ORIGINAL ARTICLE

High-throughput sequencing reveals altered expression of hepatic microRNAs in nonalcoholic fatty liver disease–related fibrosis

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Recent evidence suggests that microRNAs (miRNAs), small, noncoding RNA molecules that regulate gene expression, may play a role in the regulation of metabolic disorders, including nonalcoholic fatty liver disease (NAFLD). To identify miRNAs that mediate NAFLD-related fibrosis, we used high-throughput sequencing to assess miRNAs obtained from liver biopsies of 15 individuals without NAFLD fibrosis (F0) and 15 individuals with severe NAFLD fibrosis or cirrhosis (F3–F4), matched for age, sex, body mass index, type 2 diabetes status, hemoglobin A1c, and use of diabetes medications. We used DESeq2 and Kruskal-Wallis test to identify miRNAs that were differentially expressed between NAFLD patients with or without fibrosis, adjusting for multiple testing using Bonferroni correction. We identified a total of 75 miRNAs showing statistically significant evidence (adjusted *P* value <0.05) for differential expression between the 2 groups, including 30 upregulated and 45 downregulated miRNAs. Quantitative reverse-transcription polymerase chain reaction analysis of selected miRNAs identified by sequencing validated 9 of 11 of the top differentially expressed miRNAs. We performed functional enrichment analysis of dysregulated miRNAs and identified several potential gene targets related to NAFLD-related fibrosis including hepatic fibrosis, hepatic stellate cell activation, TGFB signaling, and apoptosis signaling. We identified FOXO3 and FBXW7 as potential targets of miR-182, and found that levels of FOXO3, but not FBXW7, were significantly decreased in fibrotic samples. These findings support a role for hepatic miRNAs in the pathogenesis of NAFLD-related fibrosis and yield possible new insight into the molecular mechanisms underlying the initiation and progression of liver fibrosis and cirrhosis. (Translational Research 2015;■:1–11)

Abbreviations: ■■■ = ■■■

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AT A GLANCE COMMENTARY

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Background

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of conditions resulting from excessive accumulation of fat in hepatocytes not because of overconsumption of alcohol. Many NAFLD patients will also develop steatohepatitis, fibrosis, and/or cirrhosis, which are associated with increased liver-related morbidity and mortality. At present, the ability to predict which NAFLD patients are more likely to develop coincident inflammation and fibrosis and/or cirrhosis is limited by an incomplete understanding of the pathogenesis underlying progression to more severe manifestations of the disease.

Translational Significance

The findings reported here yield new insights into the pathologic mechanisms underlying the development of fibrosis and cirrhosis in NAFLD.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of conditions resulting from excessive accumulation of fat in hepatocytes (ie, steatosis) not because of overconsumption of alcohol. Obesity, type 2 diabetes (T2D), and insulin resistance often contribute to the development of NAFLD and many patients with these disorders will have increased hepatic fat storage, a condition known as steatosis. A subset of these patients will also develop steatohepatitis, fibrosis, and/or cirrhosis, collectively representing nonalcoholic steatohepatitis (NASH), an advanced form of NAFLD that is associated with increased liver-related morbidity and mortality.[1] NAFLD can be categorized into nonalcoholic fatty liver representing hepatic steatosis and NASH.[2] Although clinical outcomes for NAFLD patients with coincident hepatocyte injury, liver inflammation, and fibrosis are substantially worse compared with those with hepatic steatosis,[3,4] there are currently no accurate laboratory measurements or clinical characteristics that predict disease severity in NAFLD patients. In addition, the molecular mechanisms contributing to the heterogeneous outcomes of NAFLD remain poorly understood. At present, the ability to predict which NAFLD patients are more likely to develop coincident inflammation and fibrosis and/or cirrhosis is limited by an incomplete understanding of the pathogenesis underlying progression to more severe manifestations of the disease.

NAFLD can develop in response to environmental and genetic factors, and recent studies also suggest a strong role for epigenetic influences, such as microRNAs (miRNAs) in the pathogenesis of the disease. MiRNAs are endogenous, single-stranded RNAs that regulate gene expression primarily through post-transcriptional mechanisms and less commonly via transcriptional targeting of the promoter region.[5] In humans, miRNAs silence the expression of target genes predominantly at the post-transcriptional level by imperfectly base-pairing to the 3′ untranslated region of target messenger RNAs (mRNAs), leading to translational inhibition and/or mRNA deadenylation and decay.[5] Several miRNAs have been shown to play a role in the pathogenesis of NAFLD in animals. Expression levels of miR-122, miR-451, miR-27a, miR-429, and miR-200a/b were dysregulated in rats with diet-induced NASH compared with animals fed a standard diet.[6] Altered levels of several miRNAs, including miR-146a, miR-210, miR-29c, miR-103, miR-20b-5p, miR-106b, miR-212, miR-31, miR-10a, miR-203, miR-27b, miR-199a, miR-107, let-7b, miR-33, miR-145, miR-196b, miR-93, let-7d, and miR-19 were found to differentiate between steatohepatitis and steatosis in diet-induced NASH.[7] Other studies in animals have identified a number of miRNAs associated with NAFLD and related outcomes[8-14]; however, very few miRNAs have been replicated across studies. Discrepancies in findings are most likely because of differences in dietary composition and regimen, phenotypic endpoint, and strain of animal used.

In humans, investigations of NAFLD-related miRNAs have been conducted using both liver tissue and serum. In one of the first studies of NAFLD-related miRNAs, Cheung et al[15] observed differences in hepatic levels of miR-34a, miR-146b, and miR-122 between patients with NASH and individuals with normal liver histology, although none of these miRNAs were associated with differences in disease severity. Another investigation of 84 circulating miRNAs reported upregulated serum levels of miR-122, miR-192, miR-19a/b, miR-125b, and miR-375 in patients with either simple steatosis or NASH.[16] However, similar to the results from animal studies, there has been little replication of findings across studies in humans. Some of the inconsistencies among findings may be attributed to differences in study design and/or method of statistical evaluation, as well as clinical variability between cases and controls. In addition, different microarrays

interrogate different populations of miRNAs, oftentimes resulting in limited overlap between studies.

High-throughput sequencing approaches circumvent the limitations of microarrays by providing unbiased measurements of expression levels of all miRNAs, known and unknown. To date, a high-throughput sequencing strategy has not yet been implemented in the discovery of NAFLD-relevant miRNAs, although such an approach represents a powerful strategy for identifying new candidates related to the disease process. We undertook this study, therefore, to determine whether any previously uncharacterized miRNAs contribute to the development of NAFLD-related fibrosis, focusing on this particular phenotype to lessen the potential impact of clinical variability from more heterogeneous, intermediate levels of fibrosis. We performed miRNA sequencing in a well-characterized group of individuals with (F3–F4) and without (F0) advanced NAFLD fibrosis, matched for age, sex, body mass index (BMI), T2D status, hemoglobin A1c (HbA1c), and diabetes medication use. We observed several miRNAs showing statistically significant evidence for differential expression between cases and controls, and validated a number of candidates showing the strongest changes using quantitative polymerase chain reaction (PCR). The findings reported here are concordant with a role for miRNAs in mediating changes that accompany the development of fibrosis in NAFLD patients.

MATERIALS AND METHODS

Study sample. Study participants comprised Caucasian individuals enrolled in the Bariatric Surgery Program at the Geisinger Clinic Center for Nutrition and Weight Management. All research participants had undergone a standardized, multidisciplinary preoperative program in which a large amount of clinical data and biospecimens were collected.[17] In all participants, wedge biopsies of the liver were obtained intraoperatively from similar anatomic locations. The tissue was sectioned so that one-fourth to one-third of it was submerged directly in RNA*later* (Applied Biosystems/Ambion, Austin, Texas) and stored at −80°C for subsequent miRNA sequence analysis, and the remainder was fixed in neutral buffered formalin, stained with hematoxylin and eosin, and histologically evaluated as part of clinical standard of care using NASH CRN criteria,[18] as previously described.[19] Patients with histologic or serologic evidence for other chronic liver diseases were excluded from this study. Clinically significant alcohol intake was determined via a 1–2 hour comprehensive clinical interview and psychological evaluation that were conducted by certified psychology professionals and included a multiquestion determination of drugs, alcohol, and smoking behaviors. Patients who were assessed to manifest either definite or possible evidence of addictive behaviors were not allowed to proceed to surgery. In addition, we also evaluated available clinical data, including diagnostic ICD-9 codes, medical, and medication history, for any indication of drug or alcohol abuse, and if identified, those patients were removed from the study. Following medical history, psychological evaluation, and histologic assessment, individuals with clinically significant alcohol intake and drug use were also excluded from participation in the program.

Variables were obtained from an electronic database as described previously,[17] and included basic clinical measures, demographics, diagnostic ICD-9 codes, medical and medication history, and common laboratory results. Individuals were selected for inclusion in the sample based on histologically defined liver fibrosis stage. Cases were defined by the presence of liver fibrosis stage 3 or 4 (F3–F4), whereas controls were defined as those showing no evidence of liver fibrosis (ie, liver fibrosis stage 0 or F0) and were otherwise histologically normal. Cases and controls were matched for sex, age, BMI, T2D status, HbA1c levels, and use of diabetes medications (33% of F0 and 67% of F3–F4 patients were taking diabetes medications at the time of biopsy). Demographic data and laboratory measures were obtained from all patients at the time of liver biopsy. The research was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The Institutional Review Boards of the Geisinger Clinic and the Translational Genomics Research Institute approved the research, and all participants provided written informed consent.

miRNA sequencing analysis, quality control, quantification, and normalization. Frozen liver tissue from 30 NAFLD patients with fibrosis stage F0 or F3–F4 was minced and resuspended in Cell Disruption Buffer from the mirVana PARIS RNA and Native Protein Purification Kit (Life Technologies; Grand Island, New York) according to the manufacturer's protocol, and then homogenized in conjunction with a S220 ultrasonicator (Covaris, Inc, Woburn, Massachusetts). We extracted miRNA using the mirVana miRNA Isolation Kit (Life Technologies) according to the manufacturer's protocol and quantified products using the Quant-iT RiboGreen RNA Assay Kit (Life Technologies) before sequencing. We sequenced samples using the Illumina HiSeq 2000 (Illumina, San Diego, California) sequencing platform for miRNA-Seq. Small RNA libraries were loaded onto the sequencer and sequenced for 50 cycles on a single-read flow cell.

One lane of the flow cell was dedicated to a PhiX control to account for the low amount of nucleotide diversity in small RNA samples and to allow for the calculation of phasing and prephasing of the sequenced sample. Samples were sequenced to the same approximate depth/number of reads to allow quantitative comparisons to be made between samples. Sequencing data were processed using the Illumina pipeline CASAVA v1.8.2 to generate raw fastq reads. We performed quality control checks on raw sequence data using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). All data passed FastQC quality check. To ensure that fastq reads were in normal range (green tick ≥Q28), we discarded reads containing sequencing errors. We also performed other prealignment processing steps (ie, adapter clipping and read collapsing) using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit). After quality checks, we discover that 2 F0 samples did not yield usable data and 3 F0 samples showed a low number of mapped reads (<100,000). These 5 samples were removed from further analysis.

We used the miRDeep2 software package for the identification of novel and known miRNAs from deep sequencing data.[20] We used the Mapper module to process raw sequences and map processed reads to the reference genome (GRCh37/hg19). Once aligned, the miRDeep2 module excised genomic regions covered by the sequencing data to identify probable secondary RNA structure. Potential precursors were evaluated and scored based on their likelihood of being genuine miRNAs. The quantifier module produced a scored list of known and novel miRNAs with quantification and expression profiling. We used recommended default parameters and allowed one single nucleotide variation.

We measured the expression level of miRNAs generated by high-throughput sequencing as the number of sequenced fragments mapped to the miRNA transcript, which we expected to correlate directly with its abundance level. To identify miRNAs that were differentially expressed, we used DESeq2[21] and Kruskal-Wallis, comparing F0 and F3–F4, and adjusting for age, T2D status, HbA1c, and BMI. We corrected for multiple comparisons using the Bonferroni method. The level of statistical significance, represented by corrected *P* value, and the degree of fold change were used to identify miRNAs showing the strongest differential expression between phenotypic categories.

Analysis of individual miRNAs using quantitative reverse-transcription PCR. To analyze individual miRNAs, we performed quantitative reverse-transcription PCR (RT-qPCR) using miRNA from the same liver samples used in miRNA sequencing. We converted miRNA to complementary DNA using the TaqMan MicroRNA Reverse-Transcription Kit according to the manufacturer's protocol (Life Technologies). We assessed miRNA levels using TaqMan MicroRNA Assay primers in conjunction with ABI Prism 7900 HT Sequence Detector apparatus (Life Technologies). Assay names and mature sequences for the validated miRNAs are shown in Supplementary Table S1. All assays were performed in triplicate. Cycle threshold values were generated using Expression Suite Software v1.0 (Life Technologies) and means were normalized against miR-26a, which showed stable levels among all samples. The $-\Delta\Delta$Ct method was used to determine fold change of miRNA expression between samples.

Validation of FOXO3 and FBXW7 transcript and protein levels. For prediction of target genes for miR-182, we interrogated the miRanda,[22,23] miRWalk,[24] and miRTarBase[25] databases and used a combination of overlap analysis, relevance to liver fibrosis, and literature mining to select specific genes for functional analysis. We performed first-strand complementary DNA synthesis from 100 ng of total RNA using the TaqMan RNA-to-Ct 1-Step kit and TaqMan Gene Expression Assays according to the manufacturer's protocol, followed by qPCR analysis as described previously. Ct values of *FOXO3* and *FBXW7* were normalized using glyceraldehyde-3-phosphate dehydrogenase.

We determined protein concentrations in tissue lysate preparations using the bicinchoninic acid assay protocol (ThermoFisher Scientific). We used a commercial sandwich enzyme-linked immunosorbent assay kit (Cusabio; Wuhan, China) to determine concentrations of FOXO3 and FBXW7 protein in liver tissue lysate.

Pathway and functional enrichment analysis. We used the Ingenuity Pathways Analysis software (Qiagen, Redwood City, California; www.qiagen.com/ingenuity) to identify canonical signaling pathways and establish network connections between all dysregulated miRNAs and their respective predicted targets. The miRNA target prediction was limited to experimentally validated targets in humans present in the Ingenuity Pathways Analysis library (Inequity Expert Findings, Inequity ExpertAssist Findings, miRecords, TarBase, and TargetScan Human) and expressed in liver tissue. The significance of the association between miRNA targets and the canonical pathway was assessed using 2 criteria: (1) the ratio of the number of molecules mapped to the pathway and total number of molecules involved in the canonical pathway and (2) the Benjamini-Hochberg corrected *P* value from the right-tailed Fisher Exact test.

Table I. Patient demographics and clinical characteristics Q28

	F0 (N = 15)	F3–F4 (N = 15)	*P* value
Female, n (%)	15 (1.00)	15 (1.00)	NS
Mean age in years at biopsy (SD)	47.1 (6.3)	51.3 (8.2)	NS
BMI at biopsy (SD)	41.0 (4.6)	44.4 (6.6)	NS
Diabetes mellitus, n (%)	6 (0.40)	6 (0.40)	NS
Laboratory measures, mean (SD)			
Serum AST, U/L	23.2 (4.2)	47.5 (23.3)	0.0004
Serum ALT, U/L	23.7 (8.1)	47.3 (26.5)	0.0027
AST/ALT, U/L	1.0 (0.3)	1.0 (0.3)	NS
Alkaline phosphatase	71.2 (15.9)	91.6 (80.5)	NS
Total bilirubin	0.49 (0.24)	0.47 (0.18)	NS
Glucose (mg/dL)	100.9 (31.9)	120.2 (53.5)	NS
Insulin	18.0 (13.2)	39.7 (29.8)	0.0155
HbA1c, % (SD)	6.0 (1.0)	6.6 (1.3)	NS
Triglycerides	142.9 (64.5)	186.9 (67.2)	0.04
Total cholesterol	179.7 (26.1)	197.9 (33.9)	NS
LDL-C	107.3 (14.4)	116.5 (17.2)	NS
HDL-C	55.9 (28.3)	46.7 (32.4)	NS
Histologic characteristics			
Steatosis, mean grade (SD)	0	2.1 (0.7)	<0.0001
Lobular inflammation, % = grade 1	0	0.47	<0.0001
Lobular inflammation, %>grade 2	0	0.4	<0.0001
Portal inflammation, (%)	0	0.4	<0.0001
Ballooning (mean score)	0	1.3 (0.7)	<0.0001

Abbreviations: ALT, alanine aminotransferase; *AST,* aspartate aminotransferase; *BMI,* body mass index; *HbA1c,* hemoglobin A1c; *NS,* nonsignificant; *SD,* standard deviation. Q29

RESULTS

Study sample characteristics. Two groups of biopsy-proven NAFLD patients comprised the study sample: 15 individuals with stages 3 and 4 (F3–F4) of NAFLD fibrosis and 15 without fibrosis (F0). The demographic data and clinical characteristics of the NAFLD patients comprising the study sample are shown in Table I. As expected, individuals with NAFLD fibrosis had higher levels of aspartate aminotransferase and alanine aminotransferase compared with individuals without fibrosis. Fibrosis cases also had significantly higher levels of insulin and triglycerides compared with controls. Histologic features representing disease severity showed significant differences between the 2 groups. NAFLD patients with fibrosis had greater lobular inflammation, portal inflammation, and hepatocyte ballooning compared with individuals without fibrosis.

Differentially expressed hepatic miRNAs NAFLD-related fibrosis. We used high-throughput sequencing to examine differences in hepatic miRNA expression between 15 F0 and 15 F3–F4 NAFLD patients. Of these, 2 F0 samples did not yield usable data and 3 F0 samples were removed because of a low number of mapped reads (<100,000). Therefore, the total number of samples analyzed comprised 15 F3–F4 and 10 F0. Sequencing results of these samples detected a total of 777 miRNAs with normalized average count across all samples >1, of which >19.7% showed dysregulated expression (fold change ≥1.0, ie, doubling or halving of expression). The differential expression analysis resulted in 75 miRNAs significant at adjusted *P* value <0.05, at varying level of expression. Results from hierarchical clustering analysis including all differentially expressed miRNAs are shown in Supplementary Fig S1.

Using a base mean ≥5.0, a Bonferroni-corrected *P* value <0.05, and a 1-fold $\log_2$ (F3–F4/F0) expression difference as a cutoff, 43 miRNAs showed statistically significant evidence for differential expression in liver samples from patients with NAFLD fibrosis. Among these, 29 were downregulated and 14 were upregulated (Supplementary Table S2). Among the downregulated species, the 10 most differentially expressed miRNAs were miR-219a, miR-373, miR-378c, miR-590, miR-3611, miR-376b, miR-186, miR-17, miR-1286, and miR-5699 (Table II). Among the upregulated species, the 10 most differentially expressed miRNAs were miR-183, miR-31, miR-150, miR-182, miR-200a, miR-224, miR-92b, miR-3613, miR-708, and miR-766 (Table III).

Validation of selected miRNAs by RT-qPCR. To validate the differential expression of hepatic miRNAs in NAFLD fibrosis identified by sequencing, we selected several miRNAs to analyze based on fold change, statistical significance, and biological relevance for RT-qPCR

Table II. Top microRNAs (miRNAs) showing significant downregulation in hepatic expression in nonalcoholic fatty liver disease fibrosis

Name	Base mean*	Fold change†	*P* value	Adjusted *P* value‡
hsa§-mir-219a-2	7.0	−2.54	1.55×10^{-4}	4.68×10^{-3}
hsa-mir-373	7.4	−2.48	2.24×10^{-5}	1.08×10^{-3}
hsa-mir-378c	1252.7	−2.33	3.68×10^{-8}	8.91×10^{-6}
hsa-mir-590	5.5	−2.32	5.64×10^{-4}	1.24×10^{-2}
hsa-mir-3611	9.3	−2.31	6.45×10^{-4}	1.30×10^{-2}
hsa-mir-376b	7.3	−2.17	1.29×10^{-3}	2.02×10^{-2}
hsa-mir-186	6.2	−2.16	7.84×10^{-4}	1.39×10^{-2}
hsa-mir-17	133.1	−2.04	1.53×10^{-6}	1.12×10^{-4}
hsa-mir-1286	5.4	−1.98	3.30×10^{-3}	3.65×10^{-2}
hsa-mir-5699	9.7	−1.92	7.65×10^{-4}	1.39×10^{-2}
hsa-mir-378i	704.5	−1.89	2.55×10^{-7}	4.63×10^{-5}

*Base mean: average of normalized count values, taken over all samples.
†$\log_2$ ratio of miRNA (F3–F4)/miRNA (F0).
‡Bonferroni-corrected *P* value.
§hsa refers to Homo sapiens-specific miRNA per the standard miRNA nomenclature system.

Table III. Top microRNAs (miRNAs) showing significant upregulation in hepatic expression in nonalcoholic fatty liver disease fibrosis

Name	Base mean*	Fold change†	*P* value	Adjusted *P* value‡
hsa-mir-183	27.1	1.99	4.23×10^{-10}	1.54×10^{-7}
hsa-mir-31	35.1	1.77	1.10×10^{-6}	9.95×10^{-5}
hsa-mir-150	4516.7	1.69	6.92×10^{-7}	8.96×10^{-5}
hsa-mir-182	1130.5	1.68	5.69×10^{-11}	4.14×10^{-8}
hsa-mir-200a	12.3	1.66	1.46×10^{-5}	8.17×10^{-4}
hsa-mir-224	189.0	1.62	7.40×10^{-7}	8.96×10^{-5}
hsa-mir-92b	268.5	1.30	4.00×10^{-5}	1.71×10^{-3}
hsa-mir-3613	6.9	1.25	2.58×10^{-3}	3.11×10^{-2}
hsa-mir-708	8.3	1.20	5.96×10^{-4}	1.28×10^{-2}
hsa-mir-766	14.3	1.17	5.46×10^{-4}	1.24×10^{-2}

*Base mean: average of normalized count values, taken over all samples.
†$\log_2$ ratio of miRNA (F3–F4)/miRNA (F0).
‡Bonferroni-corrected *P* value.

analysis. These miRNAs included miR-182, miR-183, miR-150, miR-31, miR-224, miR-200a, miR-378c, miR-378i, miR-17, miR-373, miR-590, and miR-219a. As shown in Fig 1, *A*, we confirmed upregulation of miR-182, miR-183, miR-150, miR-31, and miR-224, but not miR-200a. We also confirmed significant downregulation of miR-378i, miR-17, miR-590, and miR-219a, but not miR-378c and miR-373 (Fig 1, *B*).

Functional enrichment analysis. We used functional enrichment analysis to identify pathways potentially affected by dysregulated hepatic miRNAs. The core analysis of the predicted targets resulted in 110 pathways with $-\log$(adjusted *P* value) >1.36, out of which we highlighted 9 because of their potential association with disease pathogenesis including hepatic fibrosis, hepatic stellate cell activation, TGFB signaling, and apoptosis (Table IV).

Expression changes in downstream targets of miR-182 in fibrotic liver. Because of the known role of miR-182 in mechanisms related to hepatocellular carcinoma,[26-28] we sought to investigate this miRNA in NAFLD-related fibrosis by looking at relevant target genes. We used 3 algorithms (miRanda,[22,23] miRWalk,[24] and miRTarBase[25]) to identify potential mRNA targets of miR-182, and found FOXO3 and FBXW7, both of which have been implicated in hepatic metabolism.[29,30] We measured levels of both FOXO3 and FBXW7 in F0 and F3–F4 samples, and as shown in Fig 2, *A* and *B*, we observed a significant decrease in both transcript and protein levels of FOXO3 ($P = 0.0001$), respectively. In contrast, we did not find any significant differences in FBXW7 expression (Fig 2, *C* and *D*, respectively) between cases and controls.

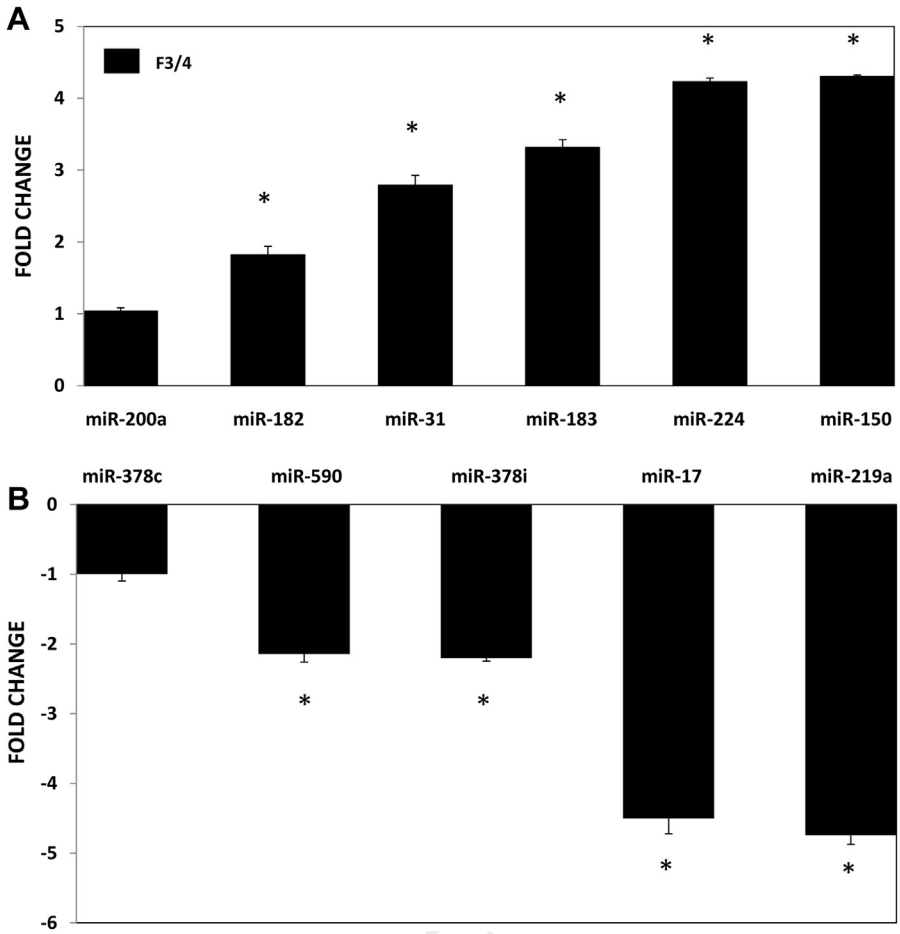

Fig 1. Validation of hepatic expression of individual miRNAs by RT-qPCR. (**A**) miRNAs showing upregulated expression in fibrotic liver. (**B**) miRNAs showing downregulation in fibrotic liver. miRNAs were extracted from biopsied liver tissue from the same 30 individuals comprising the discovery cohort as described in the Methods section. Relative quantification of each miRNA was performed by TaqMan RT-qPCR. Results are shown as F3–F4 fold change relative to F0 levels, which were set to a value of 1. All results represent averages from 3 independent experiments, normalized against miR-26a. Data are expressed as the means ± standard deviation. miRNA, microRNA; RT-qPCR, quantitative reverse-transcription polymerase chain reaction. Q25

DISCUSSION

It is now widely accepted that the dysregulation of miRNA expression or action contributes to the development of many different human diseases including certain cancers,[6] neurologic disorders,[7] and heart disease.[8] In this study, we used high-throughput sequencing to identify differentially expressed hepatic miRNAs associated with NAFLD-related fibrosis. We observed evidence for differential expression of 43 hepatic miRNAs in biopsy-proven NAFLD fibrosis and confirmed dysregulation of 10 of these, including miR-182, miR-183, miR-150, miR-31, miR-224, miR-378c, miR-378i, miR-17, miR-590, and miR-219a. To our knowledge, this is the first study to apply a high-throughput sequencing approach to the investigation of hepatic miRNAs in NAFLD-related fibrosis.

Some of the present results corroborate findings of dysregulated levels of miRNA identified in other studies. In one study, hepatic levels of miR-200a and miR-224 were upregulated 1.25-fold and 1.35-fold, respectively, in individuals with NASH.[15] Expression of miR-150 was dysregulated in visceral adipose tissue in NAFLD patients, although in this study, levels were decreased (−1.86-fold) in contrast to the results reported here.[31] This discrepancy may reflect effects of tissue-specific differential expression, although additional studies will be necessary to address this possibility. Hepatic levels of miR-224, miR-17-5p, and miR-378 were also dysregulated in *ob/ob* mice.[9] Interestingly, the strongest replication was found with miR-182, which has been previously associated with hepatocellular carcinoma.[26-28] Dolganiuc et al[32] observed

Table IV. Functional enrichment analysis results

Pathway	−log(adjusted *P* value)	Molecules
EMT regulation	9.60	MAP2K4, RELA, ID2, FZD3, SMAD3, PIK3R1, HIF1A, FGFR3, TGFBR2, SMO, SMAD4, CTNNB1, FGF7, ETS1, SMAD2, FGF16, MAP2K7, PIK3C2A, ZEB1, MET, RHOA, ZEB2, FGFRL1, JAG1, NOTCH1, TCF7L2, MMP9, WNT5A
Apoptosis signaling	8.50	TP53, MAP2K4, RELA, MAP2K7, APAF1, PLCG1, IKBKE, BAX, FAS, BAK1, CASP6, CAPN8, PLCG2, TNFRSF1B, TNF, MAP4K4, CASP7, BCL2L11, FASLG
Hepatic fibrosis/hepatic stellate cell activation	5.18	RELA, SMAD2, CCR5, CTGF, SMAD3, IL6R, BAX, FAS, PDGFB, TGFBR2, MET, VEGFA, ACTA2, IGFBP3, IGF1R, SMAD4, CD14, ILIB, TNFRSF1B, TNF, MMP9, AGTR1, FASLG
TGFB signaling	4.07	TGFBR2, MAP2K4, SMAD2, JUN, RUNX2, SMAD3, ACVR1, SMAD4, BMPR2, BMP7, SMAD5, BMPR1B, SMAD1, ACVR1B
Hepatic cholestasis	3.46	MAP2K4, PRKACB, PPARA, RELA, MYD88, ADCY6, IKBKE, IL13, PRKC1, JUN, ILIRN, NR1I2, CD14, IL1B, TNFRSF1B, TNF, ESR1, IL11
IGF-1 signaling	2.74	PRKACB, SOCS1, JUN, PRKC1, CTGF, FOXO1, PIK3C2A, PIK3R1, FOXO3, IGFBP3, IGF1R, CYR61, SOCS5
T2D signaling	2.00	MAP2K4, SOCS1, RELA, MAP2K7, PIK3C2A, PIK3R1, IKBKE, CEBPB, MTOR, PRKC1, TNFRSF1B, TNF, SOCS5
Growth hormone signaling	1.98	SOCS1, PRKC1, PIK3C2A, PLCG2, PIK3R1, IGF1R, IGFBP3, PLCG1, RPS6KA5, SOCS5
ERK/MAPK signaling	1.92	PRKACB, ETS1, PIK3C2A, PPP2R2A, PIK3R1, ITGA2, PLCG1, ITGA5, RPS6KA5, MYC, TLN2, PRKC1, PPP2R4, PLCG2, CREB1, ATF4, ESR1

hepatic upregulation of miR-182 and its miR sibling, miR-183, in a mouse model of diet-induced NASH compared with control animals. In mice, miR-182, miR-183, and miR-96 are expressed from a unique primary transcript,[33] which is highly conserved in humans and directly activated by sterol regulatory element binding protein 2.[29] Conversely, miR-182 was shown to regulate sterol regulatory element binding protein expression through the direct inactivation of its target gene, *Fbxw7*, thereby contributing to a regulatory loop for intracellular lipid homeostasis.[29] However, when we investigated hepatic Fbxw7 levels, we did not observe significant differences in the expression between fibrotic and nonfibrotic tissue, suggesting that other mechanisms may compensate for reduced expression of this gene.

We identified *FOXO3* as a potential target gene of miR-182. Previous studies have shown that deletion of *FOXO3* increases hepatic lipid secretion and causes hepatosteatosis.[30] Increased FOXO3 levels are associated with upregulation of the proapoptotic BH3-containing protein, PUMA, resulting in cholangiocyte injury through lipoapoptosis in NAFLD and NASH patients.[34] FOXO3 may also protect against other forms of liver disease[35,36] and regulate hepatic glucose metabolism.[37] Although miR-182 is recognized as an important regulator of FOXO3 expression in skeletal muscle and lung cancer cells,[38,39] a role for this miRNA/mRNA relationship has not been demonstrated in NAFLD-related fibrosis. We observed significant downregulation of FOXO3 transcript and protein levels in fibrotic compared with nonfibrotic liver ($P > 0.0001$), suggesting a potential role for this gene in fibrogenesis. Additional studies, including validation of a direct interaction between miR-182 and FOXO3 and knockdown/overexpression experiments will be necessary to confirm this relationship.

The role of tissue-specific and circulating miRNAs in NAFLD has been explored in previous studies (Table V). Cheung et al[15] profiled liver miRNA in 15 individuals with NASH and 15 individuals with normal liver histology and found that out of the 474 miRNAs represented on the array, only 6 showed differential expression between the 2 groups. Of these, upregulation of miR-34a and miR-146b and downregulation of miR-122 were confirmed using RT-PCR. However, in this study, miRNA levels were not associated with NASH severity, suggesting a role in biological processes not related to liver fibrosis. A similar analysis profiled miRNA expression in visceral adipose tissue of patients with NAFLD.[31] A total of 113 miRNAs were differentially expressed between groups, but only 7 of these remained significant after correction for multiple testing. In an investigation of circulating serum miRNAs, Pirola et al[16] observed significantly upregulated levels of miR-122, miR-192, miR-19a, miR-19b, miR-125b, and miR-375 in patients with either simple steatosis or NASH. Levels of miR-122 and miR-192 showed the most dramatic changes in NASH patients vs controls.

As shown in Table V, results from miRNA studies in NAFLD performed to date have been largely inconsistent. Lack of reproducibility among studies likely stems from a number of variables including differences in study design, clinical variability between cases and

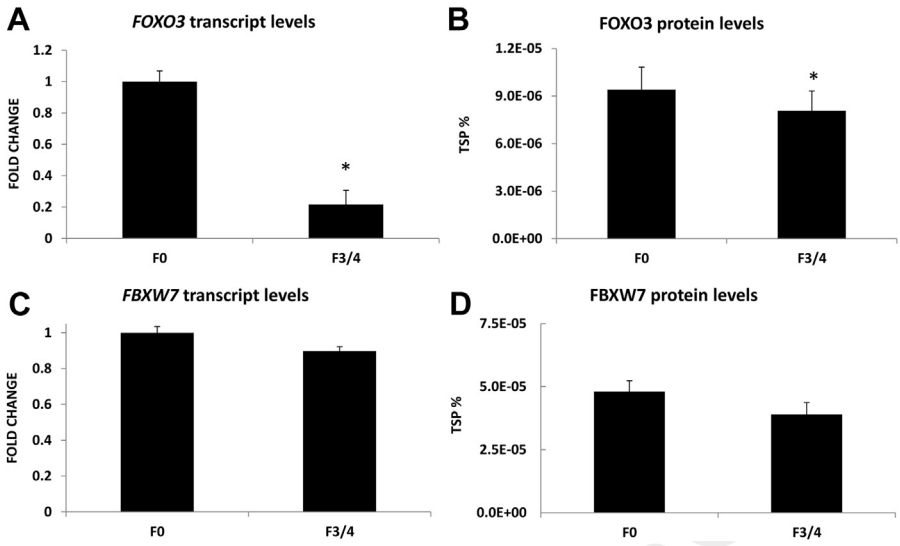

Fig 2. Hepatic expression of FOXO3 (**A**) and FBXW7 (**C**) transcript and of FOXO3 (**B**) and FBXW7 (**D**) protein. Total RNA and protein were extracted from the biopsied liver tissue from the same individuals comprising the discovery cohort as described in Methods section. Relative quantification mRNA was performed by TaqMan RT-qPCR and normalized against ACTB. Protein concentrations were assessed via enzyme-linked immunosorbent assay and are depicted as percentage of total soluble protein (TSP). Results represent averages from 3 independent experiments. Data are expressed as the means ± standard deviation. mRNA, messenger RNA; RT-qPCR, quantitative reverse-transcription polymerase chain reaction.

Table V. Summary of miRNAs previously associated with NAFLD-related parameters

Population	Phenotype	Method	Source	Major miRNAs identified	Reference
Caucasian†	NASH	Array	Liver	↑**miR-34a**, miR-146b ↓**miR-122**‡	15
Caucasian†	NASH	Array	Adipose tissue	↓miR-132, miR-150, miR-433, miR-28-3p, miR-511, miR-517a, miR-671, miR-197, miR-99b	31
Unknown	NAFLD	qPCR	Serum	↑**miR-122**, **miR-34a**, miR-16	40
Chinese	NAFLD	RT-PCR	Serum	↑miR-15b	14
Unknown	NAFLD	Array	Serum	↑**miR-122**, miR-192, miR-19a, miR-19b, miR-125b, miR-375	16
Caucasian	NAFLD fibrosis	NGS†	Liver	↑miR-182, miR-183, miR-150, miR-224, miR-200a, miR-31 ↓miR-378c, miR-17, miR-373	Present study

Abbreviations: miRNA, microRNA; *NAFLD,* nonalcoholic fatty liver disease; *NASH,* nonalcoholic steatohepatitis; *RT-qPCR,* quantitative reverse-transcription polymerase chain reaction.
*Whole genome next generation sequencing.
†≥80% Caucasian.
‡Bold font represents miRNAs identified in more than one study.

controls, methods of statistical evaluation, and experimental approaches. Each study used a different technology platform with a dissimilar representation of selected miRNAs, which presents significant difficulties for interstudy comparisons. For example, Pirola et al[16] used the MIHS 106Z PCR array, which interrogates 84 miRNAs. Of the 12 validated miRNAs in the present study, only 4 (miR-150, miR-224, miR-200a, and miR-17) were present on this array. The other studies were published several years ago, when far fewer miRNAs had yet been discovered, including many of the miRNAs identified in the present study. Differences in source miRNA may also contribute to poor reproducibility among studies because each of the studies published to date analyzed miRNA from different sources (ie, liver, visceral adipose tissue, and serum). Like Cheung et al,[15] we used liver-derived miRNA in our analyses. However, the lack of corroboration between studies is not surprising, given that, unlike the present study, the authors excluded patients with extreme fibrosis and cirrhosis and used normal, healthy individuals as controls. Overall, the present study differed from previous reports by implementing a high throughput, unbiased sequencing approach that did not rely on a

preselected panel of miRNAs for interrogation, analyzing a carefully stratified study sample comprising biopsy-proven NAFLD with or without fibrosis, and using human liver as the source for miRNA.

Despite the significance of these findings, we recognize that the present study has some limitations that are important to acknowledge. First, this study comprises solely of obese females who are candidates for gastric bypass surgery and in whom the pathophysiology of hepatic fibrosis may be unique. However, we designed this study sample for 2 reasons: (1) obesity is a significant risk factor for worse liver-related outcomes in NAFLD,[41] and (2) the value of obtaining liver tissue from individuals without clinical indication, which effectively removes bias toward clinically suspect liver disease. Despite these considerations, conclusions with regard to NAFLD-related fibrosis obtained from this group may not be readily relevant to other populations without additional validation.

Second, the sample size in the present study, although comparable with other reports,[15,31] is still considered small, which may limit our ability to detect real differences in miRNA levels. For this reason, we consider this study an exploratory one and acknowledge that validation in a larger, independent dataset will be necessary to confirm our findings. In addition, we acknowledge that the stratification of the study sample into phenotypic extremes does not allow assessment of intermediate levels of perisinusoidal fibrosis (F1A and F1B) or portal fibrosis (F1C). We dichotomized our cohort into extreme histologic phenotypes to increase our power to identify clinically relevant differences in miRNA levels. Additional studies including individuals spanning the spectrum of NAFLD fibrosis will be critical to further validate these findings. We also note that the cross-sectional design does not allow associations with disease progression to be drawn. In the absence of serial liver biopsies in the present cohort, we were not able to use a prospective design. Assessment of miRNA levels in longitudinal biopsies will be necessary to determine the roles of specific candidates in disease progression.

Finally, the results reported here do not allow us to make specific conclusions about miRNAs and biological pathways. Additional studies, including those showing direct interactions between miRNAs and target genes, and functional consequences of miRNA effects on putative targets, will be necessary to confirm the role of specific miRNAs in liver fibrosis.

CONCLUSIONS

The results obtained in the present study provide evidence of differential levels of a number of hepatic miRNAs in NAFLD-related fibrosis. These data may help to yield new insights into the pathologic mechanisms underlying the development of fibrosis and cirrhosis in NAFLD. Future investigations including validation in independent cohorts and functional characterization of miRNA gene targets and pathways will be important to extend these findings.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.trsl.2015.04.014.

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