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MicroRNA changes, activation of progenitor cells and severity of liver injury in mice induced by choline and folate deficiency☆

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Abstract

Dietary deficiency in methyl-group donors and cofactors induces liver injury that resembles many pathophysiological and histopathological features of human nonalcoholic fatty liver disease (NAFLD), including an altered expression of microRNAs (miRNAs). We evaluated the consequences of a choline- and folate-deficient (CFD) diet on the expression of miRNAs in the livers of male A/J and WSB/EiJ mice. The results demonstrate that NAFLD-like liver injury induced by the CFD diet in A/J and WSB/EiJ mice was associated with marked alterations in hepatic miRNAome profiles, with the magnitude of miRNA expression changes being greater in WSB/EiJ mice, the strain characterized by the greatest severity of liver injury. Specifically, WSB/EiJ mice exhibited more prominent changes in the expression of common miRNAs as compared to A/J mice and distinct miRNA alterations, including the overexpression of miR-134, miR-409-3p, miR-410 and miR-495 miRNAs that were accompanied by an activation of hepatic progenitor cells and fibrogenesis. This *in vivo* finding was further confirmed by *in vitro* experiments showing an overexpression of these miRNAs in undifferentiated progenitor hepatic HepaRG cells compared to in fully differentiated HepaRG cells. Additionally, a marked elevation of miR-134, miR-409-3p, miR-410 and miR-495 was found in plasma of WSB/EiJ mice fed the CFD diet, while none of the miRNAs was changed in plasma of A/J mice. These findings suggest that miRNAs may be crucial regulators responsible for the progression of NAFLD and may be useful as noninvasive diagnostic indicators of the severity and progression of NAFLD.
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Keywords: Liver injury; Methyl-donor deficient diet; Mouse; MicroRNA; Progenitor cells

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States and other Western countries, with a rising prevalence worldwide [1,2]. NAFLD refers to a broad spectrum of related hepatic disorders, ranging from simple steatosis (a benign form of the disease) to nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (the most advanced disease stage) [3]. Uncomplicated hepatic steatosis is generally considered to be a benign form of NAFLD and has a favorable outcome [3–5]; however, 10% of individuals diagnosed with simple hepatic steatosis will progress to NASH and have a consistently greater risk of developing hepatocellular carcinoma [2,5]. In light of this, it is critical to identify this susceptible cohort at early stages of the disease progression; however, most of the current studies have focused on identifying the underlying molecular mechanisms of the pathogenesis of NAFLD and less attention has been given to (i) clarify the determinants for the disease progression to NASH in certain individuals and (ii) identify these individuals. This is critical not only for identifying subpopulations sensitive to NAFLD but also for its treatment and prevention.

We reported previously that dietary choline and folate deficiency induces fatty liver injury in mice, which resembles many histopathological features of human NAFLD [6,7]. Importantly, it has been demonstrated that humans eating low-choline diets and/or having genetic polymorphisms in choline and one-carbon metabolism genes develop NAFLD-like liver injury [8]. The results of our previous study showed that the severity of NAFLD-associated liver injury in mice induced by a choline- and folate-deficient (CFD) diet was strongly associated with marked dysregulation in gene expression [6], aberrations in the intracellular one-carbon [7] and iron metabolism [9] and altered microRNA (miRNA) expression [10].

Comprehensive research efforts in recent years have demonstrated the involvement of a number of miRNAs in lipid and glucose metabolism,

Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; miRNA, microRNA; mRNA, messenger RNA; CFD, choline- and folate-deficient; cDNA, complementary DNA; IPA, Interactive Pathways Analysis; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse-transcription PCR.

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endoplasmic reticulum and oxidative stress, apoptosis and inflammation, each of which are key pathophysiological processes associated with the development and augmentation of NAFLD. This suggests that NAFLD, in addition to the well-established molecular abnormalities, may exhibit disease-associated miRNA alterations. Indeed, several reports have shown that miRNAs are differentially expressed in both human and rodent NAFLD liver tissue samples [11–13], indicating that miRNAs may not only have significance in the pathogenesis of NAFLD but may also be involved in the progression of disease.

In light of this, the goal of the present study was to investigate the role of miRNAs in the molecular events and mechanisms associated with the pathogenesis of NAFLD and, especially, progression of the disease from uncomplicated NAFLD state to the advanced form of the disease. We demonstrate that differences in the severity of NAFLD-like liver injury induced by a CFD diet in A/J and WSB/EiJ mice were associated with marked alterations in hepatic miRNA expression profiles. Specifically, WSB/EiJ mice, the strain characterized by the greatest severity and magnification of NAFLD, exhibited more prominent changes in the expression of common miRNAs as compared to A/J mice and distinct changes in miRNA expression that were accompanied by an activation of hepatic progenitor cells and fibrogenesis. This suggests that miRNAs may be crucial regulators responsible, at least in part, for the progression of NAFLD.

2. Methods

2.1. Animals and experimental design

The in-life portion of this study, diet description, mouse treatment, tissue collection and results of histopathological and clinical chemistry analyses are described in detail in Tryndyak *et al.* [10]. In our previous reports, we demonstrated that feeding inbred mice a CFD diet caused an interstrain variability in NAFLD-like liver injury, which magnitude increased in the following A/J≈C57BL/6J≈C3H/HeJ<129S1/SvImJ≈CAST/EiJ<PWK/PhJ<WSB/EiJ [6,10]. Hence, for the further analyses, only A/J and WSB/EiJ mice, the strains that exhibited the greatest differences in the extent of NAFLD-associated liver injury induced by the CFD diet, were selected. All experimental procedures were reviewed and approved by the National Center for Toxicological Research Animal Care and Use Committee.

2.2. Histopathology and immunohistochemistry

Histopathological examination of the liver tissue sections and immunohistochemical evaluation of extent of cell proliferation were performed as described previously [10,14].

2.3. Cells and cell culture

The human liver HepaRG cell line was obtained from the Biopredic International (Overland Park, KS), and the cells were maintained according to the manufacturer's recommendations. Briefly, the cells were seeded at a density of $1.3\times10^4/cm^2$ and maintained in William's E medium supplemented with growth additives for HepaRG cells (Biopredic International) for 14 days, and then the cells were differentiated by maintaining cells in William's E medium supplemented with differentiation additives (Biopredic International) for an additional 14 days. The medium was changed every other day. HepaRG cells were microphotographed using an Olympus CK2 phase-contrast inverted microscope (Olympus America Inc., Center Valley, PA) and harvested by mild trypsinization after 3, 7, 14 and 28 days of the initial seeding, washed in phosphate-buffered saline and immediately frozen at −80°C for subsequent analyses.

2.4. miRNA expression profiling

Total RNA, including miRNA, was extracted from liver tissues (n=3 per strain per dietary treatment) using miRNeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg total RNA from livers using the TaqMan microRNA reverse-transcription kits and Megaplex RT Rodent pool A version 2.0 primers according to the manufacturer's instructions (Life Technologies, Grand Island, NY), loaded onto a TaqMan Array Rodent microRNA card A (v2.0) (Life Technologies) and amplified using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) running SDS software. SnoRNA202 and U6 snRNA were used as endogenous controls. The relative amount of each miRNA transcript was determined using the $2^{-\Delta\Delta Ct}$ method [15].

2.5. Pathway analyses of significant miRNAs

Interactive Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) was used to identify functional processes and pathways associated with deregulated miRNAs as determined by a two-tailed Student's t test analysis (P<.05). Using the IPA software, lists of miRNAs were compared against the Ingenuity Knowledge Base to determine disease and functions with which the miRNAs were directly and indirectly associated, at a significance of P<.05. The pathways with the largest portion of associated miRNAs determined by *in silico* analyses were used to generate association maps via the Pathway Finder tool of IPA, based on miRNA name and sequence.

2.6. Quantitative reverse-transcription quantitative PCR

Total RNA, including miRNA, was extracted from liver tissues, white blood cells and plasma (n=5 per strain per dietary treatment) and HepaRG cells (n=5 per timepoint) using miRNeasy Mini kits (Qiagen) according to the manufacturer's instructions. Reverse transcription was conducted using a High-Capacity cDNA Reverse-Transcription kit and a TaqMan MicroRNA Reverse-Transcription Kit (Life Technologies) following the manufacturer's instructions. The level of messenger RNA (mRNA) transcripts of protein-coding genes in the livers and miRNAs in the livers, white blood cells and plasma (n=5 per strain per dietary treatment) was determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using TaqMan gene expression assays and TaqMan microRNA assays (Life Technologies) as described in Tryndyak *et al.* [10]. Level of mRNA transcripts was normalized to *ACTB*. SnoRNA202 was used as an endogenous control for relative quantification of miRNA level in livers, HepaRG cells and white blood cells. Plasma miRNA was normalized to mmu-miR-16, an ubiquitous nonliver-specific miRNA. The relative amount of each mRNA transcripts of protein-coding genes and miRNA was measured using the $2^{-\Delta\Delta Ct}$ method [15]. All qRT-PCR reactions were conducted in triplicate.

2.7. Statistical analyses

Significant differences (P<.05) between groups were evaluated using unpaired two-tailed Student's t tests. For a graphical representation of data, error bars indicate the standard deviation of experimentally determined mean values. For data that failed the Shapiro–Wilk normality test, values were log transformed prior to statistical analyses. Linear regression analysis was used to determine time-related trends. All experimental values have been normalized to those of control samples.

3. Results

3.1. miRNA expression changes in the livers of mice fed a CFD diet

The miRNA expression profiles were examined in the livers of control A/J and WSB/EiJ mice and mice fed a CFD diet. Fig. 1 shows that feeding A/J