Association of Equiliberative Nucleoside Transporter (ENT) with liver fibrosis stage in Hepatitis-C

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Abstract

Objective: To evaluate the pattern of equiliberative nucleoside transporters in viral hepatitis responding and non-responding patients, to correlate the liver fibrosis stage with the pattern among the non-responders, and to correlate the equiliberative nucleoside transporter status with housekeeping hypoxanthine-guanine phosphoribosyltransferase gene.

Method: The comparative cross-sectional study was conducted at the Molecular Biology and Genetics Department, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan, from March to August 2018, and comprised adult hepatitis C virus patients of either gender who completed six months of treatment. They were assessed for response to therapy in terms of the presence of the viral load in their serum by using real time polymerase chain reaction, and divided into responder group A and non-responder group B. The groups were compared and correlation between equiliberative nucleoside transporter expressions and liver fibrosis was evaluated. Data was analysed using SPSS 23.

Results: Of the 80 patients, 33(41.3%) were males and 47(58.8%) were females. The overall mean age was 37.46±10.61 years. In terms of response to treatment, there were 40(50%) in each of the two groups. Mean post-treatment duration was 15.38±30.09
weeks. Age was not significantly different with respect to gender (p>0.05), but the age pattern was significantly different between the two groups (p<0.001). Also, non-responders had significant post-treatment duration compared to the responders (p<0.001). Hypoxanthine-guanine phosphoribosyltransferase gene showed no significant difference between the groups (p=0.144). Equiliberative nucleoside transporter was significantly down-regulated in the non-responders (p<0.001) and showed correlation with the degree of liver fibrosis (p<0.034) compared to the responders.

**Conclusion:** There was a significant association between equiliberative nucleoside transporters and liver fibrosis stage in hepatitis C virus non-responding patients.

**Key Words:** Hepatitis C, ENT, HPRT gene, Liver fibrosis, Responders, Non-responders.

**Introduction**

Hepatitis C is one of the important blood-borne pathogens. According to the World Health Organisation (WHO), approximately 3-4 million new cases are detected every year globally and about 120 to 130 million people are infected with hepatitis C virus (HCV); about 3% of the global population (1). HCV is composed of 9.6-kb single-strand ribonucleic acid (RNA) genome with ‘5’ untranslated region (UTR) that acts as the interior site of entry of ribosomes. Various genotypes have been identified based on their characteristic geographic distribution and clinical course, including 1, 2, 3, 4, 5, 6 and 7, where 1, 2, 3 and 4 are most common genotypes.

Liver fibrosis on the other hand is the main complication related to chronic viral hepatitis which results in cirrhosis and later raises the risk of HCC. Equiliberative nucleoside transporters (ENTs) are classified into 4 human isoforms (hENT1-4), each of them consists of transmembrane domains (TMDs) and large loops that are hydrophilic at their N-terminus (in hENT3 and 4) or in between TMD5 and TMD7 (in hENT1 to 4) (2-7). Low level of ENT expression is reported to be correlated with advanced adverse conditions, including liver fibrosis and HCC etc.(5-8).
The studies cited above demonstrated the correlation of ENT1 expressions with variable response to ribavirin-based treatment regime for HCV. Regardless of the type of therapy, the main concern is the development of liver fibrosis among the non-responders that has never been explored in correlation with the pattern of ENT. The current study was planned to evaluate the pattern of ENT in viral hepatitis responding and non-responding patients, to correlate the liver fibrosis stage (LFS) with ENT pattern among the non-responders, and to correlate ENT status with housekeeping hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene.

Patients and Methods

The comparative cross-sectional study was conducted at the Molecular Biology and Genetics Department, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan, from March to August 2018. After approval from the institutional ethics review committee, the sample was raised using consecutive sampling technique and comprised adult HCV patients of either gender who had completed six months of standard treatment. Patients with decompensated liver disease and those aged outside the 20-60-year range were excluded. After taking written informed consent from the subjects, they were assessed for response to therapy in terms of presence or absence of the viral load in their serum by using real time-polymerase chain reaction (RT-PCR). Those found negative were placed in responder group A, and those found positive were placed in non-responder group B.

Demographic and clinical data was noted using a proforma.

For RNA extraction from buffy coat, 0.5ml blood sample of each patient was transferred into 2ml tube containing 1.2ml lysis solution, and the plasma layer containing RNA was carefully aspirated after centrifuging. According to kit protocol (HCV SC-RGQ kit batch number 17200 Qiagen), lysis solution 800µl, 50µL sodium acetate and 500µl acid:phenol:chloroform were added to the mixture and stored for 5min at room temperature and centrifuged for 01min. Next, 600µl of 100% ethanol was mixed with the aqueous phase volume in a new 2ml tube. Wash solutions was
added along with centrifuging and filtering of the samples at each step. Finally, elution solution 100µl was mixed (used in two steps) and spined. The eluted RNA was stored at -20ºC for short-term storage.

Complementary deoxyribonucleic acid (cDNA) synthesis and amplification was done using reverse transcriptase-PCR and real time PCR (RT-PCR) respectively. Total RNA was used as a template that served as a measure of total ENT messenger RNA (mRNA) expression with the cDNA synthesis kit protocol (Affemertix 75780) at 37ºC, 95ºC and 04ºC for 50, 5 and 5 minutes, respectively.

ENT and HPRT primers were used for cDNA amplification by RT-PCR Standard curve method was used for ENT quantification. The cDNAs of extracted samples were used along with negative control for RT-PCR for amplification and the expression levels were normalised by HPRT as the housekeeping gene. The amplification of products was done through RT-PCR using HPRT/ENT primers and noted on the basis of fluorescent signals and standard curve (Figures 1-2).

Total volume 25µl was made including veriquest sybr green quantitative PCR (qPCR) master mix 12.5µl, forward primer 1.25µl, reverse primer 1.25µl, cDNA (1:5 dilutions) 10µl.

The expression of ENT was taken on the basis of cycle threshold (ct) values of the product samples which were directly plotted and mean ct value was noted as a measure of ENT and HPRT expressions. The desired bands were seen on 2% agarose gel by gel electrophoresis.

The interpretation of ct values was taken as: <29 = strong positive reactions suggesting abundant amount of the target nucleic acid in the sample, 30-37 = positive reactions suggesting moderate amounts of the target nucleic acid, and 38-40 = weak reactions suggesting minimal amounts of the target nucleic acid.

For gel electrophoresis of PCR product HPRT/ENT, 50ml of 1X Tris/Borate/Ethylenediaminetetraacetic (TBE) buffer and 01g of agarose powder were added and microwaved for 5-7 minutes. Then 4µl of ethidium bromide was added to the solution and poured in the gel casting tray. After placing the comb < 0.5-1.0mm, the solution
was solidified at room temperature for 30min. The comb was then gently removed. The PCR products were analysed separately on 2% agarose gel. Mixture of 2µl of the amplified product and 3µl of bromophenol blue were loaded. Voltage was set at 100v, and 100bp ladder was used. Then finally the gel was put in the Bio-Rad gel documentation system and visualised under ultraviolet light.

A number of primer dilutions were used to optimise PCR. Initially, primer conditions for HPRT were tested. A number of primer concentrations were tested like neat, 1:10 and 1:16, and, by using neat primer, results of HPRT for real time PCR were optimised. For synthesising cDNA by RT-PCR, total RNA was used as a template that served as a measure of desired mRNA expression. The stored cDNAs were first thawed and spun shortly for the settling of the contents. The cDNAs of the samples were amplified using the ENT/HPRT primers on RT PCR (Figures 3-4).

Aixplorer ultrasound system multiwave version 8.2.0 (supersonic imagine s.a, aix-en-provence, France) was used to perform elastography of the non-responders. The stiffness of liver tissues was displayed in both quantitative as well as qualitative modes. In the qualitative mode, the tissues were displayed in blue to red colour in ascending order of stiffness. In the quantitative mode, the stiffness of tissues in the region of interest (ROI) was calculated as mean, median and standard deviation. Kilo Pascal (kpa) unit was used for displaying the results and mean kpa was considered as the final score as shown in Figure 05(A-D). The interpretation was done as: F0-F1 = 5.3-7.0 (Mean 6.4) Kpa; F>2 = 7.1-8.5 (Mean 7.5) Kpa; F>3 = 9.5-11.6 (Mean 10.1) Kpa; and F=4 = 13.1-18.8 (Mean 14.4) Kpa.

Data was analysed using SPSS 23. Chi square test was used for qualitative variables, and Mann Witney’s U test was used for quantitative variables. Correlation between ENT expressions and liver fibrosis was assessed using correlation test. For all analyses, two-sided statistical significance was set at p<0.05. Linear regression model was used to assess the relationship of ct-ENT value with response to treatment.
Results
Of the 80 patients, there were 40 (50%) in each of the two groups with mean post-treatment duration of 15.38 ± 30.09 weeks. Overall, there were 33 (41.3%) males with a mean age of 39.39 ± 11.5 years, and 47 (58.8%) females with a mean age of 36.10 ± 9.77 years (p = 0.137). The mean age of the sample was 37.46 ± 10.61 years.

Among the non-responders, 14 (35%) were males with mean age 45.57 ± 8.84 years, and 26 (65%) were females with mean age 39.38 ± 9.38 years. There were 23 (57.5%) aged 41-60 years, followed by 10 (25%) aged 31-40 years, and 07 (17.5%) aged 20-30 years. There was significant difference in terms of age pattern of the groups (p < 0.001) (Table 1).

Significant difference was found in the mean ct-ENT value of the responders 30.94 ± 4.2 and the non-responders 34.86 ± 5.31 (p < 0.001) (Table 1).

Among the non-responders, 18 (45%) came to laboratory for HCV detection in 5-12 weeks, 10 (25%) in 0-4, 8 (20%) in >24 weeks and 4 (10%) in 13-24 weeks. Of them, 20 (50%) had ct value 30-37, 14 (35%) had ct value >37 and 06 (15%) had it <29. Significant difference was seen among the non-responders in relation to post-treatment duration (p < 0.001).

There was a significant association of ENT expression with the degree of liver fibrosis among the non-responders (p < 0.034).

Among the non-responders, 15 (37.5%) patients had LF S01 with mean kpa 6.2 ± 0.404, 12 (30%) LF S02 with mean kpa 7.28 ± 0.54), 9 (22.5%) were LF S03 with mean kpa 11.01 ± 1.07, and 4 (10%) were LF S04 with mean kpa 14.07 ± 1.05. No significant association was found between gender and LFS (p = 0.246) (Table 2).

The variability of ENT expression between the groups compared to the housekeeping HPRT gene was significant (p < 0.001), while no significant difference was seen in HPRT expressions of the two groups (p = 0.144).

Discussion
The study showed significant correlation of ENT with low levels among the non-
responders and high LFS among HCV non-responders. Liver cirrhosis and HCC are two main complications of chronic HCV if left untreated, or patients become nonresponsive to therapy. During liver fibrosis, the components of the extracellular matrix (ECM) are considered to be released into circulation, and some ECM-associated molecules, such as hyaluronic acid and type IV collagen, have been used as biomarkers to estimate the degree of liver fibrosis (9). Among these biomarkers, the aspartate transaminase (AST) to-alanine transaminase (ALT) ratio (AAR) is regarded as a well-known classical biomarker which increases with the advancement of liver fibrosis.

Since the introduction of direct-acting antiviral agents (DAA), the management of viral hepatitis has been revolutionised and cure rate of patients with HCV infection climbed to >90% even with advanced liver disease. Ribavirin is widely used in combination for the treatment of decompensated liver disease(10, 11). Given such excellent cure rate, the remaining 10% at potential risk of no response make a considerable number of patients. These observations may suggest that in <10%, the principal reason for development of complications is HCV-induced variability in response to therapy.

The current study compared the expressions of ENT in both responders and non-responders, and found significant difference in expressions of ENT in the groups, while the housekeeping HPRT genes were normal, showing that in non-responders there is HCV-mediated under-expressions of ENT compared to the responders. These findings are in line with another study(8)

The successful viral eradication is associated with a decline in liver stiffness measurement (LSM). According to a recent meta-analysis of 24 studies, 6-12 months after achieving sustained virologic response after 12 weeks (SVR12), patients experience a median LSM decrease of 28%, and approximately 47% of patients with baseline values compatible with advanced liver fibrosis or cirrhosis show post-treatment values compatible with lower degrees of fibrosis(12). Patients with mild fibrosis can safely be discharged after the achievement of SVR, but those with advanced fibrosis and cirrhosis remain at risk of developing complications of liver disease, thus requiring regular and life-long surveillance. Major complications
of cirrhosis that need to be monitored is HCC(1). Two major pathways exhibited high
dysregulation in early fibrosis compared to the controls or when compared with late
fibrosis. These were the transforming growth factor beta (TGFβ)-related pathway genes
and Matrix-deposition associated genes. These were considerably over-expressed at
transcriptional levels as early as F0 (13).

The current study found under-expression of ENT gene in treatment non-responders,
and identified that the serum concentrations of ENT significantly correlated with hepatic
fibrosis assessed non-invasively by elastography, demonstrating the decreased serum
concentration of ENTs as determinant of liver stiffness in HCV-infected non responders
that was independent of other potentially profibrogenic factors.

These findings are supported by various fibrosing conditions showing under-expression
of ENT, including ENT1 in epithelial to mesenchymal transition with resultant fibrosis
in kidneys(14); ENT3 in H syndrome, a form of sclerodermoid condition associated
with sclerosis of skin(15); under-expressions of ENT in idiopathic lung fibrosis(16);
atherosclerosis(17); and purinergic signalling to renal fibrosis in experimental diabetic
nephropathy (18).

Besides ENT, certain other markers, like platelets derived growth factor (PLGF),
hepatocyte growth factor (HGF) and growth differentiation factor (GDF15) might play
role in the early detection of liver fibrosis complication. This underlines the potential of
these markers to improve other non-invasive fibrosis tests based on either ultrasound or
serum marker(19). Certain studies also discovered other serum markers, including
serum concentrations of semaphorins (SEMA3A, SEMA3C, SEMA5A, SEMA6B and
SEMA6D) as well as microRNA (miRNA)-16 and miRNA-146a that were significantly
up-regulated in early and late stages of HCV-induced liver fibrosis in patients with
chronic hepatitis C (CHC) compared to the controls (10, 20).

The current study also found a significant difference in the distribution of ENT among
age groups of the non-responders, indicating that old-age patients were at high risk of
resistance to therapy, but no significant gender difference was seen. A study
demonstrated low ENT1 expressions as poor predictor to response to gemcitabine in
pancreatic carcinoma(21). The current study found poor response to ribavirin in treatment non-responders to be associated with under-expression of ENT. Moreover, up-regulation of ENT1 was seen in the brain of knockout mice with Huntington disease with resultant low adenosinergic transmission in the striatum as adenosine, which is metabolic product of adenosine triphosphate (ATP), is mainly removed by ENT1 transporters(22). Experimental studies found that targeting microRNA (MiR-26b) mimics played a role in the treatment of rats with cirrhotic portal hypertension by targeting hENT1 to inhibit the Rho-associated protein kinase (RhoA/ROCK-1) signalling pathway(20). The current study demonstrated that measurements of serum markers ENTs in patients with chronic liver diseases add further information to the results obtained non-invasively by elastography presenting with advanced fibrosis and/or liver stiffness values that correspond to cirrhosis.

The current study identified that down-regulation of ENT gene was associated with the development of HCV-related complications in non-responders. As such, early detection of this down-regulation in HCV-infected individuals will help as a non-invasive tool for the identification of these life-threatening complications.

As the sample size was small and new advancements in therapies against HCV have reduced the rates of development of non-responsiveness, such studies should be performed on a large scale.

**Conclusion**

An under-expression of ENT and significant correlation with LFS were identified in treatment non-responders.

**Disclaimer:** The text is based on a Ph.D thesis.

**Conflict of Interest:** None.

**Source of Funding:** Liaquat University of Medical and Health Sciences (LUMHS), Jamshoro.
References


Table 1: Equiliberative nucleoside transporter (ENT) status in viral hepatitis patients.

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
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<tbody>
<tr>
<td><strong>Mean Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>34.84 ± 11.43</td>
<td>45.57 ± 8.84</td>
</tr>
<tr>
<td>Female</td>
<td>32.04 ± 8.84</td>
<td>39.38 ± 9.38</td>
</tr>
<tr>
<td><strong>P=0.351</strong></td>
<td></td>
<td><strong>P=0.137</strong></td>
</tr>
<tr>
<td><strong>ENT value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30.94 ± 4.27</td>
<td>34.87 ± 5.67</td>
</tr>
<tr>
<td>Female</td>
<td>31.26 ± 3.92</td>
<td>35.6 ± 5.04</td>
</tr>
<tr>
<td>**Male=30.58 ± 4.71</td>
<td>33.47±5.67</td>
<td><strong>P &lt;0.001</strong></td>
</tr>
<tr>
<td><strong>Female=31.26 ± 3.92</strong></td>
<td><strong>Female=35.6 ± 5.04</strong></td>
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Table 2: Equiliberative nucleoside transporter (ENT) correlation with liver fibrosis stage (LFS).

<table>
<thead>
<tr>
<th>Non responders</th>
<th>Liver fibrosis stage</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male= 14(35%)</td>
<td>LFS 01 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Female=26(65%)</td>
<td>LFS02 (30%)</td>
<td>0.034</td>
</tr>
<tr>
<td>Total=40</td>
<td>LFS03 (22.5%)</td>
<td></td>
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<td></td>
<td>LFS04 (10%)</td>
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Figure 1: HPRT standard curve. Real-time polymerase chain reaction (PCR) standard curve representing 100% PCR efficiency.

Figure 2: Hypoxanthine-guanine phosphoribosyltransferase (HPRT) amplification plot showing relative fluorescence versus cycle number.
Figure 3: Equiliberative nucleoside transporter (ENT) standard curve on real-time polymerase chain reaction (PCR) representing 100% PCR efficiency.

\[ y = -0.229x + 8.64, \text{ r-squared } = 0.883 \]

Figure 4: Equiliberative nucleoside transporter (ENT) amplification plot showing relative fluorescence versus cycle number.
Figure 5A: Shearwave elastography of liver showing fibrosis stage 1 in non-responders.

Figure 5B: Shearwave elastography of liver showing fibrosis stage 2 in non-responders.
Figure 5C: Shearwave elastography of liver showing fibrosis stage 3 in non-responders.

Figure 5D: Shearwave elastography of liver showing fibrosis stage 4 in non-responders.