Negative Correlation Between Hepatitis C Virus (HCV) and Let-7 MicroRNA Family in Transplanted Livers: The Role of rs868 Single-Nucleotide Polymorphism

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Background: Genetic alterations of TGF-β pathway members, including its transmembrane receptor, TGFBR1, may influence the course of HCV infection. Rs868 is a single-nucleotide polymorphism of the 3'UTR region of TGFBR1, located in a binding site for the conserved let-7/miR98 microRNA family. Previously, we demonstrated a favorable course of hepatitis C recurrence after liver transplantation in rs868 AG genotype of the transplanted liver when compared to rs868 AA. The aim of the present study was to confirm the biological effect of rs868.

Material/Methods: HepG2 cell line was transfected with luciferase vectors cloned with 3’UTR of TGFBR1 gene encompassing different rs868 alleles. Post-transplant liver biopsies from 61 patients with HCV-related end-stage liver disease were evaluated histopathologically and analyzed for the expression of TGFBR1 mRNA, let-7/miR98 microRNAs, HCV RNA load, and rs868 genotype.

Results: Luciferase expression was significantly lower in the A allele-containing vector. TGFBR1 mRNA and HCV RNA load were correlated negatively with let-7/miR98 microRNAs and this correlation was significantly stronger for rs868 AG compared to AA genotype. A strong positive correlation was demonstrated between TGFBR1 and HCV in both genotypes. In AG heterozygotes, let-7/miR98 microRNAs showed a strong negative correlation with peribiliary or periporal interface hepatitis (Ishak A score).

Conclusions: There is a negative correlation between let-7/miR98 microRNAs and HCV viral load and TGFBR1 mRNA after liver transplantation. In the rs868 AG heterozygotes, this correlation was stronger and there was a negative correlation between let-7/miR98 and Ishak A score, which is in concordance with the previously demonstrated protective role of this genotype in post-transplant hepatitis C recurrence.

MeSH Keywords: Hepacivirus • Liver Transplantation • MicroRNAs

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Hepatitis C virus (HCV)-associated end-stage liver disease is currently one of the leading indications for liver transplantation (LT) [1,2]. WHO estimates that 3% of the world population is infected with HCV [3]. Approximately 75–85% of infected patients develop chronic hepatitis and 10–20% of them progress to cirrhosis within 20 years [4]. According to the European Liver Transplant Registry, 13% of all LT procedures in the period from 1988 to 2009 were performed in patients in whom HCV was the sole etiology of liver cirrhosis [2]. As well as being an established risk factor for hepatocellular carcinoma (HCC), HCV infection is also a cofactor in the progression of other liver diseases such as those related to hepatitis B infection or alcohol abuse, all of them being among the most frequent indications for LT [5]. Graft failure secondary to recurrent HCV is the most frequent cause of death, graft failure, and need for retransplantation in HCV-infected recipients [6]. All patients who undergo LT with detectable serum HCV RNA experience graft re-infection, with the HCV viral load reaching pretransplant levels in a significant portion of patients as early as on the 4th day after transplantation [7]. The high rate of hepatitis C recurrence and the accelerated course of HCV infection in LT are partially due to immunosuppressive regimens, which enable escape of the virus from specific anti-HCV immune surveillance [8–10].

Several host factors were identified that affect HCV entry, replication, assembly, and release. Among them, microRNA molecules are emerging as playing important roles [11]. MicroRNAs (miRNAs) are small non-coding RNAs, about 22-nucleotides in length, that regulate gene expression [12]. They exert their regulatory function after base pairing with messenger RNA using standard Watson-Crick rules [13]. The best characterized docking sites for miRNAs are located in 3’ untranslated regions (3’UTRs) of mRNAs [14]. Thus, genetic diversity in 3’UTR region may affect the miRNA–mRNA interaction, leading to genome-dependent changes in gene expression. Single-nucleotide polymorphisms (SNPs) located in 3’UTR have been described in numerous studies to alter this interaction, with a significant clinical effect [15–17]. In our previously published study, we found an association between the severity of hepatitis C recurrence after LT and rs868 SNP genotype of the donor liver, with the AG genotype carriers presenting a significantly more favorable outcome over the AA genotype carriers [18]. Rs868 is located in the 3’UTR region of TGF-β receptor type 1 gene (TGFBR1). The in silico analysis of TGFBR1 3’UTR sequences has revealed that rs868 SNP maps within a conserved binding site for miR98/Let-7 microRNA family and TGFBR1 mRNA. We also examined the clinical effect of these microRNAs with respect to the rs868 genotype of the transplanted liver.

**Material and Methods**

**Patients and sample collection**

The study was approved by the Ethics Committee of the Medical University of Warsaw and was conducted according to strict institutional guidelines in accordance with 1975 Declaration of Helsinki. All patients gave informed consent.

The study group consisted of 61 chronic hepatitis C patients who underwent LT and had a liver biopsy performed between January 2013 and May 2014 at the Department of General Surgery and Transplantology, Transplantation Institute, Medical University of Warsaw. Only biopsies in which HCV was the main cause of primary liver disease were included. Patients with acute rejection, confirmed post-transplant alcohol abuse, diffuse steatosis in liver biopsy, and postsurgical biliary tract complications were rejected. Possible graft arterial and venous thrombosis and cholestasis in the study group were excluded by ultrasound examination and angio-CT or cholangio-MRI.

The biopsy samples were stored immediately after the procedure in RNA later (Ambion, Austin, TX) according to the manufacturer’s protocol.

**Laboratory tests**

Evaluations of the level of AST, ALT, bilirubin, ALP, albumin, prothrombin time, and other blood parameters were routinely performed in a certified laboratory at the Warsaw Medical University Hospital.

**Figure 1.** Predicted target region for miR98/Let-7 family of miRNA at TGFBR1 3’ UTR (position 59–82) containing rs868 SNP (figure previously published by authors in reference 16).

The biopsy samples were stored immediately after the procedure in RNA later (Ambion, Austin, TX) according to the manufacturer’s protocol.
Histopathological evaluations

HCV-related graft necroinflammatory changes were scored semiquantitatively and are expressed as histological activity index (HAI) according to Ishak et al. [26]. All histological evaluations were routinely made by experienced pathologist unaware of the study design.

DNA, RNA, and microRNA extraction of the biopsy samples

DNA, RNA, and microRNA were extracted from stored donor liver biopsies using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

For mRNA quantification, RNA samples were reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI) according to the directions provided by the manufacturer.

Expression of \textit{TGFBR1} gene was evaluated using specific TaqMan® gene expression assay (Applied Biosystems, Foster City, CA) according to instructions provided by the manufacturer. Reactions (25 μl) were run in triplicates on an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA) using TaqMan® Universal Master Mix, specific primer set, and MGB probe, and 50 ng of cDNA. To compensate for differences in total mRNA amount, the samples were normalized using the human GAPDH Endogenous Control FAM/MGB probe.

For miRNA quantification, RNA samples were reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and the expression of each of the let7 family miRNAs was evaluated using the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA) in the manner described above. The \textit{RNU6B} and \textit{RNU43} levels were used as internal controls. The relative expression levels were calculated using the 2^{-ΔΔCT} method.

Cloning of 3’UTR of \textit{TGFBR1} Gene

The 3’UTR of \textit{TGFBR1} gene encompassing rs868 SNP was cloned from the genomic DNA of a GG homozygote for rs868 SNP. In brief, a 744 bp fragment of \textit{TGFBR1} 3’ UTR region between 49174 and 49917 (NG_007461.1) was amplified by PCR using hot start Taq polymerase (Promega, Madison, WI) and sense 5’GAAACGTGATTTACAGTTGCGCTGAAC-3’ and antisense 5’TAAATAAGCTTTTGAATTCAACAC-3’ primers containing \textit{Hind III} restriction sites. Amplification products were cloned into plet vector ( Fermentas Inc., Glen Burnie, MD). Minipreps from positive clones were \textit{Hind III} digested, the released inserts were isolated from agarose gel, purified and subcloned into \textit{Hind III} site of the pMIR-REPORT vector (Ambion, Austin, TX). Resulting colonies were screened by PCR to select clones with correctly oriented inserts. The selected colonies with the correctly oriented inserts were sequenced to confirm the G genotype in the rs868 locus and to rule out any other polymorphisms and mutations.

To acquire a plasmid with the A allele at the rs868 locus, the G allele-carrying vector was used as a template for mutagenesis. G to A mutagenesis was carried out using the QuickChange II Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The outcome of mutagenesis was confirmed by sequencing. Plasmids carrying G and A variant of the rs868 SNP were designated pMIR-G and pMIR-A, respectively. An empty pMIR-REPORT vector was designated as pMIR-poly.

Luciferase reporter gene assay

HepG2 cells were transfected in triplicates with pMIR-poly, pMIR-G, and pMIR-A plasmids using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. Each transfection was performed with 0.8 µg of the respective pMIR construct and 0.05 µg of the pRL-TK vector encoding Renilla luciferase (Promega, Madison, WI), which was used to normalize the assay. The cells were harvested on the following day and assayed using reagents of Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and the FLUOstar Omega luminometer (BMG Labtech, Offenburg, Germany). Background luminescence of the lysate from untransfected HepG2 cells was subtracted from the luminescence of samples. The results are expressed as a ratio between luminescence driven by pMIR and pRL-TK.

HCV RNA load quantification in liver biopsies

HCV RNA load was evaluated using the RNA isolated from liver biopsy samples by RT-PCR, as described previously [27]. The results were normalized to GADPH mRNA level.

Statistical analysis

Variables were tested for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. Nonparametric variables were analyzed by Mann-Whitney U test. Correlation values were
calculated using Spearman’s rank correlation coefficient. All statistical analyses were carried out with Statistica 10.0 software (StatSoft Inc., Tulsa, OK). The differences between the tested variables were considered significant at \( p < 0.05 \).

### Results

#### Laboratory tests and correlation to rs868 genotype

Basic demographics and clinical data of the evaluated patients are shown in Table 1. Of the 61 patients included in the study group, 36 had the AA genotype and 25 had AG genotype for rs868 SNP. There were 3 and 5 protocol biopsies in the AA and AG groups, respectively. The rest of the biopsies were performed due to clinical indications, based on the “first event” strategy [28]. No statistically significant differences were observed between the 2 groups.

### Assessment of functional significance of rs868 SNP

For the evaluation of the functional significance of rs868 SNP, we obtained luciferase vectors containing a fragment of TGFBR1 3′UTR DNA with each of the G and A alleles and evaluated their effect on luciferase expression in reporter gene assay in HepG2 cells. As shown in Figure 2, the A allele-containing plasmid (pMIR-A) conferred a strong (about 5-fold) inhibitory effect on reporter gene expression when compared to control (pMIR-poly) and G allele containing (pMIR-G) plasmids (\( p = 0.005 \) and \( p = 0.0002 \), respectively).

### TGFBR1 mRNA and let-7 family microRNA expression levels

There was no significant difference in the level of TGFBR1 mRNA normalized to GAPDH between the 2 genotypes. However, rs868 genotype strongly influenced the correlation between TGFBR1 mRNA level and the levels of different members of the let-7 miRNA family. A negative correlation was observed in both genotypes; it reached statistical significance and was more prominent in the AG genotype (Table 2).
The correlation between let-7 microRNAs, TGFBR1 mRNA, and HCV RNA load in liver biopsies

No statistically significant difference was found between the HCV RNA load normalized to GAPDH in liver biopsies between the 2 genotypes. However, a strong negative correlation was observed between let-7 miRNAs and HCV levels only in the AG group and it reached statistical significance for let-7d, e, f, and miR98. A strongly positive and statistical significant correlation was detected between TGFBR1 and HCV RNA levels in both groups (Table 3).

The correlation between let-7 family members and clinical markers

The level of let-7 miRNA molecules expression, TGFBR1 mRNA, and HCV viral load was correlated with clinical data. A negative correlation was observed between the let-7 miRNA expression levels and Ishak A, B, and C scores in the AG genotype carriers, and it reached statistical significance for let-7a, b, c, d, and f normalized to RNU6B in the Ishak A score. This correlation was mainly positive in the AA genotype carriers and reached statistical significance for let-7e normalized to RNU43 in the Ishak A score and for let-7i normalized to RNU43 in Ishak C score.

Discussion

Because miRNA molecules are an important part of cell regulatory systems, there is much potential for interplay between host cell miRNAs and viruses [29,30]. Hepatocytes are the major site of HCV replication [31]. Thus, HCV and miRNA interaction involves, most importantly, direct targeting of the HCV genome by liver-abundant miRNAs, with miR-122 being a prominent example [31,32]. MiR-122 is a liver-specific miRNA that binds directly to 5' UTR of the virus genome in 2 adjacent sites in association with Ago2 [32]. It forms an oligomeric complex in which 1 miR-122 molecule binds to the 5' UTR of HCV RNA with 3' overhanging nucleotides, masking the
S' terminal sequences from nucleolytic degradation, thereby promoting viral RNA stability and propagation of the HCV genome [32,33]. On the other hand, miRNA molecules binding directly to the HCV genome can also exert an antiviral activity. miR-199a* binds to the 5'UTR region of HCV genome and has been shown to inhibit HCV replication in an immortalized hepatocyte model [34]. Other examples of miRNAs that interact directly with HCV RNA and suppress viral replication are let-7b, miR-196, and miR-448 [35].

Alternatively, HCV virus and miRNA interaction can be based on the alteration of host signaling pathways by HCV molecules and miRNAs. For instance, HCV infection leads to overexpression of miR-130a, which in turn facilitates HCV replication by inhibiting the interferon (IFN)-induced transmembrane protein IFITM1 [36].

In the present study, the expression levels of let-7/miR-98 microRNA family members, TGFBR1 mRNA, and HCV RNA load were analyzed in liver biopsy samples taken from a set of patients in whom the sole etiology of the native liver end-stage disease was HCV infection. The influence of rs868 SNP genotype of the donor liver on these expression levels and the clinical data were assessed. Rs868 is located in the 3'UTR region of TGFBR1 mRNA. In silico analysis has demonstrated that it is located in the complementary binding site of let-7/miR-98 miRNAs, thus suggesting a biological mechanism for this effect. This mechanism was proved using the luciferase model. The clinical effect of polymorphisms located in the let-7 complementary binding site has been previously demonstrated for an SNP located in the 3'UTR region of the KRAS gene in nonsmall cell lung cancer [37]. In our previous study, we showed that HCV-infected liver transplant recipients carrying the rs868 AG genotype have a more favorable outcome in comparison to the AA genotype carriers in terms of severity of hepatitis C recurrence [18]. Recently, the clinical effect of rs868 has been also demonstrated in hereditary mismatch repair-proficient colorectal cancer (MSS HNPCC), where the A allele was a risk-associated genotype, proving the potential of this SNP for modification of the course of diseases [38].

In the present study, a negative correlation between HCV RNA load and let-7/miR-98 microRNA expression levels was observed. This correlation was influenced by rs868 genotype of the donor liver. It reached statistical significance and was more prominent in the AG genotype in comparison to AA genotype.

It has been shown previously that let-7b microRNA targets the HCV genome, leading to a decrease in HCV RNA accumulation and viral production [39]. However, although it interacts directly with HCV RNA, this effect is independent from regulation of HCV translation, as it targets the coding region of HCV NS5B protein. In the same study, let-7b and IFNα-2a was reported to express a synergistic anti-HCV activity. The exact mechanism of this effect remains unknown [39]. The findings of the present study suggest that the TGF-β signaling pathway may play a role in this effect. Interestingly, the same miRNA molecule has been shown to repress TGFBR1 protein expression in a mouse model of kidney fibrosis. Knockdown of let-7b elevated TGFBR1 expression and mimicked some of the profibrotic effects of TGF-β1 [40]. In our study, we demonstrated a strong negative correlation between let-7/miR-98 miRNA family members and TGFBR1 mRNA (Table 2). Again, it was more prominent and reached statistical significance in the AG genotype carriers for rs868, the same genotype that was advantageous in our previous study in terms of hepatitis C recurrence severity [18].

The level of TGFBR1 protein expression was not analyzed and the level of TGFBR1 mRNA expression did not show any significant difference between the 2 genotypes. However, in an ideal setting, this comparison should have been performed between AA genotype carriers and GG genotype carriers. In our study group, there were no individuals with GG genotype and the comparison was performed between AA homozygote and AG heterozygote patients, which reduces the differences in mRNA levels caused by the A allele compared to G allele. Similar difficulties in the interpretation of the influence rs868 on TGFBR1 mRNA levels has been reported previously [38]. On the other hand, the interaction between let-7 miRNAs and TGFBR1 mRNA may exert its biological effect by inhibiting translation, not leading to the degradation of mRNA molecules, thus not affecting the mRNA expression levels [41]. Further studies at the protein level are necessary to elucidate this point.

The interplay between HCV and TGF-β signaling is a well-known phenomenon. While normal hepatocytes secrete only small amounts of TGF-β1, studies suggest that HCV core proteins and subgenomic replicons can directly induce TGF-β1 gene expression in hepatocytes, leading to a significant increase in TGF-β1 expression in both serum and liver [42–44]. On the other hand, the positive effect of TGF-β1 on HCV proliferation has also been demonstrated [44]. The latter is independent of the TGF-β1 effect on immunity [45]. Thus, a positive correlation between the TGF-β signaling pathway and HCV viral load can be expected. This is consistent with the results of our study, showing a strong, statistically significant correlation between TGFBR1 mRNA expression and HCV viral load (Table 3). As mentioned above, let-7 miRNA family members showed a negative correlation with TGFBR1 mRNA level and HCV viral load (Tables 2, 3). The significance of this correlation was altered by the TGFBR1 3'UTR region SNP rs868, being more prominent in the AG genotype carriers (Tables 2, 3). Thus, the previously described negative effect of let-7b miRNA on HCV RNA accumulation, also shown in our study for other let-7 miRNA molecules, may be mediated through TGF-β1 signaling.
rs868 was also influential at the clinical level. The comparison of the pathological data revealed an apparent and significant negative correlation between let-7 miRNA expression and inflammation score (Ishak A) in the AG genotype carriers for rs868, consistent with the previously reported better clinical outcome of this genotype in comparison to AA genotype (Table 4) [18]. Ishak A score is a semiquantitative measure of periportal or periseptal interface hepatitis (piecemeal necrosis)[26]. Interestingly, in a study performed on a group of 72 hepatitis B anti-Hbe positive patients by Shafaei et al., interface hepatitis was the only parameter of the Ishak grading score that correlated significantly with the serum viral load [46].

Conclusions

For the first time, we showed a negative correlation between let-7/miR98 miRNA family and HCV RNA and TGFBR1 mRNA in post-transplant livers. This effect is regulated by rs868 SNP located in the 3'UTR region of TGFBR1 mRNA and directly alters the miRNA/mRNA interaction. The G allele carriers exhibited a higher level of negative correlation between let-7/miR98 miRNA family members and each of the TGFBR1 mRNA levels, HCV RNA load, and Ishak A score. This is in concordance with the previously suggested protective role of rs868 G allele in the recurrence of hepatitis C after liver transplantation. Thus, the rs868 allele genotyping can be used as a marker for predicting HCV recurrence severity. Additionally, the proven negative correlation between HCV RNA and let7/miR98 miRNAs in post-transplant livers can be a basis for the development of miRNA-based therapies. The rs868 genotype could serve as a marker of better response for such therapies.

Table 4. Correlation between Ishak grading score and let-7 miRNA molecule expression normalized to RNU43 and RNU6B. The correlation was calculated using the Spearman's rank correlation coefficient (p values <0.05 are indicated in bold: * p<0.05, ** p<0.01).

| Ishak Score | A   | AG  | AA   | AG  | AA   | AG  | AA   | AG  |
|-------------|-----|-----|------|-----|------|-----|------|-----|
| Genotype    |     |     |      |     |      |     |      |     |
| let7a/43    | 0.1090 | 0.0857 | −0.0768 | 0.1004 | 0.3293 | −0.2003 | −0.4003 | −0.0409 |
| let7b/43    | 0.1040 | 0.0115 | −0.1801 | 0.0167 | 0.2283 | −0.0187 | −0.2785 | 0.0409  |
| let7c/43    | 0.0946 | 0.0362 | −0.0320 | −0.0753 | 0.1721 | −0.3193 | −0.2617 | 0.0819  |
| let7d/43    | 0.2759 | 0.1285 | 0.1195 | −0.1004 | 0.3895 | −0.1659 | −0.1253 | 0.3279  |
| let7e/43    | 0.4476* | 0.3725 | 0.1819 | −0.2259 | 0.3989 | −0.0500 | −0.0651 | 0.2869  |
| let7f/43    | 0.2211 | 0.1071 | 0.2662 | 0.2008 | 0.3553 | −0.2755 | −0.0890 | −0.0409 |
| let7g/43    | 0.2485 | 0.3230 | 0.1921 | 0.0836 | 0.3255 | −0.1878 | −0.0245 | 0.4919  |
| let7i/43    | 0.3028 | 0.4483 | 0.1360 | −0.0920 | 0.4347* | 0.1847 | −0.0651 | 0.4919  |
| miR98/43    | 0.1447 | 0.2439 | 0.2177 | −0.0291 | 0.3197 | −0.2630 | −0.0295 | −0.0409 |
| let7a/6B    | 0.1444 | −0.5456* | −0.1445 | −0.2008 | 0.0832 | −0.3350 | −0.1044 | −0.4099 |
| let7b/6B    | 0.2039 | −0.6198** | −0.0775 | −0.0920 | 0.1316 | −0.1784 | 0.0000  | −0.2869 |
| let7c/6B    | 0.1172 | −0.6198** | −0.0261 | −0.2594 | 0.0507 | −0.3882 | −0.1136 | −0.2869 |
| let7d/6B    | 0.2985 | −0.5208* | 0.0298 | −0.2677 | 0.2582 | −0.2630 | 0.0301  | −0.1229 |
| let7e/6B    | 0.3642 | −0.2522 | 0.1291 | −0.3012 | 0.3222 | −0.1784 | −0.0989 | −0.0819 |
| let7f/6B    | 0.1683 | −0.5225* | 0.2187 | 0.0251 | 0.1804 | −0.2630 | −0.0626 | −0.3279 |
| let7g/6B    | 0.2227 | −0.3544 | 0.1200 | −0.3179 | 0.0457 | −0.1565 | 0.0319  | 0.0409  |
| let7i/6B    | 0.3012 | −0.3758 | 0.1264 | −0.2594 | 0.2069 | 0.0313  | 0.0571  | 0.0000  |
| miR98/6B    | 0.1815 | −0.3758 | 0.2045 | −0.2594 | 0.0333 | −0.3162 | 0.0536  | −0.4919 |
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