Gastroenterology 2018;154:238–252

MicroRNA 122, Regulated by GRLH2, Protects Livers of Mice and Patients From Ethanol-Induced Liver Disease

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BACKGROUND & AIMS: Chronic, excessive alcohol consumption leads to alcoholic liver disease (ALD) characterized by steatosis, inflammation, and eventually cirrhosis. The hepatocyte specific microRNA 122 (MIR122) regulates hepatocyte differentiation and metabolism. We investigated whether an alcohol-induced decrease in level of MIR122 contributes to development of ALD. **METHODS:** We obtained liver samples from 12 patients with ALD and cirrhosis and 9 healthy individuals (controls) and analyzed them by histology and immunohistochemistry. C57Bl/6 mice were placed on a Lieber-DeCarli liquid diet, in which they were fed ethanol for 8 weeks, as a model of ALD, or a control diet. These mice were also given injections of CCl4, to increase liver fibrosis, for 8 weeks. On day 28, mice with ethanol-induced liver disease and advanced fibrosis, and controls, were given injections of recombinant adeno-associated virus 8 vector that expressed the primary miR-122 transcript (pri-MIR122, to overexpress MIR122 in hepatocytes) or vector (control). Two weeks before ethanol feeding, some mice were given injections of a vector that expressed an anti-MIR122, to knock down its expression. Serum and liver tissues were collected; hepatocytes and liver mononuclear cells were analyzed by histology, immunoblots, and confocal microscopy. We performed in silico analyses to identify targets of MIR122 and chromatin immunoprecipitation quantitative polymerase chain reaction analyses in Huh-7 cells. **RESULTS:** Levels of MIR122 were decreased in liver samples from patients with ALD and mice on the Lieber-DeCarli diet, compared with controls. Transgenic expression of MIR122 in hepatocytes of mice with ethanol-induced liver disease and advanced fibrosis significantly reduced serum levels of alanine aminotransferase (ALT) and liver steatosis and fibrosis, compared with mice given injections of the control vector. Ethanol feeding reduced expression of pri-MIR122 by increasing expression of the spliced form of the transcription factor grainyhead like transcription factor 2 (GRHL2) in liver tissues from mice. Levels of GRHL2 also were increased in liver tissues from patients with ALD, compared with controls; increases correlated with decreases in levels of MIR122 in human liver. Mice given injections of the anti-MIR122 before ethanol feeding had increased steatosis, inflammation, and serum levels of alanine aminotransferase compared with mice given a control vector. Levels of hypoxia-inducible factor 1 alpha (*HIF1α*) mRNA, a target of MIR122, were increased in liver tissues from patients and mice with ALD, compared with controls. Mice with hepatocyte-specific disruption of *Hif1α* developed less-severe liver injury following administration of ethanol, injection of anti-MIR122, or both. **CONCLUSIONS:** Levels of MIR122 decrease in livers from patients with ALD and mice with ethanol-induced liver disease, compared with controls. Transcription of MIR122 is inhibited by GRHL2, which is increased in livers of mice and patients with ALD. Expression of an anti-MIR122 worsened the severity of liver damage following ethanol feeding in mice. MIR122 appears to protect the liver from ethanol-induced damage by reducing levels of HIF1α. These processes might be manipulated to reduce the severity of ALD in patients.

Keywords: miR-122; AAV; Mouse Model; Ethanol; Gene Expression.

BASIC AND TRANSLATIONAL LIVER

Chronic alcohol consumption accounts for nearly 50% of liver-related deaths in the United States; however, no effective therapies exist for patients. Although early steatosis in alcoholic liver disease (ALD) is reversible, chronic, excessive alcohol consumption leads to steatohepatitis and fibrosis. Acute alcoholic hepatitis has a 30% to 50% 30-day mortality and the standard of care with steroids has limited benefits and significant side effects. Alcoholic cirrhosis (Liennec cirrhosis) is the single greatest cause of hepatocellular cancer (HCC).[1,2] Thus, identification of novel therapeutic targets is a major clinical need in ALD.[2]

ALD is characterized by liver steatosis, inflammation, and progressive fibrosis.[2–4] Alcohol-triggered hepatocyte steatosis and cell death in combination with bacterial lipopolysaccharide due to alcohol-induced "leaky gut" results in the activation and infiltration of immune cells. The subsequent

Abbreviations used in this paper: **ALD, alcoholic liver disease; ALT, alanine aminotransferase; CCl4, carbon tetrachloride; FL, full length; GLuc, Gaussia Luciferase; GRHL2, grainyhead like transcription factor 2; GRHL2-S, splice variant of GRHL2; HCC, hepatocellular cancer; HCV, hepatitis C virus; HIF1α, hypoxia-inducible factor 1 alpha; miRNA, microRNA; MIR122, microRNA 122; pri-MIR122, MIR122 primary transcript; rAAV8, recombinant adeno-associated virus 8; Scr, scrambled control vector; TuD, Tough Decoy; WT, wild-type.**

Most current article

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2017.09.022

EDITOR'S NOTES

BACKGROUND AND CONTEXT

Chronic, excessive alcohol consumption leads to alcoholic liver disease (ALD) characterized by steatosis, inflammation, and eventually cirrhosis. The acute severe manifestation of ALD, alcoholic hepatitis, has limited treatment options and is associated with significant mortality.

NEW FINDINGS

The authors define a novel pathway regulating the pathogenesis in alcoholic liver disease whereby chronic alcohol reduces MIR122 through upregulation of its direct transcriptional inhibitor, grainyhead-like 2. Restoration of MIR122 levels in a murine model of advanced alcoholic fibrosis is beneficial and reduces liver injury.

LIMITATIONS

The study defines a potential role of MIR122 overexpression as beneficial in ameliorating ALD but is limited to a murine model where alcoholic fibrosis was established in an accelerated model using CCl4.

IMPACT

MIR122 appears to protect the liver from ethanol-induced damage by reducing levels of HIF1A. These processes might be manipulated to reduce the severity of ALD in patients.

release of inflammatory cytokines causes further hepatocyte cell death and results in perpetuation of liver injury.[5]

Hepatic microRNAs (miRNAs) have crucial roles in maintaining liver homeostasis, mitochondrial function, and regulating oncogenesis.[6,7] MIR122 constitutes 70% of all miRNAs in mature hepatocytes, or approximately 130,000 copies per cell, with negligible expression in other cells and tissues.[8] Mice with germline and liver-specific deletion of MIR122 display steatosis at birth, spontaneous progression to fibrosis, and HCC.[9] In humans, liver MIR122 expression inversely correlates with HCC survival and metastasis, whereas MIR122 inhibition reduces hepatitis C virus (HCV) viremia, serum triglycerides, and cholesterol.[10–12] These observations suggest that MIR122 has diverse and pleiotropic effects on hepatocytes and liver diseases and prompted us to explore the role of MIR122 in ALD.

Encoded on chromosome 18, MIR122 is transcribed as an approximately 4.7-kb noncoding pri-miRNA transcript by RNA polymerase II that is then rapidly processed into a 66-nucleotide (nt) pre-MIR122 by Drosha. Subsequently, the pre-miRNA is shuttled into the cytoplasm where it is processed into its mature 23-nt form. Although factors that maintain the high level of expression of MIR122 in the healthy liver have been well studied, little is known about the regulation of MIR122 expression in disease states.

In this study, we explored the hypothesis that alcohol modulates MIR122 expression in the liver and contributes to pathogenic features of ALD. We demonstrate a significant reduction of MIR122 expression in hepatocytes and show restoration of MIR122 levels had therapeutic benefits in ALD. Further, we discovered that the decrease in MIR122 was due to inhibition of MIR122 transcription by chronic alcohol-induced increases in the grainyhead like 2 (GRHL2) transcriptional regulator. We found that alcohol-induced MIR122 reduction is hepatocyte-specific and it mediates steatosis and inflammation through its primary target, hypoxia-inducible factor-1 alpha (*HIF1α*).

Materials and Methods

Human Liver Samples

Human liver samples were provided by the National Institutes of Health–funded Liver Tissue Procurement and Cell Distribution System (N01-DK-7–0004/HHSN26700700004C). Demographics can be found in Supplementary Table 1. Healthy liver samples were provided as age-matched controls.

Animal Use

All animals received care in compliance with protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School. As previously described,[13] mice were acclimated to a Lieber-DeCarli liquid diet of 5% ethanol (vol/vol) over a period of 1 week, then maintained on the 5% diet for 4 weeks. Wild-type (WT) mice (C57/BL6), Alb-Cre, and $HIF1^{flox/flox}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME).[14] Mice were treated by tail vein injection with adeno-associated virus (AAV) vectors at 6×10^{11} genome copies per mouse or approximately 3×10^{13} genome copies/kg.[15]

Murine Model of Advanced Alcoholic Fibrosis

Model of advanced fibrosis was adapted from previously published work.[16] Mice were gradually started on a Lieber-DeCarli liquid diet with 2% ethanol (vol/vol) for a period of 2 weeks, which was increased to 4% and then 5% each 2 weeks. During this time, 0.5 μL/kg carbon tetrachloride (CCl4) or corn oil was given every third day for 8 weeks. On day 28, CCl4 injections were held for 1 week and 6 x 10^{11} viral particles of AAV8 containing either pri-MIR122 or scrambled (Scr) vector were administered intravenously. CCl4 injections resumed on day 35 and continued every 3 days until day 56. Mice were killed 48 hours after the last dose of CCl4.

Statistical Analysis

Statistical significance was determined using 2-tailed *t*-test; 2-way analysis of variance with Dunnett's multiple comparison posttest were used to compare the means of multiple groups. Outliers were determined using the ROUT method and a q of 1%. Data are shown as mean ± SEM and were considered statistically significant at $^{*}P < .05$, $^{**}P < .005$, and $^{***}P < .0005$. GraphPad Prism 7.02 (GraphPad Software Inc, La Jolla, CA) was used for analysis.

Additional methods are found in the Supplementary Materials.

Results

Chronic Alcohol Decreases Hepatic MIR122 Expression in Humans and Mice

Because the role of MIR122 in ALD is unknown, first, we hypothesized that alcohol may regulate MIR122 levels