Hepatic and serum levels of miR-122 after chronic HCV induced fibrosis.

Jonel Trebicka#, Evrim Anadol#, Natalia Elfimova+, Ingo Strack+, Michael Roggendörf, Sergei Viazov†, Inga Wedemeyer+, Uta Drebber+, Jürgen Rockström#, Tilman Sauerbruch#, Hans-Peter Dienes+, Margarete Odenthal†

*Department of Pathology, University of Cologne, Germany.
#Department of Internal Medicine I, University of Bonn, Germany.
†Institute of Virology, Essen University Hospital, Essen, Germany.

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Short title: Low levels of miR-122 correlate with severe CHC fibrosis.

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Abbreviations:

- ALT: alanine aminotransferase
- AST: aspartate aminotransferase
- CHC: chronic hepatitis C virus infection
- HCV: hepatitis C virus
- miR: micro ribonucleic acid
- SEM: standard error of the mean

Contact address:

Jonel Trebicka, Department of Internal Medicine I, University of Bonn, Sigmund-Freud Str. 25, D-53105 Bonn, Germany. jonel.trebicka@ukb.uni-bonn.de, Tel: +49 228 287 15507, Fax: +49 228 287 19718
Abstract

Background/Aims: The progression of liver fibrosis in patients with chronic hepatitis C (CHC) is important in deciding on the treatment of the virus. As liver biopsy and liver stiffness measurement for staging of fibrosis present limitations, circulating levels of miR-122 have been suggested as a novel biomarker to predict the extent of liver injury. We evaluated the potential of miR-122 as an indicator of fibrosis progression in CHC infection and performed for the first time a comprehensive analysis of hepatic and circulating miR-122 levels in patients with CHC.

Methods: Patients with well-documented CHC infection were selected from the database of HepNet, the German-Competence-Network on Viral Hepatitis. All patients underwent blood sampling and liver biopsy with grading of inflammation and staging of fibrosis. RNA was extracted from 84 liver biopsies and 167 serum samples of CHC patients. miR-122 levels in liver and serum samples were quantified by Real Time PCR normalized by RNU6 or spiked-in RNA, respectively.

Results: Hepatic levels of miR-122 decreased significantly with the severity of fibrosis (p=0.001). In addition, circulating miR-122 levels correlated negatively with increasing stages of fibrosis, although inverse correlation was moderate due to a two-phase miR-122 pattern during fibrosis progression. Thus, circulating miR-122 levels decreased in patients with severe fibrosis (F3, F4), while in early stages with distinct fibrotic structures (F2) and high inflammatory activity, miR-122 serum levels were elevated.

Conclusions: We conclude that during progression of fibrosis less miR-122 is released into the blood stream due to the loss of liver cells and to the decrease of hepatic miR-122 levels. Although the release of circulating miR-122 possibly mirrors acute liver injury, in chronic liver disease and fibrosis, the loss of liver cells and decreased hepatocellular miR-122 expression render miR-122 an inappropriate marker when exclusively used for interpretation of fibrosis progression.

Word count: 296
Introduction

Hepatic fibrosis, defined as excessive accumulation of extracellular matrix components, develops after chronic liver injury mainly due to chronic viral hepatitis B and C, alcoholism and fatty liver disease [1]. Liver fibrosis may progress to liver cirrhosis, the end-stage liver disease, which has been a globally increasing major health problem with high mortality and morbidity in the past twenty years [2, 3]. The progression of fibrosis to cirrhosis in response to chronic hepatitis C infection (CHC) differs with rapid development in some patients and slower development in others [4]. The identification of “rapid fibrosers” is clinically important as elimination of the hepatitis C virus (HCV) possibly attenuates liver fibrosis thus preventing progression to cirrhosis [5, 6]. Liver biopsy is the gold standard for grading and staging of fibrosis and diagnosis. However, this invasive technique has a mortality rate of 0.1–0.01 % and harbors the risk of severe complications [7-10]. Measurement of liver stiffness using transient elastography can detect severe fibrosis [11, 12], but this technique is hampered by several limitations (e.g. ascites, obesity, cholestasis, hepatic inflammation) [13, 14]. MicroRNAs (miRNA) are 19-24 nucleotides long, non-coding RNA molecules that repress gene expression postranscriptionally [15]. Since extracellular miRNA was also identified in serum, plasma and other body fluids, miRNA has been proposed as biomarker to predict presence and severity of different pathologies [16-18]. Indeed, the circulating miRNA pattern was shown to change after initiation and progression of different cancers [19-22] as well as after various liver diseases [23-29]. Circulating miR-122 was identified in serial serum specimens from mice with liver pathology induced by paracetamol intoxication. Interestingly, miR-122 was observed before the increase of alanine aminotransferase (ALT) values suggesting that miR-122 may be considered a biomarker of liver pathology [23]. miR-122 is the most abundant miRNA in liver cells, which is known to be involved in
cholesterol synthesis [30]. In addition, miR-122 triggers replication of HCV by repression of the heme oxygenase-1 [31] or by interaction to the 5´untranslated region (UTR) of HCV RNA [30, 32-38]. Recent studies have shown that hepatic miR-122 is markedly decreased after liver injuries independent on the etiology [23, 39-41]. However, miR-122 levels in serum samples of patients with chronic liver disease have not been studied extensively to date. Extracellular miR-122 levels were shown to be increased after development of hepatocellular carcinoma [28, 42] and a recent study on 53 HCV-positive serum samples suggested that circulating miR-122 levels might also serve as a biomarker in the evaluation of severity of fibrosis in CHC [43]. However, data of hepatic as well as circulating miRNA levels after CHC in a large, well-documented cohort are still missing. Here, we analyzed hepatic and circulating miR-122 levels in liver biopsies of 84 CHC patients and miR-122 in serum of 167 CHC patients, collected by the German Competence Network on Viral Hepatitis (www.kompetenznetz-hepatitis.de). Interestingly, we found that circulating as well as hepatic miR-122 levels decrease with severity of liver fibrosis in CHC patients.
Patients and Methods.

Patients

Patients with well-documented chronic hepatitis C infection were selected from the database of HepNet, the German Competence Network on Viral Hepatitis (www.kompetenznetz-hepatitis.de). All patients gave their written informed consent prior to blood sampling and liver biopsy. Grading of inflammation (G1-G4), staging of fibrosis (F0–F4), and histological assessment were independently conducted by two experienced pathologists (UD, HPD) according to the method of Desmet [44, 45]. Eighty-four liver biopsies from the central HepNet tissue bank as well as 164 serum samples from the HepNet serum bank were used to conduct the analysis as described below.

RNA isolation from liver biopsies and serum samples

From a total of 84 biopsies RNA was extracted as previously described [46]. In a further set of 167 patients, RNA was isolated from serum samples using the Qiazol reagent following the instructions of the supplier (Qiagen, Hilden, Germany). SV40-miRNA (Qiagen) was added to serum samples (2 pmol/200 µl) prior to the RNA isolation procedure for later normalization of extracellular miR-122 levels, RNA quantity was determined by A$_{260}$-measurement using the ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and quality was assessed by microcapillary electrophoresis (2100 BioAnalyser, Agilent Technologies, Waldbronn, Germany).

miR-122 quantification by real-time PCR

miRNA was analyzed by a two-step real-time PCR using the miScript-Reverse Transcription Kit and the miRNA-SYBR Green PCR Kit (Qiagen, Hilden, Germany).
miR-122, RNU6 and SV-40 primers used for cDNA synthesis and real-time PCR were selected and purchased from the GeneGlobe Search Center (Qiagen). All steps were performed in triplicate and in agreement with the supplier’s guidelines. Cellular miRNA levels were normalized using RNU6 as reference. For normalization of extracellular miR-122 levels, spike-in SV40-miRNA (Qiagen) was used.

**Statistical analysis.**

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed by non-parametric tests using the Wilcoxon test for comparison of paired data, the Kruskal-Wallis test for more than two groups and the Mann-Whitney test for unpaired comparisons. Correlations were analyzed with the Spearman rank correlation coefficient. P-values<0.05 were considered statistically significant. Statistical analyses were performed using SPSS 18.0 for Windows (SPSS Inc. Chicago, IL, USA).
Results

Characteristics of the patients included for hepatic miR-122 analysis

The hepatic levels of miR-122 were analyzed in the cohort of 84 CHC patients. Of this cohort, 58% of the patients were male, 78% were HCV genotype 1 and 19% genotype 3 positive. The clinical characteristics of this cohort were previously reported by Wedemeyer et al. [47] and are shown in Table 1.

The different stages of fibrosis were assessed by histology and were equally distributed among the selected samples (F0=2%, F1=27%, F2=31%, F3=26%, F4=13%). The majority of the biopsies also showed significant inflammation (G0=0%, G1=8%, G2=75%, G3=13% and 4% unspecified samples). In the liver biopsies of these patients, the grade of inflammation correlated positively (Rho=0.44) and significantly (p<0.001) with the stage of fibrosis.

Hepatic miR-122 levels in patients with CHC infection.

Interestingly, in these patients, the hepatic levels of miR-122 decreased significantly with increasing stage of fibrosis (Figure 1A, B). This correlation was highly significant with p<0.001 and Rho=−0.38, while in only two of these patients, the biopsies showed no fibrosis (Table 2). There was also a decrease depending on the grade of inflammation in these patients, though this data were not significant (data not shown).

Circulating miR-122 levels in patients with CHC infection.

The levels of circulating miR-122 were analyzed in a further cohort of CHC patients, who underwent liver biopsy and who were characterized by histology. The severity of fibrosis (F1=27%, F2=37%, F3=25%, F4=11%) in these patients was of similar distribution as the corresponding severity of inflammation (G1=20%, G2=70%, G3=9%, G4=0% and 1% unspecified samples), and it was comparable to the cohort.
of patients that we first analyzed. In the second cohort, 55% of patients were male, 80% were HCV genotype 1 and 13% genotype 3 positive. Available clinical data, grade of inflammation and stage of fibrosis in these patients are summarized in Table 1.

Grade of inflammation correlated positively (Rho=0.54) and significantly (p<0.001) with the stage of fibrosis (Figure 2A). In addition, ALT levels of these patients showed a significant correlation with increasing grades of inflammation (Rho=0.324) and increasing stages of fibrosis (Rho=0.325) (Figure 2B).

Circulating levels of miR-122 revealed a moderate, but significant (p=0.043) inverse correlation (Rho=-0.158) with increasing stages of fibrosis (Figure 2C). No significance of miR-122 serum alterations could be assessed between the different stages of fibrosis (Figure 2D). Interestingly, serum miR-122 levels showed a two-phase pattern during progression of fibrosis. Thus, miR-122 was more present in serum samples of CHC patients with early, but significant fibrosis (F2) than in patients with severe fibrosis stage F3 and F4.

Circulating miR-122 and relationship to fibrosis in patients with CHC infection.

The patients with stage 1 fibrosis had a tendency towards lower levels of circulating miR-122 (p=0.073) compared to the patients with significant fibrosis as defined previously [48-50]. Similarly, patients with stage 1 fibrosis had significantly lower ALT levels compared to patients with significant fibrosis (p=0.002).

In contrast, levels of circulating miR-122 in patients with fibrosis stage 1 and 2 were higher than those in patients with more severe fibrosis (Figure 3A, p=0.088). Also, levels of ALT significantly increased with increasing fibrosis stage (Figure 3B).
Discussion

The current study demonstrates a pronounced loss of miR-122 in the liver after chronic liver disease, which inversely correlates with progression of fibrosis. However, circulating miR-122 levels, recently considered as a biomarker for liver injury in mice and humans [23, 39-41], were slightly increased in early stages of fibrosis but prominently reduced in stages of severe fibrosis (Figure 4).

Chronic HCV infection is associated with liver fibrosis, which may cause the potential to cause liver cirrhosis and/or hepatocellular carcinoma. Since progression of fibrosis varies in patients distinguishing between rapid from slow fibrosis would allow better tailoring of the treatment [4]. To date, this is best achieved by follow-up liver biopsies or repeated measurement of liver stiffness. Both methods have their limitations.

Biopsy – due to its invasiveness – may lead to relevant complications [7-10], while liver stiffness measurement has the drawback of questionable or lacking results in patients with ascites, obesity, cholestasis, or hepatic inflammation [13, 14] and a considerable overlap in patients with low or moderate fibrosis [11, 12]. Serological biomarkers could offer a non-invasive alternative for assessment of fibrosis in chronic liver disease. Yet, their diagnostic accuracy has still limitations. Recently, miR-122 was suggested as a suitable marker in a study investigating approximately 50 patients [43]. However, our present study does not support these results (Figure 2) [43]. This discrepancy might be due to the different characteristics in the patient cohort [43]. In order to minimize patient and environmental factors, we studied circulating miR-122 in serum samples from a well defined and a large cohort of HCV positive patients. miR-122 quantification demonstrated high miR-122 levels in early stage of fibrosis followed by reduced levels in advanced fibrosis stages F3 and F4.

This suggests a changing pattern of circulating miR-122 levels at different stages of fibrosis (Figure 2D). Different cell populations and degree of necroinflammation may...
affect miR-122 release into the blood stream during fibrogenesis. Thus, in the early 
stage of fibrosis, miR-122 is released into the blood stream in response to 
inflammation and injury of hepatocytes confirming previous studies. Thus, Wang et 
al. demonstrated increasing miR-122 levels after acute liver intoxication in mice [23].

However, in stages of advanced fibrosis, less miR-122 is released from liver tissue 
possibly because intrahepatic miR-122 levels are reduced as shown by our analysis 
of liver biopsies. Our findings of a marked repression of liver-specific miR-122 with 
increasing fibrosis (Figure 1) confirms previous studies of Sarasin-Filipowicz et al. 
and in particular of Morita et al., who demonstrated the liver-specific loss of miR-122 
at late stages of fibrosis [41, 51]. The persistent injury perpetuates this process 
characterized by infiltration of the liver by immune cells, death of hepatocytes, 
proliferation of myofibroblasts and accumulation of extracellular matrix. In our hands, 
hepatic miR-122 loss was not significantly correlated with grades of inflammation in 
chronic hepatitis C infection, although significantly correlated with fibrosis in these 
patients. Furthermore, recent data show that deletion of miR122 might be causally 
associated with steatohepatitis, liver fibrosis, and HCC [52, 53]. Thus, with 
progression of fibrosis, hepatic levels of miR-122 decrease because hepatocytes, 
which are the main source of miR-122 are replaced by extracellular matrix. These 
changes of liver-specific miR-122 inevitably affect the extent of release into the blood 
stream as well as serum miR-122 levels (Figure 4). In summary, reduced levels of 
circulating miR-122 are probably caused by their downregulation due to inflammation 
following liver injury on the one hand and hepatocyte loss on the other hand.

We therefore conclude that circulating miR-122 levels possibly mirror the presence of 
hepatocyte injury in patients with HCV infection. However, hepatic and serum levels 
of miR-122 – according to our data – do not appear to be suitable for assessment of 
stage of liver fibrosis and inflammation in CHC patients.
References

Low levels of miR-122 correlate with severe CHC fibrosis.


Legend to figures:
Figure 1: Hepatic miRNA122 expression in CHC patients and relationship to stage of fibrosis.
In liver biopsies of 85 CHC patients, we analyzed the hepatic expression of miR-122 normalized for the endogenous RNU6, which correlated inversely significantly with the stage of fibrosis (Spearman Rho=-0.381, p<0.001) (A). The means ± standard error of the mean of the hepatic levels of miR-122 again decrease significantly with increasing stage of fibrosis in these 85 patients compared by the non-parametric Kruskal-Wallis test (B).

Figure 2: Circulating miRNA122 levels in CHC patients and relationship to stage of fibrosis.
Grade of inflammation correlates significantly with stage of fibrosis in the second cohort of 168 CHC patients, with a Spearman coefficient Rho=0.54. For each stage of fibrosis means ± standard error of the mean of the grade of inflammation are shown and compared with the non-parametric Kruskal-Wallis test revealing significant differences between the columns (A). In these patients, levels of ALT significantly and directly correlated with grade of inflammation (B) with Spearman Rho = 0.325. In this second cohort of CHC patients, circulating levels of miR-122 normalized for SV40 and correlated inversely and significantly with stage of fibrosis (Spearman Rho=-0.158, p=0.043) (C). Means ± standard error of the mean of circulating levels of miR-122 showed no significant changes with increasing stage of fibrosis in these patients (D).

Figure 3: Comparison of circulating miRNA122 levels with ALT levels in CHC patients and relationship to stage of fibrosis.
In (A) are shown the means ± standard error of the mean of the circulating miRNA levels in patients with fibrosis stage F1-F2 compared to patients with higher fibrosis stage. Comparison by the non-parametric Mann-Whitney test showed $p=0.088$ between groups. In (B) are shown the means ± standard error of ALT levels in patients with fibrosis stage F1-F2 compared to patients with higher fibrosis stage. The comparison by the non-parametric Mann-Whitney test revealed $p=0.001$ between groups.

Figure 4: Possible scenario of changes in miRNA-122 during liver injury and fibrosis.

The hepatocyte-borne miR-122 is increased inside the liver, while in the systemic circulation in healthy subjects, it is low. However, in response to hepatic injury, an intrahepatic loss of miR-122 is observed, whereby the circulating levels of miR-122 are massively increased. The persistent injury is characterized by infiltration of the liver by immune cells, progressive loss of hepatocytes, proliferation of myofibroblasts and accumulation of extracellular matrix. Thus, with progression of fibrosis hepatic levels of miR-122 decrease, while the release in the systemic circulation is also decreased.
**Tables:**

**Table 1:** Patient characteristics.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Liver samples</th>
<th>Serum samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 84</td>
<td>n = 168</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>36/48</td>
<td>75 / 93</td>
</tr>
<tr>
<td>Fibrosis score staged 0/1/2/3/4</td>
<td>2/23/26/22/11</td>
<td>0/45/62/42/18</td>
</tr>
<tr>
<td>Inflammation graded 0/1/2/3/4</td>
<td>0/7/64/11/0</td>
<td>0/34/117/15/0</td>
</tr>
<tr>
<td>HCV genotype 1/2/3/4</td>
<td>29/1/7/0</td>
<td>98/5/16/4</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>103 ± 14 (17 – 428)</td>
<td>100 ± 7 (16 - 450)</td>
</tr>
<tr>
<td>(normal range – 45 U/L)</td>
<td>n = 43</td>
<td>n = 141</td>
</tr>
<tr>
<td>Normalized miR-122 levels</td>
<td>1.13 ± 0.08</td>
<td>0.0099 ± 0.0071</td>
</tr>
<tr>
<td></td>
<td>(0.02 – 3.16)</td>
<td>(0.0000 ± 3.0000)</td>
</tr>
</tbody>
</table>

Categorical variables are shown as number of patients

Numerical variables are shown as mean ± SEM (range)

n = number of patients

ALT, alanine aminotransferase

Hepatic miR-122 levels are normalized to RNU6 as internal control

Serum miR-122 levels are normalized to SV40
**Table 2**: Hepatic and serum levels of miR-122 for each fibrosis stage.

<table>
<thead>
<tr>
<th>Fibrosis stage</th>
<th>Hepatic levels mean ± SEM</th>
<th>Serum levels mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 84</td>
<td>n = 164</td>
</tr>
<tr>
<td>F0</td>
<td>1.75 ± 0.29</td>
<td>0.0065 ± 0.0225</td>
</tr>
<tr>
<td></td>
<td>n = 2</td>
<td>n = 0</td>
</tr>
<tr>
<td>F1</td>
<td>1.46 ± 0.15</td>
<td>0.0204 ± 0.1496</td>
</tr>
<tr>
<td></td>
<td>n = 23</td>
<td>n = 61</td>
</tr>
<tr>
<td>F2</td>
<td>1.13 ± 0.13</td>
<td>0.0020 ± 0.0069</td>
</tr>
<tr>
<td></td>
<td>n = 26</td>
<td>n = 41</td>
</tr>
<tr>
<td>F3</td>
<td>0.95 ± 0.17</td>
<td>0.0009 ± 0.0015</td>
</tr>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 41</td>
</tr>
<tr>
<td>F4</td>
<td>0.69 ± 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 11</td>
<td>n = 17</td>
</tr>
</tbody>
</table>

Data are presented as means ± standard error of the mean and compared by non-parametric testing (Wilcoxon test):

- a p < 0.05 vs. F1.
- b p < 0.05 vs. F2.
- c p < 0.05 vs. F3.

n is number of patients

Hepatic miR-122 levels are normalized to RNU6 as internal control

Serum miR-122 levels are normalized to SV40
Figure 1

Hepatic miRNA 122 levels in patients with chronic HCV infection

**Panel A**
- p < 0.001
- Rho = -0.381

**Panel B**
- p < 0.001
Serum miRNA 122 levels in patients with chronic HCV infection

Figure 2

A. Bar chart showing the grade of inflammation in different stages of fibrosis. The p-value is < 0.001.

B. Scatter plot showing the relationship between grade of inflammation and serum levels of ALT. The p-value is < 0.001 and Rho = 0.325.

C. Scatter plot showing the relationship between grade of inflammation and serum levels of miRNA 122. The p-value is 0.043 and Rho = -0.158.

D. Bar chart comparing serum levels of miRNA 122 in different stages of fibrosis. F1 to F4, with p < 0.085 vs F1.
Figure 3

A. Serum levels of miR122/SIV40 [relative units] for patients without and with significant fibrosis. The p-value is 0.088.

B. Serum levels of ALT [UL] for patients without and with significant fibrosis. The p-value is 0.001.
Figure 4

Healthy liver: High hepatic miR-122 levels.

Liver damage: Loss of hepatic miR-122.

Fibrotic liver: Low hepatic miR-122 levels.

Blood stream:

- miR-122 increasing in liver damage.
- miR-122 decreasing in fibrotic liver.

Low miR-122 levels in healthy liver.

High hepatic miR-122 levels in healthy liver.