of percutaneous needle liver biopsy was done to the studied group.

Patients with decompensated liver disease, anemia, (<10g/dL), leucopenia (<3,000/mm^3), neutropenia (<1500/mm^3), thrombocytopenia (<100,000/mm^3), high serum creatinine, autoimmunity, α 1 antitrypsin deficiency, Wilson's disease, hepatitis B infection, poorly controlled diabetes mellitus, uncontrolled thyroid disorder, or psychiatric diseases were excluded from the study.

Six ml of blood were collected in a plain sterile vacutainer and separated sera were assayed for chemistry investigations, HCV-RNA titers as well as HCV antibodies. Two ml of blood were collected in a sterile EDTA vacutainer for genotyping. DNA was extracted using fresh blood, and then was stored at −20 C till it was assayed.

The laboratory work up done for the studied patients:

1- Routine tests: Complete blood picture (on CELL-Dyn 3700, USA), routine liver function tests (on Hitachi 911*; Roche, GmbH Mannheim Germany).

2- HCV-RNA titer: Patients were tested for HCV RNA using quantitative real time PCR at baseline, 12, 24, 48 weeks after start of therapy and 24 weeks after cessation of therapy on Applied Biosystems 7500 Real time PCR System using kits supplied by Qiagen (Qiagen GmbH (Hoffmann-La Roche AG) Max-Volmer-Strabe 4-40724-Hilden-Germany).

Patients whose HCV RNA turned negative or achieved a 2 log decrease in their viral load at week 12 i.e. early virologic response (EVR) continued HCV therapy. Otherwise, the child was considered as non-responder and treatment was discontinued.

Another HCV RNA was repeated 24 weeks after therapy for responders, and if positive the child was considered non-responder and treatment was discontinued. Those who responded at 24 weeks, continued treatment till 48 weeks. At the end of therapy, HCV RNA was repeated to investigate the end of treatment response (ETR). Those who achieved ETR repeated HCV RNA after 24 weeks to investigate for sustained virologic response (SVR).

3- Genotyping of IL-10 gene polymorphisms −1082 G/A and −592 C/A by real time PCR:

DNA was extracted using QIAamp DNA blood Mini kit-Qiagen. DNA was then amplified using Taq-Man SNP Genotyping Assays to detect the polymorphisms in IL-10 gene promoter at the −1082 and −592 positions.

**Amplification and real-time PCR genotyping assays:**

Genotyping was detected on Applied Biosystem step oneTM Real-Time PCR System, using TaqMan SNP Genotyping Assays (Applied Biosystems)*. Assays perform genotyping of the G → A1082 (dbSNP ID: rs1800896, TaqMan SNP Genotyping Assays ID: C174736010), and C → A 592 (dbSNP ID: rs1800872, TaqMan SNP Genotyping Assays ID: C_1747363_10).

The working protocol:

PCR reaction mix include: Taqman universal PCR master mix (2X) 12.5 μL, patient DNA 5 μL and 20X working stock of SNP genotyping assay
1.25 µL, which was completed to 25 µL by DNase-free water.

Enzyme activation step was done at 95°C for 10 minutes, cycling: 45 cycles of PCR amplification of target DNA, with the following temperature profile, 92°C for 15 seconds for denaturation of target DNA, then annealing and extension was done at 62°C for 60 seconds and Lastly genotyping plate reading and analysis using the Sequence Detection System (SDS) Software. VIC dye and Fam-dyes were used in the allele discrimination Fig. (1).

Statistical methods:
Statistical analysis of the results was done by SPSS computer software package, version 15.0 (Chicago, IL, USA). Quantitative data were expressed either in the form of mean and standard deviation when data were Gaussianly distributed, or in the form of median and interquartile range (IQR) when the data were skewed, and the difference between responders and non responders was compared by Student t-test or using Mann whitney test accordingly. Multivariate logistic regression analysis was used to detect independent factors that could predict response to IFN therapy in CHC patients. Significant differences were considered at p≤0.05.

Results
According to the response to HCV therapy, patients were divided into two groups:

Responders: Patients who achieved SVR with normalization of ALT and AST levels and clearance of the virus as denoted by negative HCV RNA by PCR 24 weeks after the end of therapy course.

Twenty eight responders were included, 16 males and 12 females, whose mean age was 11.9±2.9 years.

Non-responders: Forty five patients were included, 31 males and 14 females, whose mean age was 10.8±3 years.

Demographic and lab data of the studied subjects are presented in (Table 1). Only quantitative HCV RNA and GGT showed statistically significant difference, being higher in non-responders (p=0.005, p=0.001 respectively) (Table 1).

On comparing the hepatitis activity index (HAI) from liver biopsy done to the tested subjects, statistically significant difference was found between the 2 studied groups (p=0.004). Patients with minimal HAI were 12.8 times more likely to respond to HCV therapy than patients with mild,
moderate and severe HAI (OR=12.8). However, pretreatment fibrosis (F) score showed no statistically significant difference on comparing the 2 studied groups (Table 2).

No statistically significant difference was detected in the genotype distribution of the two SNPs between the two groups (Table 3).

On sub grouping of the genotypes, for SNP –1082 and SNP –592 respectively to predict response to interferon therapy in HCV infected patients; no statistically significant difference was found (Table 4).

Multivariate logistic regression analysis was used to assess the effect of different factors on response to HCV therapy, and showed that IL10 gene SNP –592 A>C, and pretreatment viral load were independent factors predicting response to HCV therapy, \( (p=0.002) \), \( (p=0.017) \) respectively (Table 5).

### Table (1): Pretreatment demographic and biochemical data according to treatment response.

<table>
<thead>
<tr>
<th></th>
<th>Non responders</th>
<th>Responders</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years): (Mean±SD)</td>
<td>10.8±3.5</td>
<td>11.9±2.9</td>
<td>0.168</td>
</tr>
<tr>
<td>Gender: Males (n=47)</td>
<td>31 (68.9%)</td>
<td>16 (57.1%)</td>
<td>0.308</td>
</tr>
<tr>
<td>Females (n=26)</td>
<td>14 (31.1%)</td>
<td>12 (42.9%)</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L): [Median] (IQ range)</td>
<td>53</td>
<td>57</td>
<td>0.551</td>
</tr>
<tr>
<td></td>
<td>49.5</td>
<td>56.75</td>
<td></td>
</tr>
<tr>
<td>GGT (U/L): [Median] (IQ range)</td>
<td>33.85</td>
<td>20.0</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>46.5</td>
<td>12.45</td>
<td></td>
</tr>
<tr>
<td>Ferritin (ng/ml): [Median] (IQ range)</td>
<td>36.25</td>
<td>90</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>76.5</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Quantitative HCV RNA-PCR (IU/ml): [Median] (IQ range)</td>
<td>936.02</td>
<td>418342</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>473.592</td>
<td>3,170,500</td>
<td></td>
</tr>
</tbody>
</table>

p-value was considered significant at ≤0.05.

### Table (2): Prediction of response to HCV therapy by base line hepatitis activity index (HAI) and fibrosis score (F).

<table>
<thead>
<tr>
<th></th>
<th>Non responders</th>
<th>Responders</th>
<th>p-value</th>
<th>95th% CI</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal HAI</td>
<td>24 (63.2%)</td>
<td>22 (95.7%)</td>
<td>0.004</td>
<td>(1.5-105.8)</td>
<td>12.8</td>
</tr>
<tr>
<td>Mild, Moderate and Severe HAI</td>
<td>14 (36.8%)</td>
<td>1 (4.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis scoring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No and Minimal fibrosis</td>
<td>30 (78.9%)</td>
<td>21 (87.5%)</td>
<td>0.391</td>
<td>(0.443-7.873)</td>
<td>1.867</td>
</tr>
<tr>
<td>Mild and Moderate fibrosis</td>
<td>8 (21.1%)</td>
<td>3 (12.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table (3): Genotype distribution of the two polymorphisms in the two studied groups.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non responders</th>
<th>Responders</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP –1082 A&gt;G:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>7 (15.6%)</td>
<td>5 (17.9%)</td>
<td>0.607</td>
</tr>
<tr>
<td>AG</td>
<td>23 (51.1%)</td>
<td>11 (39.3%)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>15 (33.3%)</td>
<td>12 (42.9%)</td>
<td></td>
</tr>
<tr>
<td>SNP –592 A&gt;C:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>23 (51.1%)</td>
<td>9 (32.1%)</td>
<td>0.27</td>
</tr>
<tr>
<td>CA</td>
<td>19 (42.2%)</td>
<td>17 (60.7%)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>3 (6.7%)</td>
<td>2 (7.1%)</td>
<td></td>
</tr>
</tbody>
</table>

p-value was considered significant at ≤0.05. OR = Odds ratio. CI interval = Confidence interval.
Table (4): Prediction of response to IFN therapy in HCV infected children according to genotypes of IL10 gene.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non responders (n=45)</th>
<th>Responders (n=28)</th>
<th>p-value</th>
<th>95% CI</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL10: SNP –1082 A&gt; G:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA and AG:</td>
<td>30 (66.7%)</td>
<td>16 (57.1%)</td>
<td>0.412</td>
<td>(0.568-3.964)</td>
<td>1.5</td>
</tr>
<tr>
<td>GG:</td>
<td>15 (33.3%)</td>
<td>12 (42.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA:</td>
<td>7 (15.6%)</td>
<td>5 (17.9%)</td>
<td>0.519</td>
<td>(0.335-4.156)</td>
<td>1.18</td>
</tr>
<tr>
<td>AG and GG:</td>
<td>38 (84.4%)</td>
<td>23 (82.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL10: SNP –592 A&gt; C:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA:</td>
<td>23 (51.1%)</td>
<td>9 (32.1%)</td>
<td>0.112</td>
<td>(0.169-1.214)</td>
<td>0.453</td>
</tr>
<tr>
<td>AC and CC:</td>
<td>22 (48.9%)</td>
<td>19 (67.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA and AC:</td>
<td>42 (93.3%)</td>
<td>26 (92.9%)</td>
<td>0.641</td>
<td>(0.145-5.934)</td>
<td>0.929</td>
</tr>
<tr>
<td>CC:</td>
<td>3 (6.7%)</td>
<td>2 (7.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p*-value was considered significant at ≤0.05. OR = Odds ratio. CI interval = Confidence interval.

Table (5): Multivariate logistic regression study for predictors of response to IFN therapy in chronic hepatitis C children.

<table>
<thead>
<tr>
<th></th>
<th>p-value</th>
<th>95% CI</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL 10 SNP –592 A&gt;C:</td>
<td>0.017</td>
<td>(1.345-20.065)</td>
<td>5.196</td>
</tr>
<tr>
<td>Base line quantitative PCR:</td>
<td>0.002</td>
<td>(1.629-8.275)</td>
<td>3.671</td>
</tr>
</tbody>
</table>

Discussion

Peg-IFN/RBV is the treatment of choice for chronic HCV infection; however, results are unsatisfactory in HCV-1 carriers because only 50% of those patients achieve SVR [18]. Considering the high cost and side effects of antiviral therapy, there is a need to identify factors that can predict response to antiviral therapy [19].

The main target in this study was to assess the role of the polymorphisms in the promoter region of IL-10 gene (−1082 G/A and −592 C/A) in the response to HCV treatment.

After generating a proinflammatory immune response, IL-10 dampens the inflammation that might be harmful to the host, hence limiting potential tissue damage via inhibition of secretion of inflammatory cytokines [7,8]. IL-10 production is controlled by genetic polymorphisms especially in the promoter region, influencing transcription and translation of IL10 [9,10]. The more the polymorphisms in the promoter region, the more the effect on expression in response to HCV treatment.

The results of this study revealed that 38.4% of HCV patients were responders to Peg IFN α-2b plus ribavirin therapy (28 responders versus 45 non responders). This finding is in accordance with Reis et al., [20] who reported SVR of 39.9%.

Regarding the frequency distribution of different genotypes of the two studied SNPs, there was no statistically significant difference in the genotype distribution between the responders and non-responders with no impact on response to treatment in HCV infected children. This is in agreement with previous reports [10,21], where IL-10 gene promoter polymorphisms were not associated with response to HCV therapy.

In the current study, factors other than genotype were also investigated (host and viral factors) that determine, the therapeutic efficacy of HCV treatment. It was found that patients with SVR had significantly lower baseline serum GGT levels (p=0.001), baseline viral load (p=0.005); this agrees with Hadziyannis et al., [22] who observed that the more hepatic cells remain intact in HCV patients the more effective HCV therapy, as well as some studies [10,23] where there was a close relationship between pretreatment viral load and response to INF therapy. Patients with pretreatment viral load <2 million copies/ml are 1.5 times liable to have SVR as compared to higher viral loads. Multivariate logistic regression identified that pretreatment HCV RNA levels (p=0.04) is the most significant predictor of response. This is comparable with the work by Gewaltig et al., [24] who found that IL-10 variants were highly associated with response to HCV therapy.

As regards fibrosis stages and its relation to response to treatment, in the present study it was found that fibrosis scoring was not associated with therapeutic efficacy of HCV treatment. This finding agrees with other studies [25,26] who stated that a high pretreatment fibrosis was not associated with lack of SVR.

In partial agreement with this study, Backus et al., [27] demonstrated that absence of cirrhosis and low HCV RNA level (<500,000IU/mL) are predictors of SVR to treatment for chronic HCV infection. A pilot study of a short course (14 weeks) of therapy
with peg-IFN alpha-2b and ribavirin demonstrated that absence of bridging fibrosis/cirrhosis was a crucial factor associated with SVR [28].

Lastly, multilogistic regression was done to select the minimum combination of variables that maximally differentiate between responders and non-responders to HCV treatment. IL10 gene SNP –592 and basal viral load were used to construct a response-prediction model; while all the remaining variables that were non-significant, did not contribute to prediction of response, and were accordingly excluded from the model.

The two independent variables chosen significantly improved prediction of the response and no other variable can improve model fit.

The prediction model indicated that, a patient whose IL10 gene SNPs -592 is AA is 5.2 times more prone not to respond to HCV therapy. The model also showed that, one unit increase in log basal viral load of HCV (e.g., from 10 to 100 or from 100 to 1000) is associated with an increase in the odds of non-response to HCV therapy by a factor of 3.7; controlling for other variables.

In agreement with the present study, a multivariate analysis of HCV-4 patients observed that baseline viral load, fibrosis (OR: 0.124, 95% CI: 0.030-0.505), were significantly associated with SVR [29].

However, another multivariate analysis concluded that the strongest predictor for the final response was rapid virological response (OR: 26.00; 95% CI: 7.148-94.545, \( p=0.0001 \)) [29].

Further studies are still needed on a larger pediatric population with more SNPs to be studied to detect predictors of response to HCV therapy to avoid expensive therapy that has various adverse effects.

**Conclusion:**

In conclusion, IL10 gene SNPs –592 and basal viral load were used to construct a response-prediction model. These two independent variables significantly improved prediction of the response.

Also HCV RNA-PCR and GGT pretreatment levels were strong predictors of response to interferon therapy in HCV affected children. However there is no significant association between SNPs in the IL-10 gene (1082G/A and 592C/A) as regards response to HCV therapy in children.

---

**References**

13. BROOKS D.G., TRIFIOLO M.J., EDELMANN K.H., TETTON L. and MCGAVERN D.B.: Interleukin-10 de-


الملخص العربي

المقدمة: الالتهاب الكبدى الورمي لفيروس سي يعتبر من أحد أهم أسباب أمراض الكبد المزمنة في العالم. أكثر من 80% من حالات الالتهاب الكبدى الحاد تتحول إلى حالات مزمنة، وتشمل هذه الحالات المزمنة حالات النسيجية للأورام الكبدية أو سرطان الكبد.

ان تعرض الأطفال للاصابة بالالتهاب الكبدى لفيروس سي له أسباب كثيرة، ومنها العدوى عن طريق الام أثناء الولادة، أو عن طريق نقل الدم أو مشتقاتها.

علاج الالتهاب الكبدى الناجم عن فيروس سي يتم بواسطة عقار الانتزرولين ألفا بالإضافة إلى أقراص الريبارفيرون عند الأطفال كما هو في البالغين.

وقد أصبح العلاج بالانتزرولين بالإضافة إلى أقراص الريبارفيرون هو العلاج الأساسي لهذا المرض على الرغم من تكلفة الباولو وآثاره الجانبية العديدة. وقد وجد أنه حوالي 50-60% فقط من الحالات تستجيب للعلاج، لذلك فإن الأطباء من وجود طريقة لمعرفة توقع الاستجابة للعلاج من عدمه قبل بدء العلاج لتقليل التكالفة والأثر الجانبية.

الهدف من الدراسة: هذه الدراسة صممت لمعرفة دور التعدد الجيني لجينات الانتزرولين في الاستجابة أو عدم الاستجابة لعلاج الالتهاب الكبدى - فيروس سي - المزمن في الأطفال.

النتائج: أثبتت هذه الدراسة نتائج انتقائية:

1- ليس هناك علاقة بين التعدد الجيني لجين الانتزرولين في الاستجابة أو عدم الاستجابة لعلاج الالتهاب الكبدى -فيروس سي- المزمن في الأطفال.

2- العوامل الأخرى المؤثرة في الاستجابة لعلاج فيروس C مثل مستوى البيلي سي أو المناضج، المستوى المرتفع لـ GGT في السيروم، المستوى المنخفض للبروتين C للبروتين المنخفض. وقد أظهرت هذه النتائج جميعها أدنى نسبية.

3- بالنسبة للتحليل الانتحائي المتعدد المتغيرات، فقد أظهرت هذه الدراسة أن النقطة 595- لجين الانتزرولين 10 وكذلك مستوى البيلي سي أظهرت تأثيراتها واضحة في تجنب مدى الاستجابة للعلاج.

الاستنتاج: اختلاف الجينات الانتزرولين في النقطة 595-10 في الفيروس سي لعلاج هذه الفيروس يترتب عليه الاقتراح الكيميائي لعلاج هذا الفيروس.