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# Ribavirin Transporter [Ent1] Polymorphism is a Pretreatment Predictor of Virologic Response: The Specific Role of Donor Liver Transporter

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## Abstract

The genetic polymorphism of Equilibrative Nucleoside Transporter 1 [ENT1] is involved in ribavirin cellular uptake and it could positively enhance antiviral treatment response. The liver transplant setting offers the unique opportunity to selectively observe the effect(s) of the donor liver ENT1 gene on HCV treatment outcome. We aimed at studying donor polymorphism of ENT1 and HCV therapy outcome in transplanted patients. The role of ribavirin plasma concentration was evaluated. 39 patients after HCV recurrence were included. Genotyping of donor ENT1 and of IL-28B was performed in donor liver samples by RNA PCR. Allelic frequencies of liver ENT1 were: AA 43.6%; AG 28.2%; GG 28.2%. GG genotype was associated with rapid [RR=8; 95% CI 1.6-38; p=0.01] and sustained virological response [RR=9.5; 95% CI 1.6-53; p=0.01]. In multivariate analysis, GG genotype and a ribavirin plasma concentration >2.0 ng/mL at week 12 were independently associated with sustained virological response. In conclusion, the genetic polymorphism of ENT influences treatment response and a pre-treatment determination of its activity could help to predict treatment response in HCV patients.

**Keywords:** HCV; Ribavirin; ENT1; Liver transplant

**Abbreviations:** HCV: Hepatitis C Virus; ENT-1: Equilibrative Nucleoside Trasporter-1; RVR: Rapid Virological Response; EVR: Early Virological Response; SVR: Sustained Virological Response; IFN: Interferon; CNT: Concentrative Nucleoside Transporters; LT: Liver Transplant

## Introduction

Hepatitis C virus [HCV] infection is the most common chronic liver disease and may lead to cirrhosis and end-stage liver failure [1]. Ribavirin represents a crucial component in the combined antiviral treatment for hepatitis C; in fact it lowers the relapse rate and the breakthrough episodes when compared with peg-IFN monotherapy [2]. Even the recent introduction of the protease inhibitors has not decreased the relevance of ribavirin since a lower SVR was reported when ribavirin was not administered or employed at low dose [3-5]. The antiviral activity of ribavirin occurs within the hepatocytes, thus an appropriate ribavirin uptake by these cells is considered an essential step for anti-viral ribavirin action[s] to occur [6,7].

The hepatocyte inflow of ribavirin is regulated by two subtypes of specific membrane nucleoside transporters. The major transporter is represented by the Equilibrative Nucleoside Transporters [ENT, with its known variants 1-4], encoded by SLC29 genes, which mediate facilitated bidirectional diffusion of nucleosides [7,8]. The other transporter family is represented by Concentrative Nucleoside Transporters [CNT, with its known variants 1-3], encoded by SLC28 genes, which can transport nucleosides against a concentration gradient [9].

ENT1 has been recently identified as the primary responsible for ribavirin cellular import [8,10]. In fact, the specific inhibition of ENT1 by mercaptopurine probes led to a significant reduction in ribavirin concentration within erythrocytes isolated from humans [11].

ENT1 is an integral membrane glycoprotein with 456 amino acid residues, located both in plasma and mitochondrial membranes [12].

The gene encoding the protein stays in the short arm of chromosome 6, precisely in the 6p21.1 band. Since ENT1 single nucleotide polymorphism [SNP] may influence the efficiency of ribavirin transport [13,14], the effects of ENT1 SNP on antiviral treatment outcome have been recently evaluated.

In a retrospective study conducted in 109 HIV-HCV co-infected patients, an association between the ENT1 SNP rs760370 and rapid virological response [RVR] to antiviral treatment for HCV was reported [15]. A similar result was also obtained in a study including 526 East Asian patients mono infected with HCV genotype 1b, in whom the ENT1 SNP rs6932345 was found to be an independent predictor of RVR and of treatment outcome [16].

Liver disease due to HCV is one of the main current indications for liver transplantation [LT] [17]. The recurrence of HCV after liver transplant is almost universal and has a negative impact on patient and graft survival [18]. HCV eradication was shown to improve survival; however antiviral treatment with the interferon-based therapy is still unsatisfactory being effective only in about one third of patients after LT [19].

Aim of the study was to analyze the effect of donor hepatic ENT1 polymorphism on treatment response after HCV recurrence in liver

transplant patients. The role of ribavirin concentration in the early phase of therapy (12 weeks) was also examined.

## Materials and Methods

### Study population

Sixty-five liver transplant patients consecutively treated for HCV recurrence between June 2010 and February 2012 were considered for inclusion in the study. Shared criteria for antiviral treatment were: detectable serum HCV RNA; alanine amino-transferase [ALT] × 1.5 folds above the normal values; and histological evidence of hepatitis C recurrence with a fibrosis score between 1 and 5 according to the Ishak scoring system [20]. Exclusion criteria are described in Supplementary material. Treatment was based on peg-IFN-alpha-2b at standard dose [1.5 μg/kg/week] or Peg-IFN-alpha-2a [180 μg/week] plus weight-adjusted ribavirin [11 mg/kg/day] for 48 weeks. Growth factors [granulocyte colony-stimulating factor, Filgrastim; Amgen Dompè Spa, Milan, Italy; and erythropoietin, Eprex; Janssen-Cilag, Titusville, NJ, USA] were used when needed to avoid dose reduction of both drugs.

Informed consents were obtained from subjects (recipients) who participated in this analysis. No donor living donors were included.

### Virological analysis

HCV RNA testing was done by the quantitative real-time PCR assay HCV-Ampliprep TaqMan [sensitivity threshold of 15 IU/mL; Roche Diagnostics, Mannheim, Germany].

Serum HCV-RNA level was measured at baseline, and at weeks 4, 12, 24, 36 and 48 of therapy, as well as at week 24 following the completion of treatment.

Rapid Virological Response and Sustained Virological Response [SVR] were defined as the achievement of undetectable serum HCV-RNA level at week 4 and week 24 after treatment, respectively. The Early Virological Respose intended as the reduction of >2 log of HCV-RNA after 12 week of therapy was not considered a stopping rule for treatment.

### DNA isolation and genotyping

Genomic DNA was isolated from donor liver tissue using the X-tractor Gene system [Corbett life Science, Australia] at the time of transplantation, before reperfusion.

The DNA polymorphisms for rs760370 for ENT1 gene [SLC29A1] and rs12979860 for Il28 beta gene, were studied.

Reference sequences for ENT1 genes and Il28B were obtained from NCBI GenBank database (http://www.ncbi.nlm.nih.gov/ ).

Genotyping of ENT1 and Il28 beta SNPs was performed by Pyrosequencing technology, using the Pyrosequencer PyroMark ID system [Qiagen]. Both the amplification and the sequencing primers of each SNP were obtained by the PSQ Assay Design software [Biotage AB and Biosystems, Uppsala, Sweden].

PCR primer pairs, sequencing primers for each SNP and PCR technique are reported in Supplementary materials 1 and 2. Pyrosequensing was performed by methods previously described by our center [21].

### Ribavirin plasma concentration

Ribavirin plasma trough concentrations were measured at week 4, 12, 24, 36 and 48 of therapy. Blood samples were collected early in the morning before the intake of the first daily dose of ribavirin, which was, on average, 12 h after the last dose. Ribavirin blood concentration was quantified by methods previously described by Loustaud-Ratti et al. [22] and the HPLC mass spectrometry technique are described in Supplementary materials 3.

### Statistical analysis

Results are presented as median and ranges. Dichotomous variables were examined by Fisher's exact test, whereas continuous variables were examined by Wilcoxon and Mann-Whitney U tests for independent and paired samples, as appropriate. A ribavirin concentration greater than 2 ng/mL was considered adequate for treatment, according to what has been previously reported in the literature [15,23,24].

Univariate and multivariate logistic regression analyses were performed to assess factors independently associated with the achievement of virological response. A P value <0.05 was considered significant. Statistical analysis was done with SPSS [SPSS 19, SPSS Inc., Chicago, IL]. Graphs were done with GraphPad [GraphPad software, La Jolla, CA, USA].

## Results

Thirty-nine LT patients undergoing antiviral treatment for hepatitis C recurrence were included in the study. Main baseline characteristics are shown in Table 1. 26 patients were excluded because liver biopsy was not performed preventing the ENT-1 polymorphism on-tissue determination. The main characteristics of the 39 treated patients are reported in Table 1.

Median time from LT to antiviral treatment was 11.8 (range: 6-25) months. Median baseline HCV RNA levels were 6.3 $\log_{10}$ (range: 4.5-7.6). Liver biopsy showed mild to severe inflammatory activity (mean score 7 ± 2) and moderate fibrosis (1 patient stage 4; 11 patients stage 3; 17 patients stage 2, 10 patients stage 1). An episode of acute cellular rejection was recorded in the past history of 3 patients (7.7%). Renal function and the glomerular filtration rate were within normal limits in all patients.

### ENT1 genotype

The distribution of the different genotypes according to SNPs at the ENT1 [SLC29A1] gene was as follows: rs760370 AA 43.6% (17/39 pts), AG 28.2% (11/39 pts), and GG 28.2% (11/39 pts). Patients' characteristics according to their donor ENT1 genotype are shown in Table 2. No differences in the main predictors of response to the treatment were found among ENT1 genotypes.

**Table 1:** Characteristics of patients at baseline.

| Variable | Value |
| --- | --- |
| Age*, years (range) | 62 (45-67) |
| Male sex, % | 88 |
| MELD* at LT (range) | 17 (12-22) |
| Donor age*, years (range) | 55 (19-68) |
| Diabetes, % | 33.3 |
| Tacrolimus/Cyclosporine, % | 68/32 |
| Fibrosis*, Ishak stage before treatment (range) | 2 (1-4) |
| HCV genotype 1, % | 84 |
| genotype 4, % | 5 |
| genotype 2/3, % | 10 |
| IL28B of donor liver | - |
| TT% | 15 |
| TC% | 32.8 |
| CC% | 52.2 |
| Basal HCV-RNA load* $\log_{10}$ (range) | 6.3 (4.5-7.6) |
| Basal ALT* (U/L) (range) | 101 (52-331) |
| *Values are expressed as median and ranges | |

**Table 2**: Characteristics of patients according to ENT 1 polymorphism (rs 760370).

| Variable | AA (17 patients) | AG (11 patients) | GG (11 patients) | p Value |
|---|---|---|---|---|
| Recipient age*, years | 52 | 62 | 53 | 0.3 |
| Range | (45-64) | (49-63) | (45-64) | |
| Donor age*, years | 47 | 46 | 42 | 0.1 |
| Range | (16-68) | (14-70) | (18-64) | |
| Male sex, % | 92 | 82 | 90 | 0.4 |
| Fibrosis at baseline, Ishak stage | 2 | 2 | 2 | 0.8 |
| Range | (1-3) | (1-4) | (1-4) | |
| HCV genotype 1, % | 94 | 91 | 82 | 0.9 |
| HCV RNA load at baseline*, $\log_{10}$ | 6.45 | 6.2 | 6.5 | 0.9 |
| Range | (5.5-7.6) | (5.6-7.3) | (5.2-7.1) | |
| ALT at baseline* (U/L) | 107 | 109 | 159 | 0.1 |
| Range | (51-163) | (95-287) | (70-248) | |
| IL28B of donor liver | - | - | - | - |
| TT% | 45 | 64 | 50 | 0.2 |
| TC/CC% | 55 | 36 | 50 | |
| *Values are expressed as median and ranges | | | | |

## Virological response and ENT1 polymorphism

Table 3 shows the association between predictors of response to the treatment and SVR by univariate analysis. Only ribavirin plasma concentration at week 12 and ENT1 genotype had a significant correlation to SVR. In detail, 15 out of the 39 patients attained RVR (38.5%) and 18/39 patients (46.2%) obtained a SVR. According to rs760370 genotype, RVR and SVR were achieved in 64% (7/11 pts.) and 73% (8/11 pts.) of the patients carrying the GG genotype, respectively, whereas they occurred in only 33% (13/39 pts.) and 36% (14/39 pts.) of the non GG patients, without any significant difference between AA or AG carriers. Among patients carrying both the HCV genotype 1/4 and the ENT-1 genotype AA or AG (35 pts.) the chance of SVR was even worse resulting in 30% of total. Thus, GG genotype was significantly associated with RVR [RR=8; 95%; CI 1.6-38; p=0.01] and SVR [RR=9.5; 95% CI 1.6-53; p=0.01].

## Virological response and ribavirin concentration

The median ribavirin trough concentration at week 12 was 2.2 (0.8-3.7) mg/mL. Patients who achieved SVR reached higher levels of plasma ribavirin in a shorter time compared to non-responders (Figure 1). In particular, ribavirin plasma levels at week 12 were 2.8 ng/mL among responders vs. 1.6 ng/mL among non-responders (p=0.05; Figure 1). The ribavirin dose taken was about 11 mg/kg/day and 13% of patients (n. 5/39) were on erythropoietin treatment. The prevalence of severe anemia was independent with ENT1 SNPs (18% vs. 11%, GG vs. non GG genotypes respectively; p=0.3). At univariate analysis ribavirin dose reduction was not a predictor of SVR (p=0.08), probably due to the low number of patients involved.

Interestingly, there were no significant differences in median ribavirin mean concentrations at any observed time point during treatment in patients carrying GG genotypes of liver ENT-1 compared to non-GG liver genotypes. In particular, at week 12 the values were 2.03 (1.0-2.2) and 2.3 (0.95-2.8) ng/mL, respectively; p=0.888 (Figure 2).

## Multivariate analysis

By multivariate analysis (Table 4) only 2 factors resulted independently associated with the achievement of SVR: rs760370 GG genotype [OR, 7.8; 95% CI, 1.0-62; p=0.05] and plasma ribavirin trough concentrations ≥ 2 ng/mL at week 12 [OR, 7.4; 95% CI 1.2-45; p=0.03]. IL28B maintained only a positive trend for the achievement of SVR although it was not statistically significant (p=0.09).

## Treatment related anemia

The pretreatment baseline Hb level was 13.1 g/dL (range= 10-14.8 g/dL) in women and 14.5 g/dL (range=10.6-17.6 g/dL) in men (P<0.01). Twenty-three patients (59%) developed anemia. Severe anemia occurred in 18 patients who required correction with erythropoietin; of these, 11 achieved a SVR, three patients relapsed and five patients were non-responders. In the univariate analysis the development of anemia (Hb level <10 g/dL) was not significantly associated with SVR (p=0.09). The occurrence of anemia was not different between patients carrying donor liver GG and AA/AG genotypes.

## Discussion

It is well know that in LT patients, HCV clearance increases graft and patient survival by improving histology and decreasing the risk of cirrhosis or its complication [25]. In the setting of LT, the SVR is achieved by approximately one-third of patients treated with interferon-based therapy [19,26,27]. Sofosbuvir and RBV is the first IFN-free combination that has been assessed in hepatitis C recurrence after LT [28]. Even with the new therapy for HCV hepatitis based on the direct antiviral agents, ribavirin has still a place to reduce viral relapse, especially in genotype 2 patients [28-30]. Interestingly, the present study suggests that, in the liver transplant setting, genetic polymorphism of the ribavirin liver transporter ENT1 is capable of influencing the outcome of antiviral therapy after HCV recurrence. Reasonably, this effect can be mediated by the influence that ENT1 genotype can exert on the inflow of ribavirin inside the hepatocytes [11,14]. Regarding pharmacokinetics and pharmacodynamics characteristics, host genetic features may have relevant implications in the ability to modulate the response to drugs and may be of great help in defining tailored treatments [15,16,31,32]. Recently, a growing importance has been attributed to ribavirin, the plasma level of which seems to be a significant predictor of treatment outcome [33]. Since ribavirin is hydrophilic and its activity takes place within the cells [34], it needs to be actively transported into the cells and ENT1 has been demonstrated to be the most efficient transporter of ribavirin into hepatoma cell lines [11] and cultured hepatocytes [13]. Thus, it is conceivable that the polymorphism of ENT1 could eventually affect antiviral treatment outcome by modulating the efficiency of ribavirin transport [11,14-16].

Transplanted patients are a very attractive population because liver ENT1 polymorphism is derived from the donor, while all other tissues

express the recipient ENT1 polymorphism. Assuming that ENT1 is an important predictor of response to therapy, the transplanted liver is the best setting in which to evaluate this hypothesis without confounding factors related to the metabolism of ribavirin in other tissues. Donor liver samples were taken at the time of transplantation before blood reperfusion to avoid any DNA contamination from the recipient. Contrary to what have been already reported in literature, [23] we found that ribavirin steady state of plasma concentration was reached later than the fourth week of treatment, this was probably due to the schedule of ribavirin dose we used which considered a progressive increasing of the given daily dose during the first 2-3 weeks of treatment. In our study we confirm that LT patients who achieved SVR

**Table 3:** Predictors of SVR: results from univariate analysis.

| Variable | SVR % | O.R. (C.I) | p value |
|---|---|---|---|
| ENT1 (GG vs. AA/AG) | 73 vs. 36 | 4.8 (1.6-9.5) | 0.01 |
| Ribavirin plasma concentration (≥2 vs. <2 ng/mL) at week 12 | 57 vs. 20 | 5.1 (1.0-25) | 0.04 |
| Donor IL28B (CC vs. TT/TC) | 50 vs. 33 | 3.7 (0.6-20) | 0.09 |
| Genotype 1 vs. non 1 | 41 vs. 69 | 0.4 (0.29-4.7) | 0.4 |
| Acute organ rejection (yes vs. no) | 50 vs. 62 | 0.4 (0.05-8) | 0.3 |
| Diabetes (yes vs. no) | 40 vs. 50 | 0.7 (0.07-3.6) | 0.35 |
| Immunosuppression (tacrolimus vs. cyclosporine) | 50 vs. 42 | 0.7 (0.2-5) | 0.75 |
| Anaemia (<10 g/dL) (yes vs. no) | 59 vs. 35 | 1.7 (0.2-5) | 0.09 |
| HCV-RNA viral load at baseline ($\log_{10}$) | **SVR vs. Non SVR** | - | |
| | 6.47 vs. 6.33 | | 0.1 |

**Table 4:** Independent predictors of SVR: results from multivariate analysis.

| Variable | O.R. (C.I.) | p value |
|---|---|---|
| ENT1 GG genotype | 7.8 (1-12) | 0.05 |
| Ribavirin plasma concentration ≥2 ng/mL at week 12 | 7.4 (1.2-15) | 0.03 |
| Donor IL28B CC | 1.8 (0.03-2.4) | 0.09 |
| Genotype 1 | 0.2 (0.2-70) | 0.8 |
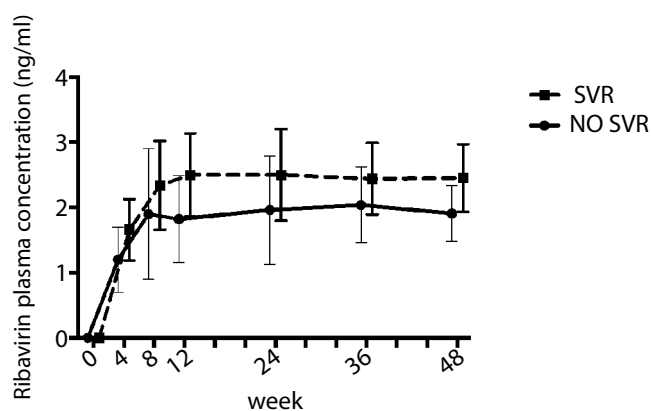


**Figure 1:** Ribavirin plasma levels; difference between responder (18 patients) and non-responder (21 patients) to anti-HCV standard therapy. Error bars represents the range of Ribavirin concentration in plasma at each control.
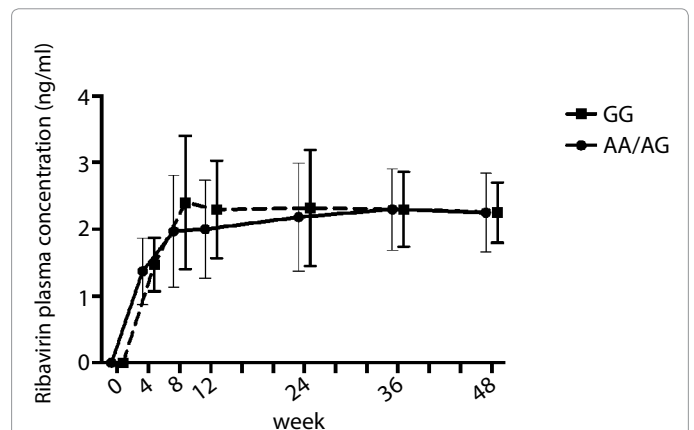


**Figure 2:** Ribavirin plasma concentration, difference between ENT1 polymorphism: GG (17 patients) vs. AA/AG (22 patients).

had a significantly higher ribavirin concentration at week 12 compared to non-responders [2.6 vs. 1.8 ng/mL; p<0.05] and that a ribavirin level higher than 2 ng/mL at week 12 is an independent predictor of SVR (Table 4).

Patients whose donor liver had GG ENT1 genotype had a 8-fold increase of SVR likelihood compared to non-GG patients (Table 4). It is not surprising that both high ribavirin levels and ENT1 genotype are independent predictors of SVR since ENT1 is an equilibrative transporter, which might modulate ribavirin distribution into cells. It has been already reported that liver ribavirin uptake in the early phase of ribavirin administration was 30 times higher in ENT1 wild compared to knockout mice [14]. And, the high serum levels of ribavirin reached during the early-phase of antiviral treatment are crucial to obtain a favorable virological response [24,33,35-37]. It may be hypothesized that the plasma ribavirin levels and intracellular concentration of the drug are in strict correlation. Indeed, ribavirin plasma levels during the first 12 weeks maintained a significant role in predicting treatment efficacy [38]. Thus, we hypothesize that the association of high ribavirin serum concentrations and favorable liver ENT1 genotype may have a synergic effect and lead to better intracellular bioavailability of the drug. The consequence of our observation may be that in patients with unfavorable ENT1 polymorphism, a higher ribavirin given dose could help to increase intra hepatocyte drug's concentration.

Our study had some limitations due to the patient's number which was not large enough to allow the analysis of the impact of other traditional baseline predictors of response such as IL28B genotype, viral load, HCV genotype and fibrosis.

In conclusion, the present study shows an association between GG genotype of liver ENT1 and increased SVR rate. This result contributes to the understanding of host genetic variants involved in ribavirin bioavailability and may help in individualizing ribavirin dosing for treatment of HCV recurrence after transplant.

### Conflict of Interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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