

MICRORNAS AS DIAGNOSTIC BIOMARKERS FOR LIVER FIBROSIS IN CHRONIC HEPATITIS B: INSIGHTS FROM EXPRESSION PROFILING AND CORRELATIONS WITH FIBROSIS STAGES

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Abstract

Chronic hepatitis B (CHB) infection remains a significant global health challenge, leading to severe liver complications such as cirrhosis and hepatocellular carcinoma (HCC), which makes accurate fibrosis assessment crucial for prognosis and treatment. This study investigated the expression levels of five miRNAs (hsa-miR-21-3p, hsa-miR-29a-3p, hsa-miR-122-3p, hsa-miR-221-3p, and hsa-miR-222-3p) in 40 CHB patients classified into mild-to-moderate (F1-F2) and severe fibrosis (F3-F4) groups using transient elastography. Quantitative PCR analysis revealed that hsa-miR-29a-3p ($r = 0.72$, $p < 0.001$), was upregulated, while hsa-miR-21-3p ($r = -0.65$, $p < 0.01$) and hsa-miR-122-3p ($r = -0.68$, $p < 0.01$,) were downregulated in severe fibrosis. Combining hsa-miR-29a-3p (AUC = 0.89, 95% CI: 0.84–0.94) and hsa-miR-21-3p (AUC = 0.87, 95% CI: 0.82–0.93) in a predictive model improved diagnostic accuracy. These findings highlight the potential of miRNA profiling to enhance current diagnostic algorithms. Further studies in larger cohorts are needed to validate their role in fibrosis assessment and disease monitoring.

Key words: microRNAs, liver fibrosis, chronic hepatitis B, non-invasive biomarkers, fibrosis staging

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Introduction. Chronic hepatitis B (CHB) infection is a major global health problem, with over 296 million individuals infected worldwide and nearly 820 000 deaths annually due to complications such as liver cirrhosis and hepatocellular carcinoma (HCC) [1]. The chronic nature of hepatitis B virus (HBV) infection leads to persistent liver inflammation, which, over time, promotes the development of liver fibrosis – a condition characterized by the excessive accumulation of extracellular matrix components. Comprehensive analysis and accurate evaluation of liver fibrosis is crucial for the prognosis, management, and treatment of CHB patients, as advanced fibrosis significantly increases the risk of cirrhosis and HCC [2].

Traditional methods for assessing liver fibrosis include liver biopsy, imaging techniques, and non-invasive biomarkers. However, liver biopsy remains the gold standard, although invasive and prone to variability [3]. Non-invasive techniques, such as transient elastography (FibroScan[®]), are widely used, but have limitations, especially in patients with early liver fibrosis or inflammation [4].

MicroRNAs (miRNAs), small non-coding RNA molecules involved in post-transcriptional gene regulation, have emerged as potential biomarkers for various diseases, including liver disorders [5]. MiRNAs are highly stable in tissues, blood, and other body fluids, making them attractive candidates for non-invasive diagnostic applications [6]. Recent studies have demonstrated that specific miRNAs are dysregulated in the liver and blood of CHB patients, reflecting underlying pathological processes such as inflammation and fibrosis [7]. Consequently, miRNAs hold promise as diagnostic tools for assessing liver fibrosis and inflammation.

The role of miRNAs in the diagnostics of liver fibrosis has gained significant attention in recent years [8]. Research highlights miR-21, miR-29a, and miR-122, as key regulators of liver fibrosis through their influence on pathways like TGF- β signalling, extracellular matrix production, and hepatic stellate cell activation [7, 9]. For instance, miR-21 is upregulated in fibrotic livers and promotes fibrogenesis by targeting *SMAD7*, a negative regulator of TGF- β signalling [8]. Similarly, miR-29a, known for its anti-fibrotic properties, is downregulated in severe fibrosis, contributing to increased collagen deposition [10]. MiR-122, a liver-specific miRNA, plays a dual role by reflecting both liver injury and fibrosis severity [7]. These dysregulated miRNAs can be detected in the circulation, offering a non-invasive approach to assess liver fibrosis stages and monitor disease progression [8].

Hence one of the most promising aspects of miRNA-based diagnostics is their potential to bridge the gap between tissue-level changes and systemic biomarkers. Since miRNAs are released into the bloodstream from damaged or stressed liver cells, their expression profiles in serum or plasma correlate with histological fibrosis stages. For example, elevated levels of circulating miR-21 and decreased levels of circulating miR-29a have been associated with advanced fibrosis in CHB patients, suggesting their utility as non-invasive diagnostic tools [11, 12]. The integration of miRNA profiling with existing methods, such as transient elastography or serum

markers, could enhance diagnostic accuracy and reduce the reliance on invasive procedures like liver biopsy.

This study aims to evaluate the role of specific miRNAs – hsa-miR-21-3p, hsa-miR-29a-3p, hsa-miR-122-3p, hsa-miR-221-3p, and hsa-miR-222-3p – as potential reliable biomarkers for liver fibrosis in CHB patients. By analyzing miRNA expression levels in patients with different fibrosis stages, we aim to establish correlations between miRNA profiles in liver tissue and blood, providing insights into their diagnostic utility and potential as key clinical tools in the management of chronic liver disease.

Materials and methods. Study population. A total of 40 patients, with a mean age of 52.4 ± 12.2 years, diagnosed with chronic hepatitis B (CHB) were enrolled in this study. All patients were evaluated prior to initiating antiviral therapy. The study cohort was divided into two groups based on fibrosis staging determined by transient elastography (FibroScan®). The control group, consists of 26 patients with mild to moderate fibrosis (FibroScan scores F1-F2). The severe fibrosis group includes 14 patients with advanced fibrosis (FibroScan scores F3-F4).

RNA extraction. Plasma samples were collected from all participants for miRNA analysis. RNA was extracted using the miRNeasy Serum/Plasma Advanced Kit (Qiagen®), in combination with miRNeasy Serum/Plasma Spike-In Control (Qiagen®), following the manufacturer's protocol. RNA concentration and purity were measured by using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific®).

Reverse transcription. Complementary DNA (cDNA) was synthesized from the extracted RNA using the miRCURY LNA RT Kit (Qiagen®). This kit utilizes locked nucleic acid (LNA) technology for efficient and accurate reverse transcription of miRNAs.

Quantitative PCR. The expression levels of five target miRNAs (hsa-miR-21-3p, hsa-miR-29a-3p, hsa-miR-122-3p, hsa-miR-221-3p, and hsa-miR-222-3p) were analyzed using miRCURY LNA miRNA PCR Assays (Qiagen®). U6 snRNA was used as the endogenous control. Quantitative PCR (qPCR) was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems®). Relative miRNA expression levels were calculated using the $2^{-\Delta CT}$ method.

Statistical analysis. The analysis of miRNA expression data was performed using the GeneGlobe Data Analysis Center (Qiagen®), an online tool designed for streamlined interpretation of PCR data.

Statistical comparisons between groups were performed using Student's *t*-tests for normally distributed data and Mann-Whitney U tests for non-normally distributed data. The GeneGlobe platform generated fold change values and provided initial statistical outputs. Further correlation analyses were conducted to evaluate the relationship between miRNA expression levels and fibrosis stages. A *p*-value < 0.05 was considered statistically significant.

Results. The expression levels of hsa-miR-21-3p, hsa-miR-29a-3p, hsa-miR-122-3p, hsa-miR-221-3p, and hsa-miR-222-3p in the two groups are shown in Table 1. The relative expression levels of the five miRNAs were compared between the mild-to-moderate and severe fibrosis groups Table 2. U6 snRNA was used as an internal control for normalization.

The result of fold change analysis of the tested miRNAs are shown in Table 3.

T a b l e 1

Δ CT values for miRNA expression levels

miRNA ID	Control group (Δ CT)	Severe fibrosis group (Δ CT)
hsa-miR-21-3p	4.34	6.01
hsa-miR-29a-3p	1.56	0.36
hsa-miR-122-3p	3.48	4.38
hsa-miR-221-3p	3.61	3.48
hsa-miR-222-3p	3.06	4.29

T a b l e 2

Relative expression ($2^{-\Delta$ CT)

miRNA ID	Control group ($2^{-\Delta$ CT)	Severe fibrosis group ($2^{-\Delta$ CT)
hsa-miR-21-3p	0.049333	0.015473
hsa-miR-29a-3p	0.338619	0.781838
hsa-miR-122-3p	0.089866	0.048064
hsa-miR-221-3p	0.081779	0.089901
hsa-miR-222-3p	0.119534	0.051215

T a b l e 3

Fold change analysis

miRNA ID	Fold change
hsa-miR-21-3p	-3.19
hsa-miR-29a-3p	+2.31
hsa-miR-122-3p	-1.87
hsa-miR-221-3p	+1.1
hsa-miR-222-3p	-2.33

Comparison with established fibrosis markers. Comparison with transient elastography scores FIB-4 showed that miRNA markers provided comparable or superior correlation with fibrosis staging. For instance:

- hsa-miR-29a-3p demonstrated a positive correlation ($r = 0.72$, $p < 0.001$) with FibroScan scores ($r = 0.56$, $p < 0.05$).

- hsa-miR-21-3p and hsa-miR-122-3p showed significant negative correlations ($r = -0.65$, $p < 0.01$ and $r = -0.68$, $p < 0.01$, respectively), highlighting their potential as non-invasive biomarkers.

Multivariate and ROC analysis. Multivariate regression analysis indicated that a combination of hsa-miR-29a-3p and hsa-miR-21-3p achieved an R^2 value of 0.81 in predicting fibrosis stages, outperforming individual markers. ROC curve analysis showed:

- hsa-miR-29a-3p: AUC=0.89 (95% CI: 0.84–0.94).
- hsa-miR-21-3p: AUC=0.87 (95% CI: 0.82–0.93).
- Combined miRNA panel: AUC = 0.92 (95% CI: 0.88–0.96), demonstrating excellent diagnostic accuracy.

These findings suggest that miRNA expression profiling not only complements established fibrosis markers but also provides enhanced diagnostic utility when combined in a panel.

Discussion. Given the global burden that CHB and its complications represent, early diagnosis and sustained follow-up of the progression of hepatic fibrosis is crucial in prevention of liver cirrhosis and end-stage liver disease. The search for reliable, sensitive and non-invasive methods for evaluating liver fibrosis is ongoing. The potential of circulating miRNAs as ideal biomarkers in HBV associated liver disease is becoming more evident [11].

The findings of this study underscore the potential of specific miRNAs as robust diagnostic biomarkers for liver fibrosis in chronic hepatitis B (CHB) patients. miRNAs such as hsa-miR-29a-3p, hsa-miR-21-3p, and hsa-miR-122-3p demonstrated significant correlations with fibrosis severity, offering insights into their biological roles and clinical applicability. Notably, other authors also report similar results [10, 12–14].

In our study, hsa-miR-29a-3p emerged as a promising biomarker, with elevated levels correlating positively with advanced fibrosis. This observation aligns with previous studies highlighting miR-29a's anti-fibrotic properties, primarily through its regulation of extracellular matrix proteins and TGF- β signalling pathways [15]. Its upregulation in advanced fibrosis may reflect compensatory mechanisms aimed at limiting fibrogenesis.

Conversely, hsa-miR-21-3p and hsa-miR-122-3p were downregulated in severe fibrosis, consistent with their known roles in promoting liver homeostasis and suppressing inflammation [16]. MiR-21, a mediator of fibrogenesis, exhibited strong negative correlations with fibrosis staging, making it a reliable marker for distinguishing between early and advanced disease stages [16]. Similarly, the downregulation of miR-122, a liver-specific miRNA, highlights its role in maintaining hepatocyte integrity and its potential as a marker for liver injury and fibrosis severity [17].

MiR-21 dysregulation is common in several types of chronic liver diseases. Nevertheless, studies have reported notable variability and heterogeneity in miR-21 expression. The main explanation may be due to the use of different disease models and study methodologies [13, 14].

Our results are in alignment with previous studies in which miR-122 is found to be downregulated in patients with significant liver fibrosis [13, 18].

The integration of miRNA profiling with established diagnostic modalities such as transient elastography and serum biomarkers demonstrated complementary strengths. Notably, hsa-miR-29a-3p outperformed traditional markers such as the AST/ALT ratio in correlating with fibrosis severity. Furthermore, multivariate analyses combining hsa-miR-29a-3p and hsa-miR-21-3p yielded a predictive model with high diagnostic accuracy, underscoring the value of miRNA panels in clinical practice.

From a clinical perspective, miRNAs offer several advantages over traditional methods. Their non-invasive nature, stability in blood, and disease-specific expression profiles make them ideal candidates for routine monitoring and early detection of fibrosis progression. However, the study also acknowledges limitations, including the small sample size and the need for validation in larger, multi-centre cohorts. Additionally, while miRNA panels demonstrated high diagnostic performance, their integration into clinical workflows requires standardization of protocols and cost-effectiveness analyses.

Future research should focus on expanding the miRNA panel to include additional fibrosis markers and explore their role in predicting treatment responses. Investigating miRNA regulation during antiviral therapy could provide valuable insights into their prognostic potential and therapeutic relevance.

Conclusion. This study highlights the boundaries of applicability and the diagnostic value of miRNAs, particularly hsa-miR-29a-3p, hsa-miR-21-3p, and hsa-miR-122-3p, as non-invasive biomarkers for liver fibrosis in CHB patients. Their integration into existing diagnostic frameworks has the potential to improve patient stratification and treatment outcomes, paving the way for precision medicine in chronic liver diseases.

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