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Associations of MicroRNA-34a Expression Profile; Serum Levels of Vascular Endothelial Growth Factor and Disease Progression in Hepatitis C Patients

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ABSTRACT

Background: Hepatitis C virus (HCV) is a leading cause of liver cirrhosis, and hepatocellular carcinoma (HCC) Noninvasive indicators for the diagnosis and follow-up of individuals with liver disorders may be found in circulating microRNAs. One such miRNA is miR-34a, which has been linked to the development of HCC. Hepatocellular carcinoma is a highly vascular tumor in which angiogenesis plays a critical role in its development. The best-known angiogenic factor, vascular endothelial growth factor (VEGF), has been proven to have a pivotal role in the development of HCC. **Objectives**: To evaluate the expression profile of miRNA-34a and the serum levels of VEGF both in patients and control group and to find their association with disease progression in HCV patients and evaluate their significance as novel markers for HCV induced HCC. **Subjects and Method:** Including 32 CHC, 23 CHC with liver cirrhosis (LC), 20 CHC with HCC patients, and 15 healthy controls, a total of 90 people participated in the study. Real-time PCR was used to examine the miR-34a expression profile. ELISA was used to assess VEGF concentrations in serum. **Results**: Serum miR-34a down-regulation was observed in patients' groups compared to control group, with lower expression in HCV infection with HCC and HCV infection with LC groups than the HCV infected group. Also VEGF level increased significantly in groups HCV infection with LC and with HCC compared to the control group, in groups HCV infection with LC and with HCC groups compared to the HCV infected group and in group HCV infection with HCC compared to HCV infection with LC group. **Conclusion**: Expression profile of miRNA-34a and serum levels of VEGF can be used as novel markers for HCV inducing HCC with prediction of disease progression in CHC patients.

Keywords: Hepatitis C patients, Hepatocellular carcinoma, Vascular endothelial growth factor, miRNA-34a.

INTRODUCTION

Viral hepatitis is a significant public health concern; it is a key global cause of mortality as well as morbidity. Hepatitis B and C are classified as identical with the same types of liver infection, commonly spread through the blood and blood products [(1)].

The prevalence of chronic hepatitis C virus (CHC) infection is high; it is estimated that 58 million persons worldwide are infected with the virus [(2)]. Egypt has a disproportionate share of the region's burden and has substantially greater prevalence rates than neighboring nations and even countries with similar socioeconomic conditions[(3)], with 92.5% of patients infected with genotype 4 [(4)]. Hepatocellular cancer, cirrhosis, as well as liver fibrosis, are all caused by the chronic inflammatory disease response triggered by CHC infection [(5)]. HCC is a major issue for public health in Egypt. Here it causes 33.63 percent of male and 13.54 percent of female malignancies [(6)]. MicroRNAs (miRNAs) are short, single-stranded RNA molecules that typically range in length from 17 to 25 ribonucleotides [(7)]. It has been found that miRNAs play crucial roles in HCC growth by regulating the expression of a wide variety of proteins [(8)]. It is possible that blood miRNAs that are differentially expressed between people with HCV and HCC might be employed as non-invasive indicators for distinguishing between people with the two diseases and healthy people [(9)]. MicroRNA-34a (miR-34a) is a marker that is often present in non-invasive human cancers[(10)]. Topping the list of selective miRNAs linked to the HCC pathway, it is one of the most widely seen miRNAs related with cancer [(7)]. Hepatocellular carcinoma (HCC) is a highly vascular tumor in which angiogenesis plays a critical role in its development. The ratio of pro- to anti-angiogenic factors is a key regulator of angiogenesis, which is controlled by the local environment [(11)]. Vascular Endothelial Growth Factor (VEGF) is the best-studied pro-angiogenic factor, and it has been linked to tumor initiation, development, and metastasis via angiogenesis in a wide range of malignancies [(12)].

To determine the value of trustworthy serum indicators, we looked at their sensitivity and specificity for illness diagnosis and prognosis, as well as their capacity to be discovered early in the course of the disease [(13)]. The purpose of this study was to examine the blood VEGF and miRNA-34a levels in HCV patients, as well as their connection with disease progression, to determine whether or not these factors may serve as a potential marker for HCV-induced HCC.

SUBJECTS AND METHOD

Seventy-five patients with chronic liver disease and fifteen healthy controls participated in this study, who served as a control group, with normal routine laboratory investigations and negative for HCV Ab. Their age ranged from 26 to 72 years old. All individuals enrolled in this study were classified into 4 groups; group A: 32 patients with HCV infection, group B: 23 HCV patients with liver cirrhosis, group C: 20 HCV patients with HCC and group D: 15 apparently healthy control group.

The Benha Faculty of Medicine's Medical Microbiology and Immunology Department was the site of this investigation. Patients at Benha University Hospital's Hepatology, Gastroenterology, and Infectious Diseases Department were the subjects of the research.

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Ethical consent: An approval of the study was obtained from Benha University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

All patients and control healthy individuals in this study underwent a full history taking, full clinical examination, and laboratory investigations to diagnose CHC, liver cirrhosis, and HCC. These included a complete blood count (CBC), alpha-fetoprotein (AFP), liver function tests, and serological markers for viral hepatitis: Testing for anti-hepatitis C virus antibodies with enzyme-linked immunosorbent assay and transabdominal sonography.

Under complete aseptic condition, 5 ml of venous blood was taken from each participant in tubes without anticoagulant. Serum from each sample was separated, divided into two parts and stored in -80^{0}C for further investigations.

Detection of VEGF by Enzyme-Linked Immunosorbent Assay (ELISA):

This kit is for the accurate quantification of human VEGF (Shanghai Korain bioassay technology laboratory ELISA kit, China; Cat. No. (E0080Hu). Measurement was according to the manufacturer's specifications, and absorbance was measured at 450nm.

Measuring miRNA 34a Expression Using Real-Time RT-PCR:

RNA extraction:

Adhering to the guidelines provided by the manufacturer, we performed an assay using the miRNeasy Serum/Plasma Mini Kit (QIAGEN, Germany) Cat. no. (217184).

RNA was extracted from 100 ml of serum by mixing it with 500 ml of QIAzol lysis reagent. after adding the same volume of chloroform as the original sample, the mixture was centrifuged. The aqueous phase at the top was drained and combined with 1.5 volumes of 100% ethanol in a fresh collecting tube. After the material was collected in a 2 ml collection tube, up to 700 µl was pipetted into an RNeasy MinElute spin column and centrifuged. The collecting tube was discarded along with the flow-through after two rounds of buffer washing and centrifugation. Finally, a fresh 1.5 ml collection tube was used to transfer the RNeasy MinElute spin column and the RNA was eluted by adding 14 µl of RNase-free water to the membrane of the spin column and centrifuging it for 1 minute at maximum speed.

Reverse Transcription (RT) and Quantitative PCR (qPCR): The miRCURY LNA RT Kit (catalogue no. 339340) was used for the RT reactions (QIAGEN, Germany). We used 4µl of miRCURY SYBR® Green RT Experiment Buffer, 10 µl of RNase-free water, 2 ul of miRCURY RT Enzyme Mix, and 4 µl of template RNA (5 ng/l) in each 20 µl RT reaction. All RT reactions were incubated at 42ºC for 60 minutes, 95ºC for 5 minutes, and 4ºC afterwards. For quantitative polymerase chain reaction (qPCR) using the miRCURY LNA miRNA SYBR® Green PCR Kit (catalogue no. 339345) and miRCURY LNA miRNA PCR Assays (catalogue no. 339306) (QIAGEN, Germany) was performed per the manufacturer's instructions. There was 3 µl of cDNA template, 1 µl of RNase-free water, 1 µl of resuspended PCR primer mix, and 5 µl of miRCURY SYBR® Green Master Mix in each 10 µl PCR reaction mix (diluted 1:60). Under these circumstances, the following reactions were performed using a Real-time PCR equipment (Roter-Gene Q): temperatures of 95ºC for two minutes, followed by 45ºC for one minute. The expression of miRNA 34a levels in each sample were finally determined after correction with U6 sn RNA expression level. Fold changes in miR 34a levels were calculated using the 2-CT (cycle threshold) approach and reported as relative units (RU) in Roter Gene software using controls as the reference samples (Applied Biosystems, USA).

Statistical Analysis

Information was entered into SPSS (Statistical Package for the Social Sciences) for Windows® version 25 for coding, processing, and analysis (IBM SPSS Inc, Chicago, IL, USA). Averages and standard deviations were used to express continuous variables with a normal distribution, which were compared by the analysis of variance (ANOVA) test. While, median and range were used to express continuous variables, with an abnormal distribution, which were compared by Kruskal-Wallis test. Categorical data were expressed as frequency and percentage and were compared by the chi-squared test. To evaluate the sensitivity and specificity of quantitative diagnostic measures, ROC analysis and curves were produced; area under the curve (AUC) values were examined and investigated. By analyzing data using a statistical technique called logistic regression, generalised linear models were able to forecast the impact of potential risk variables. For statistical significance, P value < 0.05 was considered significant.

RESULTS

The studied groups were age matched and as regard sex, there were male predominance with no significant difference. AST, ALT, bilirubin, and AFP were within the reference range for the control group but significantly elevated in the three patient groups (CHC, LC, and HCC), whereas albumin showed significantly lower levels in the patient groups than in the control group. Patients with HCC had substantially higher levels of AFP and INR than those with HCV and cirrhosis (Table 1).

Table (1): Demographic and the laboratory data of all patients and control groups

		Group A (HCV) n=32	Group B (HCV with liver cirrhosis) n=23	Group C (HCV with HCC) n=20	Group D (Control group) n=15	test of significance	within group significance#
Age (years)	**Mean ±SD**	56.6 ±10.90	58 ±9.97	57 ±8.47	49.4 ±11.98	F=2.418 P=0.073	-
Female	**N (%)**	9(28.1)	11(47.8)	5(25.0)	5(33.3)	χ^2=3.190 p=0.363	-
Male	**N (%)**	23(71.9)	12(52.2)	15(75.0)	10(66.7)		
Hemoglobin (g/dl)	**Mean ±SD**	10.49 ±1.37	9.93 ±1.91	10.7 ±2.09	13.23 ±0.96	F=15.032 P<0.001*	P1=0.044* P2=0.015* P3=0.006* P4=0.185 P5=0.126 P6=0.618
WBC ($X10^9$/L)	**Mean ±SD**	5.3 ±1.21	6.88 ±1.2	6.79 ±1.11	7.54 ±0.98	KW P=0.264	-
Platelets ($\times10^9$/L)	**Mean ±SD**	116±27.7	89±8.65	106.5±22.32	270 ±63.54	KW P<0.001*	P1<0.001* P2<0.001* P3<0.001* P4=0.183 P5=0.323 P6=0.436
INR	**Mean ±SD**	1.1±0.18	1.2±0.19	1.6±0.17	1.02±0.12	KW P<0.001*	P1=0.152 P2=0.041* P3=0.001* P4=0.104 P5<0.001* P6=0.028*
ALT (U/L)	**Mean ±SD**	43±10.21	35±8.11	65.5±13.32	26±6.33	KW P<0.001*	P1<0.001* P2=0.032* P3<0.001* P4=0.432 P5=0.047* P6=0.027*
AST (U/L)	**Mean ±SD**	51.5±7.33	48±11.44	103±22.31	25±5.42	KW P<0.001*	P1<0.001* P2<0.001* P3<0.001* P4=0.811 P5<0.001* P6=0.001*
Bilirubin (mg/dL)	**Mean ±SD**	0.85±0.18	2.1±0.41	2.95±0.51	0.9±0.17	KW P<0.001*	P1=0.908 P2=0.001* P3<0.001* P4<0.001* P5<0.001* P6=0.300
Albumin (g/dL)	**Mean ±SD**	3.1±0.61	2.6±0.32	2.3±0.11	4.5±0.91	KW P<0.001*	P1<0.001* P2<0.001* P3<0.001* P4<0.001* P5<0.001* P6=0.180
AFP (ng/mL)	**Mean ±SD**	0.7±0.11	21.8±4.33	1637.55± 94.44	0.23±0.031	KW P<0.001	P1=0.019* P2<0.001* P3<0.001* P4<0.001* P5<0.001* P6<0.001*

P1: difference between healthy control group and group A, P2: difference between healthy control group and group B, P3: difference between healthy control group and group C, P4: difference between A and B groups, P5: difference between group A and C groups, P6: difference between group B and C groups; *: significant.

In comparing miRNA-34a gene expression in studied groups, there were significant down regulation in gene expression in A, B and C groups than control group and significant down regulation in gene expression in B and C than A groups. VEGF level increased significantly in groups B, C compared to D group, in groups B, C compared to A group, and in group C compared to B group (Table 2, figs. 1, 2).

Table (2): Comparison of miRNA-34a expression and VEGF concentration among studied groups

		Group A (HCV) n=32	Group B (HCV with liver cirrhosis) n=23	Group C (HCV with HCC) n=20	Group D (Control group) n=15	test of significance	within group significance#
MiRNA-34a	Mean ±SD	0.485 ±0.11	0.14±0.021	0.065 ±0.011	0.6 ±0.11	KW P<0.001*	P1=0.030* P2<0.001* P3<0.001* P4<0.001* P5<0.001* P6=0.048*
Down regulated <0.5	N (%)	17(53.1)	23(100)	20(100)	1(6.7)	$\chi2$=49.239 P<0.001*	p1=0.002* p2<0.001* p3<0.001* p4<0.001* p5<0.001*
Normal (0.5 – 1)	N (%)	15(46.9)	0(0)	0(0)	14(93.3)		
VEGF (ng/mL)	Mean ±SD	2468 ±524.71	5896 ±370.33	9642 ±2241.12	2458 ±518.32	KW P<0.001*	P1=0.766 P2<0.001* P3<0.001* P4<0.001* P5<0.001* P6<0.001*

VEGF: Vascular Endothelial Growth Factor; X2, Chi square test; KW: Kruskal Wallis test

P1: difference between healthy control group and group A, P2: difference between healthy control group and group B, P3: difference between healthy control group and group C, P4: difference between A and B groups, P5: difference between group A and C groups, P6: difference between group B and C groups. *: significant

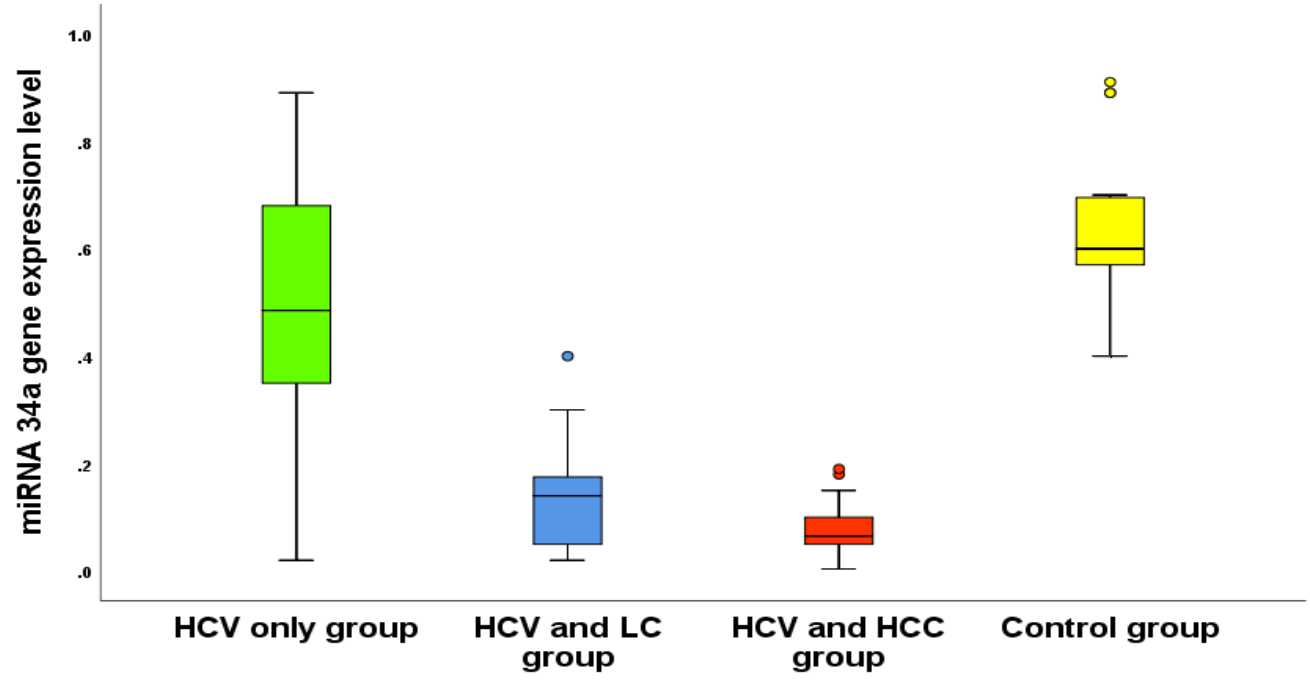

Figure (1): The miRNA-34a gene expression level among different studied groups.

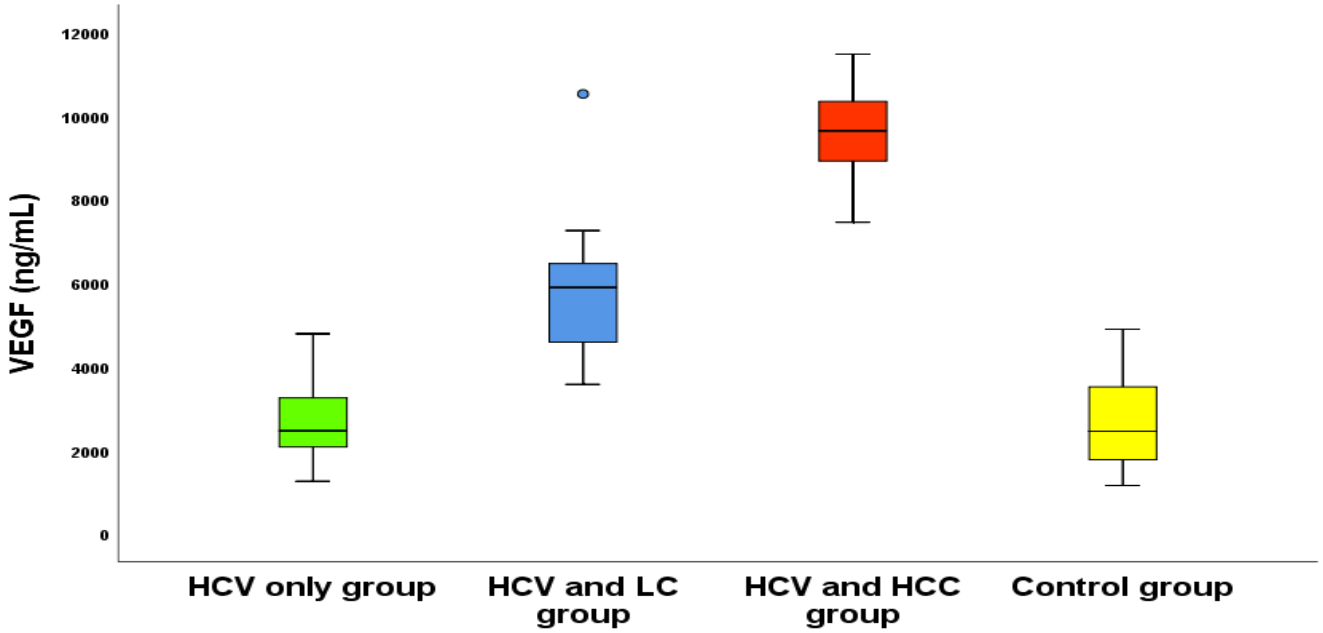

Figure (2): VEGF level among different studied groups.

Validity of miRNA-34a and VEGF in differentiating patients' groups:

Calculations of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each of the miRNA-34a and VEGF assays were performed using the AUC values so as to differentiate each patients' group from each other group (Table 3).

Table (3): Validity of miRNA-34a and VEGF serum levels in differentiating patients' groups

	AUC	Cut off point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Differentiating LC group from HCV group							
miRNA-34a	0.859	0.224	91.3	84.4	80.8	93.1	87.3
VEGF	0.984	5383.5	95.7	90.6	88	96.7	92.7
Differentiating HCC group from HCV group							
miRNA-34a	0.881	0.12	100	84.4	80	100	90.4
VEGF	1	6123.5	100	100	100	100	100
Differentiating HCC group from LC group							
miRNA-34a	0.639	0.075	60	60.9	57.2	63.6	60.5
VEGF	0.965	7358	100	95.7	95.3	100	97.7

AUC, area under ROC curve; PPV, positive predictive value; NPV, negative predictive value.

Applying regression analysis to predict HCC:

The development of HCC was predicted using a logistic regression model using miRNA-34a and VEGF as variables. When analyzed alone, higher VEGF and lower miRNA-34a gene expression levels were linked to an increased risk of HCC. However, when relevant variables from univariate analysis were included in the multivariate analysis, it was shown that greater VEGF and lower miRNA-34a gene expression levels were independent predictors of HCC risk (Table 4).

Table (4): Regression analysis for prediction of HCC development

Covariates	Univariable		Multivariable	
	P	OR(95% CI)	p	OR(95% CI)
miRNA-34a	0.003*	0.042(0.002-0.124)	0.027*	0.888(0.536-0.965)
VEGF	<0.001*	1.011(1.006-1.021)	<0.001*	1.023(1.011-1.064)

OR, odds ratio; CI, confidence interval. Logistic regression analysis was done for HCC versus LC +HCV + healthy groups; *: significant

DISCUSSION

The World Health Organization (WHO) has identified HCV, a blood-borne virus infection, as a worldwide health hazard. Persistent HCV infection is a major source of concern across the world since it can result in life-threatening complications like cirrhosis, hepatocellular cancer, and the need for a liver transplant [(14)].

It is estimated that the rate of HCV transmission in Egypt is around ten times higher than in the United States or Europe. The percentage of HCV antibody positivity in the general Egyptian population is believed to be between 10% and 13%, making Egypt the country with the greatest HCV prevalence worldwide [(15)].

Developing algorithms that combine serum indicators with noninvasive diagnostic imaging modalities is necessary for the prompt and precise detection of HCC [(16)]. Advantages of circulating miRNAs as unique and excellent diagnostic and prognostic serum indicators include their stability in bodily fluids, resistance to endogenous RNase digestion, and ease of detection using sensitive technologies like RT-PCR [(17)].

VEGF is a well-known angiogenic factor representing one of the major inducers of angiogenesis that have a role in development of liver fibrosis and carcinogenesis in CHC infection. Angiogenesis was shown to be correlated with VEGF levels in serum [(18)].

In the current study, expression of miR-34a was shown to be down-regulated in the patient groups relative to the control group; this was more pronounced in the HCV with HCC and HCV with LC groups than in the HCV infected group.

These findings corroborated those of **Shehata *et al.*** [(18)], who found a significant reduction in serum miR-34a in hepatocellular carcinoma (HCC) patients compared to controls and LC patients, with a more pronounced decrease in miR-34a expression in late HCC than in early HCC. Also, **Chen *et al.*** [(19)] found that miR-34a expression in exosomes was considerably lower in preoperative HCC patients compared to both healthy controls and postoperative HCC patients. With agreement of the present findings **Bharali *et al.*** [(20)] reported that the expression of miR-34a was considerably lower in HCC compared to non-HCC controls. MiR-34a expression was observed to be significantly different in HCC compared to healthy controls (p=0.001), HCC compared to chronic hepatitis (p=0.001), and HCC compared to liver cirrhosis (p=0.004).

On the contrary, **Cermelli *et al.*** [(21)] and **Salvoza *et al.*** [(22)] found that in HCV and non-alcoholic fatty liver disease, miR34a expression was shown to be elevated.

Several variables that influence the expression pattern of miRNAs may account for the discrepancy between the present study's findings and those of **Cermelli *et al.*** [(21)] and **Salvoza *et al.*** [(22)].

Shen *et al.* [(23)] who explained that cancer patients vary greatly in terms of tumor stage, response to treatment, and underlying cause, all of which have a role. Serum, plasma, paraffin-embedded tissue, and formalin-fixed tissue are only few of the many specimen types routinely analysed. Factors that may affect the result of the expression analysis include variations in sample collection, processing, and preservation.

In this study the VEGF serum levels increased significantly in patient's groups suffering from HCV with LC, HCV with HCC in comparison to control group. In patient groups suffering from HCV with LC, HCV with HCC groups compared to the HCV infected and in HCV with HCC group compared to HCV with LC group.

In line with these findings, **Joo *et al.*** [(11)] found that VEGF levels rose significantly with the development of liver disease from chronic hepatitis to cirrhosis and HCC (p = 0.045 for each). This significant elevation was expected, since tumor angiogenesis is essential for the development and spread of HCC since it is a highly vascular tumor [(24)].

Niu *et al.* [(25)] in his research found that VEGF is the potential target gene of miR-34a by means of TargetScan, and confirmed that miR-34a can indeed complement and combine with the mRNA 3'UTR of VEGF by using the dual luciferase reporter gene experiment. They found that up-regulation of the expression level of miR-34a in MHCC97H cells can inhibit the expression level of mRNA and protein of VEGF, indicating that miR-34a may regulate the expression of VEGF.

Furthermore, in the current study regression analysis was performed utilising miRNA-34a and VEGF as variables to predict the development of HCC. Higher VEGF, lower miRNA-34a gene expression levels were considered as independent predictors for progression of liver disease in patients' groups.

Consistent with these findings, **Niu *et al.*** [(25)] reported that miR-34a expression was up-regulated in a nude mouse xenograft model of HCC and that doing so inhibited HCC cell proliferation and significantly reduced tumor volume and mass, suggesting that miR-34a suppresses the malignant biological behaviour of HCC cells by lowering VEGF expression.

CONCLUSIONS

The expression profile of miRNA-34a and the serum levels of VEGF have important clinical significance and can be used as novel non-invasive markers for diagnosis and monitoring of HCV sequelae inducing CHC and HCC.

MiRNA-34a and VEGF are associated with disease progression and involved in hepato-carcinogenesis. This fact makes them putative targets for treating HCC.

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