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RESEARCH ARTICLE

Evaluation of the Effect of miRNA 122 and 221 Gene Expression on Lipids Profile of Patients with Liver Fibrosis

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ABSTRACT

Many biological processes depend on microRNAs (miRNAs), and disorders caused by a defect in the functioning in these genes may manifest in a variety of ways, including metabolic disorders. Therefore, the current research aims to investigate the gene expression of miRNA 122 and 221 and their impact on the lipid profile of individuals suffering from liver fibrosis. Present research is a case – control study, included blood samples collection from 44 fibrotic patients and 44 health individuals as control. After RNA extraction, gene expression of miRNA 122 and 221 evaluated by RT-qPCR. FUJI DRI-CHEM SLIDE are used to determine total Cholesterol, triglyceride, HDL and LDL concentration. The results of the current study showed higher concentration of cholesterol, triglycerides and LDL in patients (316.12 ± 96, 256.08 ± 41 and 147.48 ± 11.69 ml/dl respectively) compared to control (136.24 ± 10.5, 126.48 ± 6.96 and117.72 ± 3.59 ml/dl respectively) ($P< 0.05$) while a lower HDL concentration detected in patients (53.8 ± 4.89 ml/dl) compared to controls (59.28 ± 6.31 ml/dl). The results of the gene expression analysis showed a lower mean fold change ($2^{-\Delta\Delta CT}$) for miRNA 122 in patients (3.75) compared to controls (7.44) while we found a higher mean fold change for miRNA221 in patients (2.70) compared with control (0.04). Increase or decrease in gene expression for miRNA 122 was inversely related to the level of lipids in the patients' blood, while a moderate positive relationship appeared between the rate of gene expression for miRNA 221 and the level of lipids in patients. In conclusion, we found that 122 and 221 are associated with fibrosis, and that high or low gene expression of these genes moderately affects the level of lipids, which in turn is one of the most important causes of liver fibrosis.

INTRODUCTION

Liver fibrosis is an intricate process that involves the formation of fibrous tissue and inflammation. It occurs due to chronic liver damage and serves as the first stage in the development of liver cirrhosis. Cirrhosis is a significant worldwide health problem since there are no efficient treatments modalities (Jam et al.,2019; Schulien et al., 2019; Tashtoush et al., 2023). Liver fibrosis is caused by scar deposition and the continual accumulation of collagen I and collagen III, two extracellular matrix (ECM) components that are extremely rich in fat (Gningue et al., 2022; Tashtoush et al., 2022; Wernig et al., 2017)

Many inherited disorders can lead to the development of liver fibrosis, or occasionally cirrhosis. These might hinder the fibrogenic process's reversal. Systemic fibrosis and liver organ-specific

fibrosis are associated with several miRNAs. A useful technique for detecting liver fibrosis is examining the distinct expression of miRNAs in serum or plasma (Jarrah et al., 2022; Markovic et al., 2020; Wardatet al., 2022). Furthermore, Some miRNAs have the ability to differentiate between early and late stages of fibrosis with an accuracy that is comparable to, or better than, the APRI and Fib-4 Index in terms of both sensitivity and specificity (Roderburg et al., 2010; Su et al., 2018).

miR-122 is highly expressed in the liver and is one of the most common miRNAs there. It constitutes over 70% of the total miRNAome in adult mice livers and 52% of the entire miRNAome in human livers (Sharma et al., 2011). Therefore, the function, homeostasis, differentiation, and development of the liver are all impacted by miR-122. In living organisms, liver-enriched transcription factors (LETFs) such hepatocyte nuclear factor 6 and 4a regulate miR-122 expression and maintain an optimum amount of miR-122 during liver development. An equilibrium between hepatocyte and cholangiocyte cell proliferation and differentiation may be controlled throughout liver development by the coordinated expression of miR-122 and LETFs (Rashid et al., 2023; Rong et al 2020; Tan et al., 2022). Because of its importance in promoting hepatobiliary cell separation and in developing and maintaining a distinct liver phenotype, temporal regulation of miR-122 expression is of paramount importance. The final stage of hepatic differentiation was facilitated by miR-122, which was seen to gradually inhibit the activity of the transcription factor cut-like homeobox 1 (CUTL1) throughout mouse liver development (Kanval et al., 20244; Tsay et al.,2019).

Molecular biology refers to short RNA molecules found on the X chromosome as mir-221 microRNAs, together with their paralogue, miR-222. There are many conserved seed sequences in miR-221, much as in its homolog, miR-222. The expression of other genes may be regulated by microRNAs via multiple mechanisms. [Liu et al., 2016; Galardi et al., 2011). A previous research identified that decreasing in miRNA-221-3p in hepatocytes results in a decrease in liver fibrosis (Galardi et al., 2011).

The microRNA (miRNA) family consists of many miRNAs that have the ability to collectively regulate various signaling pathways in a synergistic way (Garofalo et al., 2009). However, our understanding of the collective functions of the miRNA family in hepatic fibrosis is lacking. Therefore, we will place more emphasis on investigating this subject. In addition, acquiring a deeper understanding of the complex genetic pathways that control the fibrogenic process might aid in the identification of epigenetic signatures as diagnostic or prognostic markers and in the development of innovative therapeutic strategies (Garofalo et al., 2009; Livak et al., 2001). A primary goal of this research is to determine how miRNA 122 and 221 contribute to liver fibrosis, in addition be looking at lipid levels and they relate to those genes.

Samples collection and methods

Study design and samples collection: The current study is a case - control study that was conducted during the period from 3/9/2022 to 15/10/2023. Samples were collected from Al-Diwaniyah Teaching hospital, outpatient clinics, Medical City in Baghdad and Gastrointestinal Unit (GIT) at Al-Hakim Hospital/Najaf Al-Ashraf. The number of cases was 44 people suffering from liver fibrosis and 44 healthy people as a control group. Moreover, consent was also obtained from the participants before collecting the questionnaire or taking blood samples. Six ml of blood were taken from all participants for conducting the required tests.

Hepatitis test: A flow chromatographic immunoassay, the one-step HBsAg/HCVAb rapid diagnostic test can qualitatively detect and differentiate between hepatitis B surface antigen (HBsAg) and anti-hepatitis C viral antibodies (IgG, IgM, IgA) in human serum. This test requires the placement of 0.5 ml of serum onto the assay plate (Raypo-Tech/USA) and the observation of any color change.

Complete blood count: The RUBY system (USA) performs a complete blood count on every blood sample. The test procedure used in this system requires inserting an EDTA tube involving a blood

sample and labeled with the patient's name and number on specified rack through the RUBY system. This system automatically collects and analyzes samplesEach patient's results were printed and labeled with their name and number after 1 to 5 minutes, after which the computer screen displayed the whole blood count result, including the white blood cell count.

Molecular study: The TRIzol® Reagent kit was used according to the instructions provided by the company to extract total RNA from blood samples. The genomic RNA that was extracted was checked using a Nanodrop Spectrophotometer, which measures the concentration of RNA and estimates its purity by measuring the absorbance at wavelengths of (260 /280 nm). The extracted RNA underwent treatment with DNase I to remove any trace amounts of genomic DNA from the total RNA that was eluted. The DNase I enzyme kit samples were used in this process, which was carried out following to the procedure outlined by the Promega company in the USA.

The present study used the miRNA Primer Design Tool and the Sanger Center miRNA database Registry to select miRNA sequences for miRNA l22 (MIMAT0000421) and miR-221 (MIMAT0004568), two qPCR primers. In contrast, this study used Primer3 plus and the NCBI-Database to design the qPCR Housekeeping gene (GAPDH) (NM_001256799.3) online. These primers in Table (1) were provided by Macrogen, a company in Korea. The qPCR master mix was made using the GoTaq® qPCR Master Mix kit. Subsequently, the Miniopticon Real-Time PCR equipment was used for mixing the qPCR master mix components in qPCR strip plate tubes using an Exispin vortex centrifuge for three minutes. After that, the qPCR plate was loaded and the following thermocycler protocol for miRNA genes or GAPDH gene in the following: Initial denaturation TM step was 95 °C for 5min- 1 cycle, The denaturation step 2 was performed. The temperature for annealing, extension, and detection was set at 95 °C for 20 seconds for 45 cycles. The temperature for detection was 60 °C for 30 seconds for 45 cycles.

The relative quantification gene expression levels (fold change) (The ΔCT method using a reference gene) described by Livak and Schmittgen was used to analyze the data results from q RT-PCR for both the target and housekeeping genes (Kazaal et al., 2024). The following equations are:

ΔCT (Test) = CT (target gene, test) – CT (HKG gene, test)

ΔCT (Control) = CT (target gene, control) – CT (HKG gene, control)

ΔΔCT= ΔCT (Test)– ΔCT (Control)

Fold change (target / HKG) = 2-CT ΔΔCT

Table (1): RT--PCR primers with their sequence

Primer		Sequence (5'-3')
miRNA universal RT primers		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTTTT TTTTTTVN
miR-122 qPCR primer	F	AACAAGTGGAGTGTGACAATGG
	R	GTCGTATCCAGTGCAGGGT
miR-221 qPCR primer	F	AACAAGACCTGGCATACAATGTAG
	R	GTCGTATCCAGTGCAGGGT
GAPDH qPCR primer	F	TCACCAGGGCTGCTTTTAAC
	R	TGACGGTGCCATGGAATTTG

Lipids test: To find out the concentrations of cholesterol, triglycerides, HDL, and LDL are in blood, that may use a FUJI DRI-CHEM SLIDE (FUJIFILM / Takeo / Japan).

Ethics approval and consent to participate: The Continuing Education Unit of Hospitals received its of approval to the study. Following the researcher's explanation of the study's purpose, objectives, and methods, the patients readily provided their written informed consent to take part in the study.

Statistical analysis

Excel 2010 and the Statistical Package for the Social Sciences, version 22, were both used to analyze data in this study. Statistically significant differences were considered as those with a probability value less 0.05 (Farrell et al., 2006).

RESULT

The current research is a case- control study, included 44 patients suffering from liver fibrosis, whose ages ranged between 29-78 years, with an average age of 61.16 ± 11.16 years, and the majority of them were male, at a rate of 68%, as in Table (2). On the other hand, the study included 44 healthy people as a control group, whose ages ranged from 25 to 76 years, with an average age of 55.6 ± 11.14 years. Statistically, we did not find significant differences when comparing patients with healthy controls in terms of age or gender ($p < 0.05$).

Table (2): Demographic properties of control and patients groups

Properties	Cases	control	*p* value
Age range	29 - 78	25 - 76	
Mean ± SD	61.16 ± 11.16	55.6 ± 11.14	0.226
SE	1.68	1.67	
Gender	N (%)	N (%)	P value
Males	30 (68%)	26 (59%)	0.077
Females	14 (32%)	18 (41%)	0.081
p value	0.0015*	0.041*	
Total number	44	44	

SD: Standard deviation, SE: Standard Error

Present study determined that 16% of patients suffer from hepatitis, especially type HBV (11%). Chronic diseases appeared in 91% of patients, and the majority of patients suffered from obesity and hypertension (34% and 27%, respectively), while diabetes appeared in 5%. Some patients were also reported to suffer from the simultaneous occurrence of more than one chronic disease at the same time. Smoking was recorded in 61% of patients, while 52% of patients were found to be alcoholics, as in Table (3). In Table (4), total number of WBC, RBC and platelets are deceased in patients (2.83, 2.47, 60.9 X 10^3/mm^3 respectively) compared to control (4.98, 4.72, 161 X 10^3/mm^3 respectively).

Table (3): Clinical assessment of hepatitis, chronic diseases, smoking and alcohol drinking of patients

Clinical assessment	Positive (%)	Negative (%)	*P* value
Hepatitis	7 (16%)	37 (84%)	0.017*
HAV	0 (0%)		
HBV	5 (11%)		
HCV	2 (5%)		
Chronic diseases	40 (91%)	4 (9%)	0.004*
Hypertension	12 (27%)		
Obesity	15 (34%)		
Diabetes	3 (7%)		
Hypertension + Obesity	3 (7%)		
Hypertension + Diabetes	2 (5%)		
Obesity + Diabetes	5 (11%)		
Smoking	27 (61%)	17 (39%)	0.038*
Alcohol drinking	23 (52%)	21 (48%)	0.333

* significant association ($P < 0.05$)

Table (4): Evaluation some blood markers of patients and control

Blood markers	Cases	Control	*T* test	95% CI	*p* value
	Mean ± SD	Mean ± SD			
WBC X 10^3/mm^3	2.83 ± 0.67	4.98 ± 0.52	16.82	-2.40 to -1.89	0.047*
RBC X 10^3/mm^3	2.47 ± 0.29	4.72 ± 0.33	33.97	-2.38 to -2.12	0.044*
PLT X 10^3/mm^3	60.9 ± 3.09	161 ± 7.99	77.51	-102.7 to -97.53	0.001*
Total number	44	44			

* significant association (p <0.05), SD: Standard deviation, CI Confidence interval

The results in Table (5) showed higher concentration of cholesterol, triglycerides and LDL in patients (316.12 ± 96, 256.08 ± 41 and 147.48 ± 11.69 ml/dl respectively) compared to healthy people (136.24 ± 10.5, 126.48 ± 6.96 and117.72 ± 3.59 ml/dl respectively) (P< 0.05) as in Table (4-12), and on the contrary, a lower rate of HDL concentration in patients (53.8 ± 4.89 ml/dl) compared with controls (59.28 ± 6.31 ml/dl) but without significant association (P = 0.227).

Table (5): Comparison case – control groups according to lipids profile

Lipid profile (ml/dl)	Cases Mean ± SD	Control Mean ± SD	*T* test	95% CI	*p* value
Cholesterol	316.12 ± 96	136.24 ± 10.5	12.355	150.94 to 208.82	0.049*
Triglycerides	256.08 ± 41	126.48 ± 6.96	20.67	117.14 to 142.06	0.029*
HDL	53.8 ± 4.89	59.28 ± 6.31	4.554	-7.872 to -3.088	0.227
LDL	147.48 ± 11.69	117.72 ± 3.59	16.143	26.095 to 33.424	0.047*
Total number	44	44			

* significant association (p <0.05), SD: Standard deviation, CI Confidence interval

The results of the gene expression analysis (Figures 1 & 2) showed a lower mean fold change ($2^{-\Delta\Delta CT}$) for miRNA 122 in patients (3.75) compared to controls (7.44) while we found a higher mean fold change for miRNA221 in patients (2.70) compared with healthy people, (0.04) which led to the emergence of significant differences (p< 0.05) as in Table (6). When distributing the gene expression rate of the studied genes according to the sex of the patients, we found that the mean fold change ($2^{-\Delta\Delta CT}$) of miRNA 221 increased in females (4.464) while the expression of miRNA 122 increased in males (3.987), as in Table (7). Pearson's linear correlation also showed that the increase or decrease in gene expression for miRNA 122 was inversely related to the level of lipids in the patients' blood, while a moderate positive relationship appeared between the rate of gene expression for miRNA 221 and the level of lipids in the blood, as in Table (8).

Table (6): comparison mean of gene expression of miRNA 122 and 221 between cases and control

Gene expression	Case-control	Mean CT(gene)	Mean CT(gapdh)	Mean ΔCT(test)	Mean ΔCT(control)	Mean ΔΔCT	Mean Fold change ($2^{-\Delta\Delta CT}$)
miRNA 122	Cases	26.22	27.23	-1.01	-0.01	-1.00	3.75*
	Control	29.31	27.78	1.53	-0.01	1.54	7.44
miRNA 221	Cases	29.12	27.23	1.88	1.88	0.00	2.70*
	Control	27.06	27.78	-0.72	1.89	-2.61	0.04

* significant association in compared with controls (p <0.05)

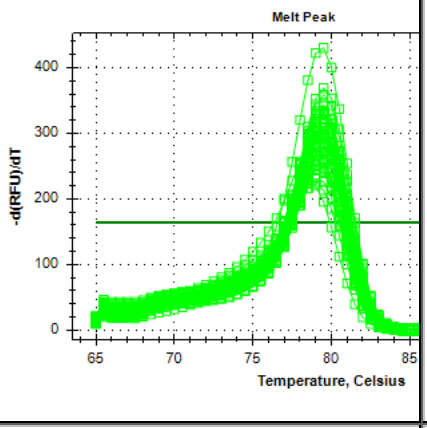

Fig 1: The Real Time PCR melting plot of miRNA 122 gene expression that showed the qPCR specifity at (79.5°C)

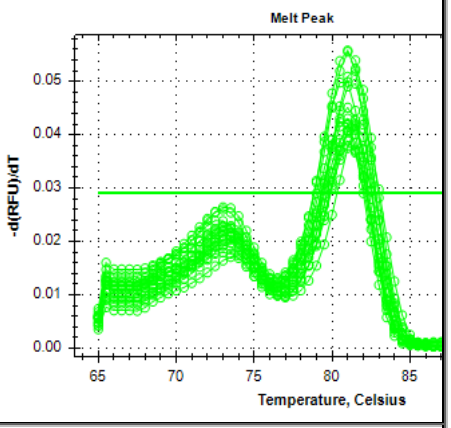

Fig 2: The Real Time PCR melting plot of miRNA 221 gene expression that showed the qPCR specifity at (81.5°C)

Table (7): Distribution of mean of gene expression of miRNA 122 and miRNA 221 according to patients gender

Gender	miRNA 122	miRNA 221	*P* value
Females	8.645	4.464	0.0392*
Males	3.987	3.324	0.1109

Table (8): Pearson correlation (r) among gene expression of miRNA 122 and 221 patients with lipid profile

Lipid profile (ml/dl)	miRNA 122		miRNA 221	
	r	p value	r	p value
Cholesterol	0.121	<0.0001	0.331	<0.0001
Triglycerides	0.135	<0.0001	0.229	<0.0001
HDL	0.103	<0.0001	0.338	<0.0001
LDL	0.117	<0.0001	0.341	<0.0001

DISCUSSION

In this study, we found that there is the close relationship between liver fibrosis and lipid profile. The potential correlation between lipids and liver fibrosis for various etiology has been extensively studied. The lipid profile may have an important role in the development of liver fibrosis, according to evidence from research. The liver plays an essential role in lipid metabolism, hence researchers have long been interested in a possible link between liver illness and lipid profile (Hirsch et al., 2010; Mena et al., 2014).

The link between lipids and liver fibrosis has been the subject of several research. Evidence from a cross-sectional study in Spain suggests that fibrosis is more common among chronic hepatitis B virus inactive carriers with lower HDL and higher TG levels (Jaafar et al., 2019). A research performed at the American University of Beirut Medical Center shown that low-density lipoprotein (LDL) is a reliable indicator of severe liver fibrosis in diabetic individuals with nonalcoholic fatty liver disease (Wu et al., 2018). In addition, a Chinese study on infected animals suggests that lipids could affect the development of *S. japonicum* infection-induced liver fibrosis by regulating the inflammatory environment, a process closely associated with scarring (Liu et al., 2022).

Liu et al., 2022 found that levels of HDL, LDL, HB, and PLT were independently associated with liver fibrosis after accounting for age, sex, BMI, FBG, AST levels, white blood cell count (WBC), red blood cell count (RBC), and other lipid profiles. The new prediction score combining HDL, LDL, and HB might also determine whether *S. japonicum* infected individuals would have liver fibrosis or without (Liu et al., 2022 ; Ghadir et al., 2010). According to Ghadir et al. (2010), cirrhotic individuals had significantly lower levels of all four studied variables (HDL, LDL, total cholesterol, and TG) than to the comparison group, indicating that patients with liver disease tend to have lower lipid levels. Moreover, the degree of liver damage was positively correlated with the amount of decrease in serum HDL, LDL, and total cholesterol (but not TG) (Mehboob et al., 2007) Prior research has revealed a significant decrease in serum total cholesterol and TG levels in cirrhotic individuals as compared to healthy individuals. This outcome is to be expected, provided liver biosynthesis is known to be reduced. For example, in 2007, Mehbob et al. obtained the same result after studying 160 individuals with chronic liver illnesses (Li et al 2013)

Patients especially those infected with HCV, showed a reduction in miR-122 in the present research. Consequently, a decreased miR-122 level in fibrotic liver cases might be explained by compromised normal hepatocytic activity or by an elimination of miR-122's inhibitory effect, which hinders fibrogenesis. In particular, miR-122 decreases collagen maturation by downregulating the production of P4HA1, an enzyme key to the process, and therefore suppressing HSCs proliferation (Halász et al., 2015) Hepatic fibrosis, according to Halász et al., is characterized by decreased miR-122 expression in advanced fibrosis, which correlates with fibrosis stage and liver stiffness values. This is especially true in cases of hepatitis virus infection (Marquez et al., 2016)

Previous studies revealed that miR-122 levels were lower in the fourth stage of fibrosis compared to the first stage, and in fibrotic liver samples, miR-122 levels were negatively correlated with fibrosis stage (Morita 2011; Wang et al., 2012) These results are substantiated by reports indicating a negative association between miR-122 and fibrosis stage in chronic HCV infection, HCV-induced HCC, and cirrhosis. Additionally, there have been observations of a reduced level of miR-122 in NAFLD [27-29]. Enhanced HBV replication was seen with reduction of endogenous miR-122, but viral generation was decreased upon overexpression of miR-122, according to study by Wang et al., which demonstrated a reverse relationship between miR-122 levels and intrahepatic viral load and hepatic necro inflammation. The research discovered a target gene of miR-122-cyclin G(1) that interacts specifically with P53, preventing P53 from binding to HBV enhancer elements. This interaction ultimately inhibits HBV transcription (Gramantieri et al., 2009)

MiR-221 increased in our patients, particularly those with HCV infection, and previous studies have supported this finding. Multiple studies have shown that miR-221 is upregulated in liver fibrosis due to various causes, including viral infections and nonalcoholic steatohepatitis (Fornari et al., 2008; Pineau et al., 2010; Ding et al., 2015), Multiple strategies to inhibit miR-221 have shown promising outcomes in terms of suppressing fibrogenic gene signatures both in laboratory settings (in vitro) and in live mice models (in vivo) of liver fibrosis. Furthermore, miR-221 has been proposed as a serum biomarker for liver cirrhosis and fibrosis . Chronic liver damage caused by HCV infection is a prevalent factor of liver fibrosis. Ding et al. (2015) found increased miR-221 levels in the serum of HCV infected individuals (Szabo et al., 2013).

Our study found that the mean fold change ($2^{-\Delta\Delta CT}$) of miRNA 221 increased in females while the expression of miRNA 122 increased in males. Szabo and Bala, in his study on miRNAs, indicated that the patient's gender was linked to the gene expression of miRNAs (Nakanishi et al., 2009)

miR-122 has an essential role in regulating cholesterol and fatty acid metabolism in the adult liver [37]. Circadian metabolic regulators of the peroxisome proliferator-activated receptor (PPAR) family and AMP-activated protein kinase (APK) were proposed as potential effectors of miR-122-mediated metabolic control, although the exact molecular mechanisms by which miR-122 regulates lipid

homeostasis remain unclear (Gerlach et al., 2009). There may be a link between miR-122, circadian gene expression, and hepatic lipid metabolism since transcription of the miR-122 locus happens in a circadian manner.

The miR-221 gene plays an essential role in regulating the metabolism of fats in patients with fibrosis of the liver was not found in a previous study, and the effect of gender on gene expression has not been addressed in other research to our knowledge. In any case, we did not find sufficient studies on this subject, so we need other studies that include a larger number of samples to determine the extent of the effect of gender on the functioning of the mentioned genes, in addition to continuing to evaluate the level of lipids according to the expression or genetic polymorphism of miR-122 and 221.

CONCLUSION

The findings of our research displayed a clear contradiction in the action of the studied genes, as it was found that gene expression for miR-122 decreased in patients compared to the control, while the rate of gene expression for miR-221 increased in patients compared with the control. However, we found an increase in the rate of harmful lipids in patients and a decrease in the concentration of HDL. We found a minor or indirect influence of miR-122 and 221 on the lipids rate in patients with liver fibrosis.

Declarations of interest

There is no conflict of interest.

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