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Original article

Association of miR-122 and miR-21 with the severity of HDV infection

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Abstract. *Background and aim:* Hepatitis D is the most severe manifestation of chronic viral hepatitis, characterized by significant clinical ramifications. These repercussions encompass an elevated susceptibility to hepatic decompensation and the development of hepatocellular carcinoma (HCC), ultimately culminating in fatal outcomes. The role of miRs in the pathogenesis of this disease remains largely unexplored. *Methods:* The study enrolled 102 treatment naïve chronic hepatitis D patients. 31 patients had chronic hepatitis D and 71 patients liver cirrhosis of HDV aetiology. The EIA confirmed HDV infection with the following quantitative PCR. *Results:* Compared to LC patients, those with CHD showed significantly higher levels of miR-122 and lower levels of miR-21. miR-122 expression inversely correlated with the severity of liver cirrhosis, showing lower numbers in the group with LC Class A compared to the LC Class B. Meanwhile, the group of patients without liver cirrhosis had the highest values. miR-21 was also higher in the LC Class A group compared to LC class B and patients without liver cirrhosis. *Conclusions:* miR-122 and miR-21 could serve as an effective predictor for further decompensation of liver cirrhosis and exacerbation of the HDV infection process. This assumption requires further study in a larger sample. (www.actabiomedica.it)

Key words: HDV infection, miR-122, miR-21, liver cirrhosis

Introduction

MicroRNAs (miRNAs or miRs) are small non-coding RNA molecules that form during RNA splicing and significantly regulate gene expression (1, 2). Their mechanism of action involves complementary interactions by binding to the 3'-untranslated regions (UTRs), coding sequences, or 5'-UTRs of target messenger RNAs (mRNAs), leading to the suppression of mRNA translation or the promotion of mRNA degradation (2). miRs constitute 1-5% of the human genome and are implicated in the functioning of 30% of protein-coding genes, participating in various cellular processes, such as cell development, differentiation, proliferation, metabolism, and immune responses (2, 3). Due to their involvement in numerous biological pathways, it is anticipated that altered miR expression is linked to a range of non-infectious and infectious diseases, including viral infections. Based on their targets and mechanisms of action, miRs can either act as proviral factors, enhancing viral replication and infection, or as antiviral factors, inhibiting viral replication, suppressing pro-viral proteins, or inducing viral latency (2-4).

Recent data increasingly indicate the crucial role of miRs in normal liver functioning and in liver diseases, particularly viral hepatitis B and C (5). Research has demonstrated the expression of 277 miRNAs in the liver (6), with miR-122 being among the most abundant and liver-specific, accounting for up to 70% (7). Research into the mechanisms of miR involvement in the pathogenesis of viral hepatitis D remains

significantly limited. The uniqueness of the HDV infection's pathogenesis stems from the fact that the hepatitis D virus is a satellite virus of hepatitis B virus and cannot replicate independently (8,9). Consequently, miRs expression within hepatitis D liver cells is intrinsically linked to the simultaneous presence of both viruses. The relevance of hepatitis D is notably pronounced in Uzbekistan, a nation where hyperendemicity of this infection prevails. Superinfection involving HDV is observed in more than 80% of cases exhibiting HBsAg-positive cirrhosis, with the prevalence of HDV infection among HBsAg-positive individuals reaches 49.1%. This prevalence significantly surpasses HBV mono-infection (9.3%) within Uzbekistan (10).

Our study also delved into miR-21 as a subject of investigation. This miR has been implicated in the induction of fibrosis in various organs, encompassing the lungs, kidneys, and heart (11,12). While not as prominently expressed in the liver as miR-122, miR-21 exerts a notable influence on liver biology and diseases through multiple ways. Its impact encompasses metabolic and regenerative processes that play a role in conditions like non-alcoholic steatohepatitis and hepatocellular carcinoma (HCC) (13). Like other miRs, miR-21 directly binds to the 3'-UTR of its target genes, exerting a negative modulation on their expression (14). The available data concerning miR-21 expression levels in the context of viral hepatitis is limited, and the outcomes obtained are occasionally inconclusive.

Our review did not uncover any information regarding the behavior of miR-122 and miR-21 in the context of hepatitis D from the available literature. One study (15) compared the expression levels of miR-222 in hepatitis D and hepatitis B monoinfection, which indicated a substantial increase in miR-222 expression in hepatitis D. However, it is important to avoid generalizing these findings to other miRs, as each miR exhibits distinct characteristics. Additionally, it's worth noting that the behavior of circulating and tissue miRs may not always align consistently. In this study we focused on the expression of miR-21 and miR-122 in chronic HDV infection to better understand the potential of these miRs as candidate non-invasive markers of progression and severity of the chronic HDV infection.

Materials and methods

Subject of the study

We retrospectively studied 102 serum samples of treatment-naïve adult patients with chronic hepatitis D admitted to the Research Institute of Virology clinical department between July 2021 and July 2022. Chronic hepatitis D was diagnosed based on the history of the disease, clinical symptoms, laboratory tests by ELISA (HBsAg, anti-HDV), PCR (HBV, HDV) and instrumental (ultrasound, fibroscanning) methods. Among 102 hospitalized patients, 31 patients were diagnosed chronic hepatitis D and 71 patients had liver cirrhosis of HDV etiology. Patients with a positive HIV or anti-HCV test, a history of significant intake of alcohol or hepatotoxic drugs within the past 6 months prior to admission, a history of chronic liver disease secondary to causes other than hepatitis B were excluded. For comparative analysis of circulating miRNAs, 30 healthy individuals were enrolled in the study. The study was approved by the Ministry of Health Ethics Committee of the Republic of Uzbekistan, No.2/11-1506, 17 April 2021. The study was performed in accordance with the 1964 Declaration of Helsinki.

Blood sampling

Blood samples were centrifuged at 1500·g for 10 min at 4°C, and then, the sera were aliquoted and additionally centrifuged at 5000·g at 4°C to remove remaining cells. Serum samples were stored at minus 80°C until further processing.

Clinical chemistry and virology

According to patients' records all subjects underwent biochemistry analysis using Semi-Auto Chemistry Analyzer BA-88A (China). According to the manufacturer's recommendations, normal ALT levels were defined as up to 35 IU/L; values greater than 35 IU/L were considered elevated. HBsAg and anti-HDV were determined by EIA EIA-HBsAg and EIA-anti-HDV commercial kits (Vector-Best, Nizhny Novgorod, Russia), respectively. HBV DNA

and HCV RNA were detected by quantification PCR kits AmpliSens®HBV-FL and AmpliSens®HDV-FL (Russia).

RNA isolation and quantitative real-time Polymerase Chain Reaction assay

Total RNA was extracted from 200 μL of serum samples using a Qiagen miRNeasy Mini kit according to the manufacturer's instructions. MiRNeasy Serum/Plasma Spike-In Control containing C. elegans miR-39 miRNA mimic was added to each sample during the RNA extraction step to allow sample-to-sample normalization of miRNA recovery and reverse transcriptase efficiency. The concentration of isolated RNA was quantified using Qubit® RNA HS Assay Kit by Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY, USA). The relative abundance of specific miR was analyzed by synthesizing cDNA using the Qiagen miScript II RT. Next, real-time PCR was performed using the miScript SYBR® Green PCR Kit (QIAGEN, Germany) on a Rotor-Gene Q platform (Qiagen, USA). A melting curve analysis resulted in single product-specific melting curves for each sample. The relative serum miR-122 and miR-21 expression levels were calculated using the 2–ΔΔ Ct method.

Statistical analysis

Demographic characteristics, laboratory data and clinical diagnosis from medical records were entered into a spreadsheet. Excel (version 2202 Build 16.0.14931.20806) was used for coding and documenting the data. The data were analyzed with DATAtab Team (2022) (DATAtab: Online Statistics Calculator. DATAtab e.U. Graz, Austria. URL https:\\datatab .net).

Numerical variables are represented in the form of mean with standard deviation as well as median or inter-quartile range whenever applicable. Correlation was analyzed using the non-parametric two-tailed Spearman rank correlation test. Expression levels of plasma miRNAs between groups were compared using the Mann-Whitney U test or the Kruskall-Wallis test with Dunn-Bonferroni post-hoc analysis. All p-values less than 0.05 are considered significant.

Results

Patient characteristics

A total of 102 plasma samples were collected from treatment naïve chronic hepatitis D patients during the study period (Table 1). Among them, 31 patients were diagnosed with chronic hepatitis D (CHD; 51.6% men; mean age 37 years), and 71 patients were diagnosed with liver cirrhosis of HDV aetiology (60.6% men, mean age 42.4 years). HDV infection was confirmed by the EIA, followed by quantitative PCR. The control group (healthy individuals) consisted of 28 patients (men 53.5%; mean age 37.5 years) with a negative status for markers of HBV, HCV and HDV infection.

miR-21 and miR-122 expression profiles in different group of patients according to clinical diagnosis

To investigate whether serum levels of miR-21 and miR-122 might be useful for differentiating patients with minimal and mild or severe liver injury, we compared their expression profiles in a group of patients with liver cirrhosis (LC) and patients without cirrhosis. Compared to LC patients, those with CHD showed significantly higher levels of miR-122 (99.5 vs. 13.0, p-value <0.05, Figure 1. A) and lower levels of miR-21 (0.75 vs. 1.77, p-value <0.001, Figure 1. B). Spearman's rank correlations with miRNAs expression levels are showed that miR-122 was positively correlated with HDV and HBV replication activity and inversely correlated with ALT and severity of liver cirrhosis. miR-21 expression levels positively correlated with ALT serum levels and inversely correlated with severity of liver cirrhosis.

Serum miR-122 and miR-21 levels differentiate different groups of patients according to severity of liver damage

To determine if ALT activity and expression of miR-122 and miR-21 could be useful in discriminating the severity of liver cirrhosis, the group of patients with LC was divided into two subgroups with different degrees of severity according to the Child-Pugh

Table 1. Characteristics of patients according to clinical diagnosis.

Variables		Liver Cirrhosis	Chronic Hepatitis Delta	Healthy Individuals
Gender	F	28	15	13
	M	43	16	15
	Total	71	31	28
Age	Mean±SD	39.15±7.4	42.42±8.8	38.86±6.7
ALT	Mean±SD	42.75±16.8	38.1±20.1	30.48±3.7
	Median (IQR)	36.2 (11.75)	35 (4.35)	30.5 (5.7)
HDV	Mean	$1.9x10^6$	$1,9x10^5$	
	Median (IQR)	0.19 (16575)	156.65 (2191.3)	
HBV	Mean	330.65	13314.94	
	Median (IQR)	0 (150)	94.5 (141.15)	
miR-122	Mean	12.93	99.58	1.2
	Median (IQR)	7.9 (14.8)	39.03 (91.87)	1.15 (0.42)
miR-21	Mean	1.77	0.75	0.53
	Median (IQR)	1.6 (1.5)	0.8 (0.3)	0.4 (0.43)

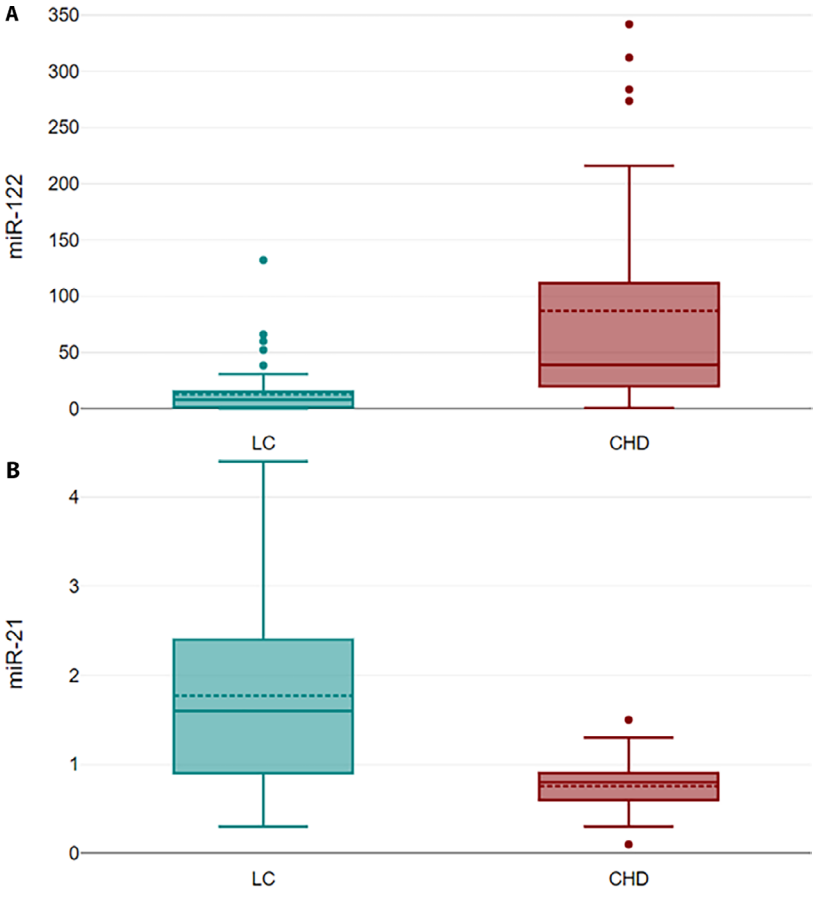

Figure 1. A. miR-122 level in patients with LC and CHD. B. miR-21 level in patients with LC and CHD.

Table 2. Kruskal-Wallis and Dunn-Bonferroni post-hoc test statistics for patients' groups divided according to severity of liver damage.

Variable	Chi^2 (p)	LC A Mean Rank	LC B Mean Rank	CHD Mean Rank	Pair-wise Comparisons [a]		
					LC A - CHD	LC A – LC B	CHD – LC B
miR-122	44.77 **(p-value <0.001)**	54.12	21.77	76.19	-22.07 **	32.35 ***	54.42 ***
miR-21	52.46 **(p-value <0.001)**	72.34	23.75	39.17	48.59 ***	33.17 ***	-15.42 NS
ALT	8.04 **(p-value <0.05)**	56.52	39.03	57.67	17.49 *	-1.15 NS	-18.64 *
N		45	31	26			

Abbreviations: LC A: liver cirrhosis Class A; LC B: liver cirrhosis Class B; CHD: chronic hepatitis Delta; [a]: Top value are mean difference between groups; (*): significant level at 0.05; (**): significant level at 0.01; (***): significant level at 0.001.

scale and compared with CHD and LC groups. Data were analyzed by the Kruskal–Walli's test with further pare-wise comparison (Table 2). Significant differences were found between subgroups in terms of ALT scores and expression of studied miRNAs: miR-122 (Chi^2 =44.77, df 2, p-value <0.001); miR-21 (Chi^2 =52.46, df 2, p-value <0.001); ALT (Chi^2 =8.04, df 2, p-value <0.05). To examine the differences between groups, we performed pair-wise comparison. Patients with LC class A showed significantly higher serum levels of miR-122 and miR-21 if compare with LC Class B (p-value <0.001). Pairwise comparisons indicated that miR-122 and miR-21 levels in LC Class A group observed to be significantly different from those of group LC Class B and CHD. There was also significant difference in ALT activity scores and miR-122 levels between CHD and LC Class B.

Discussion

The circulating miRs have recently attracted much attention in various physiological and pathological conditions and disease differentiation as diagnostic and prognostic biomarkers. A growing body of evidence suggests that the expression pattern of several miRs, including miR-21 and miR-122, are associated with the severity of liver damage. miR-21 and miR-122 are the most abundantly expressed liver miRNAs (16). miR-21 is hypothesized to regulate the genes in hepatic cholesterol and triglyceride metabolisms and its expression was associated with the degree of liver fibrosis (17). miR-122 is involved in many physiological processes and liver pathologies and plays a central role in various aspects of liver function and developing liver diseases (18). Most studies emphasize the role of miR-122 associated with the severity of the disease course, making it a potentially useful blood marker to detect liver damage, including that associated with HBV infection (19,20). The correlation between miR expression and HBV viral load has already been reported in numerous studies (21,22). Some studies have shown that the circulating miR-122 level is significantly decreased in acute HBV infection. (23). In patients with HBV infection, miR-122 is reduced in liver cells. However, its serum levels were increased, possibly because miR-122 is released from damaged hepatocytes (24).

In our study, we also found a positive correlation of miR-122 expression levels with HDV and HBV replication activity and negative correlation analysis with ALT showed that ALT scores were inversely correlated with miR-122 levels and directly correlated with miR-21 expression levels.

Blood alanine aminotransferase (ALT) enzymatic activity values are the most widely used biochemical indicators of the development of liver cell damage, although they have been reported to have debatable specificity (25). The degree of increase in hepatic transaminases reflects the intensity of hepatocyte cytolysis, so the presence of a correlation between ALT and miR-122 and miR-21 is quite expected. Compared

to the increase in blood aminotransferase activity, the change in miR-122 concentration appears earlier. In addition, this change is more specific for liver damage than for damage to other organs and is more reliable because it correlates with the histologic stage of liver disease (26). Both miR-21 and miR-122 were inversely correlated with the severity of liver cirrhosis.

The comparative analysis of miR-122 and miR-21 levels demonstrated that the development of LC was accompanied by a 6-fold decrease in miR-122 levels compared to the CHD patient group (13.0 vs 99.5, $p<0.001$). Expression of this miR-21 was also upregulated in the group with LC compared to the CHD patient group (1.77 vs. 0.75, $p<0.001$). The lower plasma miR-122 levels in patients with severe disease courses may be secondary to a lack of functional liver cells. On the contrary, their active release from damaged liver cells occurs in earlier phases of the disease. In our study, miR-122 expression inversely correlated with the severity of cirrhosis, showing lower numbers in the group with LC Class A compared to the LC Class B. Meanwhile, the group of patients without cirrhosis had the highest values. miR-21 was also higher in the LC Class A group compared to LC class B and patients without liver cirrhosis. Pairwise group comparisons showed that these miRs effectively distinguished the severity of liver damage in patients with viral hepatitis Delta.

Conclusion

The study of miR-122 and miR-21 in chronic viral hepatitis B and D will make it possible to understand their role in various liver pathologies and evaluate the potential value of these miRs as noninvasive markers of liver diseases of viral aetiology. It may be of practical interest for developing risk groups for liver fibrosis development. By the level of miR-122 and miR-21 investigated in the dynamics, one can judge the rate of progression of liver damage. Together, miR-122 and miR-21 could serve as an effective predictor for further decompensation of liver cirrhosis and exacerbation of the HDV infection process. This speculation requires further study of quantitative changes in miR-122, miR-21 in a larger sample to understand the effectiveness of their use as a prognostic and differential marker in various groups of patients with chronic hepatitis Delta.

Limitation

Due to the study's retrospective nature, we could not analyze the sensitivity and specificity of miR-122 and miR-21 for discriminating the severity of the liver cirrhosis process and determining the stage of liver fibrosis. Nevertheless, to the best of our knowledge, this is the first study of quantitative changes in circulating miR-122 and miR-21 in patients with chronic hepatitis Delta.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: EJ conceived and designed the study, drafted the first version of the manuscript; NI editing, drafted the final version of the manuscript; AKh conceived and designed the study; MA analyzed the data; MKh collected the data; MB drafted the first version of the manuscript; EI review, supervision. All authors have read and agreed to the published version of the manuscript.

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