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A micro-RNA expression-signature for human NAFLD progression

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Abstract

Background—The spectrum of nonalcoholic fatty liver disease (NAFLD) describes disease conditions deteriorating from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) to cirrhosis (CIR) to hepatocellular carcinoma (HCC). From a molecular and biochemical perspective, our understanding of the etiology of this disease is limited by the broad spectrum of disease presentations, a thorough understanding of the factors contributing to disease susceptibility, and ethical concerns related to repeat sampling of the liver. To better understand factors associated with disease progression, we investigated by next generation RNA sequencing the altered expression of microRNAs (miRNAs) in liver biopsies of Class III obese subjects (body mass index ≥ 40 kg/m²) biopsied at the time of elective bariatric surgery.

Methods—Clinical characteristics and unbiased RNA expression profiles for 233 miRs, 313 transfer RNAs (tRNAs) and 392 miscellaneous small RNAs (snoRNAs, snRNA, rRNAs) were compared among 36 liver biopsy specimens stratified by disease severity.

Results—The abundance of 3 miRNAs (miR-301a-3p and miR-34a-5p increased and miR-375 decreased) found to be differentially regulated with disease progression was validated by RT-PCR. There were no tRNAs or miscellaneous RNAs identified to be associated with disease severity. Similar patterns of increased miR-301a and decreased miR-375 expression were observed in 134 hepatocellular carcinoma (HCC) samples deposited in The Cancer Genome Atlas (TCGA).

Conclusions—Our analysis results suggest that NAFLD severity is associated with a specific pattern of altered hepatic microRNA expression that may drive the altered lipid and carbohydrate metabolism hallmark of this disorder. The three identified miRNAs can be potentially used as biomarkers to assess the severity of NAFLD. The persistence of this miRNA expression pattern in an external validation cohort of HCC samples suggests specific microRNA expression patterns may permit and/or sustain NAFLD development to HCC.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a condition of increased lipid deposition in the liver and it is associated with obesity, insulin resistance, hepatic dysfunction, and other features of the metabolic syndrome. By the year 2020, NAFLD is expected to be the leading indication for liver transplantation [1]. Nonalcoholic steatohepatitis (NASH) is a particularly worrisome stage of NAFLD characterized by liver inflammation and hepatocellular damage on a background of simple steatosis. [2]. NASH occurs in ~40% of Class III obese subjects (body mass index ≥ 40 kg/m²) by mechanisms that are not entirely understood[3]. If left untreated, NASH can progress to irreversible liver damage in the form of cirrhosis and hepatocellular carcinoma (HCC). NASH is discriminated from NAFL by the additional presence of liver inflammation and hepatocyte damage (ballooning) identified in a liver biopsy. Our data from ~200 bariatric subjects (BMI ≥ 35 kg/m²) places the incidence of NAFL (hepatic fat content >10%) at approximately 80%, with 40% having SS and 40% displaying NASH.

Many eukaryotic organisms have evolved miRNAs, which are single-stranded non-coding RNAs of 19–25 nucleotides, as a mechanism to modulate translation. Transcription of miRNA genes by RNA polymerase II (RNA pol II) results in the production of monocistronic, bicistronic, and polycistronic miRNA precursors. Transcription patterns of miRNAs during differentiation, fibrosis and insulin resistance are tightly regulated. Once fully processed, miRNAs regulate gene expression through post-transcriptional mechanisms that include blocking translation or promoting cleavage of specific target mRNAs or targeting promoter regions. miRNAs play critical roles in lipid metabolism, apoptosis, inflammation, as well as cell growth and differentiation in both endocrine and paracrine manners on multiple tissues [4]. The regulatory role of miRNAs is suggested by unique miRNA signatures for different tissues.

It has been shown that miRNA sequencing is capable of capturing all species of small RNAs, including tRNA, snoRNAs, snRNA, rRNAs[5] [6]. tRNAs are often the primary product of miRNA sequencing with abundance exceeding miRNA. tRNAs have been found to be associated with various diseases, including cancer [7–9], diabetes [10–12], and mitochondria disorders [13–15]. The potential functionality of tRNAs and other species of small RNAs with NAFLD remains largely unexplored. We hypothesized that certain small RNAs are associated with the severity of NAFLD. To test this hypothesis, we applied an unbiased miRNA sequencing and bioinformatics strategy to liver biopsy samples from 36 NAFLD patients.

METHODS

Human Subjects

Class III obese subjects scheduled for elective bariatric surgery and between 26 and 59 years old were recruited from the Center for Surgical Weight Loss and the Endocrinology Clinics at Vanderbilt University Medical Center to participate in this study. Exclusion criteria included presence of viral hepatitis, autoimmune hepatitis, hemochromatosis, significant alcohol use, intercurrent infections, and active cancer diagnosis within five years. Subjects

gave their informed written consent before participating in this study, which was approved by the Internal Review Board of Vanderbilt University (090657) and registered at ClinicalTrials.gov (NCT00983463). All studies were conducted in accordance with NIH and institutional guidelines for human subject research. The study protocol conformed to the ethical guidelines of the 2013 Declaration of Helsinki, as reflected in *a priori* approval by Vanderbilt University Medical School. All subjects, in preparation for bariatric surgery, were fasted. Wedge liver biopsies of the left lateral lobule were collected by harmonic scalpel at the time of laparoscopic bariatric surgery (Roux-en Y gastric bypass). Samples were blotted free of blood, dissected free of unusable tissue, and immediately frozen in liquid nitrogen prior to storage at -80°C .

Scoring of Liver Specimen

The presence of steatosis, ballooning, fibrosis, inflammation, and iron were determined by a pathologist using two independent histology specimens. Samples were categorized using the NAS scoring criteria: [16] steatosis ($<5\%$ - 0; 5–33% - 1; 34–66% - 2; $>66\%$ - 3), ballooning degeneration (none - 0; few - 1; many - 2), lobular inflammation (none - 0, <2 foci/200 HPF - 1; 2–4 foci/200 HPF - 2; >4 foci/200 HPF - 3), fibrosis (none - 0; zone 1, 2 or 3 - 1; zone 3 and periportal - 2; bridging - 3; cirrhosis - 4). Four cohorts of subjects were established: those with normal livers ($n = 10$), NAFL ($n = 12$), NASH ($n = 11$) and cirrhosis ($n = 3$) based on histology (Table S1).

RNA Isolation and Sequencing

Total RNA from liver biopsies were isolated using mirCURY miRNA isolation kit (Exiqon, Grand Island, NY) per manufacturer's instructions. The Exiqon miRNA isolation kit was used because a previous study determined this RNA isolation kit performed better than the TRIzol miRNA isolation kit [17]. Sequencing libraries were prepared using the TruSeq Small RNA sample preparation kit (Illumina, San Diego, CA). The small RNA protocol specifically ligates RNA adapters to mature miRNAs that have a 5'-phosphate and 3'-hydroxyl group resulting from enzymatic cleavage by RNA processing enzymes like Dicer. In the first step, RNA adapters were ligated onto each end of the RNA molecules, and a reverse transcription reaction was used to create single stranded cDNA. This cDNA was then PCR amplified with a universal primer and a second primer containing one of 48 uniquely indexed tags to allow multiplexing. Size selection of the cDNA constructs were performed using a 3% gel cassette on the Pippin Prep (Sage Sciences, Beverly, MA) to reduce the library to mature miRNAs and other regulatory RNAs in the 20–30 bp size range. The resulting cDNA libraries then underwent a quality check on the Bioanalyzer HS DNA assay (Agilent, Santa Clara, CA) to confirm the final library size and on the Agilent Mx3005P qPCR machine using the KAPA library quantification kit (Illumina, San Diego, CA) to determine concentration. A 2 nM stock was created, and samples were pooled by molarity for multiplexing. From the pool, 10 pM was loaded into each well for the flow cell on the Illumina cBot for cluster generation. miRNA sequencing was performed using Illumina HiSeq 2500 utilizing v3 chemistry and HTA 1.8. The raw sequencing reads were processed through CASAVA-1.8.2 for FASTQ conversion and demultiplexing.

Data Processing

We implemented a custom analysis pipeline [18] for small RNA sequencing data processing. We used Cutadapt [19] to trim 3' adapters for raw reads. Quality control on raw data was performed using QC3 [20]. All reads with length less than 16 were discarded. The adaptor-trimmed reads were formatted into non-redundant FASTQ file where the read sequence and copy number was recorded for each unique tag. The usable unique reads were mapped to the whole genome using Bowtie1[21] with only one mismatch allowed. In addition, our pipeline takes into the consideration non-templated additions [22–25] at the 3' end of miRNA during alignment, which results in more accurate miRNA expression quantification. The tRNA database was prepared by combining the latest UCSC tRNA database GtRNAdb [26] with the tRNA loci of mitochondria from the ensembl database [27]. The reads mapped with tRNA loci were used not only for tRNA quantification but also for tRNA mapping position coverage analysis. Small RNAs were divided into three major categories: miRNAs, tRNAs, and other small RNAs (including lincRNAs, snoRNAs, snRNAs, rRNAs and miscellaneous RNAs in the Ensembl database). Expression data quality control was performed using MultiRankSeq [28]. The elastic net method [29] was used to select a panel of small RNAs that together explain a large part of the variation in NAFLD progression. Differential RNA expression analysis was conducted using DESeq2 [30]. Samples were classified by liver histology into four conditions: normal, NAFL, NASH, and cirrhosis. Ordinal regression with elastic net was used to identify the small RNA that can best explain the variation of liver conditions. Elastic net is a variation of the Lasso regression method [31], a technique for L1-norm regularization, which has been used for variable selection (or dimensional reduction) in problems where the number of features p exceeds the number of cases N , in order to produce parsimonious and interpretable models. The elastic net method provides variable selection in the $p \gg N$ case without being severely limited by the sample size. This method greatly improves performance in the case of highly correlated predictor variables (as we expect to have in the clinical data), through the identification of significant highly correlated groups of phenotypes that contribute most to the variation in the data. R package glmnet was developed for elastic net analysis and was used to evaluate the overall association of the small RNA and treatment response. Because ordinal regression model does not produce adjusted R^2 , Mcfadden's R^2 [32] was computed as a surrogate to estimate overall variation explained. Mcfadden's R^2 is usually less than adjusted R^2 ; a value between 0.2 and 0.4 is considered a well fitted model. Trend analysis of small RNA expression across the four liver conditions (normal $\rightarrow$ NAFL $\rightarrow$ NASH $\rightarrow$ cirrhosis) was conducted using Mann-Kendall trend test [33]. Due to the lack of a functional database for tRNA and other small RNAs, we only conducted functional analysis on candidate miRNAs using Ingenuity Pathway Analysis (IPA)

Additional analyses performed on the three candidate miRNA using HCC patients from The Cancer Genome Atlas (TCGA). TCGA is a comprehensive and coordinated consortium project by National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) to accelerate the understanding of the molecular basis of cancer through the application of genome analysis technologies. TCGA contains miRNA expression data from 377 HCC patients with clinical information. We performed correlation analysis between the three candidate miRNAs and various clinical variable, including pathological

stage, fibrosis stage, etc. Because our NFALD patients were majority female, we only conducted the analysis on female HCC patients with matching age to our NFALD patients.

RT-PCR

Total RNA was reverse transcribed using the Universal cDNA Synthesis Kit II (Exiqon, Woburn, MA) per manufacturer's protocol, utilizing the UniSp6 RNA spike in control primer set. miRNAs were amplified using LNA technology with hsa-miR-301a, hsa-miR-34a-5p and hsa-miR-3375 primers, using Exilent SYBR green reagents (Exiqon). Amplificants were analyzed for distinct melting curves, and the T_m was checked to be within known specifications for the assay. RT-PCR data were analyzed using the 2^{-CT} method and normalized to 18S[34]. Spearman's correlation between RT-PCR data and miRNA sequencing gene expression data was computed to evaluate the accuracy of miRNA sequencing.

RESULTS

Table 1 shows physical, anthropometric, and biochemical measurements of all subjects in the study. All Class III obese (body mass index [BMI] ≥ 40 kg/m²) subjects were female, and our four cohorts (Normal, NAFL, NASH and Cirrhosis as defined by NAFLD Activity Scoring [16]) were composed of similar ethnicities (almost exclusively Caucasian), body weights, and BMIs. Total RNA extracted from liver biopsy tissues obtained intraoperatively during elective bariatric surgery was subjected to next generation sequencing. As reported in previous studies, NASH subjects were slightly older.[35] NASH subjects also displayed significantly elevated levels of alkaline phosphatase without significant differences in serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels.

The sequencing data were thoroughly quality controlled, and the detailed alignment statistics can be found in Table S1. The overall mapping rate of all samples was 67%. In small RNA sequencing, the majority of the alignments will be to tRNA rather than miRNA. In our data, on average, 4% of all mapped reads were mapped to miRNA. This is consistent with previous findings on miRNA sequencing [36]. On average, 80% of all mapped reads were mapped to tRNAs and the rest mapped to other RNAs, including snoRNA, tRNA, snRNA, rRNA, etc. After thorough quality control, we identified 233 miRNAs, 313 tRNAs and 393 other small RNAs that were suitable for additional analyses.

miRNA Expression Differences

Differential expression analyses were performed for three major types of small RNA: miRNA, tRNA and other small RNAs. Four cohort comparisons were made using DESeq2[30], a robust methodology for differential analysis of count data. We compared expression profiles (miRNA, tRNA or other small RNAs) for specimen spanning the spectrum of NAFLD: 1) Cirrhosis vs. Normal, 2) Cirrhosis + NASH vs. Normal, 3) NASH vs Cirrhosis, and 4) NASH vs. NAFL. For miRNA and tRNA, we found the greatest abundance differentials between Cirrhosis and Normal cohorts. The least abundance differentials were between NASH and NAFL, where no miRNAs were found to be significantly different after adjusting the P value for multiple comparisons. Four miRNAs

(miR-10b-5p, miR-143-3p, miR-301a-3p and miR-34a-5p) were increased while three miRNAs were decreased (miR-181a-5p, miR-28-5p and miR-375) in Cirrhosis versus Normal. Of these, the expression levels of miR-301a-3p, miR-34a-5p and miR-375, along with three others (miR-122-3p, miR-1248 and miR-301a-3p) were also significantly different when a Cirrhosis+NASH versus Normal comparison was made. There were no miRNAs significantly different between NASH and NAFL, and only miR-375 was different (decreased) comparing NASH versus Cirrhosis. The summary results of differential expression analysis can be found in Table 2. The complete differential expression analyses results for miRNA can be found in Table S2.

tRNA Expression Differences

We found that with a Cirrhosis versus Normal cohort comparison, there was significant differential expression of 23 tRNAs with Cirrhosis, most notably tRNA7, tRNA9, tRNA11 and tRNA13 for lysine (anticodon CTT), tRNA10, tRNA12, tRNA38, tRNA45 for aspartate (anticodon GTC) and tRNA3, tRNA5, tRNA11, tRNA20 for glutamate (anticodon TTC). The majority (15) of these tRNAs were down-regulated with Cirrhosis. Notably, one mitochondrial tRNA2 for valine was significantly up-regulated. The down-regulation of tRNA3 and tRNA11 for GluTTC also distinguished Cirrhosis from NASH. Two mitochondrial tRNAs, tRNA11-TY and tRNA18-TS2, encoding mitochondrial tRNAs for the amino acids tyrosine and serine, respectively, were significantly up-regulated. The complete differential expression analyses results for tRNA can be found in Table S3. Seven AAG tRNAs for leucine and two RNAs for aspartate (codon GTC) fell just short of being significantly reduced ($P = 0.054$ and 0.056) in Cirrhosis+NASH vs Normal.

Other Small RNAs

For other small RNAs, the most differences again were found between Cirrhosis + NASH vs. Normal. Two small Y RNAs (ENSG00000252316 and ENSG00000201778), rare types of small non-coding RNAs that are components of Ro ribonucleoproteins, were found down-regulated when comparing NASH to Cirrhosis. The complete differential expression analyses results for other small RNA can be found in Table S4.

miRNA Trend Analysis and Validation

Trend analyses using Mann-Kendall trend test identified many monotonically increasing and decreasing small RNAs: miRNA 16 increasing and 37 decreasing; tRNA 35 increasing and 31 decreasing; other small RNAs: 35 increasing and 32 decreasing. The summary of trend analyses can be viewed in Table S5, and the detailed trend analyses for miRNA, tRNA and other small RNA can be viewed in Tables, S6, S7, S8, respectively. We conducted functional analysis using the 16 increasing and 37 decreasing miRNAs using Ingenuity Pathway Analysis and identified several hepatotoxicities, including hepatocellular carcinoma, liver damage, liver steatosis, etc. (Table S9). These results suggest that the linear trend of miRNA expression change across the four liver conditions is closely related to hepatotoxicities.

Elastic net method with ordinal regression was performed to identify the subset of small RNAs that can explain the most variation in the liver conditions. Elastic net identified three miRNAs (hsa-miR-301a-3p, hsa-miR-34a-5p, hsa-miR-375), and no tRNA or other small

RNA. This suggests that tRNA and other small RNA might not play an as important role as miRNA in NAFLD progression. The three selected miRNAs were further fitted into an ordinal cumulative link model and showed reasonable significance (hsa-miR-301a-3p $P=0.09$, hsa-miR-34a-5p $P=0.06$, hsa-miR-375 $P=0.13$) (Table 3). Mcfadden's R^2 was computed to be 0.31, which suggests that at least 31% of variation within the four liver conditions can be explained by the three miRNAs. It is known that Mcfadden's R^2 usually underestimates the true variability explained, thus the true variability explained might be higher than 31%.

Further functional analyses of these three miRNA were conducted manually. All three miRNAs were also identified in the trend analyses (Figure 1). The first miRNA, miR-301a-3p, which was also found to be monotonically increasing as the liver condition progressed from Normal to Cirrhosis in the trend analysis, has been identified to be up-regulated in HCC by multiple studies [37] [38] [39] [40] [41]. The second miRNA, miR-34a-5p, which was found to be monotonically increasing in trend analysis, was found to be upregulated in hepatic fibrosis in rat [42] and mouse [43]. The third miRNA, miR-375, which was found to be monotonically decreasing in trend analysis, has been identified to be down-regulated HCC [44] [45]. The functional analysis results are in close concordance with the results from trend analysis, suggesting these three miRNA may be used as biomarkers to predict liver conditions.

RT-PCR validation of miR-34a-5p, miR-375 and miR-301-5p was carried out on 14 samples for which remaining RNA was available (Normal, $n=5$; NAFL, $n=5$; NASH, $n=4$). All three miRNAs were observed to have the same general patterns of gene expression as those observed with RNA-seq (Figure 1B).

Patients with NAFLD are at an increased risk for HCC [46–48]. We compared our miRNA expression pattern with miRNA expression profiles from hepatocellular carcinoma (HCC) datasets from TCGA, miRNA sequencing data of 110 HCC samples (female) from various HCC stages (Stage I $N=49$, average age = 63.6, Stage II $N=27$, average age = 64.7, Stage III&IV $N=34$, average age = 53.7) were analyzed. Furthermore, in TCGA, miR-34a was reported without distinction between its 5p and 3p forms. We found that out of the three candidate miRNAs, two (miR-301a-3p and miR-375) expressed the same expression pattern (in log2 scale) as HCC progress from early stage to late stage (Figure 1C). The expression pattern of miR-301a-3p in our specimen staged for progressive NAFLD was nearly identical to has-miR-301a in TCGA HCC data. These results demonstrate a persistence of this specific pattern of miRNA expression in more advanced forms of liver disease such as HCC. Additional correlation analyses were performed between the three candidate miRNAs and 18 clinical variables such as fibrosis stage, alpha fetoprotein test. No significant correlation was detected (Table S10).

DISCUSSION

In this study, we measured relative miRNA, tRNA and other small RNA abundance levels in liver biopsy tissues from Class III obese human subjects ($BMI \geq 40 \text{ kg/m}^2$) by an unbiased next-generation RNA sequencing and bioinformatics strategy. We found that the levels of

three miRNAs were significantly correlated with NAFLD severity, suggesting these miRNAs may contribute to the progression of NAFLD. In addition, several tRNAs and other small RNAs identified in our analysis exhibited differential abundance patterns specific for a certain stage of NAFLD. These differences afford new insight into the pathophysiology of NAFLD progression.

The differential abundance of human miRNAs with NAFLD has been examined most extensively in human serum. Pirola *et al.* identified significant fold differences in serum levels of miR-122 (7.2-fold change in NASH vs controls and 3.1-fold change in NASH vs simple steatosis) and miR-102 (4.4-fold change in NASH vs controls) [49]. Cermelli *et al.* identified by targeted Taqman miRNA qRT-PCR, increased serum levels of miR-122, miR-34a and miR-16 in NAFLD (NAFLD Activity Score; NAS = 4) and NASH (NAS = 5) patients compared to controls [50]. Yamada *et al.* identified in a cohort of low BMI (26.2 kg/m^2) with ultrasound-diagnosed NAFLD increased serum abundance of miR-21, miR-34a, miR-122 and miR-451 relative to controls [51]. Interestingly, only the elevations in miR-34a and miR-122 were significant in both men and women. Tan *et al.* identified miR-122-5p, miR-1290, miR-27b-3p and miR-192-5p as a panel of high diagnostic accuracy for NAFLD in a population with low BMI (25.2 kg/m^2) and low NAS scores [52]. Similarly, Celikbilek *et al.* quantified 9 miRNAs in serum of histologically proven NAFLD moderately obese ($n = 20$; BMI 31.9 kg/m^2) subjects and healthy age-match controls ($n = 20$; BMI 27.3 kg/m^2) [53]. They observed in NAFLD significantly decreased expression of miR-197, miR-146b, miR-181d and miR-99a.

In the current study hepatic levels of miR-301a-3p and miR-34a-5p were monotonically increased, and miR-375 was monotonically decreased from simple steatosis to NASH to cirrhosis. While some studies have linked miRNA dysregulation to the pathogenesis of NAFLD by profiling adipose tissue as a surrogate for difficult to obtain liver tissue [54, 55], our study is one of the few examining hepatic miRNA expression differences in biopsy-staged NAFLD. Cheung *et al.*, 2008 used μ Paraflo to quantify differential expression of 474 microRNAs in NASH (BMI 35 kg/m^2) versus controls (BMI 39.5 kg/m^2) [4]. Forty-six miRNAs were differentially expressed in subjects with NASH with half over-expressed and half under-expressed in the advanced form of the disease. miR-34a-5p was among those microRNAs significantly overexpressed, and miR-375 was among those significantly under-expressed with NASH; interestingly, miR-301a did not change. Other studies consistent with the altered pattern of microRNAs we detected come from studies in hepatocellular carcinoma [56, 57] and NAFLD [58], where differential expression of miR-34a seems to play a contributing role in disease progression, possibly through a SIRT1-AMPK-HMGCR mechanism [59]. However, the extent to which these profiles are concordant with hepatic gene miRNA expression is unknown.

The molecular mechanisms leading to HCC are not well understood, but multiple risk factors including obesity, insulin resistance and hepatic steatosis have been proposed as mediating. While HCC generally arises on a background of cirrhosis, NAFLD without cirrhosis is an emergent and independent risk factor of hepatocellular carcinoma [60]. Aberrant miRNA expression patterns borne of some or all of these risk factors may underlie NAFLD progression and potentiate the development of HCC in some individuals. Increased

expression of miR-301a and down-regulation of the homeobox gene Gax was associated with metastasis [61]. Down-regulation of miR-375 altered ATG7 expression and inhibited mitochondrial autophagy in HCC cells [62]. Decreased expression of miR-34a regulated by TGF- β targeted c-MET [63] and CCL22 [64]. These and other findings strongly suggest a role for these miRNAs in both NAFLD and HCC progression.

While there are several known and predicted biological targets of miR-34a-5p, miR-301-3p and miR-375, two genes, including DNAJC16 (DNAJ homolog, subfamily C, member 16) and TNRC6B (trinucleotide repeat containing 6B) which exhibit 8- and 7-mer seed regions matching all three of these miRNAs (TargetScan; <http://www.targetscan.org>). Another 48 targets are predicted to pair with miR-34a and miR-301a, which exhibited similar monotonic increases in expression with NAFLD progression. Targets of miR-375 in the liver include astrocyte elevated gene-1 (AEG-1) [65] and yes-associated protein (YAP) [66], the former having a role in mediating steatosis [67] and the later a mediator of hepatic proliferation and differentiation [68].

In conclusion, this study describes a novel pattern of miRNA expression in biopsy-staged NAFLD characterized by increased miR-301a-3p, miR-34a-5p and decreased miR-375 with worsened liver histology. The pattern of increased miR-301a-3p and decreased miR-34a-5p abundance was additionally present in early- to late-staged HCC suggesting these miRNAs as important modulators of liver disease progression. Alone, or in tandem with other clinical findings, these miRNAs inform on NAFLD severity and may serve as effective targets to modulate liver function for therapeutic benefit or prevent NAFLD progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

C

Figure 1.

A. Expression of the three candidate miRNAs over the severity of NAFLD measured by sequencing. **B.** Expression of the three candidate miRNAs over the severity of NAFLD measured by RT-PCR. **C.** Expression of the three candidate miRNAs over the HCC progression stages of female subjects. Note that in TCGA, only has-miR-34a was reported, there was not distinction between the 3p and 5p of this miRNA. *: t-test p-value < 0.05, **: t-test p-value < 0.01.

Table 1

Characteristics of Class III obese patients stratified by liver histology

	Normal	NAFL	NASH	CIRR	P
n	10	12	11	3	-
Gender (F/M)	10/0	12/0	11/0	3/0	-
Caucasian/African American	9/1	12/0	11/0	3/0	-
Age	38.7 ± 1.5	40.3 ± 3.1	48.6 ± 1.9*	49.3 ± 2.3	0.014
Weight (kg)	128.0 ± 5.7	117.2 ± 5.2	114.8 ± 4.0	121.3 ± 19.0	0.383
BMI (kg/m ²)	47.0 ± 2.0	44.3 ± 2.1	43.4 ± 1.4	44.9 ± 4.8	0.607
AST (normal range: 0–65 U/mL)	19.1 ± 1.9	33.7 ± 10.2	25.1 ± 3.0	34.3 ± 14.8	0.539
ALT (normal range: 0–65 U/mL)	23.0 ± 3.2	33.4 ± 8.4	29.9 ± 3.3	46.3 ± 18.3	0.420
Alkaline Phosphatase (U/L)	61.0 ± 7.4	89.0 ± 7.8	93.1 ± 6.7*	90.7 ± 10.1	0.032
Albumin (g/dL)	4.1 ± 0.2	4.0 ± 0.1	4.1 ± 0.1	4.4 ± 0.4	0.446
Bilirubin (mg/dL)	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.3	0.3 ± 0.1	0.597

Statistically significant changes are written in bold italics. Data presented as mean ± SEM. AU, arbitrary units. Asterisks represents * P<0.05. Abbreviations: BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 2

Differential Expression Analysis (DESeq2)

Comparison	<i>P</i> < 0.05	FDR < 0.05	FC > 2
miRNA Cirrhosis vs Normal	30		7
miRNA Cirrhosis + NASH vs Normal	27	6	2
miRNA NASH vs Cirrhosis	24	2	2
miRNA NASH vs NAFL	13	0	0
tRNA Cirrhosis vs Normal	88	23	83
tRNA Cirrhosis + NASH vs Normal	46	0	0
tRNA NASH vs Cirrhosis	85	8	81
tRNA NASH vs NAFL	11	0	0
other small RNA Cirrhosis vs Normal	47	8	41
other small RNA Cirrhosis + NASH vs Normal	89	36	11
other small RNA NASH vs Cirrhosis	20	2	19
other small RNA NASH vs NAFL	28	0	0

FDR - false discovery rate; FC – fold change.

Table 3

Ordinal Regression Results Based on Elastic Net Output

Ordinal Regression				Trend Analysis		
miRNA ID	Estimate	Error	P	Direction	P	Known Association
<i>miRNAs McJadden's R²= 0.31</i>	miR-301a-3p	1.047	0.626	0.095	Increase	0.089 up in HCC
	miR-34a-5p	0.673	0.352	0.056	Increase	0.089 up in hepatic fibrosis
	miR-375	-0.298	0.130	0.022	Decrease	0.089 down in HCC