Expression of circulating miRNA-21 and miR-99b in HBV-related liver cirrhosis and hepatocellular carcinoma

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Summary

Objective: Our primary objective was to assess whether these miRNAs could serve as potential biomarkers for distinguishing hepatocellular carcinoma (HCC), even in its early stages, from other liver diseases, particularly liver cirrhosis (LC). *Subject and method:* We conducted a study involving two patient groups: 50 LC and 50 HCC patients. We employed a screening approach utilizing open data sources (PubMed, Science Research, Google Scholar…), alongside bioinformatics analysis tools to identify miRNAs exhibiting high expression levels in cirrhosis and liver cancer. The miRNAs we selected for evaluation included miRNA-21, miRNA-34a, miRNA-99b, and miRNA-224. *Result:* The expression levels of miRNA-21 in plasma were found to be significantly higher in the HCC group compared to the cirrhosis group ($p<0.001$). The combination of miRNA-21 or miRNA-99b and AFP had improved diagnostic performance in distinguishing between these two groups compared to single markers. The area under the ROC curve was significantly high as expected when combining miRNA-99b and AFP with AUC = 0.85 (single miRNA-99b: AUC = 0.715). Similarly for the combination of miRNA-21 and AFP, this simple panel also had a good performance with the AUC = 0.794 (single miRNA-21: AUC = 0.715). *Conclusion*: miRNA-21 and miRNA-99b hold promise as potential biomarkers for the diagnosis of HCC.

Keywords: Hepatocellular carcinoma, cirrhosis, hepatitis B virus, microRNA.

1. Background

Chronic hepatitis B virus (HBV) infection remains a globally significant health concern, with an estimated 296 million individuals affected worldwide. Individuals with chronic HBV infection face a significantly elevated risk of developing hepatocellular carcinoma (HCC), a form of liver cancer, with the risk being approximately 100 times higher compared to those without HBV infection. According to Globocan 2020 [1], in Vietnam, HCC ranks as the leading cancer in terms of both new incidence and mortality rates.

The development of HCC typically occurs after a prolonged period of chronic HBV infection, and among these cases, 70-90% progress from liver fibrosis. Therefore, early screening for HCC in individuals with liver fibrosis due to HBV infection is of paramount importance, offering patients the opportunity for timely intervention through precision treatment modalities. However, currently available HCC screening methods such as alpha-fetoprotein (AFP) levels and abdominal ultrasound exhibit rather low sensitivity and specificity.

In recent years, numerous studies have highlighted the involvement of microRNAs (miRNAs) in liver diseases, particularly in the context of chronic hepatitis B, fibrosis progression, and hepatocarcinogenesis [2]. MiRNAs are small RNA

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molecules, typically consisting of 19-24 nucleotides, which do not encode proteins but play pivotal regulatory roles in modulating gene expression [3]. This regulation contributes to alterations in cellular signaling pathways and aids in the development of cancer.

In Vietnam, there remains a scarcity of research regarding miRNAs in liver disease patients, particularly concerning the comparative assessment of miRNA profiles between individuals with liver fibrosis and those with liver cancer. Consequently, our study endeavors to evaluate the expression levels of selected miRNAs in patients afflicted with liver fibrosis and liver cancer, aiming to elucidate their roles in supporting the screening of hepatocellular carcinoma among high-risk individuals.

2. Subject and method

Study populations

One hundred adult participants were divided into three groups: 50 patients with chronic HBV-related HCC, 50 patients with chronic HBV-related LC were enrolled in this study. Criteria for selecting subjects with chronic hepatitis B infection were either positive for HBsAg and/or HBV DNA for ≥ 6 months, or positive for HBsAg and negative for anti-HBc IgM. Patients with liver cirrhosis were diagnosed based on clinical and subclinical manifestations (abdominal ultrasound, biochemical tests, hematology, and liver elasticity assessed by Fibroscan). Patients diagnosed with HCC were identified through dynamic magnetic resonance imaging (MRI) or triphasic computed tomography (CT), with or without the presence of α-fetoprotein (AFP) or confirmation by pathological evidences. The histological classification and clinical staging of HCC were conducted in accordance with the Barcelona Clinic Liver Cancer staging system. Exclusion criteria include: Patients with alcoholic liver cirrhosis, HCV or HIV infection, drug-induced liver toxicity, etc.

RNA selection procedures: We employed a screening method to identify potential miRNAs by leveraging open data sources such as PubMed, Science Research, Google Scholar, and bioinformatics analysis tools like miRTarbase and TargetScan. This approach allowed us to analyze and pinpoint miRNAs with significantly higher expression levels in primary liver tumor tissues compared to normal liver tissues in patients with liver cancer. Our selection criteria focused on miRNAs that are involved in key signaling pathways associated with the development of hepatocellular carcinoma (HCC), including the Wnt, TGF-B, MAPK, and PI3K-AKT pathways (Figure 1). In this preliminary study, we chose four miRNAs, namely miRNA-21, miRNA-34a, miRNA-99b, and miRNA-224, for further investigation. Our primary objective was to assess whether these miRNAs could serve as potential biomarkers for distinguishing HCC, even in its early stages, from other liver diseases, particularly liver cirrhosis. This differentiation is crucial since liver cirrhosis often precedes the development of HCC in the disease progression continuum.

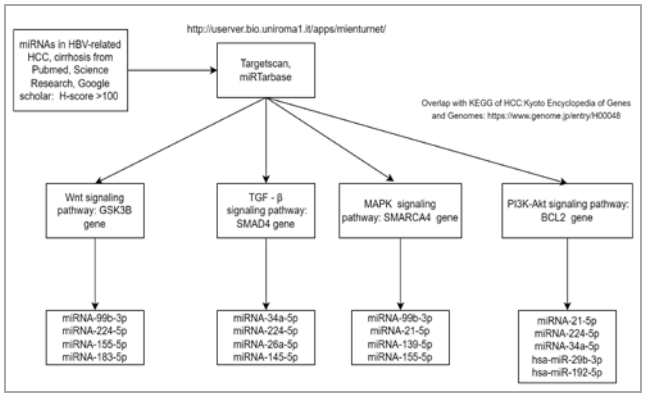

Figure 1. RNA selection scheme RNA extraction and qRT-PCR procedures

Serum total RNA was isolated by phenol-chloroform extraction, followed by cDNA synthesis for miRNA using miRNA-specific stem-loop primers. The authentic mature miRNA sequence was acquired from the miRBase website (http://www.mirbase.org/). Stem-loop RT-qPCR primer design was carried out using the web tool miRNA primer design (http://genomics.dote.hu:8080/mirnadesigntool/index.jsp). Oligo Analyzer were used for checking the the melting temperature of the chosen primers.

Real-time Quantitative PCR (qRT-PCR) for miRNAs was conducted using Real-time qPCR equipment (Roche lightcycler 96). Specific sequence primers were employed for each miRNA target, namely miRNA-34a, miRNA 21-5p, miRNA-224 and miRNA-99b. The expression of each miRNA was standardized using the stably expressed housekeeping miRNA-16. The specific forward primers, sourced from miRbase (https://www.mirbase.org/), were as follows:

miRNA-34a: 5'-GTG TTT TTT GGC AGT GTC TTA G-3'.

miRNA-21-5p: 5'-GCC CGC TAG CTT ATC AGA CTG ATG-3'.

miRNA-224: 5'-GTG TGT TTC AAG TCA CTA GTG GTT -3'.

miRNA-99b: 5'-TGT TTT TCA AGC TCG TGT CTG T-3'.

The reagent for performing real-time qPCR miRNA was Bioline SYBR Green master mix. Reaction compositions consist of 5µl SYBR Green master mix, 0.4µl PCR primer mix, 4.6µl cDNA sample which has been diluted with a ratio of 1:10.

The relative expression of the target gene was determined through quantification cycle (Cq) analysis employing Livak's method [4]. The formula for miRNA relative expressions is as follows:

ΔCq HCC group = Cq (miRNA target, HCC group) - Cq (miRNA-16, HCC group). ΔCq LC group = (miRNA target, LC group) - Cq (miRNA-16, LC group). ΔΔCq = ΔCq HCC group - ΔCq LC group. Fold change expression miRNA target = $2^{-\Delta\Delta Cq}$.

Statistical analysis

Analysis was performed with SPSS 20.0 to assess between-group differences with Student's t test. ROC curves were calculated to assess the diagnostic efficiency of serum miRNA-21, miRNA-34a, miRNA-99b, miRNA-224 levels and AFP levels. The AUC was used to measure the predictive value. Two-sides p values of < 0.05 were considered with a statistical significance.

Ethics committee and funding

The study received approval from the Hanoi Medical University Institutional Ethical Review Board, with approval number: 894/GCN-HĐĐĐNCSYH-ĐHYHN. Financial support for this study was provided by the Vingroup Innovation Foundation (VINIF) under project number VINIF.2021.DA00212, with Dr. Nghiem Xuan Hoan serving as the principal investigator. The funding source had no involvement in the study's design, data collection and analysis, the decision to publish, or the preparation of the manuscript.

3. Result

3.1. Characteristics of study subjects

Table 1. Characteristics of study subjects

	HCC	LC
Age: Mean ± SD	60.33 ± 9.77	58.58 ± 11.25
Gender		
Male	49 (97.6%)	39 (78%)
Female	1 (2.4%)	11 (22%)
BCLC:		
A n (%)	10 (25%)	
B	22 (55%)	
C	8 (20%)	
D	0 (0%)	
AFP (ng/ml)	20857.49 ± 14063	45.38 ± 22.73

The mean age of patients in the HCC group was 60.33 ± 9.77. The majority of patients were male (97.6%), while females make up a small portion (2.4%). In the study, 25% of patients were in the early stage of the HCC (BCLC-A). The mean AFP level in the HCC group was 20857.5 ± 14063ng/mL, in the LC group, the mean age was 58.58 ± 11.25.

3.2. The miRNA expression levels in HCC and LC groups

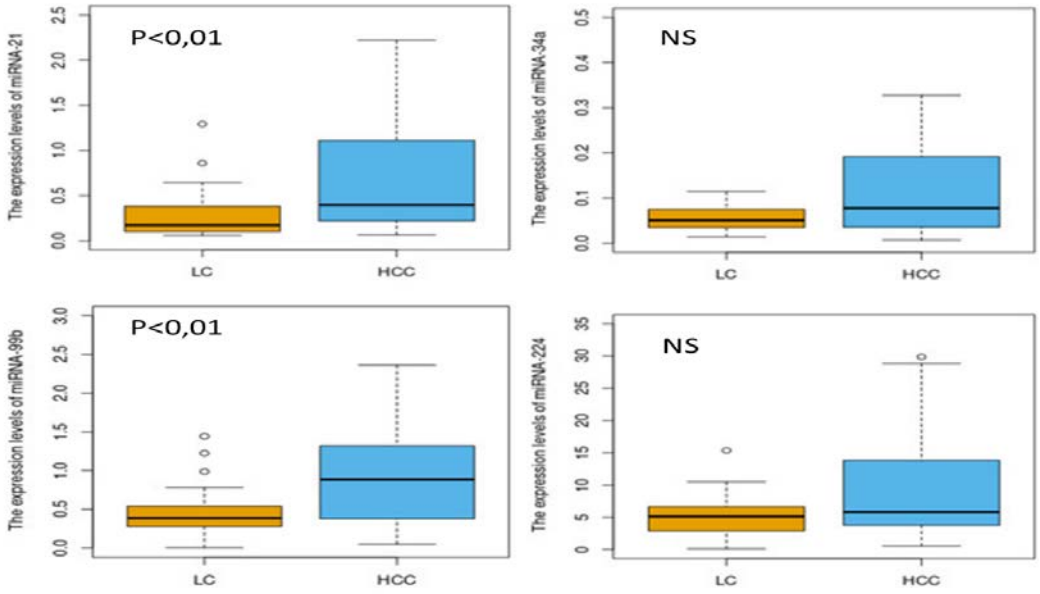

Figure 2. Expression levels of miR-21, miR-34a, miR-99b and miR-224 in HCC and LC patients

The expression levels of all four miRNAs were higher in HCC patients compared to those with LC. However, a statistically significant difference between HCC and LC was observed for miRNA-21 and miRNA-99 ($p<0.01$).

3.3. Performance of single miRNA in differentiating HCC from LC

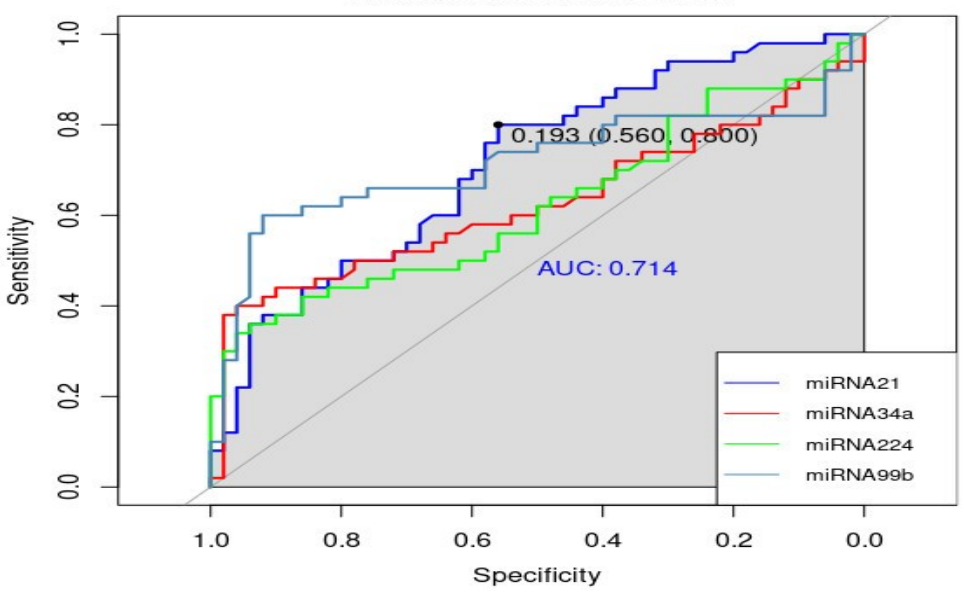

Figure 3. Performance of single miRNA in differentiating HCC from LC

MiRNA	AUC	p	Cut-off	Sensitivity (%)	Specificity (%)
miRNA-21	0.714	<0.001	0.193	80%	56%
miRNA-34a	0.627	0.029	0.11	40%	96%
miRNA-99b	0.715	<0.001	0.7	60%	92%
miRNA-224	0.622	0.035	8.96	36%	94%

In the diagnostic differentiation between the HCC and LC patient groups, at the cut-off point of 0.193, miR-21 exhibits a sensitivity of 56% and a specificity of 80%. The area under the ROC curve for miRNA-21 was greater than that for miRNA-34a and miRNA-224, with an AUC of 0.714 (p<0.001). MiRNA-99b also held significance in distinguishing between the HCC and LC groups, with an AUC of 0.715 (p<0.001), a sensitivity of 60%, a specificity of 92%.

3.4. Performance of miRNA-21 or miRNA-99b combining with AFP in differentiating HCC from LC patients

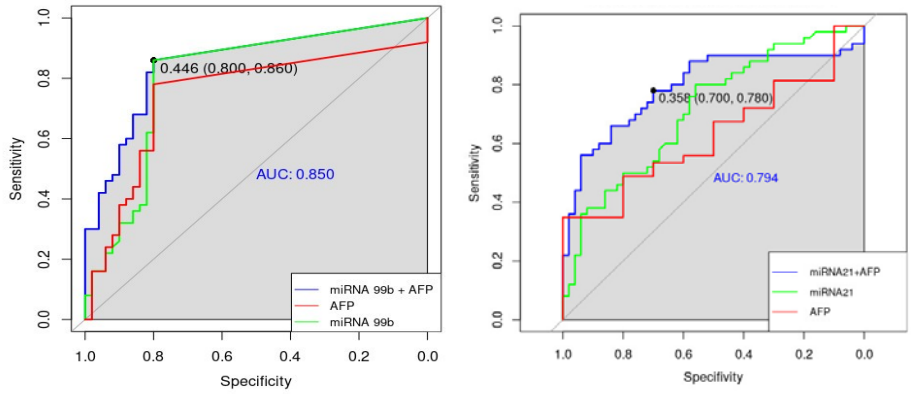

Figure 4. Performance of miRNA-21 or miRNA-99b combining with AFP in differentiating HCC from LC patients

The combination of miRNA-21 and miRNA-99b with AFP increased performance of differentiation between HCC and LC when compared to single markers. The area under the ROC curve was significantly high as expected when combining miRNA-99b and AFP with AUC = 0.85. At the cutoff point of 0.446, the combination of miRNA-99b and AFP had a sensitivity of 80% and a specificity of 86% (p<0.05). Similarly for the combination of miRNA-21 and AFP, at the cutoff point of 0.385, this simple panel had a sensitivity of 70% and a specificity of 78% with the AUC = 0.794; p<0.05. The analyzed data have shown that the combination of miRNA-21, miRNA-99b and AFP had improved diagnostic performance in distinguishing between these two groups compared to single markers.

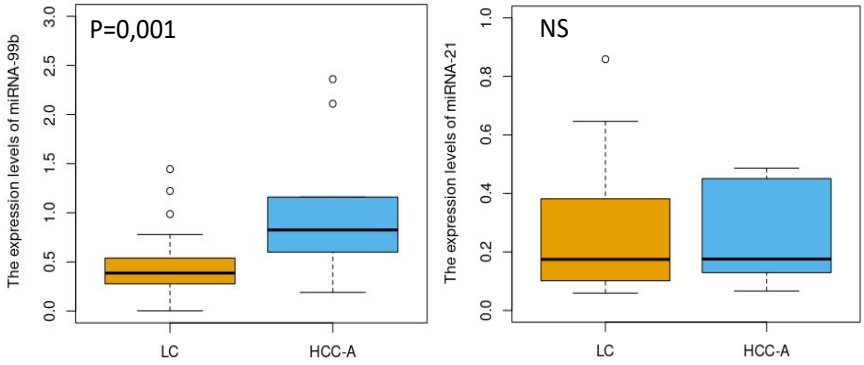

Figure 5. Expression of miRNA-21 and miRNA-99b in the early-stage HCC (BCLC-A) and LC

Significantly increased expression of miRNA-99b (but not for miRNA-21) was found in early stage of HCC (HCC-A) compared to LC group indicating significant value in diagnosing early-stage HCC.

4. Dicussion

MiRNAs can be considered non-invasive biological markers to support the diagnosis of various types of cancer. They exhibit high stability in cells and serum, are resilient to room temperature, freezing conditions, and the presence of RNAse enzymes. In the case of hcc, miRNAs can be utilized in diagnosis, targeted therapy, and prognosis. MiRNAs can function as tumor suppressors [5, 6] or oncogenes [7].

This study initially screened and identified four potential miRNAs: miRNA-21, miRNA-34a, miRNA-99b, and miRNA-224 from a list of miRNAs associated with HBV infection status in LC and HCC patients. The selection criteria included miRNA expression levels in the HCC group compared to healthy individuals ≥ 2, H-score ≥ 100, data from miRNA databases (miRTarbase, Targetscan, etc.), and bioinformatics analysis tools, all sourced from open data repositories like Pubmed, Science Research, and Google ScholarMiR-21 has been demonstrated to play a role in promoting fibrogenesis in multiple organs [8]. Clinical data have also indicated that miR-21 expression is increased in the livers of patients with liver fibrosis, miR-21 induces fibrosis by activating hepatic stellate cells (HSCs) and stimulating collagen synthesis [9, 10]. The analysis of miRNA expression levels reveals that miRNA-21 is significantly higher, being 2.5 times greater in the LC group (p=0.01). These results are in line with previous studies by Juan Qu and colleagues (2019) [11] and the research conducted by Dr. Dang Chieu Duong in 2020 [12] at 108 Military Central Hospital. At the cutoff point of 0.193, miRNA-21 shows the ability to differentiate between HCC and LC with a sensitivity of 56% and a specificity of 80%, and an area under the ROC curve (AUC) of 0.714 (p<0.001). These findings are consistent with the study by Juliane Malik [13] in 2022, which reported an AUC of 0.71 (p<0.0001), and the research by Ngo Tat Trung [14] in 2018 with an AUC of 0.698 (p<0.05). MiRNA-21 exhibits a higher diagnostic value than AFP, and when combined with AFP to distinguish between HCC and cirrhosis, the AUC increases significantly compared to miRNA-21 and AFP individually (AUC = 0.794). This finding aligns with the report by Ngo Tat Trung [14] in 2018, where the combined AUC was 0.714 (p<0.05).

MiRNA-99b demonstrates the ability to distinguish between the HCC and LC groups with an AUC of 0.715 at a cutoff point of 0.7, showing a sensitivity of 60% and a specificity of 92%. The 2014 study by Youwen Tan revealed that the expression level of miRNA-99b-3p in the HCC group was 4.6 times higher than in the healthy group (p<0.05) [15]. MiRNA-99b demonstrates superior diagnostic efficacy compared to AFP. When utilized alongside AFP to differentiate between HCC and cirrhosis, the combined AUC shows a substantial increase in performance, reaching 0.85 with a sensitivity of 80% and a specificity of 86%, individually (p<0.05). Recent research has indicated that members of the miR-99 family exhibit enhanced expression in liver tissues infected with HBV. Their upregulation stimulates HBV replication and antigen expression in cells from HCC patients. The increased expression of miR-99 inhibits the activation of the IGF-1R/AKT/mTOR pathway and promotes HBV replication after transcription through an autophagy process mediated by mTOR/Unc-51-like kinase 1 (ULK1) signaling pathway [16]. Furthermore, in the HCC early-stage group (BCLC: A), miRNA-99b proves to be diagnostically significant in distinguishing from the LC group, with miRNA-99b in the early-stage HCC group being 2.3 times higher than in the LC group (p=0.001). However, it's important to note that the sample size of the early-stage HCC group in our study is relatively small. To precisely determine the differentiation capability between the early-stage HCC group and LC group for miRNA-99b, further research with a larger number of patients will be conducted in the later phases of the study. There is no clear distinction between the HCC and LC groups in terms of two miRNAs: miRNA-34a and miRNA-224.

5. Conclusion

MiRNA-21 and miR-99b have the potential to be utilized as non-invasive biomarkers for the early detection of HCC. When combined with AFP (Alpha-fetoprotein), miRNA-21 and miRNA-99b can be employed as a biomarker to improve the accuracy of HCC diagnosis in individuals with hepatitis B virus (HBV) infection. Nonetheless, further comprehensive research is essential to validate the roles of miR-21 and miR-99b and to develop highly effective diagnostic models for the early detection of HCC.

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