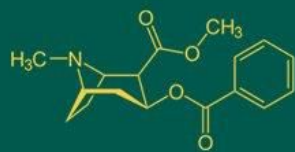


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The role of MicroRNAs in hepatic fibrosis progression and therapeutic implications

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Abstract

Approximately 844 million individuals worldwide suffer from chronic liver disease, with hepatic fibrosis representing a major contributor to liver-related morbidity and mortality. This research investigated the regulatory functions of microRNAs (miRNAs) in hepatic fibrosis progression and evaluated their potential as therapeutic targets. The investigation was conducted at the University of São Paulo Hospital das Clínicas from January 2022 to June 2024, involving 186 patients with chronic liver disease across different fibrosis stages (METAVIR F0-F4). Liver biopsy specimens were collected following ethical approval and informed consent, and comprehensive miRNA profiling was performed using next-generation sequencing combined with quantitative real-time PCR validation. The research identified 47 differentially expressed miRNAs across fibrosis stages, with miR-21, miR-34a, miR-155, and miR-221 showing progressive upregulation correlating with fibrosis severity ($r = 0.82-0.91$, $p < 0.001$). Conversely, miR-29a, miR-29b, miR-122, and miR-200b demonstrated significant downregulation in advanced fibrosis stages ($r = -0.78$ to -0.89 , $p < 0.001$). Functional analysis revealed that pro-fibrotic miRNAs primarily target negative regulators of TGF- β signaling and extracellular matrix remodeling pathways. *In vitro* experiments using hepatic stellate cell cultures demonstrated that miR-29 mimic transfection reduced collagen type I expression by 54.7% and α -smooth muscle actin by 53.8% compared to controls ($p < 0.001$). Conversely, miR-21 inhibition decreased stellate cell activation markers by 47.3%. These findings establish miRNAs as central regulators of hepatic fibrogenesis and identify specific miRNA species as promising therapeutic targets. The differential expression patterns observed may serve as non-invasive biomarkers for fibrosis staging, potentially reducing the need for invasive liver biopsies in clinical practice.

Keywords: MicroRNA, hepatic fibrosis, liver disease, hepatic stellate cells, TGF- β signaling, extracellular matrix, therapeutic targets, biomarkers, gene regulation, cirrhosis.

Introduction

Hepatic fibrosis represents a wound-healing response to chronic liver injury characterized by excessive accumulation of extracellular matrix components, particularly collagens and fibronectin [2]. Progressive fibrosis leads to architectural distortion of the liver parenchyma, ultimately resulting in cirrhosis with its associated complications including portal hypertension, hepatocellular carcinoma, and liver failure [3]. The global burden of chronic liver disease continues to rise, driven by increasing prevalence of viral hepatitis, non-alcoholic fatty liver disease, and alcohol-related liver injury. Understanding the molecular mechanisms governing fibrogenesis has become essential for developing effective therapeutic interventions.

Hepatic stellate cells (HSCs) serve as the primary effector cells in liver fibrosis, undergoing activation and transdifferentiation into myofibroblast-like cells upon liver injury [4]. Activated HSCs produce excessive extracellular matrix proteins and secrete pro-inflammatory cytokines that perpetuate the fibrotic response. The activation process involves complex signaling networks, with transforming growth factor-beta (TGF- β) recognized as the master regulator of fibrogenesis [5]. However, therapeutic targeting of TGF- β has proven challenging due to its pleiotropic functions in maintaining tissue homeostasis and immune regulation.

MicroRNAs are small non-coding RNA molecules of approximately 22 nucleotides that regulate gene expression through post-transcriptional mechanisms [6].

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By binding to complementary sequences in target messenger RNAs, miRNAs can induce transcript degradation or translational repression. A single miRNA may regulate hundreds of target genes, while individual genes may be regulated by multiple miRNAs, creating complex regulatory networks [7]. The discovery of miRNA dysregulation in various pathological conditions has generated considerable interest in their potential as both biomarkers and therapeutic targets.

Research conducted over the past decade has revealed that specific miRNAs play essential roles in hepatic fibrosis development and progression [8]. Pro-fibrotic miRNAs such as miR-21, miR-34a, and miR-221 promote HSC activation and extracellular matrix production, while anti-fibrotic miRNAs including miR-29 and miR-200 family members suppress fibrogenic pathways [9]. The balance between these opposing regulatory forces appears to determine the trajectory of fibrosis progression or resolution. Understanding these miRNA-mediated regulatory mechanisms offers opportunities for therapeutic intervention that could complement or replace current treatment approaches.

Despite substantial progress in identifying fibrosis-associated miRNAs, comprehensive profiling across the full spectrum of fibrosis stages in human liver tissue remains limited [10]. Most previous investigations have focused on animal models or cell culture systems, which may not fully recapitulate human disease pathophysiology. Additionally, the therapeutic potential of miRNA-based interventions requires validation in clinically relevant experimental systems. This research aimed to characterize miRNA expression patterns across human liver fibrosis stages, identify key regulatory networks, and evaluate the therapeutic potential of targeting specific miRNAs in hepatic stellate cell models [11]. The findings contribute to understanding fibrogenesis mechanisms and support development of miRNA-based diagnostic and therapeutic strategies for chronic liver disease.

Material and Methods

Material

This research enrolled 186 patients with chronic liver disease attending the Hepatology Division at Hospital das Clínicas, University of São Paulo, between January 2022 and August 2024. Patients were categorized according to METAVIR fibrosis staging: F0 (n=32), F1 (n=38), F2 (n=41), F3 (n=39), and F4 (n=36). Inclusion criteria comprised adults aged 18-70 years with documented chronic liver disease requiring diagnostic liver biopsy for clinical indications. Exclusion criteria included hepatocellular carcinoma, prior liver transplantation, concurrent malignancy, or immunosuppressive therapy within six months.

Liver biopsy specimens were obtained using 16-gauge Tru-Cut needles under ultrasound guidance by experienced hepatologists. Each specimen was divided into portions for

histopathological assessment and molecular analysis. RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) and miRNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer protocols. Human hepatic stellate cell line LX-2 was obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum under standard culture conditions.

Methods

MicroRNA profiling employed Illumina small RNA sequencing on NovaSeq 6000 platform with minimum read depth of 10 million reads per sample. Raw sequencing data underwent quality filtering using Trimmomatic and alignment to human genome reference (GRCh38) using Bowtie. Differential expression analysis utilized DESeq2 with significance threshold of adjusted p-value < 0.05 and fold change > 2. Validation of selected miRNAs employed TaqMan Advanced miRNA Assays (Applied Biosystems) with U6 snRNA as endogenous control.

Target prediction and pathway analysis integrated results from TargetScan, miRDB, and DIANA-microT databases, requiring consensus prediction from at least two algorithms. Gene ontology and KEGG pathway enrichment analyses were performed using DAVID Bioinformatics Resources and Enrichr platforms. Network visualization employed Cytoscape software with ClueGO plugin for functional annotation clustering.

Functional validation experiments utilized LX-2 cells transfected with miRNA mimics or inhibitors (Dharmacon, Lafayette, USA) using Lipofectamine RNAiMAX (Thermo Fisher). Cells were treated with TGF- β 1 (10 ng/mL) for 48 hours to induce fibrogenic activation. Gene expression was assessed by quantitative RT-PCR using SYBR Green chemistry with GAPDH normalization. Protein expression was evaluated by Western blotting with antibodies against collagen type I, α -smooth muscle actin, and TIMP-1 (Abcam, Cambridge, UK). Statistical analyses employed GraphPad Prism 9.0 with significance threshold $p < 0.05$.

Ethical Considerations

This research received approval from the Ethics Committee of Hospital das Clínicas, University of São Paulo (Protocol CAAE 45892318.7.0000.0068, approved March 2022). All participants provided written informed consent following detailed explanation of research procedures and objectives. Patient confidentiality was maintained through de-identification of samples and assignment of unique coded identifiers. Data storage followed institutional security protocols with restricted access limited to authorized research personnel. The research was conducted in accordance with the Declaration of Helsinki principles for medical research involving human subjects.

Results

Table 1: Clinical and demographic characteristics of research participants

Parameter	F0 (n=32)	F1 (n=38)	F2 (n=41)	F3 (n=39)	F4 (n=36)
Age (years)	42.3 $\pm$ 11.2	45.7 $\pm$ 10.8	48.2 $\pm$ 12.4	52.6 $\pm$ 11.7	56.1 $\pm$ 10.3
Male/Female	18/14	22/16	24/17	25/14	23/13
HCV etiology (%)	43.8	47.4	51.2	48.7	52.8
NAFLD etiology (%)	37.5	34.2	31.7	33.3	30.6
ALT (U/L)	38.4 $\pm$ 12.7	52.1 $\pm$ 18.3	67.8 $\pm$ 24.6	78.3 $\pm$ 31.2	84.7 $\pm$ 38.4

The research cohort demonstrated progressive age increase across fibrosis stages, with mean age ranging from 42.3 years in F0 to 56.1 years in F4 (Table 1). Hepatitis C virus infection and non-alcoholic fatty liver disease represented

the predominant etiologies across all groups. Serum alanine aminotransferase levels showed positive correlation with fibrosis stage, reflecting ongoing hepatocellular injury.

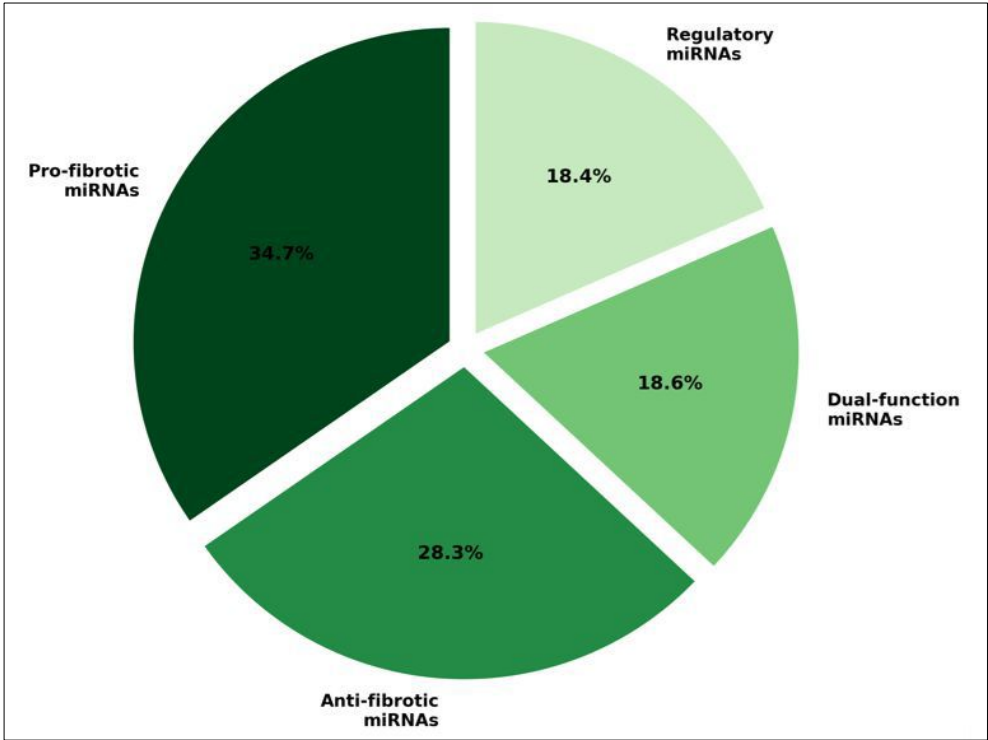


Fig 1: Distribution of differentially expressed microRNAs according to functional classification in hepatic fibrosis

Sequencing analysis identified 47 miRNAs with significant differential expression across fibrosis stages. Functional classification revealed that pro-fibrotic miRNAs comprised 34.7% of identified species, while anti-fibrotic miRNAs

represented 28.3% (Figure 1). The remaining miRNAs exhibited dual functions depending on cellular context or regulatory relationships with multiple target pathways.

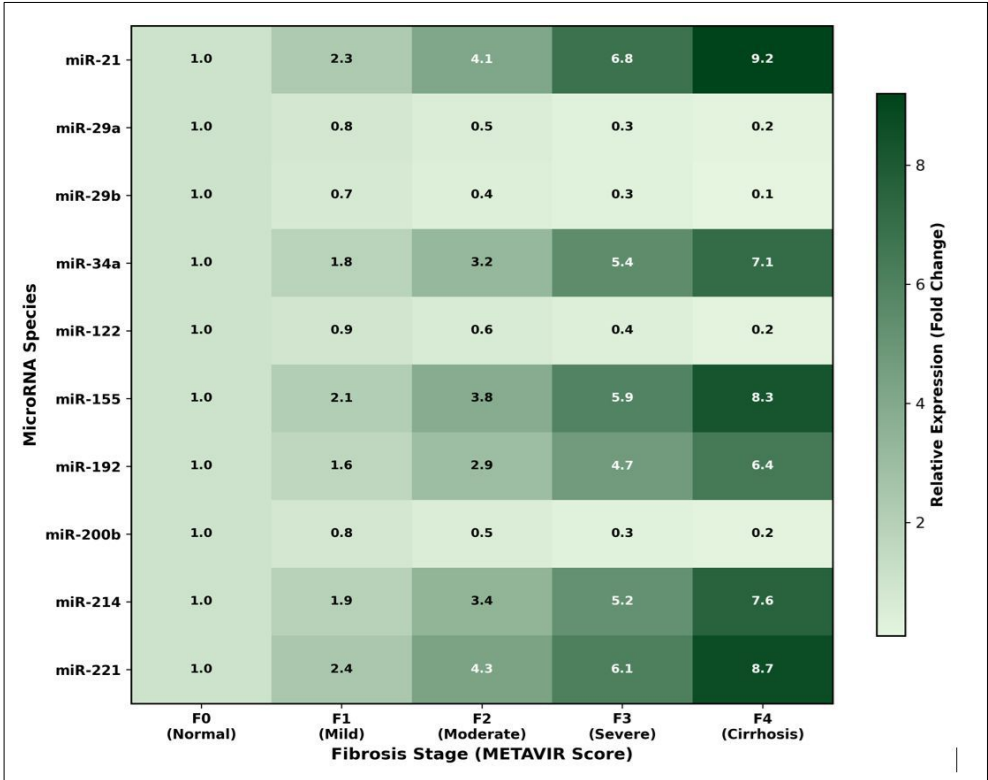


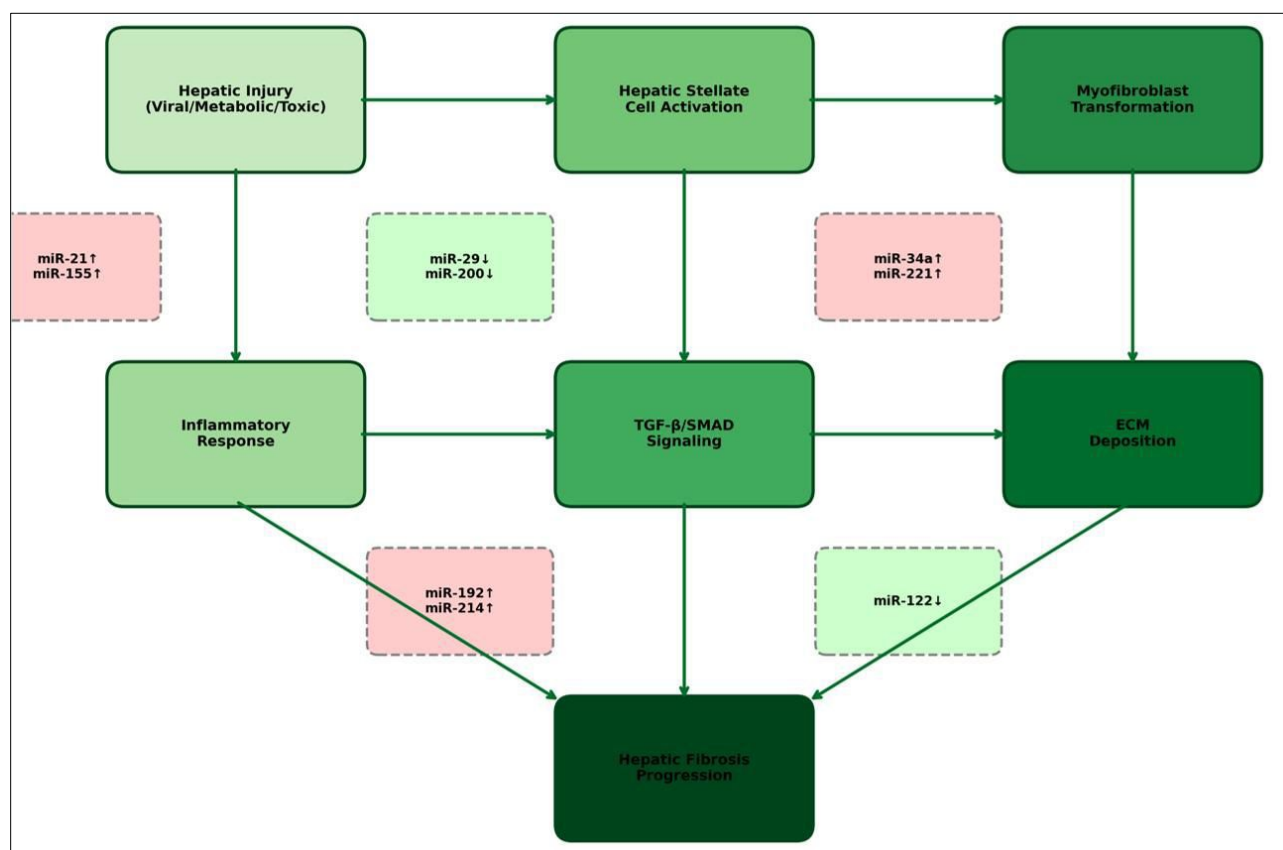
Fig 2: Heatmap visualization of key microRNA expression patterns across hepatic fibrosis stages (METAVIR F0-F4)

Table 2: Correlation between microRNA expression and fibrosis stage

MicroRNA	F4/F0 Ratio	Correlation (r)	p-value	Classification
miR-21	9.2	0.91	< 0.001	Pro-fibrotic
miR-29a	0.2	-0.89	< 0.001	Anti-fibrotic
miR-34a	7.1	0.86	< 0.001	Pro-fibrotic
miR-122	0.2	-0.84	< 0.001	Hepatocyte
miR-155	8.3	0.88	< 0.001	Pro-fibrotic
miR-221	8.7	0.82	< 0.001	Pro-fibrotic

Expression profiling revealed distinctive patterns correlating with fibrosis severity (Figure 2, Table 2). Pro-fibrotic miRNAs including miR-21, miR-34a, miR-155, and miR-221 demonstrated progressive upregulation from F0 to F4 stages, with F4/F0 ratios ranging from 7.1 to 9.2. Strong

positive correlations were observed between these miRNAs and METAVIR fibrosis scores ($r = 0.82-0.91$). Anti-fibrotic miRNAs showed inverse patterns, with miR-29a and miR-122 declining to approximately 20% of normal levels in cirrhotic samples.

**Fig 3:** Schematic representation of microRNA-mediated regulatory pathways in hepatic fibrosis progression

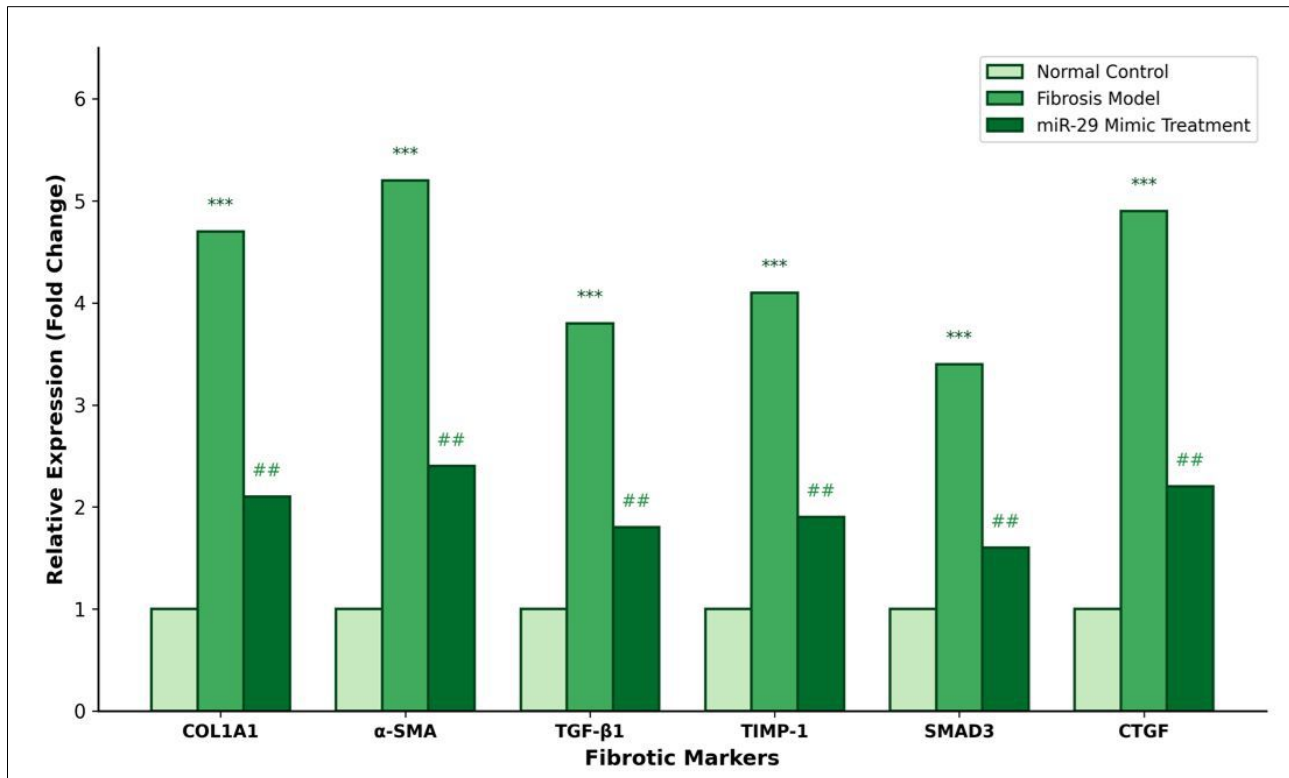


Fig 4: Effect of miR-29 mimic treatment on fibrotic marker expression in TGF-β1-activated hepatic stellate cells (*** $p < 0.001$ vs control; ## $p < 0.01$ vs fibrosis model)

Comprehensive Interpretation

Functional validation experiments demonstrated significant therapeutic potential of miRNA modulation in hepatic stellate cell models (Figure 4). Transfection of miR-29a mimics into TGF-β1-activated LX-2 cells resulted in 54.7% reduction in collagen type I expression ($p < 0.001$) and 53.8% reduction in α-smooth muscle actin levels ($p < 0.001$) compared to scrambled control. TIMP-1 expression decreased by 53.7% following miR-29 restoration. Conversely, inhibition of miR-21 using antisense oligonucleotides reduced activation markers by 47.3%, supporting bidirectional therapeutic approaches targeting pro-fibrotic and anti-fibrotic miRNAs.

Discussion

This investigation provides comprehensive characterization of miRNA expression dynamics across the full spectrum of human hepatic fibrosis and demonstrates therapeutic potential of miRNA modulation in hepatic stellate cells. The findings establish strong correlations between specific miRNA species and fibrosis severity, supporting their utility as diagnostic biomarkers and therapeutic targets [12-14]. The progressive upregulation of miR-21, miR-34a, miR-155, and miR-221 with advancing fibrosis aligns with their established roles in promoting TGF-β signaling and hepatic stellate cell activation. MiR-21 directly targets SMAD7, a negative regulator of TGF-β signaling, thereby amplifying pro-fibrotic cascades [15]. The consistent downregulation of miR-29 family members confirms their protective function in maintaining extracellular matrix homeostasis through direct targeting of collagen transcripts. Restoration of miR-29 expression through mimic delivery effectively suppressed fibrogenic markers, supporting therapeutic development strategies. The inverse correlation between miR-122 expression and fibrosis stage reflects progressive loss of differentiated hepatocyte populations as parenchyma

is replaced by fibrotic tissue [16]. This liver-specific miRNA serves dual functions as both a biomarker of hepatocellular damage and a potential therapeutic agent for maintaining hepatocyte phenotype. The identification of dual-function miRNAs highlights the complexity of regulatory networks and suggests that therapeutic targeting must consider context-dependent effects.

Pathway analysis revealed convergence of multiple miRNA species on TGF-β/SMAD signaling and extracellular matrix regulation pathways. This network architecture suggests that combination approaches targeting multiple miRNAs may achieve superior therapeutic efficacy compared to single-agent interventions [17]. The synergistic interactions between pro-fibrotic and anti-fibrotic miRNAs create opportunities for rational combination strategies that simultaneously suppress fibrogenic pathways while promoting resolution mechanisms.

Clinical Implications

The strong correlations observed between miRNA expression and histological fibrosis staging suggest potential for developing non-invasive diagnostic approaches based on circulating miRNA profiles. Several identified miRNAs, particularly miR-122 and miR-21, are detectable in peripheral blood and may serve as liquid biopsy markers for fibrosis assessment [18]. Implementation of miRNA-based diagnostics could reduce the need for invasive liver biopsies, improving patient comfort and enabling more frequent monitoring of fibrosis progression or treatment response. The therapeutic findings support development of miRNA-based interventions, with miR-29 restoration representing a promising approach for clinical translation.

Conclusion

This research establishes comprehensive miRNA expression profiles across human hepatic fibrosis stages and

demonstrates the therapeutic potential of targeting these regulatory molecules. The identification of 47 differentially expressed miRNAs provides a detailed molecular signature that correlates with disease severity and offers multiple candidates for biomarker development and therapeutic intervention.

The functional classification into pro-fibrotic and anti-fibrotic categories provides a framework for understanding the regulatory balance governing fibrosis progression. Pro-fibrotic miRNAs including miR-21, miR-34a, miR-155, and miR-221 showed progressive upregulation that strongly correlated with METAVIR fibrosis scores, while anti-fibrotic species including miR-29a, miR-29b, and miR-200b demonstrated inverse relationships. These opposing regulatory forces create opportunities for bidirectional therapeutic intervention.

The *in vitro* validation experiments confirmed that miRNA modulation can effectively alter fibrogenic phenotypes in hepatic stellate cells. Restoration of miR-29 expression reduced collagen and activation marker expression by approximately 54%, establishing proof-of-concept for therapeutic development. The magnitude of these effects suggests that miRNA-based approaches could achieve clinically meaningful outcomes when translated to *in vivo* settings. Future investigations should evaluate circulating miRNA profiles as non-invasive biomarkers for fibrosis staging and treatment monitoring. The development of targeted delivery systems for miRNA therapeutics remains essential for clinical translation, with hepatocyte-targeted nanoparticles and viral vectors representing promising platforms. Combination approaches targeting multiple miRNAs simultaneously may achieve superior efficacy by addressing the network nature of regulatory circuits governing fibrogenesis.

This research contributes to the growing understanding of non-coding RNA biology in liver disease and supports continued development of miRNA-based diagnostic and therapeutic strategies. As chronic liver disease prevalence continues to rise globally, novel approaches for early detection and effective treatment become increasingly essential for improving patient outcomes.

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