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Micro RNA Let7c Induced Monocyte Activation in Patients with Chronic HCV Induced HCC Via Regulation of IL10

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Abstract:

Background Monocytes, critical components of the immune system, act as a link between innate and adaptive immunity. In the context of chronic hepatitis C virus infection, progressing to liver cirrhosis and hepatocellular carcinoma (HCC), monocytes differentiation into macrophages is thought to be crucial in altering microenvironment of the tumor. Recent data emphasizes the critical role of microRNAs (miRNAs) in the regulation of gene expression, influencing monocyte activation, immune responses, and cancer progression. Chronic liver disorders, such as HCV-related cirrhosis and HCC, have been linked to the dysregulation of certain miRNAs. Particularly miRNA let-7c has attracted attention because of its role in immune regulation and tumor biology.

Objectives Here, we investigated the role of miRNA let-7c in controlling monocyte activation in chronic HCV-related cirrhosis and HCC, with an emphasis on its relationship to the immune-regulatory cytokine IL10. **Methods** The study included 54 patients with chronic HCV infection, divided into 3 groups; chronic HCV, liver cirrhosis, and HCC, as well as, 15 healthy individuals as controls. MiRNA let-7c and IL10 gene expression were assessed by RT-PCR, and CD16 and CX3CR1 expression on monocytes was assessed by flowcytometry. **Results:** The results showed that whilst IL10 expression was significantly elevated in HCC patients in comparison to HCV, cirrhosis and controls (P-value < 0.05), miRNA let-7c expression was gradually downregulated from chronic HCV to cirrhosis and HCC (P-value < 0.01). Furthermore, the percentage of CD16+CX3CR1+ monocytes was significantly increased the disease with disease severity, indicating their potential role in HCC progression. **Conclusion** Our findings suggest that decreased miRNA let-7c expression may contribute to monocyte activation and immune modulation in HCC via the regulation of IL10. IL10 overexpression in HCC may facilitate immune evasion and tumor progression, making it a promising biomarker for HCC diagnosis and monitoring. miRNA let-7c and CD16+CX3CR1+ monocytes could be valuable biomarkers for following the development of cirrhosis and HCC from a persistent HCV infection. Targeting the IL10 pathway could offer new therapeutic strategies for HCC treatment.

Keywords: miRNA let-7c, IL10, monocytes, HCV, liver cirrhosis, hepatocellular carcinoma (HCC), biomarkers.

1. Introduction

Monocytes play an important role in the immune system, acting as bridges between innate and adaptive immune responses. These cells have a profound effect on the body's immune system by differentiating into macrophages, especially in chronic diseases [1]. Hepatitis C virus (HCV) infection is a main health problem worldwide, with the progression to cirrhosis and hepatocellular carcinoma (HCC) [2]. Interactions between the immune system and the tumor microenvironment are essential for understanding the pathogenesis of these conditions. In this context, activation and differentiation of monocytes are key determinants of disease progression [3]. Monocytes make up a significant portion of the infiltrating leucocyte population at the tumor site. Once they have homed to the malignant tissue, they undergo differentiation into TAMs, or tumor-associated macrophages, which vary in type based on the type of tumor. Monocytes and macrophages plasticity is regulated at transcriptional and translational levels [4]. So far, recent researches have investigated the important role of microRNAs (miRNAs) in regulating gene expression, especially in immune cells. miRNAs, small non-coding RNAs, modulate a variety of biological processes including immunity, inflammation, and cancer development [5]. MiRNAs have been studied as potential therapeutic targets and biomarkers, although there are some variations between studies [6]. MiRNA dysregulation has been linked to chronic liver diseases, such as HCV-related cirrhosis and HCC [7]. Among these, miRNA let-7c has a considerable interest due to its involvement in tumorigenesis and immune modulation [8]. The role of miRNAs in immune responses regulation, especially within the monocyte-macrophage axis, is still being studied [9]. MiRNA let-7c, in particular, has been shown to affect the activation and differentiation of monocyte [10]. However, its significance in the advancement of HCV-related liver disorders, including HCC, is not fully understood. Moreover, the link between miRNA let-7c and the immune-regulatory cytokine interleukin 10 (IL10) is still to be fully investigated [11]. Our goal in this work is to better understand how miRNA let-7c controls monocyte activation and function as chronic HCV infection progresses to cirrhosis and HCC, focusing on the relationship that may support immune evasion in HCC between miRNA let-7c and IL10 production, a cytokine with immunosuppressive properties. By examining monocyte subsets, including CD16+CX3CR1+ cells, we aim to elucidate their potential involvement in HCC pathogenesis and their value as potential biomarkers for predicting the progression of the disease.

Material and Methods

Patients' selection

This study conducted on 54 patients, recruited from the department of hepato-gastroenterology at Theodor Bilharz Research Institute (TBRI), in addition to 15 healthy controls. Patients were classified into 3 groups as follows; chronic HCV group, liver cirrhosis group and HCC group. The study was approved by TBRI Research Ethics Committee (FWA 00010609) no. PT 815, after obtaining informed consents. Inclusion Criteria: Patients with chronic HCV infection, diagnosed by serological and molecular testing. As well as patients with HCC post HCV infection. Exclusion Criteria: Patients with chronic liver diseases other than HCV infection.

Sample collection

Peripheral blood samples were collected aseptically by clean venipuncture. About 5 ml of blood was withdrawn and distributed as follows: In a vacutainer tube containing EDTA anticoagulant, 2 ml of blood was delivered to perform the flow cytometric analysis of CD16 and CX3CR1. And 3

ml of blood was collected on plain tube. The serum was then collected, divided and kept in tightly closed vials at -80°C for quantitative reverse transcription PCR (qRT-PCR) analysis of miRNA let-7c, IL10 gene and routine analysis.

Bioinformatics methods

The gene cards database (<https://www.genecards.org>) reports that the IL10 gene encodes a cytokine produced by monocytes and lymphocytes. GeneMANIA tool (<http://www.genemania.org>) was used to predict the potential functional relationships between the IL10 and the other gene. MiRNet (<https://www.mirnet.ca>) version 2.0, was used to predict IL10 target miRNAs with hsa-let-7c-5p. Conserved region between IL10 3' and hsa-let-7c-5p were predicted using TargetScan V.8 database, which acts as a prediction tools for the miRNAs-targets (<https://www.targetscan.org>).

Gene expression by RT-PCR

The expression of let-7c miRNA and the target gene IL10 by qRT-PCR was performed in the following steps: total RNA extraction was done using RNA Isolation Kit (Roche, catalog number 11828665001). The RNA were quantitatively and qualitatively checked by a NanoDrop spectrophotometer (NanoDrop Technologies, USA). The FIREScript RT cDNA synthesis KIT, SOLIS BIODYNE, catalog number 06-15-00050) was used for Reverse transcription (RT) reaction. The expression pattern of miRNA let-7c and IL10 gene was evaluated using EvaGreen HOT FIREPol qPCR Mix Plus, SOLIS BIODYNE, catalog number 082400001) using LightCycler EvoScript RNA SYBR Green I. All miRNAs and mRNAs expression values will be evaluated using relative quantification analysis by $\Delta\Delta C_t$ method and will be normalized to Beta-actin U6 and as endogenous controls for IL10 gene and miRNA let 7c, respectively.

Flow cytometric study

For flow cytometric assessment of circulating CD16⁺ CX3CR1⁺ monocytes, 10 μ L of Percp- anti-human CD16 antibody (BioLegend, catalog no. 302006) and 10 μ L of PE-anti-human CX3CR1 (BioLegend, catalog no. 355704) were added to 100 μ L of whole blood. After 15 minutes incubation in the dark at room temperature, 500 μ L of RBC lysing solution (Biolegend, catalog no.422401) was added for 10 minutes, washed twice in PBS and finally re-suspended in 300 μ L of PBS. Data were analyzed using a Beckman Coulter flow cytometer (Beckman Coulter Epics XL-MCL, USA), after acquisition of 10000 events, data analysis was done through the Kaluza analysis software (Beckman Coulter Inc., USA).

Statistical analysis

The data were analyzed using SPSS Statistics (IBM Corp., Armonk, N.Y., USA), version 28. Non parametric data were represented as median and 25% - 75% range (IQR) with 95% confidence interval; a p-value < 0.05 was considered statistically significant. Mann-Whitney U test was used to compare medians of abnormally distributed data. The ROC curve was used for analysis of the diagnostic performance by calculating the area under the curve (AUC). Stratification and the logistic regression model were used to evaluate the prognostic performance.

Results

Bioinformatics analysis

Results obtained from the gene cards database and the online database GeneMANIA demonstrated that IL10 cytokine encoded by its specific gene and produced by monocytes and lymphocytes. The results indicated that IL10 gene is the main regulator to the leukocyte differentiation, programmed necrotic cell death, leucocyte apoptotic process, response to IL4, positive regulator for protein secretion, and endothelial cell regulation. At the same time, the IL10 directly interact with many other genes which are working as the main regulator of immune system (Figure 1).

Analysis of the miRNet database showed that the hsa-let-7c-5p can be directly targeted to IL10 (Fig. 2A). The binding site between IL10 and hsa-let-7c-5p was conserved with a 7mer-A1 seed region and a Context++ score of -0.32 (Table 1), according to the TargetScan V.8 database <https://www.targetscan.org>.

According to the previous bioinformatics results, we have selected the miRNA let 7c and IL10 gene to test their validity as diagnostic and prognostic biomarkers for HCC as shown in Figure 2B).

Gene expression results by qRT-PCR

Our results revealed that miRNA let-7c gene expression was significantly upregulated in HCV and liver cirrhosis groups in comparison to the control (5.0, 3.0, and 1.0 respectively) (P-value < 0.01). However, its expression was downregulated in HCC group when compared to the control (0.6 vs. 1.0 respectively) (P-value<0.05). MiRNA let-7c expression was significantly downregulated in cirrhosis and HCC groups (3.0 and 0.6) compared to HCV group(5.0), and in HCC group (0.6) compared to cirrhosis (3.0) and CLD group (Table 2, Figure 3a). In addition, our findings also showed that, IL10 expression was significantly downregulated in the HCV and cirrhosis groups, while it was significantly upregulated in the HCC group in comparison to the control. In comparing the diseased groups with each other, there was a significant upregulation of IL10 gene expression with the progression of the disease (0.1, 0.5 and 2.1in HCV, cirrhosis and HCC groups respectively) with P-value < 0.05 (Table 2, Figure 3b).

Flow cytometric analysis results

The percentage of monocytes that positively express CD16 and CX3CR1 was significantly increased in accordance with the progression of the disease when compared to the control group (P- value < 0.01), as well as in the cirrhosis and HCC groups when compared to the HCV group. Moreover, the percentage of CD16+ CX3CR1+ monocytes was significantly elevated in the HCC group in comparison to cirrhosis or HCV groups (P-value < 0.01) (Table 2, Figures 3c and 4).

Analysis of the diagnostic and prognostic values of the studied biomarkers

The ROC curve study for the diagnostic performance of the selected biomarkers revealed: MiRNA let-7c showed there were a significant difference between the HCV group and the control group (sensitivity = 100.0, specificity=100.0, p- value <0001), the cirrhosis group and the HCV group (sensitivity =100.0, specificity =89.0, p- value=0.035), the HCC group and the Cirrhosis group (sensitivity =100.0, specificity =83.0, p-value=0.011), and the HCC group and the CLD group (sensitivity = 100.0, specificity =83.0, p-value = 0.001).

The IL10 gene showed significant discrimination between the HCV group and the control group (sensitivity = 100.0, specificity =89.0, p<0001), the cirrhosis group and the HCV group (sensitivity

=72.0, specificity =56.0, p=0.018), the HCC group and the Cirrhosis group (sensitivity =72.0, specificity =73.0, p=0.05), and the HCC group and the CLD group (sensitivity = 72.0, specificity =81.0, p= 0.002).

It is noteworthy that the CD16+CX3CR1+ monocytes showed significant difference between the HCV and control groups (p- value = 0.005), between the HCC group and the cirrhosis group (p-value<0001), and between the HCC group and the CLD group (p-value < 0.0001). Despite this, there was no statistical difference between cirrhosis and HCV groups (p = 0.351) (Table 3, Figure 5).

The prognostic performance of the selected biomarkers was assessed using the logistic regression analysis and showed a significant positive correlation between miRNA let-7c and progression of the disease in HCC when compared with the CLD group p- value = 0.032 (Table 4).

Table 1 Bioinformatics of the binding site between IL10 and hsa-let-7c-5p

	Predicted consequential pairing of target region (top) and <u>miRNA</u> (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	PCT	Predicted relative KD
Position 140-146 of IL10 3' UTR hsa-let-7c-5p	5' ...AUUUUUUACCUCUGAUACCUCAA... 3' UUGGUAUGUUGGAUGAUGGAGU	7mer-A1	-0.32	90	-0.32	6.938	0.95	N/A

Table 2: The expression pattern of miRNA let-7c, IL10 gene, and CD16+ CX3CR1+ monocyte

Groups	Control	CLD		HCC
		HCV	Cirrhosis	
miRNA let-7c	1a	5.5(3.1-15.0) bb	3.0(1.5- 8.0) bb, c	0.6(0.1- 4.5) b, cc, d, ee
IL10	1 a	0.1(0.02- 0.3) bb	0.5(0.1- 1.6) b, c	2.1(0.2- 20.9) bb, cc, d, ee
CD16+ CX3CR1+ monocyte	4.6±2.7 a	8.4±3.7 bb	9.9±4.8 bb	33.5±22.4 bb, cc, dd, ee

miR let-7c and IL10 expression is represented as median and (25% -75%) range; the data were analyzed by Mann-Whitney Test. While CD16+ CX3CR1+ monocyte expression is represented as Mean±SD; the data were analyzed by t Test. Based on flowcytometry percent, expression of CD16+ CX3CR1+ monocyte was evaluated. Values with different symbols (a, b, c, d, e) are significantly different at P<0.05. 1 initial P value < 0.05 is significant, 2 initials P value < 0.01 is highly significant.

Table 3 ROC curve analysis for the performance of the selected biomarkers

	Biomarker(s)	Cut-off	sensitivity	specificity	AUC (C.I 95%)	S.E	P-value
HCV Vs Control group	miRNA let-7c	>1.5	100.0	100.0	1.0 (1.0-1.0)	0.000	<0.0001**
	IL10	<0.98	100.0	89.0	0.11(0.0-0.25)	0.074	<0.0001**
	CD16+ CX3CR1+ Monocyte	<6.3	67.0	67.0	0.79(0.63-0.94)	0.079	0.005**
Cirrhosis Vs HCV group	miRNA let-7c	>0.78	100.0	89.0	0.29(0.12-0.47)	0.091	0.035*
	IL10	<0.14	72.0	56.0	0.73 (0.56-0.89)	0.084	0.018*
	CD16+ CX3CR1+ Monocyte	<8.4	67.0	56.0	0.59 (0.4-0.78)	0.097	0.351
HCC Vs Cirrhosis group	miRNA let-7c	>0.09	100.0	83.0	0.25 (0.08-0.42)	0.087	0.011*
	IL10	<1.2	72.0	73.0	0.68 (0.51-0.87)	0.092	0.05
	CD16+ CX3CR1+ Monocyte	<15.9	83.0	89.0	0.90 (0.79-1.0)	0.058	<0.0001**
HCC Vs CLD group	miRNA let-7c	>0.09	100.0	83.0	0.214(0.06-0.37)	0.081	0.001**
	IL10	<1.2	72.0	81.0	0.758(0.61-0.91)	0.076	0.002**
	CD16+ CX3CR1+ Monocyte	<15.9	83.0	95.0	0.908(0.79-1.0)	0.057	<0.0001**

AUC: Area under curve and C.I: 95% Confidence Interval. *: significant p- value <0.05, **: highly significant p-value <0.01.

Table 4 The regression analysis for the prognostic performance of the selected biomarkers

		OR	C.I 95%		P-value
			Lower	Upper	
HCV Vs Control group	miR let-7c	0.706	0.511	0.975	0.035*
	IL10	0.396	0.15	1.043	0.061
	CD16+ Monocyte CX3CR1+	1.097	0.992	1.213	0.071
Cirrhosis Vs HCV group	miR let-7c	1.016	0.973	1.06	0.469
	IL10	1.583	0.737	3.401	0.239
	CD16+ Monocyte CX3CR1+	1.016	0.951	1.084	0.641
HCC Vs Cirrhosis group	miR let-7c	1.021	0.965	1.081	0.469
	IL10	1.022	0.987	1.059	0.224
	CD16+ Monocyte CX3CR1+	1.037	1.003	1.072	0.031*
HCC Vs CLD group	miR let-7c	1.071	1.006	1.14	0.032*
	IL10	1.021	0.987	1.056	0.223
	CD16+ Monocyte CX3CR1+	1.019	0.994	1.044	0.143

OR; Odd Ratio, C.I; Confidence Interval, p value of Prognostic viability is calculated depending on logistic regression analysis. * p. value <0.05 is significant, ** p. value <0.01 is highly significant.

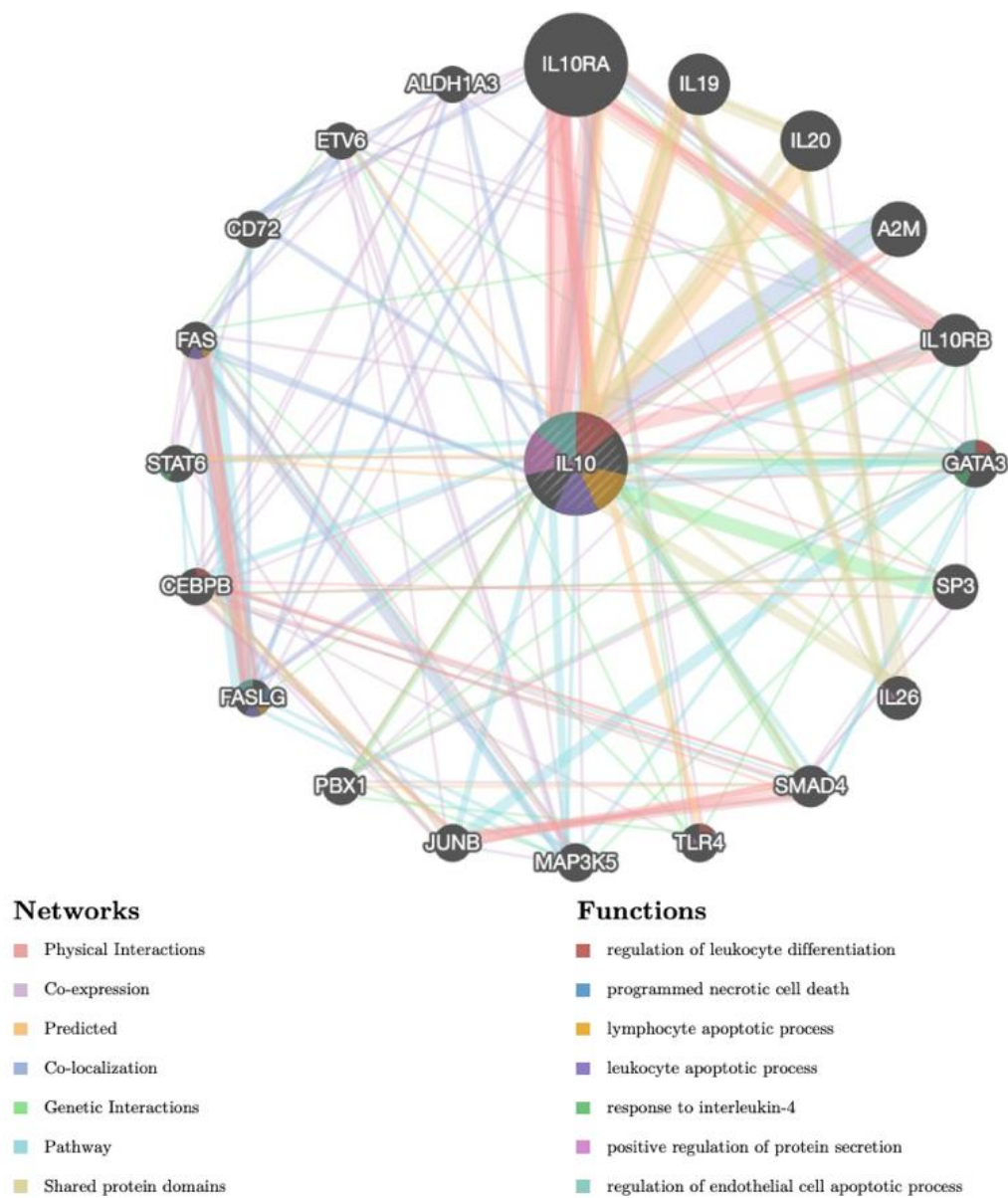


Figure 1. GenMANIA database showing network of IL10 gene interaction with other genes.



Figure 2 Study design. a) miRNet database showing targeted miRNAs for IL10 gene. b) Summary of the study design showing regulatory gene to the targeted cell.

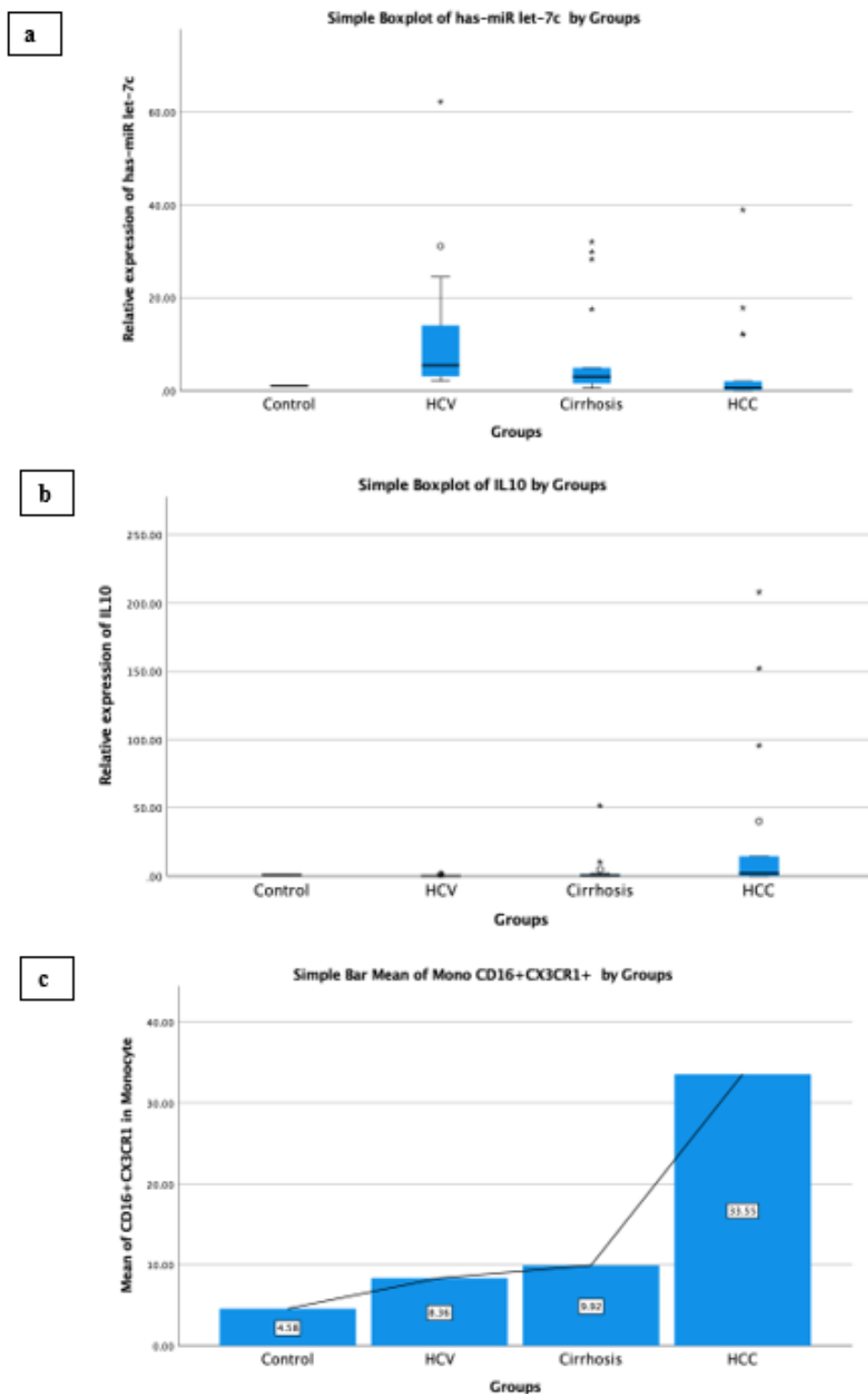


Figure 3. Expression pattern of the biomarkers in the different groups. a) let-7c-5p, b) IL10, c) CD16+CX3CR1+ Monocyte

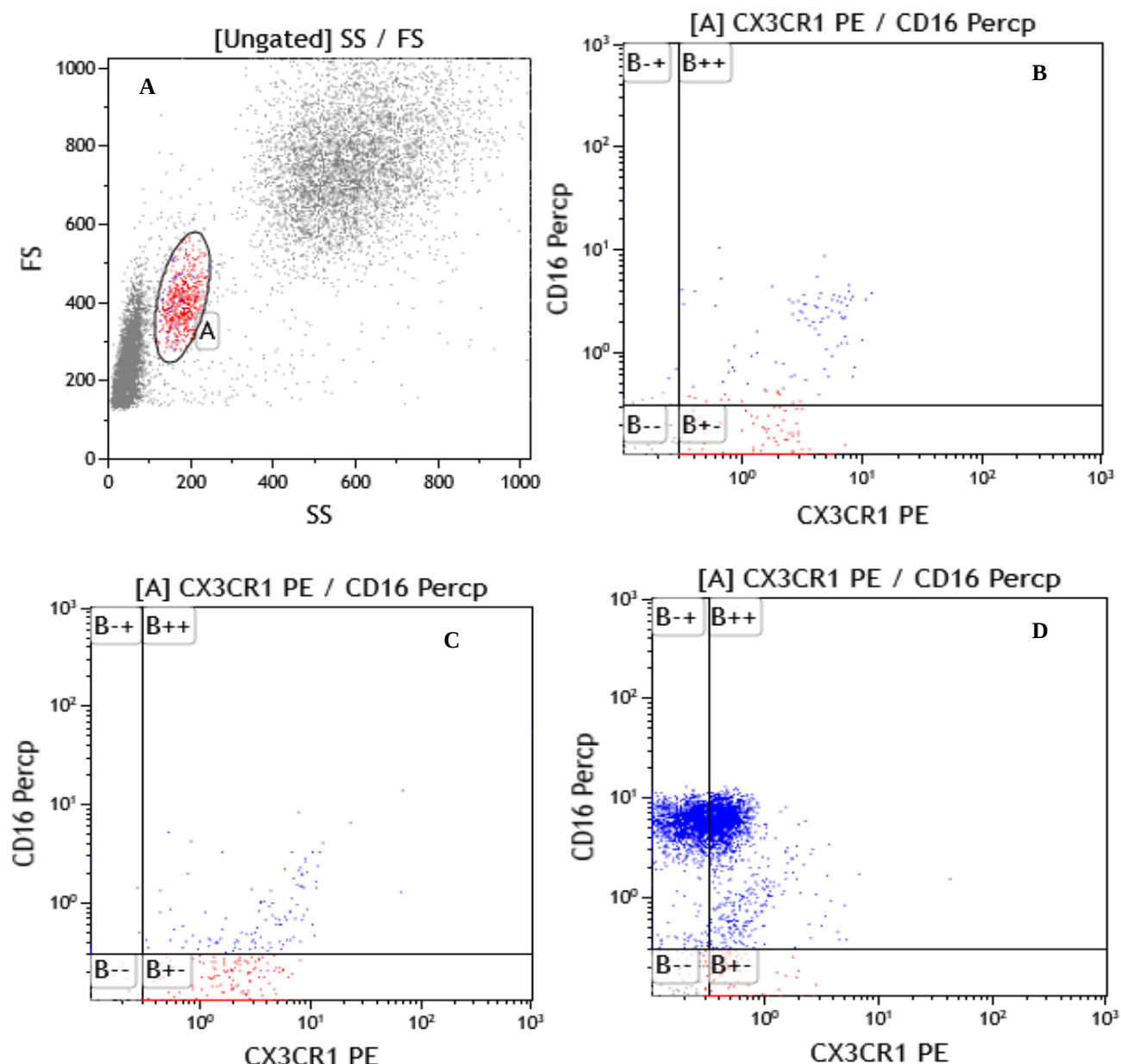


Figure4 Flow cytometry showed: **A.** scatter plot that display gating strategy depending on forward vs side scatter of peripheral blood Monocytes. **B.** scatter plot represents the dual expression of CD16⁺ CX3CR1⁺ monocytes in HCV patients. **C.** scatter plot showed the dual expression of CD16⁺ CX3CR1⁺ monocytes in patients with liver cirrhosis. **D.** scatter plot reveal the dual expression of CD16⁺ CX3CR1⁺ monocytes in HCC patients.

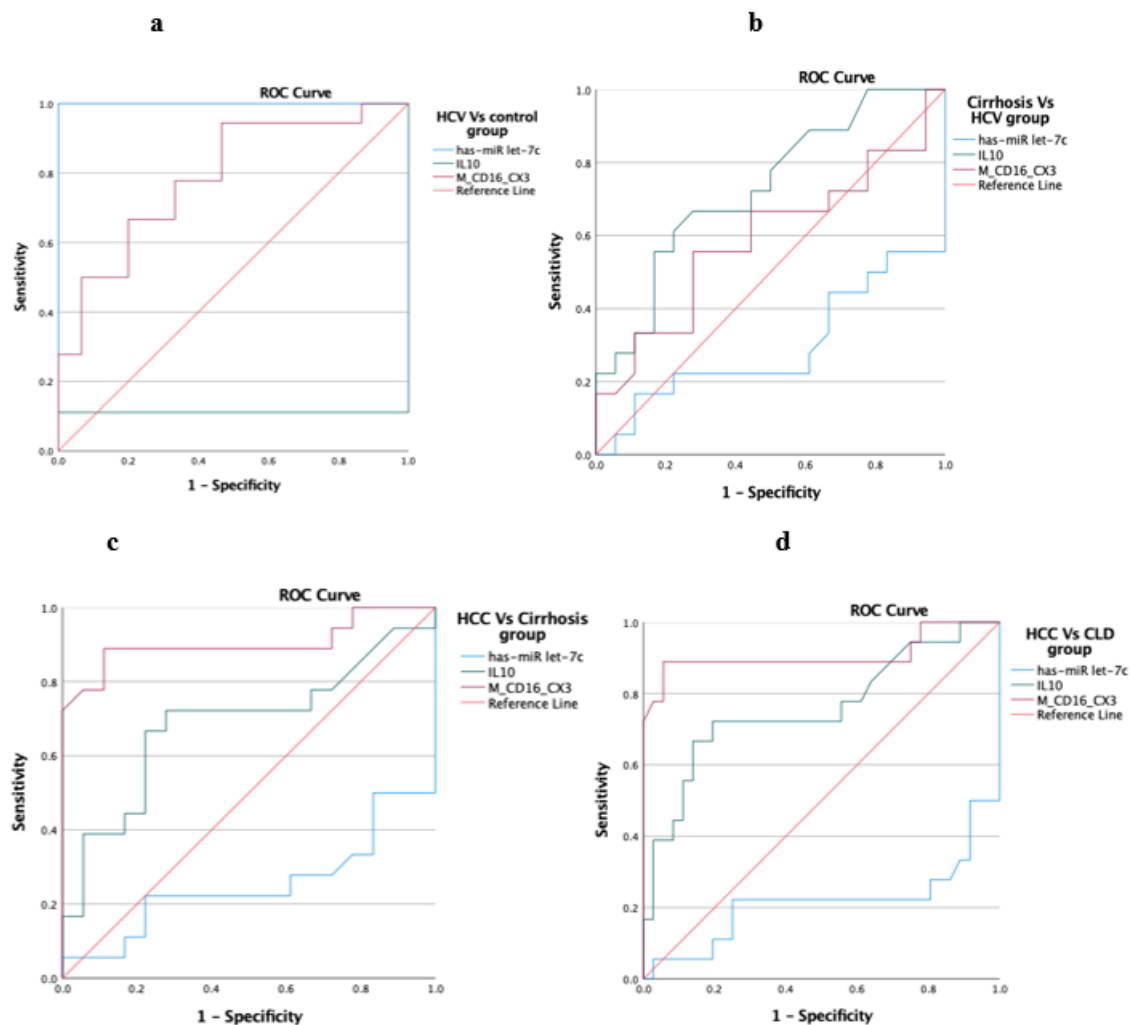


Figure 5. ROC Curve of the biomarkers in the research groups. a) The discrimination between HCV and control group, b) The discrimination between Cirrhosis and HCV group, c) The discrimination between HCC and Cirrhosis group, d) The discrimination between HCC and CLD group,

Discussion

Monocyte recruitment and activation had a significant role in both inflammatory and immunological responses. Inflammatory chemokines play an important role in this process [12]. Monocytes make up a significant portion of the infiltrating leucocyte population at the tumor site. Once they have homed to the malignant tissue, they undergo differentiation into TAMs, or tumor-associated macrophages, which vary in type based on the type of tumor. Monocytes and macrophages exhibit high plasticity, which is closely regulated at transcriptional and translational levels [4]. MicroRNAs, or miRNAs are essential for the post-transcriptional control of several cellular activities, including host defense mechanisms. Let-7 miRNA plays a crucial role in regulating immune cell activity. Let-7 is linked to various human disorders, including cancer and viral infections. Viruses can alter miRNA expression through many mechanisms [8].

The present study investigates the role of let-7c in activating peripheral blood monocytes in chronic HCV and hepatocellular carcinoma (HCC), via its regulation of IL10 expression. Our findings reveal significant insights into the molecular mechanisms involved in liver disease progression, highlighting let-7c, IL10, and CD16+ CX3CR1+ monocytes as possible treatment targets and biomarkers for HCV-induced HCC.

A key result from this study is the inverse correlation between miRNA Let-7c expression and disease progression. We observed that while let-7c expression was upregulated in chronic HCV patients, it was significantly downregulated in cirrhosis and further reduced in HCC patients. This downregulation of let-7c in advanced stages of HCV infection and liver cirrhosis is consistent with previous studies that reported the role of let-7c in suppressing HCV viral replication. These studies found that overexpressing miR-let-7c could markedly reduce HCV replication targeting the transcriptional repressor Bach1 to increase the expression of hemoxygenase 1. In contrast, MiR-let-7c inhibitor therapy reduced its antiviral efficacy by overexpressing Bach1 and decreasing HO-1 action [12]. In addition the downregulation of let-7c in HCC in our study is in agreement with other studies that demonstrated the tumor-suppressive role with the frequent downregulation of the Let-7 family across various cancers like lung cancer [13], breast cancer [14], acute lymphoblastic leukemia [15], prostate cancer [16] and HCC [17]. In addition, bad prognosis of cancer patients is related to the loss of the tumor-suppressor activity of let-7 [18, 19]. Moreover, it has been reported that in vitro, let-7c over expression lead to cell apoptosis and inhibition of HCC cell proliferation [20].

The role of Let-7c in regulating immune responses through its interaction with IL10 further underscores its importance in the tumor microenvironment, where immune evasion is a hallmark of the development of cancer. IL-10 plays a crucial function in immune regulation, therefore disorders like cancer or viral infections that are marked by a hyperinflammatory state may be affected by this cytokine [21].

In our study, in contrast to let-7c, IL10 expression followed an opposing trend, being significantly upregulated in HCC patients. This suggests that IL10 might contribute to the immune-modulatory environment in HCC, possibly aiding in the suppression of effective antitumor immunity. IL10 is well known for its anti-inflammatory properties and its ability to downregulate the activation of monocytes and macrophages [21].

In the context of HCC, increased IL10 expression could favor tumor growth by inhibiting the body's natural immune surveillance mechanisms. The observed increase in IL10 gene expression with disease progression aligns with reports that IL10 promotes tumor growth and immune tolerance in other cancers [22]. Moreover, a previous study found that malignant hepatocytes secreted more IL-10 than normal one [23].

IL-10 has potent anti-inflammatory effects via the Jak/STAT signaling pathway and is essential for regulation of immune responses. On the other hand, IL-10 has an immunosuppressive effect in response to pathogens, facilitating their own survival with persistence and progression of the disease [24]. This aligns with our results that showed a significant IL10 upregulation in advancement of the disease from chronic HCV infection to cirrhosis. In addition, previous studies stated that IL-10 is very important in the advancement of liver diseases and a higher level of IL10 and its high gene expression could influences the susceptibility to HCV infection and liver fibrosis [25-27].

The chemokine C-X3-C Receptor 1 (CX3CR1) is a transmembrane receptor present on a subset of immune cells, it promotes the chemotaxis of immune cells, particularly monocytes and macrophages [28]. The upregulation of CD16+ CX3CR1+ monocytes with disease progression is another noteworthy finding. Monocytes and macrophages, particularly tumor-associated macrophages (TAMs), are pivotal in shaping the tumor microenvironment [29]. In our study, the increased presence of CD16+ CX3CR1+ monocytes in patients with cirrhosis and HCC suggests that these cells may play a role in the inflammatory processes that facilitate tumor growth. Their increased expression correlates with both the downregulation of Let-7c and the upregulation of IL10, suggesting that these immune cells could be influenced by miRNA-mediated regulation of immune responses.

In accordance with our findings, previous studies have demonstrated that tumor-associated macrophages had a significant effect in regulating tumor growth through controlling immune suppression and angiogenesis [30].

There is a controversy regarding the alteration of expression of CX3CR1 in tumour progression , while some studies reported that CX3CR1 signaling could enhance proliferation of tumour, progression of the cell cycle, and failure of apoptosis [31- 33], other researches revealed that CX3CL1 expression correlates with longer survival in patients with lung adenocarcinoma [34] and Yu et al found that in a melanoma mouse model, mice with deficient CX3CR1 had larger tumors than in normal counterparts [35].

In the current study, the bioinformatics analysis further supported the relationship between let-7c and IL10, with let-7c targeting IL10's 3'UTR region. This interaction highlights the regulatory role of miRNAs in the immune system and their potential use as biomarkers for disease progression. Our findings are in agreement with earlier studies that demonstrated let-7 miRNAs' involvement in modulating immune responses and their potential as diagnostic markers in cancer [6].

From a clinical perspective, our study identifies let-7c and IL10 as promising, noninvasive biomarkers for tracking the liver disease progression from chronic HCV to cirrhosis and HCC. The significant diagnostic performance of Let-7c in distinguishing between different stages of liver disease, as evidenced by the ROC analysis, underscores its potential utility in clinical settings. Similarly, the expression of CD16+ CX3CR1+ monocytes could serve as an additional biomarker for monitoring disease progression.

In conclusion, this study provides compelling evidence for the involvement of let-7c and IL10 in the progression of HCV-induced liver disease, with implications for their use as diagnostic and prognostic biomarkers. Moreover, targeting the let-7c/IL10 axis and the immune-modulatory pathways of monocytes could represent new therapeutic avenues for treating HCV-related HCC.

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