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Molecular Evaluation of miR-122 and miR-221 Gene Expression in Liver Fibrosisa

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ABSTRACT

miRNAs play role in regulating and managing many body functions, and the disorders that result from a defect in the functioning of this genes may have multiple effects, such as metabolic disorders. Therefore, the current study attempts to investigate gene expression of miRNA 122 and 221 in liver fibrosis and evaluate their effects on the function of hepatocytes. present research is a case – control study, included blood samples collection from 88 fibrotic patients and 88 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 concentration of liver enzymes (TSB, ALP, ALT, AST), Albumin, C- reactive protein (CRP).

The results showed an increase in the concentration of TSB, ALP, ALT, AST and CRP in patients compared to controls and on the contrary, the concentration of albumin increased in healthy people compared with patients ($P<0.05$). The results of the gene expression analysis determined by real time PCR showed a lower mean fold change for miRNA 122 in patients ($3.76 \times 2^{-\Delta\Delta CT}$) compared to controls ($7.45 \times 2^{-\Delta\Delta CT}$) while we found a higher mean fold change for miRNA221 in patients ($2.71 \times 2^{-\Delta\Delta CT}$) compared with healthy people, ($0.04 \times 2^{-\Delta\Delta CT}$) which led to the emergence of significant differences ($P< 0.05$). did not find a clear relationship between miRNA 122 with the tests performed to evaluate liver function, as the Pearson linear relationship was positive but very weak. In addition, gene expression for miRNA 221 was associated with a clear change in the activity of ALP, AST and Albumin ($r= 0.260$, 0.312 and 0.338 respectively). Gene expression of miRNA 122 and 221 significantly associated with liver fibrosis although we didn't find clear correlation between these genes expression and liver function..

Keywords: Liver fibrosis, miRNA 122, miRNA 221, RT-qPCR

1. INTRODUCTION

Liver fibrosis is a complex fibrogenic and inflammatory process that results from chronic liver injury and represents an early step in the progression of liver cirrhosis. Cirrhosis is a major health problem worldwide, owing to the lack of effective treatment methods (Chen et al., 2013). During hepatic fibrosis, continuous accumulation of extracellular matrix (ECM) extremely rich in fats, collagen I and III leads to scar deposition and liver fibrosis (El-Kamary et al., 2013).

A number of genetic disorders contribute to an increased risk of developing liver fibrosis, and in certain instances, may progress to cirrhosis. These genetic factors might hinder the regression of fibrotic changes in the liver. Various microRNAs (miRNAs) have been linked to both liver-specific and systemic fibrotic conditions. The measurement of specific miRNAs in plasma or serum serves as a valuable tool for identifying liver fibrosis. Moreover, certain miRNAs are capable of differentiating between early and advanced stages of fibrosis, demonstrating sensitivity and specificity that are comparable to, or even exceed, those of traditional markers such as the APRI and Fib-4 Index (Karakatsanis et al., 2013 & Su et al., 2018).miR-122 is highly enriched in the liver and represents one of the most predominant microRNAs in this organ. It constitutes approximately 70% of the total hepatic miRNA profile in adult mice and about 52% in adult humans (Su et al., 2018 Sharma et al., 2011). As a result, miR-122 plays a pivotal role in liver development, cellular differentiation, homeostasis, and overall hepatic function. Its expression is regulated by liver-enriched transcription factors (LETFs), such as hepatocyte nuclear factors 6 and 4α, which also modulate the levels of miR-122 during in vivo liver development. Notably, the coordinated expression of miR-122 and these transcription factors is believed to maintain the appropriate balance between cell proliferation and differentiation within both hepatocyte and cholangiocyte lineages during liver form (Sharma et al., 2011 & Tan et al., 2020). The time-dependent regulation of miR-122 expression is especially critical, as it supports the segregation of hepatobiliary lineages and contributes to the establishment and preservation of a liver-specific phenotype. In

fact, studies in mouse liver development have demonstrated that miR-122 progressively suppresses the transcription factor cut-like homeobox 1 (CUTL1), thereby facilitating the final stages of liver cell differentiation (Rong et al., 2020)

In molecular biology, mir-221 microRNA (and its paralogue, miR-222) is a short RNA molecule which located on the X chromosome. miR-221 has several conserved seed sequences which are identical to its homologous miRNA, miR-222. MicroRNAs function to regulate the expression levels of other genes by several mechanisms [Tsay et al., 2019 & Liu et al., 2016). Pervious report discovered miRNA-221-3p, whose downregulation in hepatocytes results in reduced liver fibrosis (Galardi et al., 2011).

The microRNA (miRNA) family includes multiple members that may act in a coordinated manner to regulate key signaling pathways through synergistic mechanisms (Liu et al., 2016). However, it is poor to understand the roles of miRNA family as a whole in hepatic fibrosis; therefore we will focus more on this topic. Furthermore, gaining deeper insight into the intricate genetic mechanisms that govern the fibrogenic process may aid in the identification of epigenetic markers with diagnostic and prognostic value, as well as in the development of innovative therapeutic approaches [(Galardi et al., 2011 & Garofalo et al., 2009). The current study aims to determine the role of miRNA 122 and 221 in the occurrence of liver fibrosis, in addition to determine their effects on some blood markers.

2. MATERIALS AND METHODS

Study design and data collection: The current study is a case - control study that was conducted during the period from 7/2/2022 to 10/4/2023. Samples were collected from Al-zhara Teaching Hospital, and outpatient clinics in AL- Kut city. The number of cases was 88 people suffering from liver fibrosis and 88 healthy people as a control group. Moreover, consent was also obtained from the participants before collecting the questionnaire or taking blood samples. Five ml of blood were taken from all participants for conducting the required tests.

Clinical and laboratory examinations were conducted on the patients to determine the period and degree of liver cirrhosis by specialist doctors. The necessary tests were also conducted to detect chronic diseases such as diabetes, blood pressure, and viral hepatitis to confirm the causes that contributed to the occurrence of liver fibrosis. The study was ethically approved by the Continuing Education Unit of the affiliated hospitals. Prior to participation, patients were provided with a clear explanation of the study's purpose, objectives, and methodology, after which they voluntarily signed a written informed consent form.

Liver function test: FUJI DRI-CHEM SLIDE are used to determine total concentration of liver enzymes (TSB, ALP, ALT, AST), Albumin, C- reactive protein (CRP). Serum samples analysis according to FUJI DRI-CHEM SLIDE user manual.

Complete Blood Count: A complete blood count (CBC) was performed for each blood sample using the RUBY system (USA). The procedure involved placing an EDTA tube—containing the blood sample and labeled with the patient's name and identification number—into a designated rack within the RUBY analyzer. The system then automatically processed and analyzed the samples. Within 1 to 5 minutes, the CBC results, including white blood cell (WBC) count, were displayed on the computer screen. Subsequently, each patient's results were printed and labeled with their corresponding name and identification number.

Molecular study Molecular Study:

Total RNA was extracted from the blood samples using the TRIzol® Reagent kit, following the manufacturer's protocol. The quality and concentration of the extracted RNA were evaluated using a NanoDrop spectrophotometer, which measures RNA concentration and assesses purity based on absorbance ratios at 260/280 nm .The extracted RNA was treated with DNase I enzyme to eliminate any residual genomic DNA. This procedure was performed using a DNase I enzyme kit, following the protocol provided by Promega (USA). After that, the qPCR plate was loaded and the following thermocycler protocol for miRNA genes or GAPDH gene in the following table:

Table (1): qPCR master mix preparation

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45

Annealing\Extention Detection(scan)	60 °C	30 sec	

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) (The ΔCT Method Using a reference gene) that described by (Livak and Schmittgen, 2001). as following equation:

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

The qPCR Primers for miRNA l22 (MIMAT0000421) and miR-221 (MIMAT0004568) were designIn this study, the miRNA sequences

were selected using the Sanger Center miRNA Database Registry, and the specific primers were designed through the miRNA Primer Design Tool. Additionally, primers for the housekeeping gene GAPDH (accession number NM_001256799.3) used in qPCR were designed using the NCBI database in combination with the Primer3 Plus online design tool.

These primers were provided by (Macrogen company, Korea) as in Table (2). qPCR master mix was prepared by using GoTaq® qPCR Master Mix kit. After that, these qPCR master mix component placed in qPCR strip plate tubes and mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system. 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, denaturation step2 wad 95 °C for 20 sec-45 cycle, annealing\extention/ detection(scan) was 60 °C for 30 sec – 45 cycle.

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold Change) (The ΔCT Method Using a reference gene) that described by Livak and Schmittgen [16]. As following equation:

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 (2): RT--PCR primers with their sequence

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

Statistical analysis: the current study included data analysis using the Statistical Package for the Social Sciences, version 22. We also used Excel 2010. Significant differences smaller than 0.05 were considered statistically association (Kazaal et al., 2009).

3. RESULT

This research is a case-control study, 88 patients with liver fibrosis were included in the study; their ages ranged from 25 to 77 years, and their age mean ± standard deviation was 55.76 ± 9.16 years. As shown in Table (3), the majority of these patients were male (61%) compared to female (39%). Results from cases are contrasted with those of healthy people as a control group. 88 healthy people with an average age of 48.6 ± 11.55 years made up the control group.

Table (3): The demographic characteristics of patients and control groups

Properties	Cases	control	*P* value
Age range (years)	25 - 77	25 - 79	
Mean ± SD	55.76 ± 9.16	48.6 ± 11.55	0.499
SE	5.94	5.18	
Gender	**N (%)**	**N (%)**	**P value**
Males	54 (61%)	50 (57%)	0.382
Females	34 (39%)	38 (43%)	0.388
***P* value**	0.022*	0.044*	
Total number	**88**	**88**	

*** significant association (*P* <0.05), SD: Standard deviation, SE: standard error**

To evaluate liver function, we measured TSB, ALP, ALT, AST, albumin and CRP concentration. The results of Table (4) showed an increase in the concentration of TSB, ALP, ALT, AST and CRP in patients compared to controls and on the contrary, the concentration of albumin increased in healthy people compared with patients (*P*<0.05).

Table (4): comparison mean of liver function tests between cases and control groups

Liver function tests	Control	Patients	T test	95% CI	*P* value
	Mean ± SD	Mean ± SD			
TSB (mg/dl)	0.500 ± 0.088	16.3 ± 2.51	40.27	14.14 to 15.57	0.033*
ALP (U/L)	122.54 ± 10.86	372 ± 108.6	15.17	215.6 to 281.5	0.021*
ALT (U/L)	26.35 ± 4.96	42.40 ± 5.98	13.84	14.18 to 18.75	0.039*
AST (U/L)	10.92 ± 2.17	47.81 ± 3.00	65.03	34.77 to 36.98	0.016*
Albumin (g/dl)	3.93 ± 0.43	1.77 ± 0.41	22.99	-2.33 to -1.99	0.023*
CRP (mg/L)	6.77 ± 0.820	15.51 ± 2.01	27.11	8.05 to 9.37	0.044*
Total number	**88**	**88**			

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

Chronic diseases that associated with liver fibrosis described in figure (1). Present study determined that 16% of patients suffer from chronic HCV infection and the majority of patients suffered from obesity and hypertension (34% and 27%, respectively), while diabetes appeared in 18%.

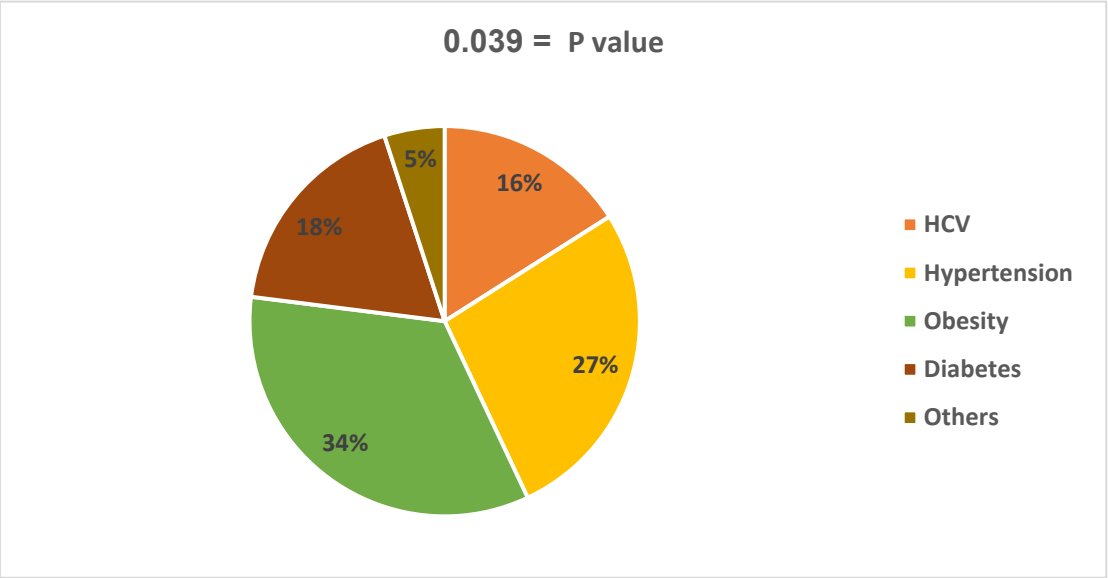

Figure (1): Clinical assessment of chronic diseases among of patients with liver fibrosis

The results of the gene expression analysis determined by real time PCR (Figures 2 & 3) showed a lower mean fold change ($2^{-\Delta\Delta CT}$) for miRNA 122 in patients (3.76) compared to controls (7.45) while we found a higher mean fold change for miRNA221 in patients (2.71) compared with healthy people, (0.04) which led to the emergence of significant differences ($P< 0.05$) as in Table (5). 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 122 and 221 increased in males (8.656±0.13 and 4.333±0.10 respectively) while decreased in females (3.989±0.009 and 3.401± 0.03 respectively), as in Table (6).

Table (5): 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**	26.21	27.24	-1.01	-0.01	-1.00	3.76*
	Control	29.30	27.79	1.52	-0.01	1.53	7.45
miRNA 221	**Cases**	29.12	27.23	1.88	1.88	0.00	2.71*
	Control	27.07	27.79	-0.72	1.88	-2.60	0.042

*** significant differences in compared with controls ($P < 0.05$)**

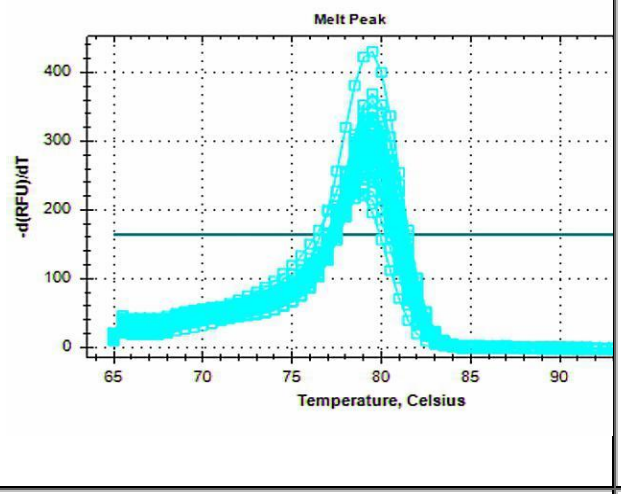

Figure (1): The Real Time PCR melting plot of miRNA 122 gene expression that showed the qPCR specifity at (80.5°C)

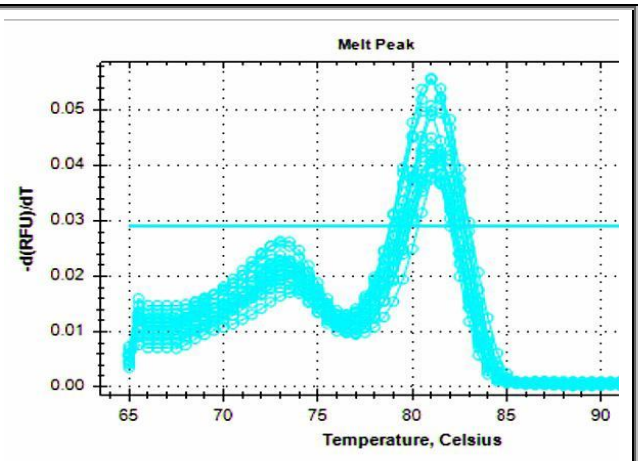

Figure (2): The Real Time PCR melting plot of miRNA 221 gene expression that showed the qPCR specifity at (82.5°C)

Table (6): Distribution of mean of gene expression of miRNA 122 and miRNA 221 according to patients gender

Patients' Gender	Mean Fold change ($2^{-\Delta\Delta CT}$) ± SD		*P* value
	miRNA 122	miRNA 221	
Males	8.656±0.13	4.333±**0.10**	0.032*
Females	3.989±**0.009**	3.401± **0.03**	0.217

The results of our study (Table 7) did not find a clear relationship between miRNA 122 with the tests performed to evaluate liver function, as the Pearson linear relationship was positive but very weak (r<0.2). In addition, gene expression for miRNA 221 was associated with a clear change in the activity of ALP, AST and Albumin (r= 0.260, 0.312 and 0.338 respectively).

Table (7): Pearson correlation (r) among miRNA 122 and 221 and liver function tests in liver fibrosis

Pearson Correlation (r)	Folding of genes expression	
Liver function tests	**miRNA 122**	**miRNA 221**
TSB (mg/dl)	0.014	0.049
ALP (U/L)	0.149	0.260
ALT (U/L)	0.021	0.173
AST (U/L)	0.044	0.312
Albumin (g/dl)	0.073	0.338
CRP (mg/L)	0.062	0.022

4. DISCUSSION

In present study, we found decreased miR-122 in patients. Thus, a decreased level of miR-122 in fibrotic liver cases may be interpreted as the result of compromised normal hepatocytic activity or as the eliminated suppressive function of miR-122 that hinders fibrogenesis. Namely, miR-122 has been found to suppress the proliferation of HSCs, resulting in decreased maturation of collagen by downregulating the expression of P4HA1, a key enzyme in collagen maturation [18,19]. In research of Halász *et al*., 2015 reduced expression of miR-122 in advanced fibrosis and its correlation with fibrosis stage and liver stiffness values seem to be characteristic of hepatic fibrosis of various etiologies [20]. Results of pervious studies revealed a reduced level of miR-122 in fourth stage fibrosis as compared with first stage, and miR-122 showed a negative correlation with fibrosis stage in fibrotic liver samples [19,21,22]. These findings are supported by reports of a negative correlation between miR-122 and Fibrosis stage and also by observations of a decreased level of miR-122 in NAFLD [19,21,22]. Many reports have demonstrated the upregulation of miR-221 in liver fibrosis caused by multiple etiologies such as viral infections and nonalcoholic steatohepatitis. Inhibition of miR-221 via different strategies has shown promising results in terms of the suppression of fibrogenic gene signatures in vitro, as well as in vivo, in independent mouse models of liver fibrosis. In addition, miR-221 has also been suggested as a noninvasive serum biomarker for liver fibrosis and cirrhosis. One of the most common causes of liver fibrosis is chronic liver damage due to HCV infection. In the serum of HCV infected patients, increased levels of miR-221 were detected by Ding *et al.,* (2015) [23].

Our study did not show a clear and reliable association of miRNA 122 and 221 with indicators of liver function, and at the same time we did not find previous studies that dealt with this in depth, as it can be said that our study is the first study that linked miRNA 122 and 221 genes expression with liver functions especially CRP. Tan *et al.,* (2014) showed that a panel including miR-122-5p, miR-141-3p, and miR-26b-5p was diagnostic for liver cancer with higher sensitivity and specificity than traditional biomarkers, such as ALP, ALP, AST and Albumin [24]. In study of Appourchaux and his coworkers, the chronic HBV cohort, serum miR-122 was correlated with ALT ($R2 = 0.28$, $p = 0.01$) and serum miR-21 was positively correlated with both ALT ($R2 = 0.23$, $p = 0.04$) and AST ($R2 = 0.23$, $p = 0.04$) [25].

In current data, miRNA122 not effects on liver function tests whereas pervious study showed circulating levels of miR-122 correlate with ALT levels in patients with NAFLD and be a better NAFLD severity indicator than classic liver function markers, including AST and cytokeratin-18 [26]. Menghini and Federici remember that miRNAs therapeutics is already in clinical trials in liver related disorders such as familial hypercholesterolemia, diabetes mellitus and hypertriglyceridemia [27]. Future work will establish whether measuring circulating miR-221/122 will improve liver enzymes and treatment of NASH and related hyperlipidemia complications [27]. However, the mechanism that miRNAs especially miRNA 122 regulates liver function remains unknown and needs further exploration [28].

Finally, it must be noted that studies on miRNAs genes are very limited, as we have not found a study that clarifies the relationship between these genes and sex hormones or age. Furthermore, studies that address the relationship between miRNAs and liver function were very limited and brief. Therefore, it is necessary to continue investigating the true role of these genes, which may contribute to the diagnosis or treatment of liver cirrhosis, in order to increase the incidence of this disease around the world.

5. CONCLUSION

The results of our study showed 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. The results of the current study did not show a close relationship between genes and liver function, especially miR-122 . We also found a clear relationship between gene expression disorders and the gender of patients. In any case, we need a broader study that addresses the true role of these genes on liver health and the extent of the relationship between sex hormones and the function of these genes.

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