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Serum miR-122 as a biomarker of **necroinflammation in patients with chronic hepatitis C virus infection**

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ABSTRACT

OBJECTIVES: The liver contains large amounts of microRNA-122 (miR-122), whereas other tissues contain only marginal amounts of this miRNA. MicroRNAs have also been found to circulate in the blood in a cell free form; their potential as readily accessible disease markers is currently evaluated. Here we investigated if the serum levels of miR-122 might be useful as disease parameter in patients with chronic hepatitis C virus (HCV) infection.

METHODS: RNA was extracted from sera of patients with chronic HCV infection and healthy controls and was analyzed for miR-122 content by quantitative real-time reverse-transcription PCR and for standard parameters of liver function. Liver biopsies from the same patients were examined for the histologic activity index (HAI) and the degree of fibrosis.

RESULTS: Sera from patients with chronic HCV infection contained higher levels of miR-122 than sera from healthy controls. Serum miR-122 levels correlated well with markers of liver inflammatory activity, i. e. serum levels of alanine leucine transaminase (ALT) and aspartate transaminase, and the HAI score. In patients with persistently normal ALT levels, serum miR-122 levels did not differ from healthy controls. There was no correlation of serum miR-122 levels with serum albumin, international normalized ratio, liver fibrosis or serum HCV RNA.

CONCLUSIONS: The serum level of miR-122 strongly correlates with serum ALT activity and with necroinflammatory activity in patients with chronic HCV infection and elevated ALT levels, but not with fibrosis stage and functional capacity of the liver.

INTRODUCTION

MicroRNAs (miRNAs) are evolutionarily conserved, small (18–25 ribonucleotides) non-coding RNAs and play important roles in the control of many biological processes, such as cellular development, differentiation, proliferation, apoptosis and metabolism (1). They are implicated in pathogenesis, diagnosis and therapeutic aspects of viral infections, cardiovascular disease, and neurological and muscular disorders (2,3). Recently, an increasing number of reports showed that circulating miRNAs are detectable in serum and plasma in a form sufficiently stable to serve as biomarkers (e. g. [4,5]). The levels of miRNAs in plasma and serum correlate strongly, suggesting that either plasma or serum can be used for investigation of these blood-based biomarkers (6). The pattern of serum/plasma miRNAs have been found to be altered in several diseases, in particular heart disease, sepsis, malignancies and autoimmune diseases (reviewed in [7]). Thus, blood-derived miRNAs have great potential to serve as non-invasive markers for various human diseases. This has not been evaluated in patients with chronic hepatitis C virus (HCV) infection.

miR-122 accounts for approximately 70% of all miRNAs species present in the liver (8), whereas other tissues show only minor expression of this miRNA (9). Therefore, cell-free miR-122 in the blood should be highly specific for liver pathology. miR-122 is known to modulate the expression of genes involved in hepatic lipid and cholesterol metabolism (10,11), HCV replication (12,13), and apoptosis (14). Persistent expression of miR-122 appears to be essential for maintaining the adult liver phenotype (9-11). miR-122 is down-regulated in a proportion of hepatocellular carcinomas (15) and appears to act as tumor suppressor (16). Toxic liver injury has been shown to lead to an

elevation of miR-122 in the plasma of rodents (17,18). A recent study indicates that miR-122 levels are enhanced in plasma from patients with hepatitis B virus infection (19). Here we investigated if the serum levels of this liver-specific miRNA correlate with disease parameters in patients with chronic HCV infection (CHC).

METHODS

Patient Selection

68 therapy-naïve patients, who had undergone liver biopsy for staging and grading of CHC in our hospital as well as 19 healthy control subjects were enrolled in the present prospective study. Their characteristics are summarized in **Table 1**. This investigation represents a sub-study of a multicenter study evaluating ultrasound-based elastography methods and serum markers for liver fibrosis in patients infected with HCV. Inclusion criteria were the consistent detection of HCV-RNA (≥ 6 months) and positive anti-HCV antibodies by third-generation enzyme immunoassay. Exclusion criteria were co-infection with hepatitis B virus or human immunodeficiency virus types 1 and 2, liver disease of other origin than HCV infection, decompensated liver disease, organ transplantation, immune suppression, autoimmune disorders, consumption of more than 20 g/day alcohol, and intravenous drug abuse within the last six months. The study was approved by the local Ethics Committee, and informed consent was obtained from all participants of the study. Histological assessment of liver fibrosis was performed by liver biopsy in all patients and blood samples were obtained on the day of presentation.

Liver histology

Liver biopsy specimens were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin and were reviewed by experienced hepatopathologists. Fibrosis stages (F0=no fibrosis - F6=cirrhosis) and the histologic activity index (HAI) in the liver were evaluated semi-quantitatively according to the Ishak criteria (20). The histologic activity index (HAI) consists of A, periportal or periseptal interface hepatitis (piecemeal necrosis 0-4), B, confluent necrosis (0-6), C, focal lytic necrosis, apoptosis and focal inflammation (0-4) and D, portal inflammation (0-4), resulting in a maximal score of 18.

Blood sampling

Peripheral blood was collected at the time of liver biopsy from each individual directly into serum tubes (Sarstedt, Nümbrecht, Germany). The tubes were centrifuged at 1500 x g for 10 min at 4°C, sera were aliquoted and additionally centrifuged at 2000 x g to completely remove any remaining cells. Sera were stored at -80°C until use.

Detection of miRNAs by quantitative real-time reverse-transcription (RT)-PCR

For isolation of RNA from serum 50 µl homogenate additive (miRVana RNA isolation kit, Ambion-ABI, Austin, TX) and 250 µl lysis buffer (miRVana RNA isolation kit) were added to 500 µl of serum sample and incubated on ice for 10 min. RNA extraction was performed with Tri®ReagentLS (Sigma-Aldrich, St. Louis, MO) and chloroform. Phase separation was achieved by centrifugation at 12000 x g for 15 min. Total RNA was isolated from the aqueous phase according to the *mirVana*TM miRNA isolation kit protocol and stored at -20°C.

5 µl of RNA was reverse transcribed using the TaqMan miRNA reverse transcription kit and the TaqMan miRNA assay specific for miR-122 or miR-16 according to the instructions of the manufacturer (ABI). For real-time PCR, 3 µl of cDNA was mixed with 12 µl master mix (7.5 µl TaqMan Universal PCR MasterMix no UNG (ABI), 3.75 µl H₂O and 0.75 µl TaqMan MicroRNA Assay probe. Real-Time PCR was performed on a StepOne™Plus Real-Time PCR System (ABI). Quantitative real-time PCR was performed in duplicates. The cycle threshold (C_T value) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR.

Statistical analysis

Data were analysed using the BiAS software for windows (version 9.07). Statistical significance for correlations were determined using Spearman's nonparametric rank test. The correlation coefficients *r* were calculated using Spearman correlation. Differences between two groups were evaluated using the Wilcoxon-Mann-Whitney-U test. P values <0.05 were considered to be significant.

RESULTS

The level of miR-122 is increased in sera from patients with CHC

To investigate if the serum level of miR-122 is altered in patients with CHC, we studied the levels of miR-122 in sera from 68 patients with CHC as well as 19 healthy controls. Total RNA was extracted from the sera and miR-122 was quantified by real-time RT-PCR using hairpin primers. The cycle threshold (C_T value), i. e. the number of

cycles required for the fluorescent signal to cross the threshold in qPCR, inversely correlates with the miRNA level. The C_T value of miR-122 in sera from healthy controls significantly differed from that in patients with CHC ($P < 0.05$) (**Figure 1a**). There was no correlation between the age and serum miR-122 levels (**Figure 1b**), indicating that the increased levels of miR-122 in sera from CHC patients and healthy controls can not be attributed to the different mean age of the two groups.

Serum levels of miR-122 and markers of liver injury

To explore the potential suitability of the serum level of miR-122 as disease parameter in patients with CHC, we correlated the level of miR-122 with surrogate parameters routinely used to evaluate CHC (21,22). The C_T value of miR-122 in the sera negatively correlated with serum levels of alanine leucine transaminase (ALT) ($r = -0.642$, 95% confidence interval (CI): $-0.83 - -0.45$, $P < 0.000001$) (**Figure 2a**) and aspartate transaminase (AST) ($r = -0.522$, 95% CI: $-0.73 - -0.31$, $P = 0.000007$) (**Figure 2b**), i. e. the serum level of miR-122 positively correlated with ALT and AST. The correlation between miR-122 and ALT was more pronounced than that between miR-122 and AST. The miR-122 serum levels also correlated with the histologic activity index (HAI) score in the liver ($r = -0.39$, 95% CI: $-0.61 - -0.16$, $P = 0.0011$) (**Figure 2c**). There was no correlation between the serum levels of miR-16, an ubiquitous miRNA which is not specific for the liver, and any of these parameters (**Figure 3a-c**).

Repeated measurements in two healthy subjects over three days revealed reproducible values for miR-122 and ALT (See Supplementary Figure 1a,b online). Observation of miR-122 and ALT levels in sera of a CHC patient who was not under

antiviral therapy showed only minor changes in both parameters over four months (See Supplementary Figure 1c online).

Approximately 20-30% of patients with CHC show persistently normal levels of serum ALT, i. e. the disease activity in these patients cannot be monitored by measuring serum ALT levels (23). In patients with persistently normal ALT levels the serum miR-122 levels were comparable to those in sera from the healthy volunteers (**Figure 1a**). These data indicate that the serum level of miR-122 reflects necroinflammatory activity in the liver in patients with CHC and elevated ALT values, but not in patients with normal ALT levels.

Serum levels of miR-122 and parameters of liver function and liver fibrosis

To investigate if the serum level of miR-122 is related to liver function, we investigated the relationship between the levels of serum miR-122 and the serum albumin concentration, international normalized ratio (INR) and the serum bilirubin concentration. However, although there were positive trends for a positive correlation between the serum miR-122 level and the serum bilirubin concentration ($P < 0.084$) (**Figure 4b**) as well as INR ($P < 0.072$) (**Figure 4c**), none of these correlations reached significance (**Figure 4a-c**), suggesting that the serum miR-122 levels did not reflect overall liver function. We also compared miR-122 serum levels and the stage of liver fibrosis. Again, there was no correlation between the miR-122 serum level and the stage of liver fibrosis ($P = 0.168$) (**Figure 4d**).

Levels of the miR-122 and HCV RNA

Given the link between miR-122 and HCV infection (12,13), we investigated if there is a relation between the serum levels of miR-122 and serum HCV RNA. However, no such relation was found (**Figure 5**).

DISCUSSION

Evidence is accumulating that cell-free miRNAs released from pathologically altered tissues (4,24,25), most notably tumors (4,26) are useful blood-based biomarkers for diseases. Cell-free circulating miRNAs are released by pathologically altered tissues (4,24,25) and tumors (4,26). A recent study indicates that the release of miRNA might be a mechanism of adaptation of cells to an altered environment (27). The identification of altered individual miRNAs or miRNA pattern in serum or plasma offers the prospect of novel readily accessible biomarkers which could be used for the assessment of diseases such as CHC. In contrast to the majority of miRNAs, miR-122 is almost exclusively expressed in the liver, rendering this miRNA particularly interesting to be exploited as biomarker for liver diseases. Thus, it was previously shown that the level of miR-122 in blood plasma increases earlier than that of ALT upon toxic liver injury in rodents (17,18). We show here for the first time that serum levels of miR-122 in patients with CHC are frequently elevated compared to healthy people. Variations of the concentration of miR-122 in serum or plasma should be more specific for liver diseases than ALT and AST, because miR-122 is almost exclusively expressed in the liver, whereas ALT and AST may originate from other tissues, most notably from skeletal muscle (28), reducing their

diagnostic value. Further analyses revealed that the concentration of miR-122 in sera from patients with CHC correlates with the serum activities of ALT and AST as well as with the HAI score in the liver, which represents the gold standard in the assessment of hepatic inflammation and cell damage. In contrast, the serum level of the non-liver-specific miR-16 does not show any differences between CHC patients and healthy volunteers.

CHC patients with persistently normal ALT serum levels show milder disease activity and lower hepatic proliferative activity index (29,30). Analyses of the patients with CHC and persistently normal serum ALT activity for miR-122 serum levels revealed that the miR-122 concentration in these patients were undistinguishable from those in healthy controls. Thus, it appears that the lower disease activity in these patients does not lead to alterations of miR-122 serum levels and that the patho-mechanisms leading to the increase in the concentrations of miR-122 and ALT activity are similar.

An important finding of the present study is that the level of serum miR-122 does not correlate with liver fibrosis, cirrhosis and parameters of liver function. Thus, the miR-122 serum level might be a useful parameter to specifically monitor ongoing damage of hepatocytes irrespective of the stage of chronic hepatitis. In contrast, the intrahepatic miR-122 level negatively correlates with the fibrotic stage and serum ALT and AST activity (31). Thus, alteration of the expression of miR-122 in the liver has no particular impact on the serum miR-122 level. A recent study showed that carbon tetrachloride-induced acute toxic liver injury leads to an elevation of the plasma miR-122 concentration in rats (17,18). An additional report shows a correlation between the plasma miR-122 and necroinflammatory activity in the liver in patients with chronic Hepatitis B virus infection (19). Together, these findings indicate that the serum level of

miR-122 reflects the rate of injury and/or turnover of hepatocytes. Mechanistically, miR-122 may leak from damaged hepatocytes or may be actively exported from altered tissues as a mechanism of adaptation to alterations of the state of a cell.

Hepatic miR-122 is crucial for HCV replication and is a feasible target for drug development (12,13). Infection of cultured Huh-7 cells with HCV J6/JFH reduces miR-122 levels in these cells (31). Thus, considerable evidence suggests that miR-122 plays an important role in HCV life cycle in the liver. However, miR-122 levels in the liver do not correlate with HCV load in the liver (31,32). In the present study, we did not observe any correlation of serum miR-122 and HCV RNA serum levels. These findings are in line with the weak correlation between the serum HCV level and liver histopathology (33) as well as serum ALT and AST levels (23,34). Thus, the relation between HCV and miR-122 in the liver and in serum appears to be complex and requires further clarification.

The routinely used surrogate parameters to evaluate patients with CHC fail to predict the progression of hepatic necroinflammatory activity and fibrosis in a considerable number of patients (23). Thus, there is a need for new serum markers for staging and grading of chronic hepatitis. The present study provides evidence that miR-122 serum levels reflect necroinflammatory activity in the liver of patients with CHC and might thus be a useful biomarker for grading of CHC and potentially other liver diseases. Nevertheless, further studies including larger and different patient collectives are necessary to fully evaluate the diagnostic potential of the serum miR-122 level. The process of examination of serum miRNAs as diagnostic biomarkers is still at its infancy, but considering the rapid progress in this field, development of miRNA-based blood diagnostics is likely to be successful in the near future.

We conclude that the serum level of miR-122 strongly correlates with serum ALT activity and with necroinflammatory activity in patients with chronic HCV infection and elevated ALT levels, but not with fibrosis stage and functional capacity of the liver. Future studies to fully elucidate the diagnostic potential of the serum miR-122 level in larger studies of CHC patients are warranted.

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CONFLICT OF INTEREST

Guarantor of the article: Albrecht Piiper, MD, PhD.

Specific author contributions: Conducted the study, performed the experiments, analyzed the data and performed statistical analyses and produced the figures: Verena Bihrer; Planned and supervised the study, analyzed the results, and wrote the manuscript: Albrecht Piiper; planned and supervised the study, analyzed the results, and contributed to writing of the manuscript: Oliver Waidmann; conducted the study and performed experiments initially: Jörg Haupenthal; recruited the patients for the study: Bernd Kronenberger, Mireen Friedrich-Rust, Nicole Forestier, Ying Shi, Christoph Sarrazin, Stefan Zeuzem, Oliver Waidmann; supervised the study: Bernd Kronenberger, Heinfried Radeke; performed statistical analysis: Eva Herrmann; critical revision of the manuscript: Bernd Kronenberger, Mireen Friedrich-Rust, Jan Peveling-Oberhag, Heinfried Radeke, and Stefan Zeuzem.

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Potential competing interests: None.

STUDY HIGHLIGHTS

What is current knowledge

miR-122 is a highly liver-specific miRNA.

Cell-free miR-122 correlates with inflammation in the liver and serum ALT in toxic liver injury in rats and in patients with chronic hepatitis B virus infection.

The role and diagnostic value of serum miR-122 in patients with chronic hepatitis C virus (HCV) infection are unknown.

What is new here

The serum level of miR-122 strongly correlates with serum ALT activity and with necroinflammatory activity in the liver in patients with chronic HCV infection and elevated ALT levels.

Serum level of miR-122 does not correlate with fibrosis stage and functional capacity of the liver.

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Table 1: Patient characteristics

Patients	n (%)	n (%)	
Sex		HAI	
Men	37 (54.4)	A0	14 (20.6)
Women	31 (45.6)	A1	29 (42.6)
Age		A2	18 (26.5)
Range, years	25-73	A3	7 (10.3)
Mean (SD)	46.8 (11.8)		
Fibrosis stage (Ishak)		B0	57 (83.8)
F0	8 (11.8)	B1	11 (16.2)
F1	21 (30.9)		
F2	4 (5.9)	C1	28 (41.2)
F3	6 (8.8)	C2	29 (42.6)
F4	10 (14.7)	C3	11 (16.2)
F5	4 (5.9)		
F6	8 (11.8)	D0	1 (1.5)
unknown	7 (10.3)	D1	20 (29.4)
ALT		D2	35 (51.5)
elevated ALT	51 (75)	D3	12 (17.6)
normal ALT	17 (25)		

Healthy subjects n (%)	
Men	11 (57.9)
Woman	8 (42.1)
Age range, years	21-61
Age mean (SD)	33.2 (10.6)

LEGENDS TO THE FIGURES

Figure 1. Increased serum miR-122 concentrations in patients with CHC. (a), miR-122 levels in sera of patients with CHC (n = 68), patients with CHC and elevated serum ALT levels (ALT > 35 IU/l) (n = 51), CHC patients with normal serum ALT levels (ALT < 35 IU/l) (n = 17) and healthy control individuals (n = 19). Boxes represent range, median and quartiles of the number of threshold cycles (C_T) required to detect miR-122 with real-time RT-qPCR. Differences were calculated with the Wilcoxon-Mann-Whitney-U-test. Asterisks (*) indicate a significant difference compared with the healthy control group; **P < 0.001, *P < 0.05; +++P < 0.001; n. s., not significant; (b), relationship between the serum miR-122 concentration and age in patients with CHC.

Figure 2. Correlation between serum miR-122 levels and ALT (a), AST (b), HAI score (c) in patients with CHC. Points represent C_T values required to detect miR-122 with real-time RT-qPCR. The boxes represent range, median and quartiles of the C_T values. Panel a: $r = -0.642$; $P < 0.000001$; panel b: $r = -0.522$; $P = 0.000007$; panel c, $r = -0.39$, $P = 0.0011$.

Figure 3. Relationship between serum miR-16 levels and ALT (a), AST (b), HAI score (c). There are no significant correlations (a, b) or differences between the groups (c).

Figure 4. Relationship between serum miR-122 levels and serum albumin concentration (a), INR (b), serum bilirubin concentration (c) and the stage of liver fibrosis (d). The relations are not significant.

Figure 5. Relationship between serum miR-122 levels and the serum HCV RNA in patients with CHC. The relation is not significant.

Figure 1

Figure 2

a

b

c

Figure 3

Figure 4

Figure 5

Supplementary Figure 1

Supplementary Figure 1. Time course of the serum levels of miR-122 and ALT in two healthy subjects (**a** and **b**) and one patient with CHC (**c**).