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Role of MicroRNAs in the Development of Chronic Liver Disease in Hepatitis Virus-Infected Egyptian Population

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Abstract

BACKGROUND: The identification of miRNAs that play a role in regulating the viral life cycle and its related liver diseases opens the door for the development of diagnostic biomarkers that can categorize patients at increased risk of developing end-stage liver disease.

AIM: This study investigated the role of miRNAs in the development of viral hepatitis-induced chronic liver disease (CLD) in the Egyptian population, as well as their potential as possible diagnostic biomarkers for chronic hepatitis virus infection.

MATERIALS AND METHODS: The study involved 100 CLD patients; 55 cases of hepatitis C virus (HCV) and 45 cases of non-viral hepatitis, in addition to 40 healthy controls. The expression of five miRNAs (miR-30, miR-122, miR-296, miR-351, and miR-431) was assessed using real-time PCR.

RESULTS: Serum levels of miR-30, miR-122, miR-296, miR-351, and miR-431 were significantly higher in all patients than in the control group ($p < 0.01$). Furthermore, they were significantly greater in viral hepatitis cases compared to the non-viral hepatitis group ($p < 0.01$). The sensitivities and specificities of miR-122a, miR-30, miR-296, miR-351, and miR-431 were (85.71% and 83.33%), (82.35% and 83.33%), (85.71% and 69.44%), (88.64% and 75.76%), and (87.80% and 65.79%), respectively.

CONCLUSIONS: miR-30, miR-122, miR-296, miR-351, and miR-431 play key roles in the development of CLD due to viral infection. Thus, they have the potential to be targeted for the early detection of chronic hepatitis virus infection and allow the exploration of a new frontier in the discovery of innovative therapies to combat chronic viral infection and its serious life-threatening complications, including liver cancer.

Introduction

Despite advances in the development of vaccines and antiviral agents, liver disorders have increasingly become one of the leading causes of death and illness worldwide. Almost 2 million people die per year as a result of major liver illnesses, such as hepatitis virus infections, cirrhosis, and liver cancer, accounting for 4% of all deaths worldwide [1].

One of the key reasons for end-stage liver disease is infection with viral hepatitis, including hepatitis C virus (HCV) and hepatitis B virus (HBV). Understanding the molecular pathways that mediate the chronicity of this infection and its sequelae are required for therapeutic intervention against a variety of life-threatening liver illnesses [2].

The replication of hepatitis viruses, inside the liver cells, differentially disturbs the expression of host miRNAs, which sequentially regulate the virus life cycle by directly targeting its genome and/or cellular signaling pathways, promoting the adaptation, and persistence of virus infection [3]. Therefore, accumulating evidence reveals that miRNAs could be predictive and prognostic biomarkers, as well as therapeutic targets [4]. More in-depth research on miRNAs associated with and modulating viral infection is indispensable, and their functional role has yet to be clarified [5]. The identification of miRNAs with a prominent role in the viral life cycle and the resulting hepatic disease creates a potential for the development of diagnostic biomarkers that can classify and predict early patients who are at increased risk of developing the end-stage liver disease as a result of viral hepatitis infection [6]. This will also open up a new avenue for the development of novel medications to combat chronic hepatitis virus infection and its devastating repercussions, such as liver cancer.

In the present study, the expression of five miRNAs (miR-30, miR-122, miR-296, miR-351, and miR-431) was analyzed in cases of viral and non-viral induced chronic liver diseases (CLDs) in the Egyptian population to investigate the role of miRNAs and their potential as therapeutic targets against chronic hepatitis virus infection.

Materials and Methods

Subjects of the study

The participants of the present study were recruited from the Hepato-Gastroenterology Department, Theodor Bilharz Research Institute, Giza, Egypt. They included 55 cases of HCV-induced CLD, 45 cases of non-viral hepatitis-induced CLD, and 40 healthy individuals who served as controls. CLD was diagnosed based on a history suggestive of liver disease (yellow sclera, fatigue, and pruritus), clinical stigmata (jaundice, palmer erythema, and ascites), and investigations (abnormal liver functions and abdominal ultrasound). Exclusion criteria included a history of drug use (3 months before enrollment in the study) and autoimmune diseases (thyroiditis, vitiligo, and inflammatory bowel disease).

All subjects were subjected to a detailed history, full clinical examination, routine laboratory evaluation by complete blood count, liver function tests (including ALP and GGT), and kidney function tests, and abdominal ultrasound. Informed consent forms were signed by all participants according to the guidelines of TBRI's Ethics Committee and in accordance with the 1975 Declaration of Helsinki.

Serum preparation and RNA extraction

Five milliliter of blood was spun at 1600 rpm for 5 min, and serum was aliquoted into 1.7 ml Eppendorf tubes before 15 min of high-speed centrifugation at 12000 rpm to remove all cell debris, leaving only circulating RNA. 250 μL of serum was homogenized in 750 250 μL of Trizol LS (Invitrogen) for RNA isolation. The pellet was then combined with 200 μL of chloroform before being centrifuged. After an additional chloroform extraction and isopropanol precipitation, the particle was washed twice with 70% ethanol and centrifuged. The RNA pellet was then left to dry for 10 min before being dissolved in 30 μL of diethylpyrocarbonate-treated water. DNase treatment (Qiagen) was carried out to eliminate any contaminating DNA. The concentration and quality of RNA were measured by UV absorbance at 260 nm and 280 nm (A260/280 ratio) and checked by gel electrophoresis individually. In general, 1 ml of serum yielded approximately 600 ng of RNA.

Reverse transcription (RT) and quantitative PCR (qPCR)

According to the literature, five miRNAs (miR-30, miR-122, miR-296, miR-351, and miR-431) were chosen for their distinct expression patterns during viral hepatitis infection [7]. For example, the expression of these miRNAs was found to be dysregulated in human hepatoma cell line Huh7 infected with HCV about 3–15-fold [8]. RT and qPCR kits designed specifically for miRNA analysis (Applied Biosystems, USA) were used to examine the expression of the specified miRNAs in serum samples. The TaqMan® microRNA RT Kit (Thermo Fischer Scientific, USA) was used to perform the RT reactions, according to the manufacturer's instructions. One hundred nanogram of diluted RT products (1.33 μL) was mixed with 10 μL of ×2 Taqman PCR master mix (No AmpErase UNG), 1 μL TaqMan MicroRNA assay, and 7.67 μL nuclease-free water. The miRNA assays used in the qPCR were ready-made assays provided by Thermo Fischer Scientific, USA. The following conditions were used to run all reactions on the ABI 7300 (Applied Biosystems, USA): 95°C for 10 min, then 40 cycles of 15 s at 95°C, and 1 min at 60°C. Each reaction was carried out in triplicate, including no-template control reactions. The comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) approach was used to quantify miRNA relative expression [9], with miRNA-16 serving as the endogenous control to standardize the data.

Statistical analysis

Due to the magnitude and range of relative miRNA expression levels observed, the results were log-transformed for analysis. Quantitative data were presented as mean ± SE while qualitative data were expressed as frequencies and percentages. The Kolmogorov–Smirnov test revealed no indication of deviation from normality for the log-converted data. Analysis of variance test was performed to compare the expression levels of the selected miRNAs between cases and controls.

To assess the specificity and sensitivity of miRNAs for predicting cases, receiver operating characteristic curves were created and the area under the curve (AUC) was measured. Statistical significance was defined as probability level of $p < 0.05$. SPSS 20.0 (SPSS, Inc.) was used to conduct the data analysis.

Results

Subjects of the study

This study involved 140 participants divided into 100 adult patients with CLD, 55 of whom had viral hepatitis (HCV) and 45 had non-viral hepatitis in addition to 40 healthy individuals served as normal control. Patients were assigned to the viral hepatitis group if they had evidence of CLD associated with HCV infection and to the non-viral hepatitis group if they had evidence of CLD in absence of viral hepatitis infection. The demographic and biochemical profiles of the participants enrolled in this study are described in Table 1. The age and gender of the normal controls were matched to the patient groups.

All patients in the study groups had an increase in ALT, AST, alkaline phosphatase, alpha-fetoprotein (AFP), and DCP ($p < 0.001$), compared to the control group.

Table 1: Demographic and laboratory data for all cases studied as well as controls

Variables	Control (n = 40)	Viral Hepatitis (n = 55)	Non-Viral Hepatitis (n = 45)
Age	47.0 ± 3.5	51.3 ± 4.7	48.1 ± 4.1
Gender			
Male	21 (52.5%)	27 (49.1%)	26 (57.8%)
Female	19 (47.5%)	28 (50.9%)	19 (42.2%)
Smoking			
Yes	12 (30%)	25 (45.5%)	24 (53.3%)
No	28 (70%)	30 (54.5%)	21 (46.7%)
AST (U/L)	21.32 ± 1.07	74.84 ± 5.00*	50.40 ± 7.34*,[a]
ALT (U/L)	23.16 ± 1.95	76.84 ± 5.00*	49.40 ± 7.34*,[a]
Alkaline phosphatase (U/L)	189 ± 41	336 ± 48*	320 ± 33*
Albumin (g/dL)	4.4 ± 0.5	3.8 ± 0.72	3.08 ± 0.48
White blood cells (10^3/mm^3)	7.62 ± 0.39	8.20 ± 0.56	6.65 ± 0.36
Prothrombin concentration	95.6 ± 3.4	41.5 ± 11.1[b]	59.4 ± 3.7[b]
AFP (IU/mL)	3.12 ± 0.08	10.11 ± 0.11*	12.18 ± 0.44*
DCP (mAU/ml)	30.42 ± 0.70	123.62 ± 0.38*	112.52 ± 0.66*

Quantitative data are expressed as mean ± standard error (SE), Qualitative data are expressed as frequencies (percentages), The normal range for alkaline phosphatase is up to 250 U/L, The normal range for alpha-fetoprotein (AFP) is 0.1–9.6 IU/mL, The normal range for des-γ-carboxyprothrombin (DCP) ≤ 40 mAU/ml, *$p < 0.001$ significant increase than in the control group, [a]$p < 0.001$ significant decrease than in the viral hepatitis group, [b]$p < 0.001$ significant decrease than in the control group.

Serum expression levels of (miR-30, miR-122, miR-296, miR-351, and miR-431)

Serum levels of miR-30, miR-122, miR-296, miR-351, and miR-431 were significantly higher ($p < 0.01$) in the viral hepatitis cases than in the non-viral hepatitis group (Table 2).

Table 2: Real-time PCR expression levels of serum microRNAs in the studied groups

miRNAs	Normal	Viral	Non-Viral
miRNA-122a	19.30 ± 0.69	548.99 ± 8.55**	79.28 ± 3.27**,[a]
miRNA-30	23.41 ± 0.86	95.51 ± 1.69**,[b]	39.50 ± 1.20**,[a,c]
miRNA-296	26.88 ± 0.47	111.17 ± 2.49**,[b]	55.22 ± 2.19**,[a,c]
miRNA-351	27.73 ± 1.02	121.11 ± 1.81**,[b]	63.32 ± 1.71**,[a,d]
miRNA-431	85.96 ± 1.48	374.27 ± 9.21**,[b]	125.56 ± 1.98**,[a,e]

**$p < 0.01$ significant increase than normal, [a]$p < 0.01$ significant decrease than viral, [b]$p < 0.01$ significant decrease than miRNA-122a, [c]$p < 0.01$ significant decrease than miRNA-122a, [d]$p < 0.05$ significant decrease than miRNA-122a, and [e]$p < 0.01$ significant increase than miRNA-122a.

Diagnostic performance of circulating microRNAs to differentiate between viral and non-viral hepatitis patients

To evaluate the usefulness of the studied serum biomarkers for differentiating between viral and non-viral hepatitis patients, the AUC, sensitivity, specificity, positive predictive value, and negative predictive value were calculated for each microRNA (Tables 3 and 4, and Figure 1). MiR-122a, miR-30, miR-296, miR-351, and miR-431 had sensitivity and specificity of (85.71% and 83.33%), (82.35% and 83.33), (85.71 and 69.44), (88.64 and 75.76), and (87.80 and 65.79), respectively.

Table 3: Area under the curve (AUC), confidence interval (CI), and p-values for the circulating miRNAs

MicroRNAs	AUC	CI 95%	p-value
miRNA-122a	0.803 ± 0.061	0.698–0.884	< 0.001
miRNA-30	0.766 ± 0.064	0.657–0.854	< 0.001
miRNA-296	0.777 ± 0.062	0.668–0.863	< 0.001
miRNA-351	0.736 ± 0.068	0.928–1.000	< 0.001
miRNA-431	0.796 ± 0.058	0.690–0.878	< 0.001

Discussion

Despite significant breakthroughs in the diagnosis and treatment of hepatologic disorders, such as viral hepatitis, fibrosis, and hepatocellular carcinoma, these diseases lead to considerable mortality and morbidity, as well as a high economic burden [10]. As our understanding of these disorders grew, there are numerous stages of these conditions, in which intervention can influence disease development and avoid significant mortality [11].

Table 4: Sensitivities and specificities of the different microRNAs to differentiate between patients with viral and non-viral hepatitis

Variable(s)	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value
miRNA-122a	85.71	83.33	93.98	65.79
miRNA-30	82.35	83.33	93.33	62.50
miRNA-296	85.71	69.44	86.75	67.57
miRNA-351	88.64	75.76	90.7	71.43
miRNA-431	87.80	65.79	84.71	71.43

A new approach to treat viral hepatitis infection, the most common cause of CLDs, is by targeting the host factors that enable the viral life cycle and replication [12]. Identifying these factors has emerged as a potential option since they can establish a barrier against viral invasion and can be broadly effective against all viral genotypes [13]. Using gene silencing screening techniques, several host components that aid in viral entrance, replication, assembly, and release were recognized [14]. Therefore, the identification of miRNAs that play a prominent role in the HCV life cycle and their implications for the advancement of liver disease was the aim of the current study. Five miRNAs (miR-30, miR-122, miR-296, miR-351, and miR-431) were chosen to be analyzed in cases of viral and non-viral induced CLDs to investigate their potential as possible therapeutic targets against chronic hepatitis virus infection and its catastrophic consequences.

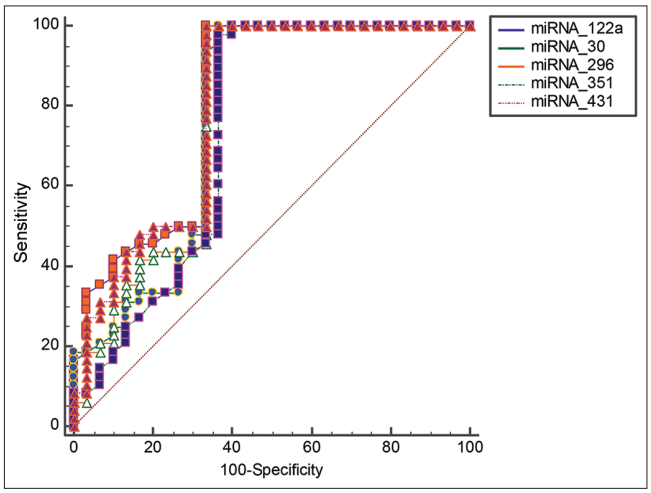

Figure 1: Receiver operating curve of the examined miRNAs for differentiating non-viral – from viral-induced chronic liver diseases

In the present study, a significant increase in the expression of miR-30, miR-122, miR-296, miR-351, and miR-431 was detected in patients with viral CLD compared to those with non-viral CLD, indicating that

these miRNAs are involved in the viral life cycle and have the potential to be targeted by inhibitors to prevent viral infection and the progression of hepatic illness.

Pedersen *et al*. [7] discovered that introducing individual miR-296, miR-351, and miR-431 mimics, induced the IFN-β-mediated reduction of viral RNA by ~75%. Furthermore, they showed that cotransfection of miRNA mimics mixed with anti-miR-122 further reduced the viraemia to >80%, indicating that modulation of expression levels of the identified miRNAs plays an eminent role in the antiviral effects of IFN-β against HCV. This was attributed to that IFN-β-induced miRNAs (miR-30, miR-296, miR-351, and miR-431) display nearly perfect complementarity in their seed sequences with HCV RNA genomes. In contrast to our findings, they found that miR-30 had no effect.

The liver specific miR-122 is one of the most promising host factors that can be targeted for viral infection management and CLD prevention. It has two target sites in the HCV genome's 5′-UTR, promoting its replication and enhancing viral protein synthesis [15]. Anti-HCV therapeutics targeting miR-122 resulted in a considerable reduction in HCV viraemia without evidence of viral resistance in chimpanzees chronically infected with hepatitis C [16]. In addition, by blocking miR-122 biogenesis, Miravirsen (miR-122 antagonist) caused a prolonged dose-dependent drop in HCV RNA levels in chronically infected patients [17]. Another study affirmed that exogenous expression of miR-122 facilitates efficient HCV RNA replication and/or virion generation in permissive cell lines, whereas its down-regulation prevents HCV replication [15].

When a human hepatoma cell line Huh7 was infected with HCV, miR-122 expression was found to be down-regulated about 3-fold, and conversely, miR-351 and miR-296 were up-regulated approximately 15- and 5-fold, respectively [8].

Wang *et al*. [18] observed that the miR-122 levels were inversely related to intrahepatic HBV burden and liver necroinflammation, and that the depletion of endogenous miR-122 led to augmented HBV replication, whereas exogenous expression of miR-122 inhibited viral replication. They stated that cyclin G1 is a target gene of miR-122 and that cyclin G1 interaction with P53 blocks the binding of P53 to HBV enhancer elements, resulting in HBV genome transcription suppression. MiR-122 has been shown to limit HBV replication by targeting the mRNA encoding the viral polymerase and sequestering the 3′-UTR region of the core protein of the HBV genome [19]. Hao *et al*. [20] noticed that IFN-α treatment caused a marked decrease in miR-122 expression in hepatocytes by upregulating the expression of the NT5C3 gene, which was identified as a target of miR-122, which could, in turn, suppress miR-122 by binding to its mRNA 3'-UTR region, promoting HBV expression and replication. This might aid in explaining why chronic HBV patients have a poor response to IFN-α.

There were certain constraints in the present study. First, the sample size was modest. Secondly, the study was performed on HCV-infected patients only. Therefore, the selected miRNAs should be investigated in further studies with a larger sample size and other types of viral hepatitis infection.

Conclusions

The results of the present study indicate the importance of miR-30, miR-122, miR-296, miR-351, and miR-431 in the development of CLD as a consequence of viral infection, as well as their promising potential as biomarkers and targets for the prevention and treatment of chronic viral infection and its serious life-threatening complications. Future investigations of the molecular mechanisms driving viral infection's modulation of miRNA expression will surely improve our understanding of the pathogenesis of hepatitis virus infection and antiviral resistance, potentially paving the way for innovative antiviral therapeutics.

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