miR-223 represents a biomarker in acute and chronic liver injury

Florian Schueller1,*, Sanchari Roy1,*, Sven Heiko Loosen1, Jan Alder1, Christiane Koppe1, Anne Theres Schneider1, Franziska Wandrer2, Heike Bantel2, Mihael Vucur1, Qing-Sheng Mi3, Christian Trautwein1, Tom Luedde1 and Christoph Roderburg1

1Department of Medicine III, University Hospital RWTH Aachen, Pauwelsstraße 30, 52074 Aachen, Germany; 2Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Hannover, Germany; 3Henry Ford Immunology Program, Henry Ford Health System, Detroit, MI, U.S.A.

Correspondence: Christoph Roderburg (croderburg@ukaachen.de)

Background: Dysregulation of miRNAs has been described in tissue and serum from patients with acute and chronic liver diseases. However, only little information on the role of miR-223 in the pathophysiology of acute liver failure (ALF) and liver cirrhosis is available.

Methods: We analysed cell and tissue specific expression levels as well as serum concentrations of miR-223 in mouse models of acute (hepatic ischaemia and reperfusion, single CCl4 injection) and chronic (repetitive CCl4 injection, bile duct ligation (BDL)) liver diseases. Results were validated in patients and correlated with clinical data. The specific hepatic role of miR-223 was analysed by using miR-223−/− mice in these models.

Results: miR-223 expression was significantly dysregulated in livers from mice after induction of acute liver injury and liver fibrosis as well as in liver samples from patients with ALF or liver cirrhosis. In acute and chronic models, hepatic miR-223 up-regulation was restricted to hepatocytes and correlated with degree of liver injury and hepatic cell death. Moreover, elevated miR-223 expression was reflected by significantly higher serum levels of miR-223 during acute liver injury. However, functional in vitro and in vivo experiments revealed no differences in the degree of liver cell death and liver fibrosis as miR-223−/− mice behaved identical with wild-type (wt) mice in all tested models.

Conclusion: miR-223 represents a promising diagnostic marker in a panel of serum markers of liver injury. Together with previously published data, our results highlight that the role of miR-223 in the pathophysiology of the liver is complex and needs further analysis.

Introduction

The term ‘liver disease’ comprises various medical conditions of different underlying aetiologies (infections, metabolic conditions, toxic agents, autoimmune diseases, vascular conditions, hereditary syndromes and others), which sooner or later all lead to a progressive limitation of the hepatic function. Acute liver failure (ALF) is a rare but still challenging clinical condition that, despite significant improvements in liver transplantation, shows mortality rates of up to 55–75% [1]. Chronic liver disease, a steadily destruction of the liver for over a period of more than 6 months, is characterized by a high, often underestimated long-term mortality rate due to liver fibrosis, cirrhosis and the development of hepatocellular carcinoma (HCC) [2]. Despite intensive research, the pathophysiology of both acute and chronic liver diseases is only poorly understood. Dysregulation of miRNAs, a novel class of endogenous, non-coding RNA has been described in different acute and chronic liver diseases, including viral hepatitis, steatohepatitis, liver fibrosis, cirrhosis and HCC [3]. Nevertheless, for most miRNAs, the understanding of their precise functional role in liver disease remains incomplete as only few studies on miRNA knockout mice are available to date.
miR-223 was firstly identified as a regulator of hematopoietic lineage differentiation and many studies indicate a role of miR-223 as a regulator of diverse immune cell functions [4,5]. miR-223 was reported as a regulator of the NLRP3 inflammasome in neutrophils [6] and shown to inhibit interleukin (IL)-1β production in primary hepatic macrophages after Concanavalin A (ConA) stimulation in vitro [7]. With regard to chronic liver diseases, miR-223 has been shown to be down-regulated in HCC tissues compared with matched non-tumour tissues [8,9] as well as in neutrophils from patients with alcoholic liver disease [10]. However, the specific function of miR-223 in the pathophysiology of acute and chronic liver diseases is still unknown. In the present study, we therefore investigated miR-223 expression and serum levels in different mice models of acute and chronic liver injury as well as in respective human patients. By using miR-223−/− mice, we further examined a potential function of miR-223 in liver injury.

Methods

Mice
In all experiments performed with miR-223−/− mice [11], the respective wild-type (wt) control mice were generated as follows. For the breeding of miR-223−/− and wt mice, heterozygous co-founders (+/−, C57BL/6J) were interbred and +/+ and −/− mice from the F1 generation were separated and interbred in parallel. In all other experiments performed only in wt mice, the respective wt (C57BL/6J) mice were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). All animal studies were approved by the Federal Ministry for Nature, Environment and Consumers’ Protection of the state of North-Rhine-Westphalia and were performed in accordance with the respective national, federal and institutional regulations.

Hepatic ischaemia/reperfusion mice model of acute liver injury
Ischaemia/reperfusion (I/R) experiments were performed as previously described [12]. All experiments were performed using male mice between 6 and 10 weeks of age. After 75 min of warm ischaemia of median and left liver lobes, reperfusion was initiated by removing the microvascular clamps on the respective branches. Blood and liver tissues were taken from mice 3, 6, 24 and 36 h after reperfusion. Sham-treated mice served as controls. To further investigate the functional role of miR-223 in the context of I/R, wt and miR-223−/− mice were treated as described above and killed 24 h after reperfusion. The numbers of mice included into the experiment are indicated in the respective figure legend.

Bile duct ligation
Bile duct ligation (BDL) was performed by tying the biliary duct of the mice with a non-absorbable suture, as previously described [13]. Sham-operated mice served as controls. All experiments were performed on male mice between 6 and 8 weeks of age. The numbers of mice included into the experiment are indicated in the respective figure legend.

Induction of acute and chronic liver damage by CCl4
Six to eight weeks old male miR-223−/− and wt mice were injected intraperitoneally with 0.6 ml of carbon tetrachloride per kg body weight (CCl4, Sigma–Aldrich, Munich, Germany; mixed with sunflower seed oil) with either (acute) a single CCl4 dose or (chronic) CCl4 twice a week for 6, 8 or 10 weeks. All mice in chronic experiments were killed 48 h after the last injection. Sera and liver tissues were collected at the indicated time points. For the investigation of liver fibrosis regression, mice were repetitively injected with CCl4, as described above, for 8 weeks and killed either 2 or 4 weeks later. The numbers of mice included into the experiment are indicated in the respective figure legend.

Fas ligand treatment
Six to eight weeks old male mice were injected intraperitoneally with 0.5 μg of Fas ligand (FasL; catalogue number: 310-03H, PeproTech, Hamburg, Germany) per g body weight and survival was monitored for 8 h.

Mice model of acetaminophen-induced liver injury
Six to eight weeks old male mice were injected intravenously with 250 mg of acetaminophen (APAP; amneal pharmaceuticals, Ireland) per kg body weight. Mice were killed after 24 or 48 h after APAP injection. Untreated mice served as controls.

Mice model of Concanavalin A-induced liver injury
Six to eight weeks old male mice were injected intravenously with 25 mg of ConA (Sigma–Aldrich, diluted in isotonic NaCl solution) per kg body weight. Mice were killed 48 h after ConA injection. Untreated mice served as controls.
Human samples
miR-223 expression was measured in human samples from a recently described cohort of patients with ALF [14], further described within the Supplementary Table S1 and a second cohort of patients with ALF due to different disease aetiologies. miR-223 serum levels were analysed in 24 serum samples from a recently described cohort of patients ([14]; Supplementary Table S2). Human fibrosis and cirrhosis cohorts are described in details in our recent publication [15].

Serum analysis
Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), alkaline phosphatase (AP) activity and serum bilirubin levels were measured by standard procedures in the Institute of Clinical Chemistry at University Hospital RWTH Aachen.

RNA isolation from tissue or serum and cDNA synthesis
RNA isolation from tissue and serum was performed as described recently [16]. Total RNA was purified from liver tissue using TRIZol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and Direct-zol™RNA MiniPrep (Zymo Research Europe GmbH, Freiburg, Germany) according to manufacturer's protocol. Quantity and quality of RNA were determined using Nanodrop (Thermo Scientific, Waltham, MA, U.S.A.). For tissue analysis, 1 μg of total RNA was reverse transcribed using cDNA synthesis Kit H Plus (Peqlab, VWR International GmbH, Darmstadt, Germany). For reverse transcription, total RNA was reverse transcribed using miRNA miScript Reverse Transcriptase Kit (miRNA, Qiagen GmbH, Hilden, Germany) or first strand cDNA synthesis kit (mRNA, Roche Diagnostics, IN, U.S.A.) according to the manufacturer's protocol. For serum analysis, sera from human samples (400 μl) or mouse samples (70 μl) were spiked with miScript miRNA mimic SV40 for sample normalization followed by phenol/chloroform isolation of RNA as described previously [16]. Later, cDNA was resuspended in suitable amounts of H2O.

Semi-quantitative reverse transcriptase PCR
Semi-quantitative reverse transcriptase polymerase chain reaction (qPCR) was performed as described recently [16]. Briefly, 2 μl of cDNA samples were added in a total volume of 25 μl using SYBR Green ET™(mRNA, Invitrogen) together with specific primers or the miScript SYBR Green PCR Kit (miRNA, Qiagen) with miRNA-specific primers (Qiagen) on a PCR machine (Applied Biosystems 7300 Sequence Detection System, Applied Biosystems, Foster City, CA, U.S.A.). All qPCR reactions were performed in duplicates. Data were generated and analysed using the SDS 2.3 and RQ manager 1.2 software packages. Primer sequences are given in Supplementary Table S3.

Hydroxyproline assay
Hepatic hydroxyproline (HP) was photometrically measured in liver hydrolysates. Analogue segments (50 mg) of snap-frozen livers were first lysated in 1 ml of HCl (6N) at 110°C for 16 h, filtered and aliquoted. Aliquots (50 μl) were incubated with 100 μl of 0.6% chloramine T solution for 10 min and subsequently with additional 100 μl of Ehrlich's reagent (600 mM) for 45 min at 50°C. Adsorption was determined in duplicates at 570 nm. HP concentration was determined using a standard curve for HP. The results are expressed as μg HP per g liver tissue.

Histological staining and quantification
As described previously [16], paraffin sections (2 mm, fixed with 4% paraformaldehyde) were stained with H/E and Sirius Red by a standard protocol for paraffin sections respectively. TUNEL staining was performed using the TUNEL kit from Promega according to the instruction manual. Paraffin sections were stained for Collagen IV using CL50451AP primary antibody (Cedarlane®, 1:300, overnight) and the ImmPRESS HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit (Vector Laboratories) according to the manufacturer's protocol. Images were obtained using a Leica DM 1000 Microscope and a Leica EC3 camera in combination with Leica Application Suite. Histological staining were quantified by analysing 12 (Sirius Red staining) or 6 (Collagen-IV staining) randomly selected magnification fields at 100× magnification for each sample with ImageJ software.

Cell separation
Hepatocytes and CD45+ cells fractions were isolated from murine livers after induction of liver damage by I/R or repetitive CCl4 injection as described recently [16]. Mouse numbers are indicted in the respective figure legend.
**miR-223 mimic transfection**

Transfection of Huh7 cells was performed as described recently [17]. Briefly, Huh7 cells were seeded in 96-well plate at a density of 300,000 cells per well. Twenty-four hours after seeding, cells were transfected with 50 nM *miScript miRNA mimic* (miR-223-3’p, Qiagen) using Lipofectamin 2000 (Invitrogen) for 48 h according to manufacturer’s protocol. Transfection with *miScript Inhibitor negative control* (Qiagen) and untransfected cells served as controls.

**H₂O₂ treatment**

Forty-eight hours after cell transfection, Huh7 cells were cultured for 4 h with 3 or 1.5 mM H₂O₂ (Sigma–Aldrich, Germany) as described previously [17].

**MTT-assay**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Thermofischer Scientific) was used to determine cell viability according to manufacturer’s instructions. After 2 h of incubation at 37°C, medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the crystals. Optical density was measured at 540 nm.

**Statistics**

Data from humans are given as median and range considering the skewed distribution of most parameters. Box plot graphics display a statistical summary of the median, quartiles, range and extreme values. The whiskers extent from the minimum to the maximum value excluding outside and far-out values, being displayed as separate points. Outside values (indicated by an open circle) are defined as values which are smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range. Far-out values (indicated by an asterisk) are defined as values which are smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range. All values, including ‘outliers’, have been included into statistical analyses. Statistical analyses on human samples were performed with SPSS version 12.0 (SPSS, Chicago, IL, U.S.A.). Differences in mice are displayed as mean + standard error of the mean (SEM). Kolmogorov-Smirnov test was used to test Gaussian distribution. T-test was used for normally distributed cohorts and Mann-Whitney U-test for cohorts, which are not normally distributed. Correlations between variables have been analysed using either Spearman (no Gaussian distribution of values) or Pearson correlation test (Gaussian distributed values). P values of P < 0.05 are considered statistically significant and are marked as follow: ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant. Graphical presentations in mice were performed with GraphPad Prism 5 (Graph-Pad, San Diego, U.S.A.). Detailed information about used tests in the respective figures is provided in Supplementary Table S4.

**Results**

**Serum and tissue levels of miR-223 are elevated in models of acute liver injury**

To analyse the specific role of miR-223 in acute liver injury, we applied the well-established model of hepatic ischaemia and reperfusion injury (I/R; Figure 1A) as a bona fide model for hepatic injury in mice and measured the expression of miR-223 in livers after induction of I/R (3 h: n=6; 6 h: n=6; 24 h: n=4; 36 h: n=6) and compared it with sham-treated mice (n=4). As demonstrated in Figure 1(B) and Supplementary Figure S1, I/R led to a significant up-regulation of miR-223 (P=0.0286). Notably, this up-regulation could also be confirmed in different models of acute liver injury such as treatment with CCl₄ (P = 0.00256, oil: n=6, CCl₄: n=7), APAP (P₂₄h = 0.0293, P₆₈h = 0.0003, n=6) and ConA (P=0.0034, n=6; Supplementary Figure S2A and B, S2D and E). In I/R and acute CCl₄ model, miR-223 expression strongly correlated with the degree of liver injury according to serum concentrations of transaminases (P=0.0046, r=0.9048, Figure 1C, Supplementary Figures S2B and S3A). Moreover, miR-223 expression strongly correlated with the amount of TUNEL positive cells (P=0.0007, r=0.9329, Figure 1D), indicating a direct link between expression levels of miR-223 and the degree of liver injury after I/R. We further examined how miR-223 is regulated during I/R and isolated hepatocytes and CD45⁺-immune cells from livers of mice after I/R or sham surgery (Supplementary Figure S3B), suggesting that the up-regulation of miR-223 in response to I/R injury is mainly caused by hepatocytes (P=0.0136, Figure 1E, sham: n=8, I/R: n=9).

Circulating miRNAs were recently proposed as biomarkers for various hepatic or non-hepatic diseases [18,19]. We therefore hypothesized that similar to alterations in intracellular miR-223 expression, miR-223 serum concentrations might also be regulated during hepatic I/R injury. We therefore compared miR-223 serum levels between mice after...
Figure 1. Serum and liver tissue levels of miR-223 are elevated in models of acute liver injury

(A) Haematoxylin and eosin (HE) and TUNEL staining of livers (scale bar = 100 μm) subjected to ischaemia and reperfusion (I/R). (B) Hepatic miR-223 expression in I/R mice model 3 (n=6), 6 (n=6), 24 (n=4) and 36 h (n=6) after liver reperfusion compared with sham-treated mice (n=4). (C) mir-223 expression after 24 h of I/R / SHAM correlated with ALT serum levels and with (D) the amount of TUNEL positive cells. (E) Hepatocytes (sham: n=8; I/R: n=9) and CD-45 immune cells (sham: n=11; I/R: n=12) were isolated from livers of mice after I/R revealing significantly higher miR-223 expression in hepatocytes but not in immune cells. (F) miR-223 serum levels in I/R mice model 3 (n=6), 6 (n=6), 24 (n=4) and 36 h (n=6) after liver reperfusion compared with sham-treated mice (n=4). (G) miR-223 expression after 24 h of I/R / SHAM correlated with ALT serum levels and with (H) the amount of TUNEL positive cells. (I) miR-223 levels were significantly higher in supernatant of hepatocytes after hypoxia and reoxygenation compared with controls (n=6 respectively) and correlate with transaminases 24 h after reoxygenation (J and K); ***P<0.001; **P<0.01; *P<0.05; ns, not significant (unpaired student’s t-test or Mann–Whitney U-test). Error bars indicate SEM.

© 2017 The Author(s). Published by Portland Press Limited on behalf of the Biochemical Society
miR-223 serum and tissue levels are elevated in patients with ALF

To analyse the transferability of these results to human liver failure (ALF), miR-223 expression levels were analysed in livers from patients with ALF (n=7) and healthy liver controls (n=10). In these analyses, miR-223 was significantly overexpressed in liver samples of ALF patients (P<0.001, Figure 2A). To investigate the potential role of miR-223 as a prognostic ALF marker, we used a second cohort of patients with ALF ([14], patients characteristic are given in Supplementary Table S1) and performed subgroup analysis with regard to the recovery of the patients (spontaneous recovery (SR), n=4; non-spontaneous recovery (NSR), n=6). Compared with SR patients, NSR patients showed (independent of the disease aetiology) elevated, hepatic miR-223 expression levels (P=0.019, Figure 2B), suggesting that analysis of miR-223 expression may allow prognosis prediction of ALF patients.

In the next step, the potential of miR-223 serum levels to serve as serum-based biomarker for human ALF was analysed. We therefore prepared RNA from sera of 24 patients with ALF (described in [14]) as well as 24 controls and
performed qPCR analysis to compare miR-223 concentrations. This analysis revealed significantly higher miR-223 levels in sera of ALF patients ($P<0.0001$, Figure 2C) compared with healthy controls. Moreover, we analysed miR-223 serum levels in the subgroup of patients which spontaneously recovered (SR, $n=6$), and in those patients which did not recover but needed liver transplantation ($n=9$) or died ($n=9$) due to the severity of their ALF. miR-223 serum concentrations were not altered between these subgroups (Figure 2D) or between the different disease aetiologies (Supplementary Figure S4). Furthermore, miR-223 serum levels did not correlate with serum levels of established ALF markers such as miR-122 ($P=0.605$, $r=0.111$) and miR-192 ($P=0.4955$, $r = -0.1462$).

**Deletion of miR-223 does not affect liver injury in I/R or toxic liver damage**

To analyse the functional role of miR-223 in acute liver injury, we used miR-223$^{-/-}$ mice (Supplementary Figure S5A) in the above-mentioned models of ALF. First, we examined the suitability of these mice to investigate liver injury. Therefore, we treated mice with Fas/CD95 ligand [20]. In line with previously published results, miR-223$^{-/-}$ mice were protected from FasL/CD95L-induced liver injury (Supplementary Figure S5B–D), demonstrating their suitability and comparability for further analysis of liver damage [20]. Of note, untreated miR-223$^{-/-}$ mice did not display any sign of liver injury (Supplementary Figure S5E).

In a first set of experiments, we subjected miR-223$^{-/-}$ as well as the respective wt mice to I/R surgery and compared the extent of liver injury. As shown in Figure 3(A), histological staining of livers from miR-223$^{-/-}$ and wt mice after I/R did not reveal any differences in liver damage. Moreover, AST, ALT and GLDH serum levels were not altered between miR-223$^{-/-}$ ($n=16$) and wt controls ($n=10$) following I/R-induced liver damage (Figure 3B). Similar to this finding, the lack of miR-223 had no influence on the dimension of liver damage upon CCl4 injection as a second model of ALF in mice (Figure 3C; wt: $n=3$; miR-223$^{-/-}$: $n=9$). Notably, these results are not in contradiction to the protective role of miR-223 in regulating FasL-induced liver injury as it was previously demonstrated that FasL is not directly involved in the pathophysiology of ischaemic liver injury [21]. To clarify these results in a better defined in vitro setting, we isolated primary hepatocytes from livers of miR-223$^{-/-}$ and wt mice ($n=12$ respectively) and cultured them under hypoxia conditions as described above. Compared with wt controls, the cell viability was slightly impaired in hepatocytes lacking miR-223 expression after 6 ($P=0.0441$) and 24 h ($P=0.0024$) of hypoxia (Figure 3D). To further investigate if increased miR-223 levels might trigger increased cell survival under hypoxia conditions, we transfected HUH7-cells ($n=3$) with an miR-223 mimic and cultured them under oxidative stress conditions. In this analysis, overexpression of miR-223 had no effect on the cell viability (Figure 3E).

**miR-223 serum and tissue levels are elevated in liver fibrosis**

Despite the fact that we were unable to identify a functional role of miR-223 in acute liver injury, we hypothesized that miR-223 might be functionally involved in the pathogenesis of liver fibrosis. Therefore, we analysed miR-223 expression in livers of wt mice which were repeatedly injected intraperitoneally with CCl4 twice a week for 6 ($n=5$) or 8 weeks ($n=5$). Similar to the results from ALF, miR-223 expression was significantly up-regulated in liver tissues after CCl4 injection compared with control mice ($n=5$ respectively; $P=0.0079$, Figure 4A). To analyse the cell specific regulation of miR-223 in hepatofibrogenesis, we isolated hepatocytes and CD45$^+$ immune cells from mice livers after repetitive CCl4 injection. In these analyses, miR-223 was significantly up-regulated in primary hepatocytes ($P=0.0005$), but not in liver-residential CD45$^+$-immune cells ($P=0.411$, Figure 4B) after repetitive CCl4 injection compared with controls. Using the BDL model, we confirmed these results in a second mouse model of hepatic fibrosis ($n=5$, $P=0.0328$, Figure 4C).

Based on these data, we next investigated whether circulating miR-223 levels might serve as biomarker for chronic liver injury. Therefore, we isolated miRNA from sera of mice after repetitive CCl4 treatment for 6 weeks and respective controls ($n=5$ respectively). qPCR analyses revealed a strong trend towards elevated miR-223 levels in sera of fibrotic mice ($P=0.0838$, Figure 4D).

As several studies have demonstrated that liver fibrosis and even cirrhosis are reversible in a considerable number of cases if the causative agent is withdrawn, we established a model of liver fibrosis regression to examine the kinetics of miR-223 expression during liver regeneration. Therefore, miR-223 expression was measured after 8 weeks (CTRL: $n=6$; Figure 4E) of CCl4 injection as well as 2 ($n=6$) and 4 weeks ($n=7$) after the last injection respectively. As seen in Figure 4(E), relative miR-223 expression was significantly decreased 2 ($P=0.0002$) and 4 weeks ($P<0.0001$) after the last CCl4 injection in comparison with the control. Interestingly, miR-223 tissue expression showed a striking correlation with the expression of the well-established fibrosis marker α-SMA ($P=0.0077$, $r=0.5912$, Figure 4F), suggesting a role of miR-223 in hepatofibrogenesis.
Figure 3. miR-223 is not functionally involved in the pathophysiology of tested acute liver injury models

(A) Representative HE staining of liver sections is depicted of miR-223−/− and wt mice subjected to I/R surgery (scale bar = 400 μm). (B) AST, ALT and GLDH serum levels were not altered in wt and miR-223−/− mice before I/R and 6 or 24 h (wt: n=10; KO: n=16) after liver reperfusion as well as (C) 24 h after a single injection of CCl4 (wt: n=3; KO: n=9) respectively. (D) Primary hepatocytes isolated from miR-223−/− and wt mice (n=12 respectively) were subjected to a hypoxia and reperfusion procedure and cell viability was analysed at the indicated time points by MTT assays. (E) Cell viability of HuH-7 cells transfected with miR-223 mimic (n=3) or neg. CTRL (n=3) and treated for 4 h with H2O2. Cell viability was determined by MTT assays; **P<0.01; ***P<0.001; *P<0.05; ns, not significant (unpaired student’s t-test or Mann–Whitney U-test). Error bars indicate SEM.
Figure 4. miR-223 levels are elevated in serum and liver tissue of mice models of chronic liver injury

(A) Hepatic miR-223 expression was significantly up-regulated after 6 weeks and showed a strong trend after 8 weeks of repetitive CCl4 injection twice a week compared with control animals (n=5 respectively). (B) miR-223 expression of primary hepatocytes (CCl4: n=7, oil: n=5) and hepatic CD45+ immune cells (CCl4: n=6; oil: n=5) isolated via MACS after repetitive CCl4 or oil injection. (C) miR-223 expression in mice subjected to BDL surgery (n=5 respectively). (D) miR-223 serum levels in mice after repetitive CCl4 injections for 6 weeks (n=5 respectively). (E) miR-223 and (F) α-smooth muscle actin (α-SMA) expression were significantly decreased during regression of liver fibrosis either 2 (n=6) or 4 weeks (n=7) after the last CCl4 injection compared with control mice (n=6, injected with CCl4 for 8 weeks) and miR-223 levels positively correlated with α-SMA expression; ***P<0.001; **P<0.01; *P<0.05; ns, not significant (unpaired student’s t-test or Mann–Whitney U-test). Error bars indicate SEM.

miR-223 serum and tissue levels were dysregulated in patients with chronic liver injury

Next, we analysed the diagnostic and prognostic potential of miR-223 in liver and serum samples of patients with liver fibrosis or cirrhosis. As presented in Figure 5(A) and (B), miR-223 tissue and serum levels are significantly altered in patients with liver fibrosis compared with healthy liver controls (P=0.0021). In contrast with the elevated miR-223 expression levels in fibrotic livers, serum levels of miR-223 were significantly lower in patients with liver fibrosis compared with healthy controls. To further clarify this finding, we analysed whether alterations of miR-223 levels correlate with clinical features of the regarding patients. However, miR-223 serum levels did not correlate with different stages of liver cirrhosis (according to the Child–Pugh score (Figure 5C), the MELD score (Figure 5D)) or disease aetiology (Figure 5E). Nevertheless, miR-223 serum levels were significantly altered between patients with AST serum levels higher or lower than 80 U/l (Figure 5F). Based on previous reports, suggesting a role of circulating miR-223 in the diagnosis of HCC, we finally analysed if serum levels of miR-223 differed in patients with or without HCC. However, at least in our cohort of patients, no differences in miR-223 concentrations became apparent between these subgroups of patients (P=0.1, Figure 5G).
miR-223 is not functionally involved in the pathophysiology of liver fibrosis

In order to investigate the function of miR-223 in the development of liver fibrosis, wt \((n=6)\) and \(\text{miR-223}^{-/-}\ \ (n=5)\) mice were repeatedly injected with CCl\(_4\) twice a week for 6 and 10 weeks. CCl\(_4\) treatment induced a robust fibrosis in both mice strains, however, no differences between \(\text{miR-223}^{-/-}\) and wt mice were observed macroscopically with regard to the severity of liver fibrosis (Figure 6A). Moreover, miR-223 knockout had no major influence on standard diagnostic markers of liver injury such as AST, ALT and GLDH (Figure 6B). Histological and immunohistological analyses (HE, Sirius Red and Col-IV) of liver tissue after 6 and 10 weeks of CCl\(_4\) injection did not reveal any differences regarding the degree of liver fibrosis in \(\text{miR-223}^{-/-}\) or wt mice (Figure 6C and D). Similarly, quantification
Figure 6. miR-223 is not functionally involved in the pathophysiology of CCl₄-induced chronic liver injury

*wt* (*n* = 6) and *miR-223⁻/⁻* (*n* = 5) mice were repetitively injected with CCl₄ for indicated time points (A). Representative macroscopic pictures of livers (scale bar = 1 cm), (B) serum levels of AST, ALT, GLDH and APs, (C) HE, Sirius Red and Col-IV staining of livers (scale bar = 200 μm), (D) quantification of Sirius Red and Col-IV staining, measurement of hepatic hydroxyproline levels as well as (E) analysis of expression levels of α-SMA, Col1A1, Tgf-β, Timp1 and (F) analysis of expression levels of Il-6, Tnf, Il-1-β as well as of the chemokines Ccl2 and Cxcl10 revealed no differences between *wt* and *miR-223⁻/⁻* mice after treatment of CCl₄ for 6 and 10 weeks respectively; ***P < 0.001; *P < 0.05; ns, not significant; unpaired student’s t-test or Mann–Whitney U-test. Error bars indicate SEM.

of hydroxyprolin content in these liver tissues revealed no difference in collagen content in livers of *miR-223⁻/⁻* or *wt* mice after induction of fibrosis (Figure 6D). In line, the severity of liver fibrosis was measured by qPCR of established fibrotic markers like α-SMA, Col1A1, Tgf-β and Timp1 all confirming that the knockout of miR-223 had no
Discussion

In the present study, we demonstrate that miR-223 is upregulated in livers and serum from mice after experimental acute liver injury as well as from patients with ALF. A similar up-regulation of miR-223 is found in mouse models of hepatofibrogenesis and in patients with histologically confirmed liver cirrhosis. miR-223 serum and tissue concentrations correlate with clinical markers of liver injury. Unexpectedly, further functional analysis using miR-223−/− mice and in vitro transfection experiments revealed that miR-223 is not involved in the pathophysiology of the applied models of acute (I/R, single CCl4 injection) or chronic liver injury (repetitive CCl4 injection), as the knockout of miR-223 have no impact on the degree of liver injury in these models.

Only few data on alterations of miR-223 expression in liver diseases are available [10,22,23]. In the present study, we provide for the first time a comprehensive analysis on the role of miR-223 in the pathophysiology of different acute and chronic liver diseases. In contrast with previous studies, which are focused on one distinct clinical entity or species, our analysis is unique as it comprises a broad set of data from different models of acute and chronic liver diseases as well as from large cohorts of patients with the respective disease entities, which are correlated to functional knockout experiments and in vitro experiments. In agreement with published data [22], we show that expression of miR-223 is up-regulated in livers from mice after acute I/R injury (Figure 1B and C). Additionally, we demonstrate that miR-223 is up-regulated in models of acute toxic liver injury (Supplementary Figure S2A and B, S2D and E), suggesting that the up-regulation of miR-223 expression represents a common feature of acute liver injury. Strikingly, in patients with acute liver injury, elevated expression of miR-223 reflects an impaired prognosis as those patients which did not spontaneously recover demonstrate a significantly higher hepatic expression of miR-223 than patients which spontaneously recovered from ALF. These data provide evidence for the first time that hepatic expression levels of miR-223 might represent a new biomarker to predict the outcome of ALF patients.

With regard to chronic liver diseases, we observe elevated hepatic miR-223 expression levels in mice after repetitive CCl4 injection or BDL-surgery compared with controls (Figure 4A and C). miR-223 expression levels correlate with the degree of liver fibrosis according to α-SMA expression as a surrogate for the activation of hepatic stellate cells (Figure 4F). Similar to the murine data we found a striking up-regulation of miR-223 in livers from patients with liver fibrosis. Elevated expression levels of miR-223 were also reported from patients with cystic lung fibrosis [24] as well as cardiac fibrosis [25], suggesting an organ-independent role of miR-223 in the pathophysiology of fibrotic diseases. Nevertheless, only very few functional data on miR-223 have been presented in the context of liver diseases. We demonstrate that the knockout of miR-223 have no impact on the severity of liver injury in different in vivo models of acute liver injury (Figure 3) and CCl4-induced chronic liver injury (Figure 6). Moreover, alterations in miR-223 expression had no effect on cell proliferation (Figure 3; Supplementary Figure S7). Similarly, the deletion of miR-223 did not affect the degree of liver damage in mice upon chronic ethanol feeding or acute ethanol gavage, representing standard models for induction of acute or chronic liver injury respectively [10]. In line, transgenic miR-223 mice with a heart-specific overexpression of miR-223 demonstrated a physiological cardiac hypertrophy but no signs of cardiac fibrosis [24], potentially arguing against a strong role of miR-223 in the development of organ fibrosis.

As miR-223 has been described to function as a regulator of immune cell function [6], it seems likely that the function of miR-223 might become more apparent in models of liver injury which feature a stronger activation of inflammation than those discussed above. Notably, we do not detect differences in immune cell infiltration or inflammation between miR-223−/− and wt mice upon induction of liver fibrosis (Figure 6F; Supplementary Figure S6). Supporting this hypothesis, miR-223 had a protective function in mice which were subjected to a combination of chronic ethanol administration and additional binge ethanol feeding (leading to a much more severe hepatic damage and increased hepatic immune cell infiltration) as miR-223−/− mice demonstrated elevated AST/ALT serum levels compared with wt mice [10]. Of note, in a closely defined H2O2 in vitro cell culture setting, not including immune cells, miR-223 does not have an impact on cell viability (Figure 3E). However, Qadir et al. [20] demonstrated that mir-223−/− mice were shown to be protected against Fas-induced hepatocyte apoptosis and liver injury, a finding which is confirmed in this study (Supplementary Figure S5B–D). Notably this is not contradictory to our results, as it was previously demonstrated that I/R liver injury is independent from Fas-signalling as Fas−/− mice behaved
similar to wt mice upon induction of hepatic I/R [21]. Thus, miR-223 is involved in a complex and specific manner in the pathophysiology of the different liver diseases. In the same context, Qadir et al. [20] pointed out that all miR-223−/− mice showed reduced activation of caspase 3, 8 and 9 after Fas-induction compared with wt mice. Interestingly, hepatocyte-specific deletion of caspase 8 however had no effect on the severity of liver fibrosis in the chronic CCl4 mice model [26]. Therefore, it is likely that observed differences in described acute and chronic liver injury models are due to involvement of different mechanistic pathways contributing to liver injury in these models.

Alterations of miR-223 serum levels have been extensively studied in different liver diseases with partially conflicting results [10,27-29]. In the present study, we correlate human and murine data to demonstrate that serum levels of miR-223 are up-regulated in acute liver diseases but down-regulated in patients with liver cirrhosis. Despite miR-223 serum levels do not allow prediction of patients’ fate in our cohorts, our data as well as previous reports suggest a ‘translational’ potential of miR-223 to function as a diagnostic biomarker for liver diseases. However, before a clinical use can be considered, several problems need to be solved: at present, there is no established consensus on standardization of sample collection, data normalization and analysis, providing a rationale for interstudy differences in the regulation of miR-223. Moreover, all available studies are based on retrospective analyses and need validation in longitudinal, prospective studies. Finally, mechanisms driving the up-regulation of mir-223 serum concentrations during liver injury are largely unknown. We demonstrate that miR-223 is up-regulated specifically in hepatocytes (Figure 1E; Figure 4B) and correlated with AST/ALT serum levels after liver injury (Figure 1G; Supplementary Figure S3C). Therefore, it is likely that hepatocytes are the major source for miR-223 release into the serum via a passive release upon hepatocyte death, despite we cannot fully exclude that miR-223 might be actively released and thus be part of a previously unrecognized system allowing intercellular communication [30]. Because miR-223 serum levels are significantly different between patients and the respective controls, but still show some overlap between the different groups, it is likely that not a single miRNA but detection of a whole panel might provide the necessary sensitivity and specificity for diagnosis and monitoring of acute and chronic liver diseases as recently demonstrated [14].

To date, it is unclear if alterations of miRNAs in the serum of patients reflect an active secretory mechanism, as hypothesized previously for other miRNAs in a different context [31]. Such a secretory process comprising the active and regulated release of miRNAs into the serum would lead to elevated intracellular miRNA levels, while extracellular (serum) miRNAs levels would decrease (and vice versa). Our data, which show an up-regulation of miR-223 in hepatocytes correlating with lower levels of miR-223 in the serum of cirrhosis patients, would thus further support this hypothesis. In line to our data, previous studies described a down-regulation of miRNAs in affected tissue but elevated levels in the serum from patients in various disease conditions (e.g. [32-35], supporting a general regulatory mechanism driving the diverging levels of miR-223 in the parenchyma and serum of patients and mice with liver diseases.

Despite we do not demonstrate a role of miR-223 in the pathophysiology of acute (I/R, acute CCl4 model) and chronic (repetitive CCl4 intoxication model) liver injury, the striking regulation of miR-223 in livers and serum of patients with liver diseases might have clinical implications. Notably, elevated miR-223 reflects an impaired clinical course in patients with ALF. Nevertheless, when considering miR-223 as a new prognostic marker, analysis on larger patient cohorts with distinct hepatic disease-causes and differential clinical states will have to be conducted to further test the potential of miR-223 expression as biomarker of ALF. Together, our data show that intra- and extracellular miR-223 is regulated in human patients with acute and chronic liver injury and in experimental liver injury in mice. Further, knockout-based studies will have to be conducted to unravel the exact role of miR-223 in the different aetiologies of liver diseases.
Clinical Perspectives

- Despite extensive studies on the expression of miR-223 in various contexts of liver injury, still little is known about the functional role of miR-223 in the pathophysiology of acute liver failure (ALF) and liver cirrhosis.

- In the present study, hepatic and circulating levels of miR-223 were shown to be dysregulated in mice models of both acute and chronic liver injury and in patients with ALF or liver cirrhosis.

- However, functional experiments revealed no differences in the degree of liver injury in our tested models.

- Although these findings, together with published data, indicate the need for further studies elucidating the complex pathophysiological role of miR-223 in acute and chronic liver damage, miR-223 might represent a promising diagnostic marker for liver injury.

Acknowledgements

The authors would like to express their gratitude to members of the Luedde Lab, Michaela Roderburg-Goor and Dr Jane Beger, for helpful discussions.

Ethics Approval

The study protocol was approved by the local ethics committee and conducted in accordance with the ethical standards laid down in the Declaration of Helsinki (ethics committee of the University Hospital Aachen, RWTH University, Aachen, Germany). All animal experiments were approved by the Federal Ministry for Nature, Environment and Consumers’ Protection of the state of North-Rhine-Westphalia and were performed in accordance to the respective national, federal and institutional regulations.

Author Contribution


Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work was supported by the German Research Foundation [grant number DFG RO 4317/4-1] and by a START grant of the medical department of the RWTH Aachen. Moreover, the work was supported by a Mildred-Scheel Endowed Professorship and a single project grant number [110043] from the German Cancer Aid (Deutsche Krebsshilfe); the German-Research-Foundation [grant numbers LU 1360/3-1 and SFB-TRR57 / P06 + P09]; an ERC Starting [grant number ERC-2007-Stg / 208237-Luedde-Med3-Aachen]; the EMBO Young Investigator Program, the Interdisciplinary-Center-for-Clinical-Research (IZKF) Aachen-Germany; and the Ernst-Jung-Foundation Hamburg. The funding bodies had no role in the design, collection, analysis and interpretation of data as well as in the writing of the manuscript and in the decision to submit the manuscript for publication.

Abbreviations

α-SMA, α-smooth muscle actin; ALF, acute liver failure; ALT, alanine aminotransferase; AP, alkaline phosphatase; APAR, acetylaminophen; AST, aspartate aminotransferase; BDL, bile duct ligation; Ccl2, chemokine (C-C motif) ligand 2; Col1A1, collagen, type I, α 1; Col-IV, collagen 4; ConA, concanavalin A; CTRL, control; Cxcl10, chemokine (C–X–C motif) ligand 10; DMSO, dimethyl sulfoxide; FasL, Fas ligand; GLDH, glutamate dehydrogenase; HCC, hepatocellular carcinoma; HE, haematoxylin and eosin; IL, interleukin; I/R, ischaemia/reperfusion; KO, knockout; miRNA/miR, micro-RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NLRP3, NLR family pyrin domain containing 3; NSR, non-spontaneous recovery; qPCR, semi-quantitative reverse transcriptase polymerase chain reaction; SEM, standard error of
the mean; SR, spontaneous recovery; Tgf, transforming growth factor; Timp1, TIMP metalloproteinase inhibitor 1; Tnf, tumour necrosis factor; wt, wild-type.

References


9 Karakatsanis, A., Papconstantinou, I., Gazouli, M., Lyberopoulou, A., Polymeneas, G. and Voros, D. (2013) Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. Mol. Carcinog. 52, 297–303


15 Roy, S., Benz, F., Vargas Cardenas, D., Vucur, M., Gautheron, J., Schneider, A. et al. (2015) miR-30c and miR-193 are a part of the TGF-beta-dependent regulatory network controlling extracellular matrix genes in liver fibrosis. J. Dig. Dis. 16, 513–524


