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Evaluation of the Potential role of MicroRNA 126 in hepatitis C infection patients treatment outcomes

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ABSTRACT

Hepatitis C virus (HCV) infection is a worldwide problem that is likely the cause of chronic hepatitis, liver cirrhosis, liver failure, and may underlie the development of hepatocellular carcinoma (HCC). This study aimed to verify the ability of plasma level of MiR-126 gene expression determination to differentiate patients with HCV infection according to the probable response to treatment was evaluated. Blood samples of 43 patients and 17 controls were obtained and processed for determination of serum aspartate transaminase (AST), alanine transaminase (ALT) and alpha-fetoprotein (AFP) in addition to plasma level of MiR-126 gene expression. The percentage of change in post-treatment sample was calculated. Results showed that the treatment significantly reduced serum AFP and plasma MiR-126 expression levels, while serum AST and ALT were non-significantly reduced. The rate of change in post-treatment serum AFP and plasma MiR-126 were positively correlated. Receiver operating characteristic (ROC) curve analysis defined high pre-treatment serum ALT and low pre-treatment plasma MiR-126 levels as sensitive and specific predictors for response to treatment, respectively with moderate accuracy. Multivariate Regression analysis defined the low pre-treatment gene expression level of MiR-126 as the significant predictor for response to treatment. In conclusion, the gene expression of MiR-126 was correlated with the results of laboratory diagnostic tests for HCV infection but showed a significantly higher diagnostic value. Low gene expression of MiR-126 can discriminate samples of HCV responders to treatment.

1. INTRODUCTION

Hepatitis C virus (HCV) is a common cause of chronic liver disease which is a progressive worldwide health problem of a major concern in underdeveloped countries (Ahmed et al., 2022). HCV is a significant pathogen for induction of hepatic fibrosis, cirrhosis as well as liver failure and hepatocellular carcinoma (HCC), which is the most common primary liver cancer (Ben Shabbir et al., 2022).

The pathogenesis of HCV chronic liver disease is still a matter of debate, one of the possible mechanisms is the induction of dysregulation of the innate and adaptive immune responses through affecting the functions of the monocytes, which play a crucial role in linking innate and adaptive immunity to control viral infection (Pang et al., 2022). The HCV-induced dysregulation of immune milieu was supposed to occur through upregulation of the T-cell immunoglobulin mucin 3 (Tim-3), which plays a vital role in suppressing cytotoxic T lymphocytes and T-helper-1 responses and the expression of cytokines such as tumor necrosis factor and interferon-γ (Das et al., 2016), following Toll-like receptor stimulation, and associated with the downregulation of interleukins 10 and 12 (Zhang et al., 2011).

MicroRNAs (miRs) are small non-coding (18-22 nucleotide) RNA molecules, which regulate gene expression and are key regulators of various biological and pathological processes including cell proliferation, development, and tumorigenesis (Solomon &Radhakrishnan, 2000). MicroRNAs are emerging as critical endogenous regulators of gene function and altered microRNAs levels is associated with various human diseases especially cancers (He et al., 2021). Several microRNAs exhibit an anti-tumorigenic activity under endogenous expression levels (Mockly et al., 2022). Tissues of diseased liver showed higher levels of altered expression of microRNA on comparison to tissue of normal liver. These alterations may activate or inhibit the genes that are regulated by these altered microRNAs (Newman et al., 2022).

Despite the shift of treatment of HCV with the advent of the direct-acting antivirals, elimination of HCV is limited by the complexity of the HCV continuous care, the expense of the new therapeutic lines (Jones et al., 2022). Moreover, the health burden of the underserved HCV population and the

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possibility of being a source of reinfection for population who became HCV-free (Kazmi et al., 2022). Another problem is persistent sustained viral response rates, which are increased in HCV infection following administration of direct-acting antiviral (DAA) agents (Öksüz et al., 2022).
MicroRNA-126 regulate the effects of HCV mostly through inhibition of interleukin-1 receptor-associated kinase 1 and tumor necrosis factor receptor-associated factor 6 and a negative feedback loop with nuclear factor-κB which controls the expression of microRNA-126 which overexpression downregulates the expression of interleukin-17 and 35 (He et al., 2015). This study aimed to evaluate the ability of estimated plasma expression levels of gene of MiR-126 to define the response to treatment of HCV patients in comparison to traditional methods.

2. PATIENT AND METHODS

2.1. Patients:
Inclusion criteria:
The study protocol was approved by the Local Ethical Committee, Faculty of Veterinary Medicine, Benha University by number: BUFVTM 03-09-22. This study included 43 patients; 26 males and 17 females within an age range of 27-55 years and had newly diagnosed uncomplicated HCV infection as judged by clinical examination, detection of HCV antibodies and ultrasonographic imaging to exclude the presence of fibrosis, cirrhosis of cancer foci. These patients were free of exclusion criteria. Seventeen healthy volunteers who were free of both inclusion and exclusion criteria and accepted to give blood samples as Control group.

Exclusion criteria:
Exclusion criteria included presence of cirrhosis, manifestations of hepatic malignancy, alcoholic or non-alcoholic steatohepatitis, history of schistosomiasis even if previously treated.

2.2. Sampling:
Two fasting blood samples were obtained from the antecubital vein under complete aseptic conditions, before and after the end of treatment. One blood sample was obtained from control subjects. Each blood sample was divided into four parts:

1. The 1st part was put directly in preservative-free clean dry tube and kept frozen at -20ºC till being assayed for the level of gene expression of MiR-126.
2. The 2nd part was collected in disodium EDTA containing tube for complete blood count.
3. The 3rd part was collected in fluoride containing tubes for estimation of blood glucose
4. The 4th part of centrifuged at 3000 rpm for 10-min and supernatant serum was collected for estimation of serum levels of aspartate transaminase (AST), alanine transaminase (ALT) and alpha-fetoprotein (AFP).

Blindness
Blood samples were collected by an assistant, who was blinded about the diagnosis, then, samples were transferred in iceboxes to the Molecular Biology Unit at Faculty of Veterinary Medicine and either worked-on immediately or kept frozen. The tubes containing the second part of the samples were sent to hospital lab for complete blood count.

2.3. Analysis:
2.3.1. Biochemical analysis: Serum AST, ALT and AFP were determined according to the methods described by Hafkenscheid and Dijt (1979) and Pettinato et al. (2016), respectively.

2.3.2. Molecular analysis of MiR-126gene expression level:
All Plasma samples were subjected to detection of gene expression level of MiR-126 by real time PCR (RT-PCR) (Qiagen, Hilden, Germany) according to the manufacturer's instructions in the following steps: extraction of total RNA from plasma samples, synthesis of complementary DNA by reverse transcription and detection of MiR-126 expression levels by quantitative RT-PCR after correction with the GADPH expression level. Controls were chosen as the reference samples, and fold changes in the levels of miRNA 126 were determined by the $2^{-\Delta\Delta CT}$ (cycle threshold) method and expressed as fold change (FC) using Step One software (Applied Biosystems, USA).

2.4. Statistical analysis
Obtained data were presented as mean, standard deviation, numbers, and percentages. Results were analyzed using One-way ANOVA for analysis of variance between groups and Chi-square test (X^2 test) for analysis of non-numeric data. Spearman's correlation analysis was applied to evaluate the relation between serum AST, ALT and AFP and plasma expression levels of MiR-126. Receiver characteristic curve was used to determine the best of the persistently significant predictors as judged by area under curve (AUC) as either sensitive (AUC<0.5) or specific (AUC >0.5) and its significance in relation to the reference area (AUC =0.5). Test validity characters of the suggested cutoff points using the median and quartile values to determine the best cutoff point with the highest characters and its value was assured using the ROC curve analysis. Statistical analysis was conducted using IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) for Windows statistical package. P value <0.05 was considered statistically significant.

3. RESULTS

During the study duration from Jan 2020 till April 2022, 89 HCV patients were evaluated, 21 patients were excluded for presence of steatohepatitis, 18 patients were excluded for presence of fibrosis of grade 3-4 or cirrhosis and 7 patients showed radiological signs suggestive for presence of HCC. Forty-three patients who fulfilled the inclusion criteria (Study group) gave blood samples before and after receiving treatment (Fig. 1). The inclusion criteria and baseline laboratory investigations (Table 1) showed non-significant differences in comparison to control data.

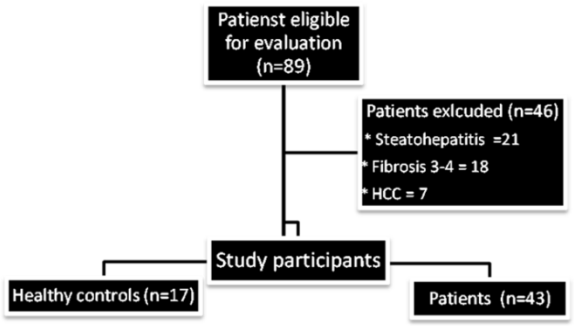

Fig. 1 Consort Flowchart

Serum AST, ALT activities and AFP level in pre- and post-treatment patients' samples were significantly higher as compared to control levels. However, serum AST and ALT in post-treatment samples were non-significantly lowered, while post-treatment serum AFP level was significantly

lowered when compared to pre-treatment levels. The pre-treatment Aspartate/Alanine transaminases ratio (AAR) was significantly lower than the control ratio, while the post-treatment AAR was non-significantly lower than control and pre-treatment ratio.

Concerning the plasma MiR-126 gene expression level, a significant upregulation was observed in pre-treatment samples than control and post-treatment samples. Moreover, significantly upregulated gene expression levels have been shown in post-treatment samples compared to control samples (Table 2).

Correlation analysis showed positive significant correlation between pre-treatment plasma level of gene expression of MiR-126 and serum AST (r=0.410, p=0.006), ALT (r=0.438, p=0.003) and AFP (r=0.379, p=0.012). Also, a positive significant correlation (r=0.356, p=0.019) was detected between the calculated percentage of change of serum AFP and plasma level of gene expression of MiR-126 after treatment (Fig. 2a-d).

Receiver operating characteristic (ROC) curve analysis defined high plasma level of MiR-126 as a specific negative predictor with moderate accuracy and significant AUC, while high serum ALT as a sensitive positive predictor with moderate accuracy and significant AUC for response to treatment. High serum level of AFP can predict the response to treatment with weak accuracy, but high serum AST could not predict the response to treatment (Fig. 3). Univariate regression analysis defined high pre-treatment serum ALT and plasma MiR-126 as significant predictors for response to treatment, but multivariate analysis defined high pre-treatment plasma levels of MiR-126 as the only significant predictor for response to treatment (Table 3).

Table 1 Demographic data of control and patients' groups

Variables		Control (n=17)	Patients (n=43)	P-value
Age (years)		40±8.1	42±8	0.387
Gender	Males	12 (70.6%)	26 (60.5%)	0.463
	Females	5 (29.4)	17 (39.5%)	
Weight (kg)		84.5±3.5	84±4	0.671
Height (cm)		170.8±4.3	169±3.8	0.115
BMI (kg/m^2)		29±1	29.4±0.4	0.156
Random blood glucose (mg/dl)		90.7±10.2	95.5±9	0.117
Hemoglobin concentration (g/dl)		12.4±1.1	12.3±1	0.641
Total leucocytic count (10^3 cells/cc)		7658.4±1188.7	8318.5±1153.3	0.052
Platelet count (10^3 cells/cc)		265.2±10.7	260.6±17.4	0.309

Data are presented as mean, standard deviation, numbers, and percentages; BMI: Body mass index; P-value indicates the significance of difference between both groups; P-value <0.05 indicates significant difference; P-value >0.05 indicates non-significant difference.

Table 2 Serum AST, ALT activities, AFP level and plasma MiR-126 gene expression in pre- and post-treatment samples in comparison to control levels

Variables		Control (n=17)	Patients (n=43)	
			Pre-treatment	Post-treatment
Aspartate transaminase (U/L)	Mean level (±SD)	12.4±1.8	50.0±29.3	46.0±28.7
	Control group (P=)		<0.001	<0.001
	Pre-treatment (P=)			0.481
Alanine transaminase (U/L)	Mean level (±SD)	19±2.3	85.0±37.2	73.0±32.9
	Control group (P=)		<0.001	<0.001
	Pre-treatment (P=)			0.117
AAR	Mean level (±SD)	0.65±0.09	0.57±0.11	0.61±0.14
	Control group (P=)		0.005	0.171
	Pre-treatment (P=)			0.152
Alpha-fetoprotein (ng/ml)	Mean level (±SD)	20.0±3.4	131.0±22.2	120.0±25.6
	Control group (P=)		<0.001	<0.001
	Pre-treatment (P=)			0.036
MiR-126	Mean level (±SD)	3177.2±852.8	5530.3±1550.3	4080.0±1331.5
	Control group (P=)		<0.001	0.012
	Pre-treatment (P=)			0.00001

Data are presented as mean, standard deviation, AAR: Aspartate/Alanine transaminases ratio; P-value <0.05 indicates significant difference; P-value >0.05 indicates non-significant difference

Table 3 The statistical analysis for prediction of the response to treatment

	Variables	AUC	SE	P-value	95% CI
ROC curve analysis	AST	0.557	0.062	0.360	0.435-0.679
	ALT	0.233	0.052	<0.001	0.131-0.335
	AFP	0.643	0.060	0.022	0.526-0.761
	MiR-126	0.755	0.051	<0.001	0.654-0.856
	Analysis	Variables	Standardized coefficient		P-value
Regression analysis	Univariate	ALT	0.314		0.001
		MiR-126	-0.377		<0.001
	Multivariate	MiR-126	-0.453		<0.001

ROC curve: Receiver operating characteristic curve; AST: Aspartate transaminase; ALT: Alanine transaminase; AFP: Alpha-fetoprotein; AUC: Area under the ROC curve; SE: Standard error; CI: Confidence interval; P-value indicates the significance of the values; P-value <0.05 indicates significant difference; P-value >0.05 indicates non-significant difference

4. DISCUSSION

The worldwide spread of HCV especially in the underdeveloped communities where health resources are limited constitutes a continuous problem through limited work-hours and low production rate. Moreover, the continuous contact of treated patients with people who were considered as responders constitutes a permanent source of re-infection. This spotlight on the problem which is how to check the responders and predict the response to treatment, so this study aimed to evaluate the ability of estimation and follow-up of the level of microRNA-126 as distinguishing modality for the responders in comparison to AST and ALT and alpha-fetoprotein as a tumor marker.

The pre-treatment serum levels of AST, ALT and AFP and gene expression plasma levels of MiR-126 were significantly higher than control levels with a positive significant correlation between the gene expression plasma levels of MiR-126 and levels of the three variables. Similarly, a recent study detected a positive significant correlation between serum levels of AST and ALT and gene expression plasma levels of the studied MiRs but was higher with ALT than AST and concluded that microRNA can be used as a marker for evaluation of liver damage in HCV infected patients (Gholami et al., 2016). Another study also detected a positive correlation between serum ALT and gene expression plasma levels of MiRs (Ullah et al., 2022).

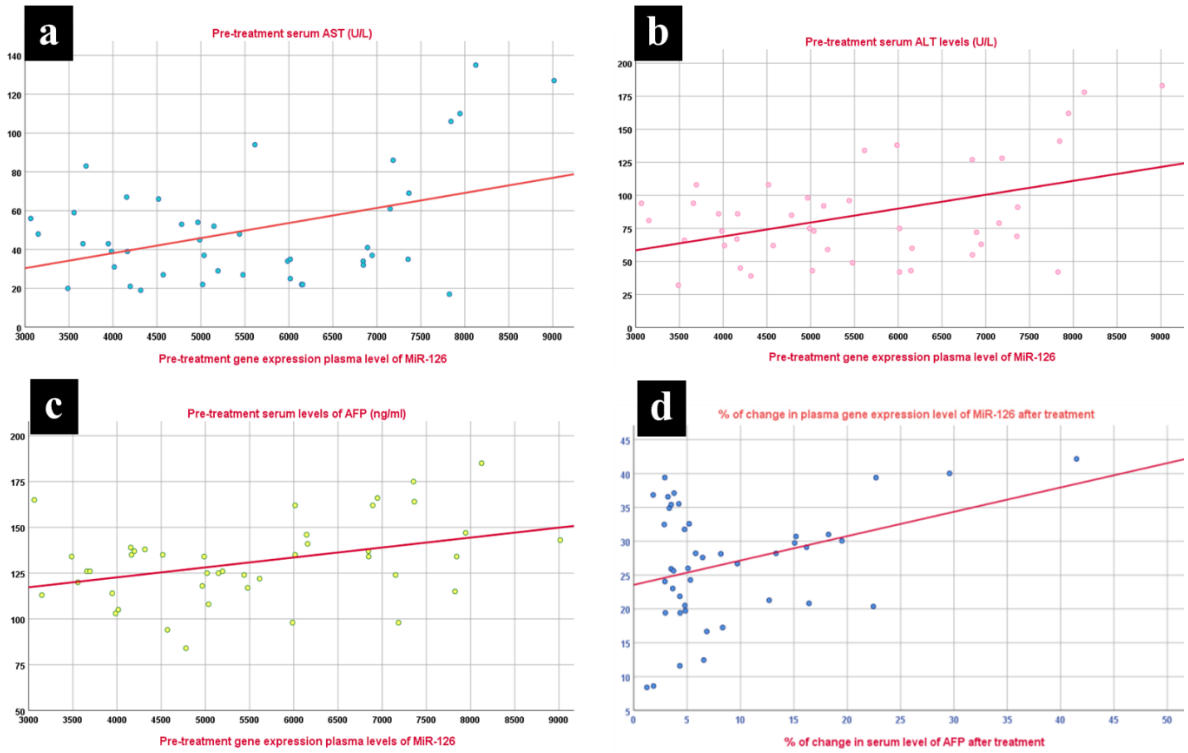

Figure (2a): Correlation analysis of pre-treatment serum levels of AST and MiR-126 plasma levels, Fig. (2b): Correlation analysis of pre-treatment serum levels of ALT and MiR-126 plasma levels, Fig. (2c): Correlation analysis of pre-treatment serum levels of AFP and MiR-126 plasma levels, Fig. (2d): Correlation analysis of post-treatment change in levels of AFP and MiR-126 in relation to pre-treatment levels

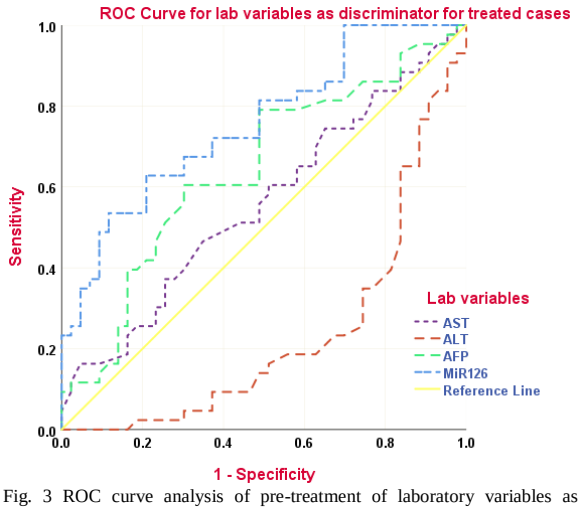

Fig. 3 ROC curve analysis of pre-treatment of laboratory variables as predictors for the response to treatment

All patients showed higher pre- and post-treatment serum levels of AST, ALT, AST/ALT, and AFP in comparison to controls; unfortunately, the difference between pre- and post-treatment serum levels of AST and ALT was non-significant. On contrary, post-treatment serum AFP was significantly decreased in relation to its pre-treatment levels. However, statistical analyses defined improved serum levels of ALT as a significant sensitive marker for response to treatment, while AFP was less significant. These findings illustrate the weak ability of these parameters to check for the response to treatment

On the other hand, post-treatment plasma levels of MiR-126 were significantly downregulated than pre-treatment levels and were non-significantly upregulated than control levels. Additionally, the percentage of change in plasma levels of MiR-126 was positively correlated with that of serum AFP. Moreover, statistical analyses defined low post-treatment plasma levels of MiR-126 as a positive significant specific predictor for the response to treatment with moderate accuracy. These data indicated the superiority of estimation of plasma levels of MiR-126 over estimation of both ALT and AFP and the possibility of using it as a follow-up marker. Similarly, a previous study found the gene expression plasma levels of MiR-126 correlated with the serum HCV load and is differentially expressed between different modes of HCV transmission (Boštjančič et al., 2015). Another study detected increased expression levels of miR-126 with severe chronic hepatic inflammation (El-Guendy et al., 2016). In addition, it was concluded that microRNA can be used as a marker for evaluation of liver damage in HCV infected patients (Gholami et al., 2016). Additionally, one study evaluated a microRNAs array and ROC curve analyses shown high sensitivity and specificity of Mir-126 and 122 to distinguish between HCV and HCC patients and normal individuals and between HCV and HCC patients (Bala et al., 2012).

The detected significantly higher plasma levels of MiR-126 before the start of treatment points to a possible role for microRNA either for pathogenesis of the pathological effects of viral infection or to combat these effects and for both conditions the expression of certain MiRs is increased in parallel to the infection severity. These suggestions are in line with the previous studies detected increased expression of miR-155 and miR-122 in the serum of HCV-infected patients and these MiRs either directly increased production of inflammatory cytokines in chronic HCV infection (Abouzeid et al., 2017) or through improving phosphorylation of the signal transducer and activator of transcription 5 (Cheng et al., 2015).Thereafter, a study found HCV infection upregulates the expression of MiR-135a which in turn mediates a more favorable environment for viral replication and possibly contributing to HCV-induced liver malignancy (Sodroski et al., 2019). Recently, in 2022,

one study detected significant increase of gene expression plasma levels of MiR-21-5p and 122-5p in the HCV-related group compared with the control group (Khairy et al., 2022). Another *in-vitro* study detected six dysregulated MiRs with the same expression trend, while another 32 dysregulated MiRs with different expression trends during different stages of HCV life cycle and some MiRs had significant promotive effect on HCV infection by suppressing retinoic acid-inducible gene 1/IFN pathway through direct binding to its mRNA (Qian et al., 2022).
In support of the ability of microRNA to be a marker for follow-up and judgment of treatment outcomes, a recent study found quantifying circulating levels of miRNAs may offer a rapid and noninvasive means of diagnosing acute rejection in human liver allografts and for discriminating between graft rejection and inflammation or fibrosis due to recurrent HCV (Muthukumar et al., 2022). Concerning MiR-126, recent studies documented its applicability for early detection of impaired response to treatment and detection of recurrences (Jia et al., 2022; Tulinsky et al., 2022).

5. CONCLUSION

The gene expression of MiR-126 is correlated with the results of laboratory diagnostic tests for HCV infection but has a significantly higher diagnostic value. Low gene expression of MiR-126 can differentiate samples of HCV responders to treatment.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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