

VIRAL HEPATITIS

microRNA changes in liver tissue associated with fibrosis progression in patients with hepatitis C

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Abstract

Background & Aims: Accumulating evidence indicates that microRNAs play a role in a number of disease processes including the pathogenesis of liver fibrosis in hepatitis C infection. Our goal is to add to the accruing information regarding microRNA deregulation in liver fibrosis to increase our understanding of the underlying mechanisms of pathology and progression. **Methods:** We used next generation sequencing to profile all detectable microRNAs in liver tissue and serum from patients with hepatitis C, stages F1–F4 of fibrosis. **Results:** We found altered expression of several microRNAs, in particular, miR-182, miR199a-5p, miR-200a-5p and miR-183 were found to be significantly upregulated in tissue from liver biopsies of hepatitis C patients with advanced fibrosis, stage F3 and F4, when compared with liver biopsies from patients with early fibrosis, stages F1 and F2. We also found miR-148-5p, miR-1260b, miR-122-3p and miR-378i among the microRNAs most significantly down-regulated from early to advanced fibrosis of the liver. We also sequenced the serum microRNAs; however, we were not able to detect significant changes in circulating microRNAs associated with fibrosis stage after adjusting for multiple tests. **Conclusions:** Adding measurements of tissue microRNAs acquired during routine biopsies will continue to increase our knowledge of underlying mechanisms of fibrosis. Our goal is that these data, in combination with studies from other researchers and future long-term studies, could be used to enhance the staging accuracy of liver biopsies and expand the surveillance of patients at increased risk for cancer and progression to advanced fibrosis.

Keywords

fibrosis – hepatitis C – liver – microRNA – sequencing

Chronic hepatitis C infection is the second most common cause of cirrhosis in the United States with approximately 130 million people infected worldwide and about three million in the United States (1). Approximately 7% of chronic hepatitis C patients develop cirrhosis after 20 years, and increases to 20% after 40 years

(2). The leading indication for liver transplantation in the United States is chronic hepatitis C infection (3).

Understanding which microRNAs are deregulated in the development of liver fibrosis and identifying the pathways affected will provide a better understanding of disease mechanisms (4–6). There have also been reports

Abbreviations

F1, F2, F3, F4, Fibrosis stage 1,2,3,4; miR, microRNA; miRNA, microRNA; ntaX, non-templated nucleotide additions.

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Key Points

- microRNAs regulate cell signalling cascades; the role of microRNAs in the pathogenesis of liver fibrosis is not well understood. We used a discovery-mode approach (sequencing) to examine microRNA deregulation in liver tissue from fibrosis stages 1–4 in hepatitis C patients.
- We identified a number of microRNAs that are expressed up or down with fibrosis.
- These microRNAs should be highlighted for continued research; to assess their involvement in underlying pathogenesis, as indicators of the disease progression and potentially the development of cancer.
- We also assessed microRNA isomiRs and microRNAs detected in serum from patients with liver fibrosis.

suggesting that circulating microRNAs may be useful biomarkers of hepatic fibrosis in chronic hepatitis C. Cermelli *et al.* (7), described a direct correlation between circulating levels of miR-122, miR-34a, and to a lesser degree miR-16, with fibrosis stage among patients with chronic hepatitis C infection. A recent publication from Shrivastava *et al.* (8), demonstrated that miR-20a and miR-92a were significantly up-regulated in sera from hepatitis C virus-infected patients; in particular, miR-20a was expressed at a higher level among patients with late fibrosis. Additionally, some microRNAs may act as biomarkers of downstream cancer risk or progression to cirrhosis; e.g. miR-183 has been shown to have high expression in diseased liver and its amplification may increase the risk for hepatocellular carcinoma (9–11).

We identified differentially expressed microRNAs in liver biopsies between early (F0–F2) and advanced (F3–F4) fibrosis stages in hepatitis C patients.

Patients and methods

Subjects

We studied 45 patients with chronic hepatitis C obtained from the Mayo and Scripps Clinics. Thirty-eight men and seven females, median age 60 years old (range: 50–76). Twelve patients had one liver biopsy sample and 33 patients had two biopsy samples (total samples, 78). Fibrosis stages were determined in liver biopsies according to METAVIR classification (12) and the distribution according to stage was as follows: F0 – I; F1 – 18; F2 – 15; F3 – 14, and F4 – 30 samples. All patients were serum HCV-RNA positive at the time of liver biopsy.

Approval

All patients were consented at the Mayo and Scripps Clinics and the Institutional Review Boards of both sites approved the collection of the samples.

RNA isolation

For liver samples, the miRVana kit (AM1560; Life Technologies) was used according to the manufacturer's protocol with a slight modification for two phenol chloroform extractions (Burgos *et al.*, 2013). RNA concentrations were measured using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). Serum samples (1–2 ml) were isolated using the same modified miRVana PARIS protocol (AM1556; Life Technologies) with sequential phenol–chloroform extractions (13).

Sequencing

The Illumina TruSeq small RNA Library preparation Kit (RS-200-0048; Illumina, San Diego, CA, USA) was used for sequencing all samples. Kit reagents were used in a half reaction (13). Each sample was assigned one of 48 different indices. Tissue samples were used with the kit at 200 ng, and 15 PCR cycles were used. For serum samples, the total RNA isolated from each sample volume (~1 mL) was used for small RNA sequencing. Indexed samples were run on a gel and purified away from the adaptor band. Fifteen to 20 samples were then pooled and placed on different lanes of a single read Illumina V3 flowcell (GD-401-3001). One lane of the flowcell was loaded with PhiX as a reference lane to help with low nucleotide diversity in microRNA.

Sequencing and statistical analysis

Reads that passed quality check were trimmed of their adaptor sequence then entered into the analysis pipeline. Samples were aligned using miRDeep2, and version 21 of MIRBASE (13). The isomiR detection and quantification was conducted through the sRNA workbench tool (14).

The miRNA read counts identified by miRDeep2 were normalized for compositional bias in sequenced libraries and library size using DESEQ2 (15) (version: 1.6.1). Samples with <100 000 mapped microRNA reads were removed from data, resulting in 43 tissue and 78 serum samples. Tissue: Stage F1 ($n = 9$), Stage F2 ($n = 9$), Stage F3 ($n = 10$), Stage F4 ($n = 15$); Serum: No Fibrosis ($n = 3$), Stage F1 ($n = 14$), Stage F2 ($n = 16$), Stage F3 ($n = 15$), Stage4 ($n = 30$). Differential expression of miRNA read counts was performed using the DESEQ2 (v1.6.1) package. For the differential expression analyses, miRNAs with average <5 counts across all samples were discarded.

Ordinal logistic regression and one-way ANOVA were used to assess monotonic relationships between fibrosis stage and normalized miRNA counts using the package ordinal (version: 2014.11-14). A graphical method for assessing the parallel slopes assumption was used to check ordinal logit requirements. Parameter confidence intervals were based on the profile likelihood function, and the estimates in the output are given in units

of ordered log odds. All analyses were performed using R version 3.1.1 (16).

We fitted a negative binomial generalized linear model for each genotype and carried out a likelihood ratio test in order to test for presence of differentially expressed microRNAs between any of the fibrosis stage groups. We found no differentially represented miRNAs with relationship to viral genotypes in varying fibrosis stages.

qPCR

TaqMan MicroRNA Reverse Transcription Kit and primers for miR-183-5p, miR-182-5p, miR-199a-5p and miR-148a-5p were run according to the manufacturer's protocol (Life Technologies) using an ABI 7900HT Fast Real-Time PCR system (Life Technologies). All qPCR reactions were performed in triplicate. Cycle threshold values for each target microRNA were normalized against U6. $2^{-\Delta\Delta Ct}$ was used to calculate the fold change of microRNA expression levels between (F0–F2) and advanced (F3–F4) fibrosis stages. A representative nine samples were chosen from each group for qRT-PCR validation. A *t*-test (two tailed, type 2) was performed to assess statistical significance between the normalized *Ct* values for the two groups.

Results

Using next generation sequencing technologies, we profiled microRNAs in liver biopsy samples from patients with hepatitis C infection. The degree of liver fibrosis was assessed by METAVIR criteria at Scripps. We obtained tissue samples from nine patients with stage F1 liver fibrosis, nine patients with stage F2, 10 patients with fibrosis stage F3, and 15 patients with stage F4. Thirty-three subjects had two liver biopsy samples. We first tested to see if there was an interaction between miRNAs and viral genotype. We did not find microRNAs in the tissue samples that were expressed differently with viral genotype, nor in association with viral genotype and fibrosis stage.

For tissue samples, the median number of reads mapped to microRNA per sample was 504 351, with a range from 121 504 to 5 708 614. The total number of microRNAs that were detected in liver tissue were 658, and those with a normalized count of ≥ 5 were 301. We obtained the following serum samples: three patients with stage F0 liver fibrosis, 13 patients with stage F1 liver fibrosis, 17 patients with stage F2, 15 patients with fibrosis stage F3 and 30 patients with stage F4. For the serum samples, the median number of reads mapped to microRNA was 2 084 067, with a range from 159 783 to 10 833 093. The total number of microRNAs that were detected in the serum were 549, and those with a normalized count of ≥ 5 were 397. Normalized counts for microRNAs detected in serum or tissue samples are displayed in Tables S1 and S2, microRNAs overlapping

Table 1. Differentially expressed microRNAs in tissue between early (F1 and F2) and advanced (F3 and F4) fibrosis. microRNAs listed have \log_2 fold change >0.60 and <-0.60 . Significant microRNAs with a smaller fold change are listed in Table S2

| microRNA | \log_2 -fold change | <i>P</i> -value | Adjusted <i>P</i> -value |
|-------------|-----------------------|-----------------|--------------------------|
| miR-183-5p | 1.2 | 3.20E-07 | 1.10E-04 |
| miR-182-5p | 1.04 | 9.09E-07 | 1.56E-04 |
| miR-200a-5p | 0.95 | 3.21E-06 | 3.68E-04 |
| miR-199a-5p | 0.74 | 1.16E-05 | 1.00E-03 |
| miR-429 | 0.89 | 4.54E-05 | 3.13E-03 |
| miR-148a-5p | -0.71 | 5.83E-05 | 3.34E-03 |
| miR-1260b | -0.8 | 1.17E-04 | 5.73E-03 |
| miR-199a-3p | 0.64 | 2.09E-04 | 7.20E-03 |
| miR-199b-3p | 0.64 | 2.09E-04 | 7.20E-03 |
| miR-200b-3p | 0.7 | 1.94E-04 | 7.20E-03 |
| miR-34c-5p | 1.02 | 2.63E-04 | 7.54E-03 |
| miR-150-5p | 0.72 | 3.41E-04 | 8.50E-03 |
| miR-200b-5p | 0.85 | 3.46E-04 | 8.50E-03 |
| miR-1260a | -0.74 | 5.57E-04 | 1.19E-02 |
| miR-200a-3p | 0.85 | 5.48E-04 | 1.19E-02 |
| miR-122-3p | -0.65 | 7.27E-04 | 1.26E-02 |
| miR-148a-3p | -0.66 | 7.67E-04 | 1.26E-02 |
| miR-378i | -0.76 | 6.81E-04 | 1.26E-02 |
| miR-10a-5p | 0.73 | 1.13E-03 | 1.76E-02 |
| miR-181d-5p | 0.61 | 1.38E-03 | 1.90E-02 |
| miR-5588-5p | -0.83 | 1.56E-03 | 1.98E-02 |
| miR-1296-5p | -0.72 | 2.95E-03 | 3.09E-02 |
| miR-192-3p | -0.65 | 2.84E-03 | 3.09E-02 |
| miR-31-5p | 0.74 | 3.06E-03 | 3.09E-02 |
| miR-452-5p | 0.62 | 2.98E-03 | 3.09E-02 |

in at least 30% of tissue and serum samples are highlighted.

We combined the microRNA data from liver biopsies classified as fibrosis F1 and F2 (early fibrosis) separately from F3 and F4 (advanced fibrosis). There were 18 samples in the early and 25 samples in the advanced fibrosis groups. We compared the microRNA between these two groups and corrected for multiple tests using Benjamini–Hochberg. There are 37 microRNAs differentially expressed between the two groups, Table 1 contains 25 significant microRNAs with a \log_2 -fold change >0.6 or <-0.6 . The remaining 12 microRNAs with smaller \log_2 -fold changes are listed in Table S3. We also assessed microRNAs differentially expressed between the two most extreme fibrosis stages, F1 and F4, 44 microRNAs were significantly different (Table S4).

The Volcano Plot in Fig. 1A displays significant microRNAs and their relative fold changes when comparing early (F1 and F2) to advanced (F3 and F4) fibrosis. miR-182 and miR-183 (green, \log_2 -fold change >1 and corrected *P*-value <0.05) and miR-200a-5p and miR-199a-5p (blue, \log_2 -fold change >0.6 and corrected *P*-value <0.05) have the greatest significance and fold change. miR-148a-5p, also pointed out in Fig. 1A, is expressed significantly lower in advanced fibrosis compared with early fibrotic stages. Box and whisker plots of normalized count data between early and

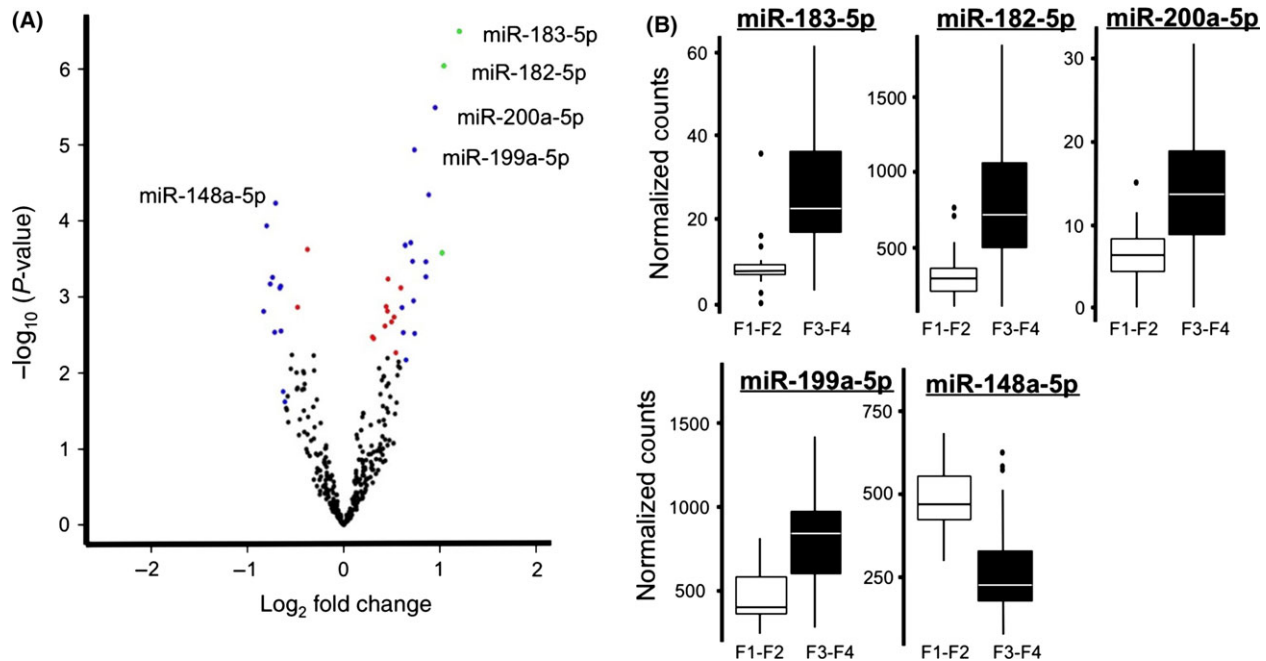


Fig. 1. Assessment of microRNAs differentially expressed in liver samples. (A) A Volcano plot of samples from F1 and F2 (early) liver fibrosis compared with samples from F3 and F4 (advanced fibrosis). \log_{10} P -value on the y-axis and \log_2 -fold change on the x-axis. microRNAs with a \log_2 -fold change >1 and significance <0.05 are indicated in green, microRNAs with fold change <-0.6 and >0.6 and adjusted P -value <0.05 are blue, smaller fold changes with adjusted P -value <0.05 are red. (B) Box and whisker plots for miR-183-5p, miR-182-5p, miR-200a-5p, and miR-199a-5p, the four microRNAs with the smallest P -values and largest fold changes. We also included miR-148a-5p, the most significantly down-regulated microRNA. The graph depicts the median and quartiles of the normalized counts for each group.

advanced fibrosis for five microRNAs are displayed in Fig. 1B.

We wanted to verify the sequencing results using a second assay platform, qRT-PCR. From liver tissue we chose the following microRNAs with increased expression in advanced fibrosis: miR-183-5p, miR-182-5p, miR-199a-5p and with decreased expression in advanced fibrosis, miR-148a-5p. We did not attempt to validate miR-200a-5p by qRT-PCR, the average count across samples was low, ~ 11 . Eighteen samples were reverse transcribed using specific TaqMan microRNA probes for the four selected microRNAs. Nine representative samples from the F1 and F2 group were compared with nine samples selected from the F3 and F4 group. Figure 2A shows the \log_2 -fold change between early and advanced fibrosis calculated using $2^{\Delta\Delta Ct}$ and U6 as the reference for normalization. microRNAs with an asterisk, miR-183-5p, miR-148a-5p and miR-199a-5p, were significantly different between early and advanced fibrosis ($P \leq 0.05$, t -test). Although the trend was the same as exhibited in the sequencing data, the change in the microRNA expression levels between early and advanced fibrosis was not significant for miR-182-5p in this subset of samples ($P \leq 0.34$; Fig. 2B).

We assessed the data using ordinal logistic regression analysis to determine if microRNA expression changes, either up or down, behaved in a progressive manner with increasing fibrosis from F1 to F4. We assessed all

microRNAs across each stage and found 35 microRNAs that indicated an ordered change in expression after multiple corrections (Table 2). miR-182-5p, miR-183-5p, miR-199a-5p, and miR-148a-5p were among the significant microRNAs that showed monotonic trend, continuous change with increasing fibrosis (Fig. S1).

We next examined serum samples to determine whether or not we could detect significant microRNA changes that indicated fibrosis stage. We assessed microRNAs that were detectable in both serum and liver (number = 495). We compared microRNA expression levels in patients with F0, F1 and F2 (combined, $n = 33$) to microRNA expression levels in patients with F3 and F4 ($n = 45$). We found 34 microRNAs that had P -value <0.05 , however, none of them survived Benjamini-Hochberg correction for multiple testing (Table S5).

By using next generation sequencing as our discovery platform, we were able to investigate the number and potential significance of variations in the mature microRNA sequence – isomiRs. We measured microRNA length heterogeneities and RNA editing. We categorized isomiRs as the following: 3' length variants (lv3p), 5' length variants (lv5p) and non-templated nucleotide additions (ntaA, ntaT, ntaC, ntaG). To provide a more detailed description of microRNA length heterogeneities, we indicated the number of nucleotides added (+) or trimmed (-) from the 3' or 5' end in the isomiR

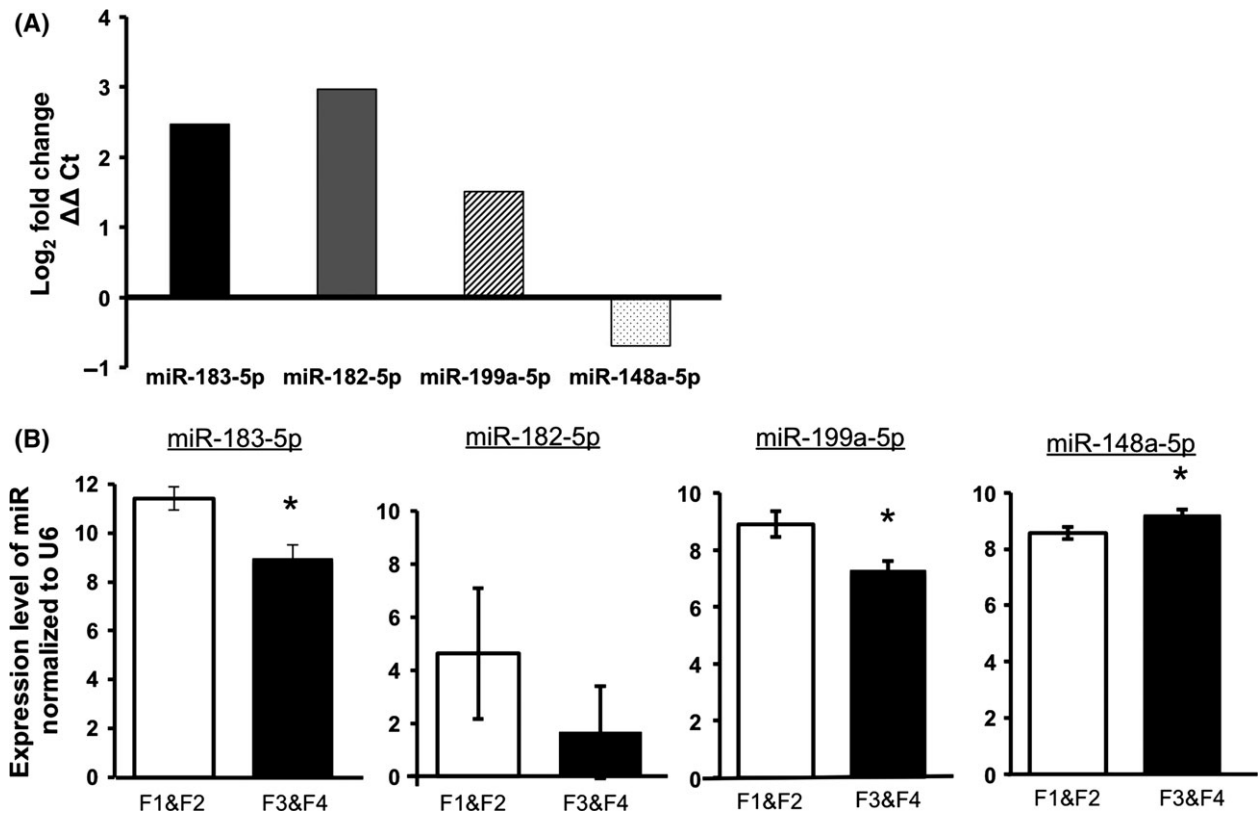


Fig. 2. qRT-PCR validation of four microRNAs with highest significance and fold change in comparison of F1 and F2 (early) with F3 and F4 (advanced) fibrosis. (A) Calculated fold change for microRNAs 183-5p, 182-5p, miR-199a-5p and miR-148a-5p. Fold changes were calculated using $2^{\Delta\Delta Ct}$. U6 was used for normalization in all samples. (B) Average Ct values normalized to U6 and the standard error of the mean. *indicates qRT-PCR changes that are statistically significant by *t*-test ($P \leq 0.05$).

categories. Of 37 differentially expressed miRNAs between early and advanced fibrosis in liver, 10 were differentially expressed for at least one of the isomiR categories: 199b-3p, 148a-3p, 150-5p, 122-3p, 199a-5p, 10a-5p, 92a-3p, 195-5p, 181c-5p, 181a-5p (Table 3). Several miRNAs with no evidence of differential expression for the exact canonical sequence were significantly differentially expressed for one or more isomiR categories (Table 3). For some microRNAs, when we combined all the counts for isomiRs and the canonical sequence, they became differentially expressed. While the functional consequences of these nucleotide changes are not yet fully understood, we report our findings in order to increase the growing knowledgebase on these types of microRNA modifications.

Discussion

We identified 37 microRNAs, 26 up and 11 down-regulated, between early and advanced fibrotic stages in liver biopsies from patients with chronic hepatitis C. Early fibrosis were METAVIR classification F1 and F2 biopsy samples grouped together. Samples classified as F3 and F4 were grouped as advanced

fibrosis. In serum, we were not able to identify microRNAs that were significant after adjusting for multiple comparisons (Table S5).

We report isomiR changes, single and multiple nucleotide changes from the canonical sequence. The impact of these single nucleotide changes on microRNA function is not well understood. With respect to microRNA expression analysis, it is also not clear how these single nucleotide changes might alter microRNA measurements using other methods such as microarray and qRT-PCR. It might cause some difficulties comparing microRNA expression levels across platforms that are sensitive or insensitive to these changes.

We focused our attention on four microRNAs with the lowest *P*-values, miR-183-5p, miR-182-5p, miR-200a-5p and miR-199a-5p, all with elevated expression levels, as well as miR-148a-5p that had significantly decreased expression when comparing early and advanced liver fibrosis. Each of these selected microRNAs demonstrated similar direction and fold changes when assessed with qRT-PCR. In addition, we used ordinal logistic regression to examine the progressive increase or decrease in each of these microRNAs with advancing fibrosis severity. Each of these microRNAs

Table 2. Ordinal logistic regression. Ordinal logistic regression was implemented in order to detect miRNAs exhibiting monotonic expression trend with fibrosis advancement. We report miRNAs with the lowest Akaike Information Criterion value significant at adjusted *P*-value <0.05

| miRNA | Estimate | SE | AIC | Adjusted <i>P</i> -value |
|---------------|----------|------|--------|--------------------------|
| miR-148a-5p | -0.01 | 0.00 | 96.73 | 6.04E-03 |
| miR-122-3p | -0.01 | 0.00 | 103.87 | 1.42E-02 |
| miR-183-5p | 0.12 | 0.03 | 101.37 | 1.42E-02 |
| miR-199a-5p | 0.01 | 0.00 | 103.30 | 1.42E-02 |
| miR-199a-3p | 0.00 | 0.00 | 108.55 | 1.98E-02 |
| miR-199b-3p | 0.00 | 0.00 | 108.55 | 1.98E-02 |
| miR-23a-3p | 0.01 | 0.00 | 108.76 | 1.98E-02 |
| miR-429 | 0.02 | 0.01 | 106.57 | 1.98E-02 |
| miR-497-5p | 0.03 | 0.01 | 106.25 | 1.98E-02 |
| miR-92a-3p | 0.00 | 0.00 | 109.00 | 1.98E-02 |
| miR-181a-2-3p | 0.04 | 0.01 | 110.87 | 2.21E-02 |
| miR-181b-5p | 0.00 | 0.00 | 110.93 | 2.21E-02 |
| miR-181c-3p | 0.18 | 0.05 | 109.75 | 2.21E-02 |
| miR-122-5p | 0.00 | 0.00 | 111.29 | 2.38E-02 |
| miR-181d-5p | 0.07 | 0.02 | 109.86 | 2.42E-02 |
| miR-192-5p | 0.00 | 0.00 | 110.69 | 2.42E-02 |
| miR-200b-3p | 0.01 | 0.00 | 111.10 | 2.42E-02 |
| miR-30c-1-3p | -0.14 | 0.04 | 112.29 | 2.79E-02 |
| miR-150-5p | 0.00 | 0.00 | 108.81 | 2.79E-02 |
| miR-181a-5p | 0.00 | 0.00 | 112.93 | 2.79E-02 |
| miR-181c-5p | 0.01 | 0.00 | 111.80 | 2.79E-02 |
| miR-182-5p | 0.00 | 0.00 | 111.40 | 2.79E-02 |
| miR-192-3p | -0.21 | 0.06 | 111.98 | 2.79E-02 |
| miR-194-5p | 0.00 | 0.00 | 113.31 | 2.79E-02 |
| miR-195-5p | 0.01 | 0.00 | 111.32 | 2.79E-02 |
| miR-455-5p | -0.06 | 0.02 | 112.41 | 2.79E-02 |
| miR-195-3p | 0.07 | 0.02 | 112.10 | 2.80E-02 |
| miR-214-3p | 0.03 | 0.01 | 111.19 | 2.80E-02 |
| miR-200a-5p | 0.16 | 0.05 | 111.85 | 2.94E-02 |
| miR-101-3p | 0.00 | 0.00 | 113.66 | 3.23E-02 |
| miR-148a-3p | 0.00 | 0.00 | 112.71 | 3.31E-02 |
| miR-194-3p | -0.11 | 0.03 | 113.92 | 3.31E-02 |
| miR-378i | -0.04 | 0.01 | 112.67 | 3.59E-02 |
| miR-215-5p | -0.01 | 0.00 | 112.21 | 3.63E-02 |
| let-7e-5p | 0.00 | 0.00 | 113.76 | 4.01E-02 |
| miR-1468-5p | -0.03 | 0.01 | 114.97 | 4.01E-02 |
| miR-1295b-5p | -0.24 | 0.08 | 115.33 | 4.31E-02 |
| miR-132-3p | 0.07 | 0.02 | 113.40 | 4.31E-02 |
| miR-214-5p | 0.05 | 0.02 | 114.54 | 4.57E-02 |
| miR-221-3p | 0.01 | 0.01 | 115.35 | 4.63E-02 |

becomes increasingly dysregulated in hepatic tissue with progressive fibrosis (adjusted *P*-value < 0.05), except miR-200a-5p (Fig. S1).

Very little is known about which microRNAs play a role in the pathogenesis of liver fibrosis, or increase the risk for downstream complications, such as hepatic cancer. Using pathway analysis to get a better understanding of the function of microRNAs in liver fibrosis can be very difficult to interpret. Not only can a single microRNA regulate many mRNA targets, but there is also a large false positive rate for the prediction of microRNAs and mRNA targets. There are currently 2588 mature

Table 3. microRNAs in tissue differentially expressed solely for one or more isomiR categories between early and advanced fibrosis. Iso-miR categories incorporate 3' length variants (lv3p), 5' length variants (lv5p) and non-templated nucleotide additions (ntaA, ntaT, ntaC, ntaG) with +/- indicating additions and trimming respectively. Additional isomiR data can be found in Table S6

| microRNA | Log ₂ -fold change | <i>P</i> -value | Adjusted <i>P</i> -value | IsomiR category |
|------------|-------------------------------|-----------------|--------------------------|-----------------|
| miR-194-5p | -1.48 | 4.57E-04 | 2.06E-02 | lv3p-4 |
| let-7e-5p | 1.10 | 1.43E-03 | 4.22E-02 | lv3p+1 |
| miR-122-5p | -1.26 | 1.03E-03 | 4.22E-02 | lv3p+1 |
| miR-21-5p | 0.33 | 1.90E-03 | 4.37E-02 | lv3p+1 |
| miR-192-5p | -1.07 | 1.29E-04 | 1.44E-02 | ntaG+1 |
| miR-142-5p | 1.39 | 3.17E-04 | 1.87E-02 | ntaT+1 |
| miR-122-5p | -1.26 | 7.38E-04 | 2.42E-02 | ntaT+1 |
| miR-30e-5p | -0.85 | 6.39E-04 | 2.42E-02 | ntaT+1 |
| miR-143-3p | 1.04 | 1.81E-03 | 3.56E-02 | ntaT+1 |
| miR-192-5p | -1.00 | 1.99E-03 | 3.67E-02 | ntaT+1 |
| miR-122-5p | -1.49 | 1.11E-04 | 9.04E-03 | ntaT+2 |
| miR-143-3p | 1.38 | 6.40E-05 | 9.04E-03 | ntaT+2 |
| miR-192-5p | -1.09 | 6.26E-04 | 1.98E-02 | ntaT+2 |
| let-7f-5p | 1.56 | 5.21E-07 | 5.42E-05 | ntaT+3 |
| miR-143-3p | 1.49 | 2.27E-05 | 1.18E-03 | ntaT+3 |
| let-7 g-5p | 1.48 | 8.80E-04 | 1.53E-02 | ntaT+3 |
| miR-192-5p | -1.34 | 1.55E-03 | 2.30E-02 | ntaT+3 |

human microRNAs listed in miRBase. Ingenuity Pathway Analysis (Qiagen <http://www.ingenuity.com>), lists 114 human microRNAs as having experimentally validated target mRNAs, most microRNAs are linked to several mRNA targets. We chose to focus our discussion on a few microRNAs identified in these experiments and the published literature that strengthens their link to liver disease. While this is not an exhaustive list, and there are many studies that found different microRNAs to be important in liver fibrosis, the microRNAs reported here have been found to be associated with liver damage in several papers. Figure 3 is a network diagram describing the relationships for each of the miRNAs and the available information from the literature and the association of each one with liver disease, injury or cancer.

miR-182

Several papers have linked high miRNA-182 expression levels to hepatocellular carcinoma (17, 18) or liver metastasis (19, 20). Metastasis suppressor 1 (18, 21, 22) was identified as a target for miR-182 in liver. MicroRNA expression measured in mouse models of alcoholic and non-alcoholic steatohepatitis (23) found miR-182 and miR-183 (both identified in our study) to be down-regulated in alcoholic liver and up-regulated in non-alcoholic fatty liver. Murakami *et al.* (24). compared liver samples from patients with chronic hepatitis and cirrhosis and found miR-182 to be one of the most differentially expressed microRNAs, up-regulated in chronic

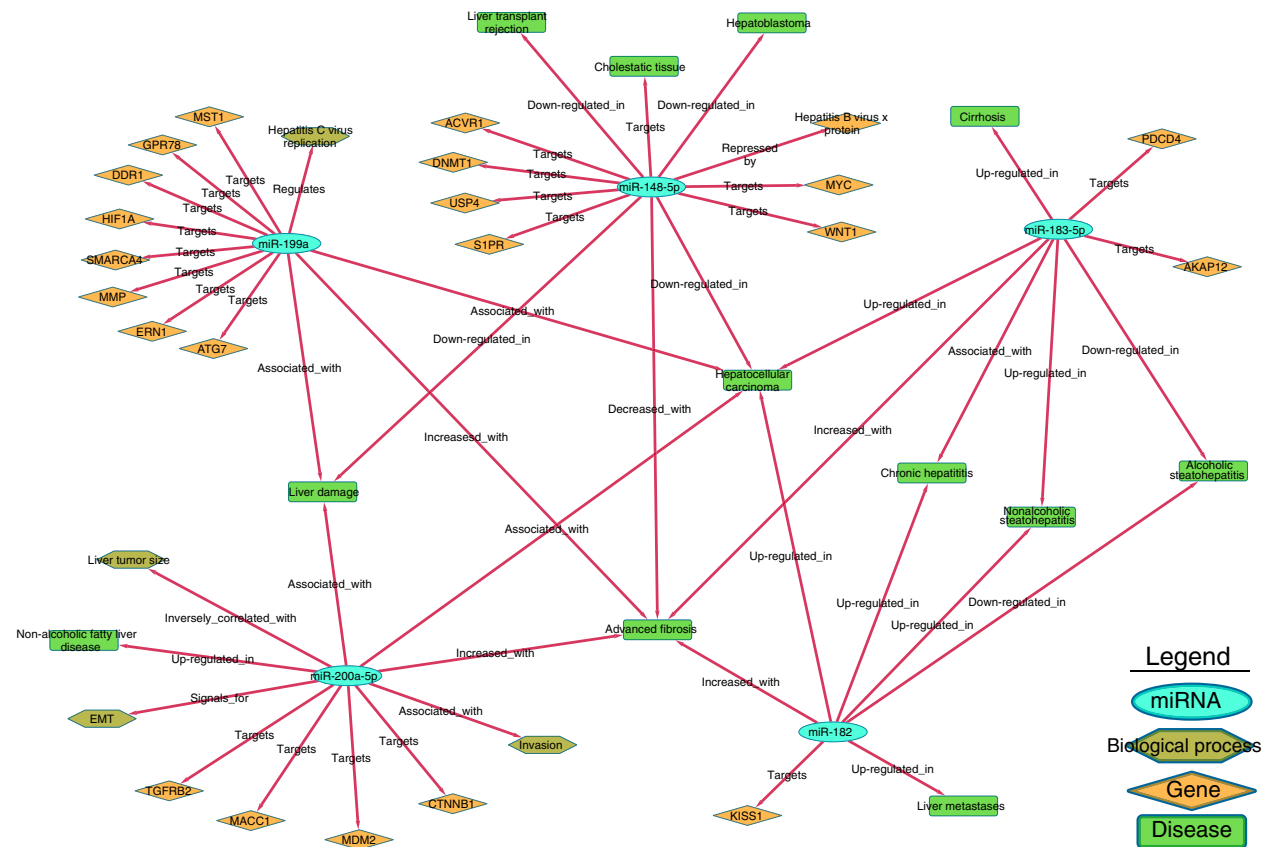


Fig. 3. Network Analysis for the five miRNAs that had the highest fold change and lowest *P*-value. Using the current literature and what is known about these miRNAs with respect to liver disease, fibrosis and hepatocellular carcinoma, we created a network diagram. The diagram indicates experimentally validated biological processes, gene targets and disease pathways affected by these miRNAs.

hepatitis. In the current paper, we also found miR-182 to increase with fibrosis stage in patients with chronic hepatitis C.

miR-183-5p

Wojcicka *et al.* (5). examined microRNAs in cirrhotic liver and hepatocellular carcinoma. miR-183-5p and miR-199a-5p (both significantly up-regulated with advanced fibrosis in our data) were among the top ten most differentially expressed microRNAs when comparing tumour and normal tissue. miR-183-5p was approximately four-fold more highly expressed in tumour samples. miR-183 was also found to be significantly up-regulated in hepatocellular carcinoma samples from patients with chronic hepatitis C (25). In a larger study examining liver biopsy samples from normal controls and patients with hepatitis C, hepatitis B, cirrhosis, hepatocellular carcinoma, they found that miR-183 increased with increasing liver damage, highest in cirrhosis and cancer. Goepfert *et al.* (10). found miR-183 to be up-regulated in cirrhotic liver and premalignant lesions, and went on to investigate potential mRNA targets for miR-183. They found

AKAP12, a tumour suppressor gene found to be down-regulated in cirrhotic liver, premalignant lesions and hepatocellular carcinoma. miR-183 was found to be up-regulated in hepatocellular carcinoma samples in a study by Li *et al.* (9), where they also found that Programmed Cell Death 4, a proapoptotic molecule was targeted by miR-183.

miR-200a-5p

In our studies the expression level of miR-200a was very low – average read counts per sample were 11. We observed a small significant increase with fibrosis stage (Table 1, Fig. 1). Several papers implicate miR-200a in liver damage and cancer, but there are discrepancies in the level and direction of expression. Perhaps these differences in findings are, in part, a reflection of the overall low abundance we found for this microRNA in tissue. In many cases, reduced expression of miR-200a predicted prognosis (26–30) and expression inversely correlated with tumour size (31). Dhayat *et al.* (32). found that miR-200a was significantly decreased in patients with hepatocellular carcinoma and in cirrhotic tissue from patients with hepatocellular carcinoma

compared with patients with cirrhosis. However, other papers found increased levels of miR-200a promoting hepatocellular carcinoma progression (33). In a rat model of non-alcoholic fatty liver disease, researchers found miR-200a to be significantly up-regulated in rats that received a high-fat diet (27, 34, 35). Feng *et al.* (27), also showed that human hepatocytes treated with free fatty acids and inflammatory factors had increased miR-200a levels. miR-200a was found to be associated with epithelial-mesenchymal transition and invasion when overexpressed (26, 36). Identified mRNA targets of miR-200a were MACC1 (metastasis associated in colon cancer1) (27), CTNNB1 (catenin, cadherin-associated protein beta1) (28, 29); TGF- β 2 (transforming growth factor beta2) (29) and MDM2 (E3 Ubiquitin-protein ligase) (37).

miR-199a-5p

There are several papers describing the relevance of both miR-199a-5p and miR199a-3p in liver disease and injury, however, there were variable findings with respect to expression levels of these microRNAs in the literature. Murakami *et al.* (38), found that miR-199a regulates hepatitis C virus replication. Several papers found the expression of miR-199a to be down with injury and disease (19, 21, 31, 39–43), and in other papers miR-199a was found to be up-regulated in hepatocellular carcinoma and liver injury (44–46) and increased with liver fibrosis progression (6, 47). Experimentally identified targets of miR199a-5p in liver disease were SMARCA4 (swi/snf-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4) and MST1 (macrophage stimulating 1) (48), MMP-9 (matrix metalloproteinase 9) (44), GRP78 (glucose-regulated protein, 78 kDa), endoplasmic reticulum to nucleus signalling 1, activating transcription factor 6 (45), clathrin heavy chain (21), ATG7 (autophagy related 7) (49), hypoxia inducible factor-1 α (41) and discoidin domain receptor-1 (43).

miR-148a-5p

In our study, miR-148a-5p was the most significantly down-regulated microRNA in advanced fibrosis. In a recent publication, Pan *et al.* (50), found miR-148a-5p was down-regulated in hepatocellular carcinoma and expression was correlated with tumour stage. Several other papers found down-regulation of miR-148a (51, 52), targeting ubiquitin specific protease 4 and sphingosine 1-phosphate receptor 1 (53), genes in the Met/Snail signalling pathway (54), Wnt1 (55), DNA methyltransferase (52, 56) and c-Myc (57). In a large study examining two subtypes of hepatocellular carcinoma (58), miR-148a was down-regulated in both cancer types compared with controls, and the more aggressive subtype had significantly less miR-148a expression.

ACVR1 (activin A receptor, type1) was identified as a downstream target in that study. Rieger *et al.* (59), found miR-148a down-regulated in cholestatic tissue. Xu *et al.* (60), found that miR-148a is repressed by hepatitis B virus X protein, and this in turn allows enhanced expression of Hematopoietic pre-B cell leukaemia transcription factor-interacting protein. miR-148a was also found to be down-regulated in liver injury and rejection with liver transplantation (61) and down-regulated in hepatoblastoma (62).

In conclusion, our data identified several microRNAs that are altered with worsening liver fibrosis in chronic hepatitis C patients. These data provide further support for the role of specific microRNAs in the pathogenesis of fibrosis and focuses attention on identifying microRNAs that could be used to predict clinical outcomes and complications.

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