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Some biochemical changes and the role of micro RNA 34 a-5p and micro RNA 365a-5p as a diagnostic marker in chronic hepatitis C (HCV)

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ABSTRACT

This study aimed to investigate Micro RNA 34a-5p and Micro RNA 365a-5p in addition to the traditional biochemical analysis as a useful diagnostic biochemical marker for chronic Hepatitis C Virus (HCV). The present study included 17 individuals with chronic liver disease, who admitted to Benha University Hospital. After investigating 5 patients from all patients, were found to be positive polymerase chain reaction (PCR) chronic HCV. 10 control negative serum samples were collected from the control healthy group. Biochemical analysis was done for investigating liver function parameters alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBil), direct bilirubin (DBil), gama glutamyl transferase (GGT), alkaline phosphatase (ALP) and albumin (Alb) and hematological analysis including total leucocytic count (TLC), hemoglobuin concentration (Hb) and platelet count (PLT), Alpha feto protein (AFP) as a tumor marker indicator. RNA was extracted from serum samples for gene expression of microRNA34 a-5p and microRNA 365a-5p. HCV group showed a significant increase in liver function parameters (ALT, AST, TBIL, DBIL, GGT, ALP) while there was a significant reduction in the albumin levels. Moreover, there was a significant decrease in TLC with non-significant decrease in the HB concentration and the platelet count. AFP concentration in HCV group was significantly increased compared to the control group. HCV group revealed a significant increase (8.98 ± 0.34) in miRNA34, with a significant decrease in miRNA365 (0.25 ± 0.03). We concluded that miRNA34a-5p and miRNA365a-5p expressions in addition to different biochemical analysis can be used as a useful diagnostic marker for HCV diagnosis.

1. INTRODUCTION

The liver is the body's principal organ for metabolism, detoxification, and secretory processes, rendering it susceptible to a variety of diseases (Kalra et al., 2018). Chronic liver disease (CLD) is characterized by the developing deterioration of liver functions over a period of more than six months (Shah et al., 2020). Many chronic liver illnesses, such as hepatitis C, can cause liver fibrosis (Piscaglia et al., 2016; Shah et al., 2020). Acute and chronic hepatitis can be caused by the hepatitis C virus (HCV). Chronic hepatitis C (CHC) patients are at a high risk of acquiring life-threatening complications, such as cirrhosis and hepatocellular carcinoma (HCC) in cirrhotic patients (WHO, 2020). HCV is a positive polarity single-stranded RNA virus that is packed by core protein and coated by a lipid bilayer comprising 2 viral glycoproteins (E1 and E2) for virion formation. It is the only member of the *Hepacivirus* genus in the *Flaviviridae* family (Pietschmann & Brown, 2019). Indirect and direct tests can be performed to diagnose HCV infections. Indirect tests check for antibodies that are produced as a result of viral infection, such as IgM for new infection and IgG for new or prior infection. Virus isolation, detection of viral antigens, and detection of viral nucleic acids are among the direct tests. In clinical practice, anti-HCV total antibody, viral genomic RNA diagnostic test and viral core antigen are currently used (Narayanamurthy et al., 2021). MicroRNAs (miRNAs) are minor noncoding RNAs with 18–24 nucleotides that influence gene expression through binding to mRNAs and interfering with the translation activity. In addition, miRNAs found in body fluids, are illness and tissue-specific, and are persistent within the circulation, implying that they could be used as disease biomarkers. A larger fibrogenic response or liver injury has been linked to altered miRNA expression (El-Ahwany et al., 2019; O'Brien et al., 2018). More than twenty microRNAs (miRNAs) have been found so far to prevent liver fibrosis from progressing and worsening, including miR-221, miR-19b, miR-222, miR-351, and others (Brandon-Warner et al., 2017; Tadokoro et al., 2021). The miR-34a, miR34b, and miR34c are the three members of the miR-34 family. The miR-34 family has also been connected to the stimulation of apoptosis and has anti-proliferative characteristics (Zhang et al., 2019). In the fatty livers of diet-induced obese mice, a rise in miR-34a levels was detected with a contemporaneous drop in Sirt1 levels, indicating that miR-34a targets hepatic *SIRT1* (Lee et al.,

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2010). In both people and mice, it was found that there was a stimulation of miR-34a in ALD; *SIRT1* and caspase-2 levels were regulated by miR-34a and it participates in hepatic steatosis in that illness (Meng et al., 2012). Prior research has shown that miR-365 acts as a tumour suppressor within a variety of cancers, involving HCC. The miR-365 appeared to be greatly expressed in invasive ductal adenocarcinoma and to cause gemcitabine resistance among the cancer cells in pancreas by directly aiming the adaptor proteins Src homology 2 domain comprising 1 and BAX, which promote apoptosis (Hamada et al., 2014). Furthermore, the expression of miR-365 was shown to be lower in colon cancer cell lines, and restoring it inhibited cell cycle progression, increased 5-fluorouracil-induced apoptosis, and reduced tumorigenicity (Wang, 2020). According to a modern study, miR-365 expression is negatively associated with weak prognosis and survival rates in individuals with HCC due to its ability to limit cell proliferation (Chen et al., 2015). Nevertheless, the role of miR-365 in regulating HCC cell death is unknown (Li et al., 2017). The aim of this work is investigating the use of Micro RNA34a-5p and Micro RNA 365a-5p and different biochemical analysis as an accurate diagnostic biochemical marker for diagnosis of chronic Hepatitis C Virus (HCV).

2. MATERIAL AND METHODS

2.1. Subjects

The present study is a cross-sectional study design that was approved by the ethical committee of the Biochemistry Department, Faculty of medicine, Benha University. The study included 17 patients with chronic liver disease, who were admitted to Banha University Hospital. five patients were found to be positive PCR chronic HCV, Patients' age ranged from 30-55 years, and they were 65-95 kg body weight. 10 control negative serum samples were collected from the control healthy group. Written informed consents were obtained from all participants

2.2. Biochemical Analysis

Serum samples were collected from all the five HCV patients for investigating:

A) Liver function parameters included serum alanine aminotransferase (ALT), aspartate amino transferase (AST), total bilirubin concentration (TBil), direct bilirubin concentration (DBil), γ-Glutamyltransferase (GGT) enzyme activity, alkaline phosphatase (ALP) and albumin (ALB).

B) Hematological parameters included total leukocytic count (TLC), HB concentration, and platelet count (Plt).

C) Analysis of alpha-fetoprotein (AFP) as a tumor marker indicator.

2.3. RNA extraction

The QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) was used to extract RNA from blood samples, by addition of [200 µl] of the sample to [600 µl] RLT buffer having [10 µl] β -mercaptoethanol per [1 ml].The cleared lysate was added to one volume of [70%] ethanol, and the processes were performed according to the QIAamp RNeasy Mini kit's Purification (Qiagen, Germany, GmbH) of Total RNA from blood samples.

2.4. SYBR green rt-PCR

A 25- reaction including [12.5 µl] of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), [0.25 µl] of RevertAid Reverse Transcriptase (200 U/L) (Thermo Fisher), [0.5 µl] of each primer in a concentration of [20 pmol], water of [8.25 µl], and RNA template with [3 µl] was used to test the primers. Stratagene MX3005P real-time PCR equipment was used to execute the experiment. The findings of the SYBR green rt-PCR were analyzed as follows:

The stratagene MX3005P program was used to determine the amplification curves and CT values. To evaluate the variance of gene expression on the RNA of the different samples, the CT of each sample was compared to that of the positive control group as shown by the "ΔΔCT" method mentioned by Yuan *et al*., (2006) via the following ratio: ($2^{-\Delta\Delta ct}$).

Where ΔΔCT = ΔCT reference – ΔCT target

Δ*CT target = CT control – CT treatment* and Δ*CT reference = CT control- CT treatment*.

2.5. Statistical analysis

Statistical analysis of the biochemical results was expressed as the mean ± standard error of the mean. A significant difference was used at the $P < 0.05$ probability level. One-way analysis of variance and the least significant difference test were carried out using the Statistical Package for Social Science Software (Version 18, SPSS Inc., USA).

Table (1): Specific oligonucleotide primer sequence used in this study

Gene	Primer sequence(5'-3')	Reference
U6	F/GCTTCGGCAGCACATATACTAAAAT R/CGCTTCACGAATTTGCGTGTCAT	Chen et al., 2003
miR-365a-5p	F/ CTG AGG GAC TTT TGG GGG CAG R/GTG CAG GGT CCG AGGT	He et al., 2019
miR-34a-5p	F/ GCAGTGGCAGTGTCTTAG R/GGTCCAGTTTTTTTTTTTTTTTACAAC	Hasakov et al., 2019

F: forward primer, R: reverse primer; U6 is housekeeping gene

3. RESULTS

3.1 Biochemical analysis

Results of the biochemical analysis presented in Table (2).

Liver function parameters:-

HCV group showed significant increase in ALT, AST, Total bilirubin concentration (TBil), Direct bilirubin concentration (DBil), GGT and ALP, Meanwhile Albumin concentration (Alb) showed a significant decrease compared to control group.

Hematological analysis:-

TLC: HCV group showed a significant decrease in comparison with control group.

Hb concentration: HCV group showed non-significant decrease in Hb in comparison with control group.

Platelet count (Plt): HCV group showed non-significant decline in Plt in comparison with control group.

Table (2): The biochemical and hematological analysis of the HCV group and comparing to control group as a significant value

Experimental Group	Control group	HCV group
Liver function parameters		
ALT (U/L)	27.50 ± 3.66	62.00 ± 1.45***
AST (U/L)	27.75 ± 2.06	61.00 ± 5.79**
ALB (g/dL)	4.73 ± 0.21	3.52 ± 0.37*
TBIL (mg/dL)	1.03 ± 0.05	1.64 ± 0.21*
DBIL (mg/dL)	0.39 ± 0.03	0.94 ± 0.11*
GGT (U/L)	22.40 ± 1.88	71.00 ± 7.97**
ALP (U/L)	35.50 ± 2.96	57.20 ± 4.73**
Hematological analysis		
TLC (10^3/cmm)	10.10 ± 1.07	6.40 ± 0.93*
Hb (g/dL)	14.03 ± 0.61	13.56 ± 0.95
PLT (10^3/cmm)	369.50 ± 26.87	301.80 38.38

AFP activity: HCV group in table (3) showed a significant increase (14.48 ± 3.34) ($P < 0.05$) in AFP concentration in comparison with control group (3.55 ± 1.28)

Table (3): The analysis of AFP activity between HCV group and control group

Experimental Group	AFP(ng/mL)
Control group:	3.55 ± 1.28
HCV group:	14.48 ± 3.34*

Data are presented as (Mean ± S.E). S.E = Standard error

3.2. Molecular analysis

The miR-34a-5p Gene Expression:-

HCV group showed a significant increase (8.98 ± 0.34) ($P < 0.001$) in mRNA34 compared to control group (1.00 ± 0.00), Table (4), Fig. (1)

The miR-365a-5p Gene Expression:-

Table (5) and Fig (2) showed that HCV group had a significant decrease in mRNA365 (0.25 ± 0.03) ($P < 0.001$) in comparison with control group.

Table (4): The analysis of miR-34a-5p Gene Expression between HCV group and control group

Experimental Group	mRNA34
Control group:	1.00 ± 0.00
HCV group:	8.98 ± 0.34***

Data are presented as (Mean ± S.E). S.E = Standard error, *** Represents statistical Significant at $P < 0.001$

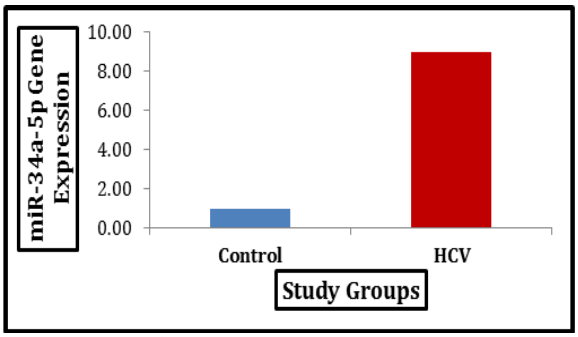

Fig (1) the analysis of miR-34a-5p Gene Expression between HCV group and control group

Table (5): The analysis of miR-365a-5p Gene Expression between HCV group and control group

Experimental Group	mRNA365
Control group:	1.00 ± 0.00
HCV group:	0.25 ± 0.03***

Data are presented as (Mean ± S.E). S.E = Standard error, *** Represents statistical Significant at $P < 0.001$

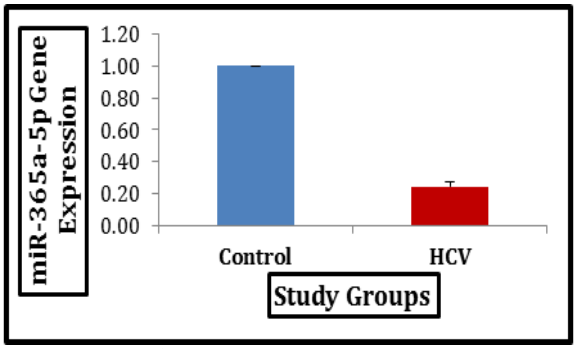

Fig (2) the analysis of miR-365a-5p Gene Expression between HCV group and control group

4. DISCUSSION

HCV is the cause of chronic liver disease around the world and it is a key risk factor for the development of liver cirrhosis as well as chronic liver failure. Chronic hepatitis C is frequently undetected, with most cases being diagnosed only through normal serologic or biochemical testing (Gupta et al., 2014). Genome sequencing, in addition to developing of high-throughput technologies that measure genome function, has provided unique prospects for developing profiles that can discriminate, detect, expect disease outcome, as well as treatment response (Asselah et al., 2009). Findings of this study showing, significant increase in ALT, AST, ALB, TBIL, DBIL, GGT, AFP, and AlkP activity in HCV group compared to control group. These findings were in agreement with Alazzawy (2018) who conducted a study to assess the influence of IL28B in the clearance of HCV. Hyder et al. (2013) who also revealed that enzymatic activity among patients' viral hepatitis had a significant increase in comparison with the control group. Mnuti, et al. (2011) found that the levels of AST and ALT were greater in the positive HCV marker group in a study of HCV infection in hemodialysis units. It has been well documented that chronic HCV is linked to a broad range of ALT levels, from normal to persistently elevated ALT, even though studies have demonstrated that patients with persistently normal ALT have a slower progression and a lower prevalence of cirrhosis (Hussein, 2016). With the damage of parenchymal liver cells in HCV infection, a leakage of aminotransferases from the liver into the bloodstream occurs, leading to high levels of these enzymes in the circulation. The obtained results were in consistent with the results of Emokpae et al. (2013). A significant increase in the AFP levels was observed in the current study, which acts as predictive marker for the development of hepatocellular carcinoma in follow up of cirrhosis. There is a correlation between serum AFP levels and sustained virological response in HCV infected patients (Masetti et al., 2018). The total bilirubin concentrations were found to be significantly increased among HCV patients, while the albumin concentrations were significantly decreased among HCV patients. These findings agreed with Ashraf-Uz-Zaman et al. (2010). Bilirubin has been shown to be a marker of liver injury. In chronic hepatitis C (CHC) patients, serum indirect bilirubin levels were found to be adversely linked with the advancement of liver fibrosis. Serum bilirubin levels, on the other hand, elevated in line with the severity of fibrosis in CHC patients (Du et al., 2016). On the other hand, a low serum albumin concentration was discovered to be an indicator of impaired liver function. Because it takes many weeks for the blood albumin level to drop after impaired albumin production, decreased serum albumin levels are not noticed in acute liver failure. Cirrhosis-related chronic liver failure is the most common cause of decreased albumin. In chronic liver disease, such as HCV, the serum albumin content is normal until cirrhosis and substantial liver damage emerge (Nagao & Sata, 2010). This study also revealed a significant low TLC in HCV patients, in addition to non-significant lower Hb levels. This was in line with the results by Hussein (2016), and Sabry, et al. (2007). The results also revealed a non-significant lower platelet count. The gene expression of mRNA34 was significantly increased among HCV patients in the present study, whereas, the gene expression of mRNA365 was significantly decreased. Parallel to these findings, Cermelli et al. (2011) and Salvoza et al. (2016) found that there was an increase in the serum levels of miR34a in chronic liver diseases such as HCV and NAFLD. Calvopina et al. (2018) revealed that miR34a-5p and miR365a-3p were up-regulated in serum of cystic fibrosis- related liver sickness in comparison with cystic fibrosis-non-associated liver sickness children. According to Feili et al. (2018), miR34a-5p was shown to be significantly down-regulated in fibrotic livers as compared to controls. Furthermore, significant differences in miR34a-5p expression within the livers of C57 as well as CCl4-treated C57 mice were detected, proposing that miR34a-5p has an important part among

fibrosis of liver. Zaldivar et al. (2010) reported that overexpression of miR34 lowered TGF-b1 production and then down-regulated p-smad3 expression, which is the downstream of TGF-b1/Smad3 pathway. Therefore, it was proposed that miR-34a-5p remained a key mediator within hepatic stellate cells pathway that controls liver fibrosis which is a major complication of chronic liver disorders and signifies the general pathway that leads to end-stage liver failure. Chronic liver disorders have been linked to miR-365's potential function, and in the plasma of children with hepatitis B, higher levels of circulating miR-365a-3p were discovered, signifying that it is possible to be as a biomarker for disease progression (Winther et al., 2013; Winther et al., 2014). It was found that miR365 was considerably down-regulated in HCC tissues and cell lines, and lower miR365 levels were strongly linked to HCC malignancy (Liu et al., 2017). Nevertheless, the biological function of miR365 among liver diseases in addition to the molecular processes by which miR-365 exerts its actions is not fully understood due to a lack of target gene information (Liu et al., 2017).

5. CONCLUSION

The current study found that in HCV patients, miRNA34a-5p expression was significantly increased while miRNA365a-5p expression was significantly decreased, implying that miRNA34a-5p and miRNA365a-5p expression, in addition to various biochemical analyses, can be used as an useful diagnostic marker for chronic Hepatitis C Virus diagnosis (HCV). Further research is needed to determine the diagnostic role of miRNA34a-5p and miRNA365a-5p for HCV.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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