Article

Diagnostic Significance of hsa-miR-21-5p, hsa-miR-192-5p, hsa-miR-155-5p, hsa-miR-199a-5p Panel and Ratios in Hepatocellular Carcinoma on top of Liver Cirrhosis in HCV Infected Patients

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Abstract. Hepatocellular carcinoma (HCC) early diagnosis is challenging. Moreover, for patients with alpha-fetoprotein (AFP)-negative HCC, this challenge is augmented. MicroRNAs (miRs) profile may serve as potential HCC molecular markers. We aimed to assess plasma homo sabines (hsa)-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, hsa-miR-199a-5p expression levels, as panel of biomarkers for HCC in chronic hepatitis C virus (CHCV) patients with liver cirrhosis (LC), especially AFP-negative HCC cases, a step toward non-protein coding (nc) RNA precision medicine. Subjects and Methods: 79 patients enrolled with CHCV infection with LC, subclassified into LC group without HCC (n=40) and LC with HCC (n=39) in comparison with 15 apparently healthy control subjects. Real-time quantitative PCR was used to measure plasma hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p. Results: Plasma hsa-miR-21-5p and hsa-miR-155-5p demonstrated significant upregulation, while hsa-miR-199a-5p demonstrated significant downregulation in the HCC group (n=39) when compared to LC group (n=40). Hsa-miR-21-5p expression was positively

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correlated with serum AFP, insulin, and insulin resistance (r=0.5, p<0.001, r=0.334, p=0.01, and r=0.303, p=0.02, respectively). According to the ROC curves, for differentiating HCC from LC, combining AFP with each of hsa-miR-21-5p, hsa-miR-155-5p, and miR199a-5p improved the diagnostic sensitivity to 87%, 82%, and 84%, respectively, vs 69% for AFP alone, with an acceptable specificity of 77.5%, 77.5%, and 80%, respectively, and AUC= 0.89, 0.85, and 0.90, respectively vs. 0.85 for AFP alone. Hsa-miR-21-5p/hsa-miR-199a-5p and hsa-miR-155-5p/hsa-miR-199a-5p ratios discriminated HCC from LC at AUC=0.76 and 0.71, respectively, with sensitivities=94% and 92%, and specificities=48% and 53%, respectively. Upregulation of plasma hsa-miR-21-5p was considered as an independent risk factor for HCC development [OR=1.198(1.063–1.329), p=0.002]. Conclusion: Combining each of hsa-miR-21-5p, hsa-miR-155-5p, and hsa-miR-199a-5p with AFP made possible to identify HCC development in LC patients' cohort with higher sensitivity than using AFP alone. Hsa-miR-21-5p/hsa-miR-199a-5p and hsa-miR-155-5p/hsa-miR-199a-5p ratios are potential HCC molecular markers for AFP-negative HCC patients. Hsa-miR-21-5p was linked to insulin metabolism in HCC patients' group as well as being an upregulated independent risk factor for the emergence of HCC from LC in CHCV patients.

Keywords: HCC; hsa-miR-21-5p; hsa-miR-155-5p; hsa-miR-192-5p; hsa-miR-199a-5p; liver cirrhosis; HCV; AFP-negative HCC

1. Introduction

Background. Globally, hepatocellular carcinoma (HCC) has the third-highest fatality rate, from a highly spreading disease, and when symptomatically diagnosed, the tumor is being in an advanced stage [1]. Serum alpha foeto protein (AFP) measurement helps in detection of HCC, however, one-third of HCC patients are AFP-negative HCC, presenting a difficult obstacle to overcome in the clinical practice setting [2]. AFP-negative HCC cases are defined as those with AFP levels lower than 20 ng/mL despite the presence of pathology confirmed HCC [3]. Therefore, finding more sensitive and reliable biomarker to forecast HCC development is mandatory. Accordingly, extensive ongoing research is focusing on identifying potential molecular markers for early HCC detection, disease prediction and/or prognosis.

Tumor epigenetics is one of the potential molecular markers that might characterize HCC [4]. Therefore, elaborating on the HCC tumor biology is of great value for HCC patients [5], namely for diagnosis or identifying possible treatment targets. Moreover, epigenetic molecular markers can be easily detected and quantified in peripheral blood liquid biopsy, being a minimally invasive tool that can identify cancer at an early stage as well as monitor disease progression [6].

Micro-RNAs (miRs) are group of the epigenetic small non-protein coding RNA (ncRNA) made of 18–22 nucleotides length [7].

According to miRBase https://www.mirbase.org/ which is one of the microRNA database Release 22.1, the miR count is 38,589 entries (accessed on October 28th, 2022).

MiRs bind to messenger RNAs (mRNA) sponging them, to control their intended-proteins production [8], a mechanism linked to carcinogenesis, being one of the different cancer hallmarks [9]. Consequently, miRs profiles may serve as possible potential HCC molecular markers being non-invasive, hopefully, sensitive, for both/either diagnosis and/or prognosis [10]. However, if miRs may, interestingly, diagnose AFP-negative HCC cases, would be a potential step toward the utility of ncRNA for precision health.

One of the most frequently dysregulated microRNAs in cancer is hsa-miR-21-5p. Tissue hsa-miR-21-5p's diagnostic and prognostic utility has been proven [5]. Altered liver tissue hsa-miR-21-5p can cause altered lipid metabolism, inflammation, and fibrosis with activation of the intracellular oncogenic signaling pathways phosphatidyl inositol 3 kinases (PI3K) to Protein kinase B (PKB:Akt), transforming growth factor-beta (TGF-ß) to the signal transducers Suppressor of Mothers Against Decapentaplegic 2 (SMAD2), and

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the transcription factor Signal transducer and activator of transcription 3 (STAT3) leading to HCC initiation [11]. However, the diagnostic significance of circulating hsa-miR-21-5p in liver cirrhosis or AFP-negative HCC cases is not studied yet.

Monitoring microRNA-155-5p expression level could be helpful in HCV cases, per CHCV develops into cirrhosis and HCC as stated earlier by Mohamed et al. [12]. Per hsa-miR-155-5p is a regulator of the pro-inflammatory precursor mediators nuclear factor kappa-B cell (NF-kB), epidermal growth factor (EGF), and others, so, hsa-miR-155-5p would be connected to both HCC and CHCV infection [13].

On the other hand, hsa-miR-192-5p was found to be downregulated in some tumor tissues [14]. However, hsa-miR-192-5p expression level in HCC tissue related to HBV infection were associated with a faster progression of HCC [15]. Therefore, exploring plasma hsa-miR-192-5p expression level in HCC related to CHCV is yet to be done.

Huang et al. [16] discovered lower hsa-miR-199a-5p expression in HCC tissues than the nearby non tumor tissues. Recently, hsa-miR-199a-5p mimics, achieved less HCC cell lines survival or colony formation via decreasing the expression of hypoxia-induced factor-1 (HIF-1) [17]. Still the role of plasma hsa-miR-199a-5p in relation to the currently mentioned miRs (hsa-miR-21-5p, hsa-miR-155-5p, and hsa-miR-192-5p) together as a panel, needs to be explored in HCC among Egyptian patients with liver cirrhosis linked to CHCV infection.

In order to improve sensitivity of HCC diagnosis, we thought to elucidate the diagnostic utility of circulating plasma hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p expression level, as non-invasive molecular markers, as panel or as ratios, for HCC in CHCV-infected Egyptian patients with liver cirrhosis. Putting in our consideration their diagnostic utility for AFP-negative HCC cases.

2. Results

2.1. Bioinformatics identification of the investigated miRs panel (Table 1) (accessed on April 28th, 2022) retrieved from miRDB https://mirdb.org/mirdb/index.html *and human ncRNA gene database GeneCaRNA* https://www.genecards.org/genecarna *as well as the miRPathDB v2.0* https://mpd.bioinf.uni-sb.de/overview.html *miRBD* *https://mirdb.org/mirdb/index.html* *and human ncRNA gene database GeneCaRNA* *https://www.genecards.org/genecarna*

Table 1: Investigated miRs information retrieved from different micro-RNA databases

Mature miR	hsa-miR-21-5p	hsa-miR-155-5p	hsa-miR-192-5p	hsa-miR-199a-5p
Sequence (5' - 3')	uagcuuaucagacugauguuga	uuaaugcuaaucgugauagggguu	cugaccuaugaauugacagcc	cccaguguucagacuaccuguuc
Length	22	24	21	23
miRBase ID	MIMAT0000076	MIMAT0000646	MIMAT0000222	MIMAT0000231
Similar miRNAs	hsa-miR-590-5p	-	hsa-miR-215-5p	hsa-miR-199b-5p
Clustered miRNAs#	-	-	hsa-miR-194-2 hsa-miR-6750	hsa-miR-3120 has-miR-214
Genomic location	chr17:59841266-59841337 (+)	chr21:25573980-25574044 (+)	chr11:64891137-64891246 (-)	chr1:172144535-172144644 chr19:10817426-10817496 (-)

clustered miRNAs are within 10kb in genome.

2.2. Demographic and Biochemical Data Analysis of the study participants are shown in Table 2.

Table 2: Demographic and clinical data (unit) of the HCC group (n= 39), liver cirrhosis group (n= 40) participants compared to each other and to the apparently healthy group (n= 15).

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	Groups (n)			*Significance*		
Characteristics (unit)	**HCC (39)**	**LC (40)**	**Control (15)**	***P*1 value**	***P*2 value**	***P*3 value**
Gender (M/F)	27/12	28/12	11/4	NS	NS	NS
Age (years)	61.0(56.0 -67.0)	58.5(54.25 -65.0)	58.0(55.0 -60.0)	NS	NS	NS
BMI (Kg/m²)	29.0(27.0 -31.0)	29.9(27.55 -33.2)	27.1(26.7 -27.8)	NS	0.008*	0.008*
D.M (Yes/No)	15/24	24/16	0/15	NS	0.005*	<0.001*
s. Insulin (mIU/L)	25.0(15.7 - 42.5)	13.5(5.98 - 20.37)	8.5(5.9 - 10.7)	0.001*	<0.001*	NS
Insulin resistance (Yes/No)	28/11	5/15	0/15	0.001*	<0.001*	0.036*
s. Albumin (mg/dl)	3.4(2.9 - 4.1)	2.7(2.12 - 3.65)	3.3(3.2 - 3.8)	0.009*	NS	0.035*
AST (U/L)	77.0(62 -105.0)	72.0(62.0 - 78.0)	33.0(30.0 - 43.0)	NS	<0.001*	<0.001*
ALT (U/L)	51.0(42.0- 65.0)	57.0(50.0 - 63.7)	28.0(24.0 - 38.0)	NS	<0.001*	<0.001*
Total Bilirubin (mg/dl)	1.2(0.9- 2.0)	1.5(1.0 - 3.07)	0.80(0.6 - 1.0)	NS	<0.001*	<0.001*
Direct Bilirubin (mg/dl)	0.70(0.40 - 1.2)	0.8(0.4 - 1.95)	0.30(0.18 -0.42)	NS	<0.001*	<0.001*
ALP (U/L)	110(82.0 -155.0)	120(99.8 - 132.8)	46.0(39.0 -58.0)	NS	<0.001*	<0.001*
GGT (U/L)	60(53.0 - 77.0)	67(55.3 - 83.5)	19.0(17.0 - 23.0)	NS	<0.001*	<0.001*
TC (mg/dl)	162(122.0 -220)	147(112-181)	155(152.0 - 162)	NS	NS	NS
TAG (mg/dl)	133(94.0 -193.0)	115(76.8 - 147)	115(99.0 - 123)	NS	NS	NS
HDL-C (mg/dl)	34(26.0 - 40.0)	36.5(30.5 - 41.75)	47(43.0 - 51.0)	NS	<0.001*	<0.001*
TAG/HDL-C ratio	4.1(2.6 - 6.7)	3.3(2.34 - 4.59)	2.35(2.2 - 2.7)	NS	<0.001*	0.01*
NLR	2.5(2.0 - 3.9)	2.1(1.38 - 3.49)	0.93(0. 33 - 1.42)	NS	<0.001*	0.001*
PLR	111.4(72.5 -240)	74.5(47.8-150.13)	128.4(82 -154.8)	NS	NS	NS
LMR	2.6(1.4 - 4.25)	2.8(1.60 - 3.92)	5.5(4.1 - 6.0)	NS	0.001*	<0.001*
INR	1.2(1.1 - 1.37)	1.5(1.30 - 2.06)	1.1(1.0 - 1.30)	<0.001*	NS	<0.001*

Data are median (inter quartile range (1st-3rd quartile)), statistics were computed using SPSS software, Mann–Whitney test was used (for non-parametric data), *p1* indicates comparison between HCC and liver cirrhosis groups, *p2* indicates comparison between HCC and the control group, *p3* denotes comparison between liver cirrhosis and the control group. * statistical significance *p*-value <0.05, NS, non-significant. [ALT, alanine aminotransferase; AST, aspartate aminotransferase, AFP, alpha fetoprotein, BMI, Body mass index; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; GGT, gamma glutamyl transferase; LC, liver cirrhosis; PLR, platelet lymphocyte ratio; NLR, neutrophil lymphocyte ratio; LMR, lymphocyte monocyte ratio; TC, total cholesterol; TAG, triacylglycerol.].

2.3. Pathological characteristics of the HCC cases are shown in Table 3.

As depicted in **Table 4**, the HCC group showed significant up-regulation of plasma hsa-miR-21-5p expression compared to LC group (median=27.66-fold change vs 8.61-fold change from average expression, $p<0.001$) and the control ($p<0.001$). In addition, hsa-miR-155-5p expression was significantly elevated in HCC patients in comparison to the cirrhotic patients (median=3.18-fold change vs 1.81-fold change, $p=0.001$) as well as the control subjects ($p=0.001$). On the other hand, no significant difference was detected between groups regarding hsa-miR-192-5p expression. However, the HCC group showed significant down-regulation of plasma hsa-miR-199a-5p in comparison to the LC group as well as the control group ($p<0.05$).

Table 3: Pathological characteristics of the studied HCC cases (n=39) and liver cirrhosis cases (n= 40)

Groups, n (%)	**HCC, 39 (100%)**	**liver cirrhosis, 40 (100%)**	**Statistics test, *P*-value**
Parameters	**Ascites**		X^2 = 7.63, **0.05***
No	24 (61.5%)	16 (40.0%)	
Minimal	8 (20.5%)	6 (15.0%)	
Moderate	5 (12.8%)	16 (40.0%)	
Marked or Massive	2 (5.1%)	2 (5.0%)	
	Lung radiological findings		N.A
Normal	38 (97.4%)	39 (97.5%)	
Abnormal findings #	1 (2.6%)	1 (2.5%)	
	LN involvement		X^2 =5.475, **0.019***
N0	34 (87.2%)	40 (100.0%)	
Yes	5 (12.8%)	0 (0.0%)	
Liver size$	16.2 (14.0 - 18.0)	12.6 (10.47 - 14.27)	U test= 223.0, **<0.001***
Splenomegaly$	16.8 (15.25 - 17.5)	15.5 (13.42 - 20.95)	U-test 719.0, NS
Portal vein dilatation	13.5 (12.4 - 15.0)	13.9 (12.5 - 15.77)	U-test 736.0, NS

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	Largest liver mass size		N.A
≤3.00 cm	8 (20.5%)	0 (0.0%)	
>3.00 cm	31 (79.5%)	0 (0.0%)	
	Portal vein patency		N. A
Patent	28 (71.8%)	40 (100.0%)	
Partially occluded	2 (5.1%)	0 (0.0%)	
Thrombosed	9 (23.1%)	0 (0.0%)	
	Subclassifications		
	Child score		X^2 = 8.9, **0.012***
A = Least severe liver disease	24 (61.5%)	12 (30.0%)	
B= Moderately severe liver disease	10 (25.6%)	14 (35.0%)	
C= Most severe liver disease	5 (12.8%)	14 (35.0%)	
	BCLC classification		N.A
A= Early stage	12 (30.8%)	-	
B= Intermediate stage	10 (25.6%)	-	
C= Advanced stage	12 (30.8%)	-	
D=Terminal stage	5 (12.8%)	-	
Total	39 (100%)	-	

Data are median (inter quartile range (1st-3rd quartile)), or as number(%), statistics were computed using SPSS software, * statistical significance *p*-value <0.05, NS, non-significant, N.A, not applicable.

Table 4: Study participants' miRs in HCC group (n= 39), liver cirrhosis group (n= 40) compared to each other and to the apparently healthy group (n= 15)

	Groups, n			***Significance***		
Parameter (unit)	**HCC, 39**	**LC, 40**	**Control, 15**	***P*1 value**	***P*2 value**	***P*3 value**
AFP (ng/mL)	80 (13 - 305)	7.4 (4.5 - 10.37)	3.2 (2.7 - 6.8)	<0.001*	<0.001*	0.002*
hsa-miR-21-5p-fold changes	27.6 (6.9 - 69.5)	8.6 (3.9 - 11.3)	0.96 (0.94 - 1.0)	<0.001*	<0.001*	<0.001*
hsa-miR-155-5p-fold changes	3.1 (1.7 - 8.12)	1.8 (0.76 - 2.2)	1.07 (0.9 -1.69)	0.001*	0.001*	NS
hsa-miR-192-5p-fold changes	0.90 (0.4 - 1.52)	1.5 (0.55 - 5.04)	1.01 (0.8 - 1.18)	NS	NS	NS
hsa-miR-199a-5p fold changes	0.16 (0.04 - 0.4)	0.37 (0.08 - 5.64)	1.03 (0.2 - 1.05)	0.046*	0.023*	NS
hsa-miR-21-5p/hsa-miR-199a-5p	85.6 (27-2759.1)	20.3 (1.1 - 66.7)	0.92 (0.89- 4.2)	<0.001*	<0.001*	0.039*
hsa-miR-155-5p/hsa-miR-199a-5p	16.5 (4.4-119.4)	2.7 (0.48 - 19.1)	1.52 (0.97 - 6.6)	0.002*	<0.001*	NS

Data are median (inter quartile range (1st-3rd quartile)), statistics were computed using SPSS software, Mann–Whitney test was used (non-parametric data), *p1* indicates comparison between HCC and liver cirrhosis groups, *p2* indicates comparison between HCC and the control group, *p3* denotes comparison between liver cirrhosis and the control group. * statistical significance *p*-value <0.05, NS, non-significant. [AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; LC, liver cirrhosis.].

According to the data presented in **Table 5**, no significant difference was observed between the HCC group with BCLC stage A and the more advanced BCLC stages concerning the expression level of hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p.

When, HCC patients were further sub-categorized according to liver mass size measured in cm. detected by CTS or sub-classification based on AFP test detection (less than 20 ng/mL) into AFP-negative HCC patients (n=12/39) and AFP-positive HCC patients (n=27/39), significant downregulation of plasma hsa-miR-21-5p levels in AFP-negative HCC patients (*p*=0.039) was evident as presented in **Table 6**.

Table 5: Blood AFP level, investigated miRs expression level and monocytes subsets frequencies in HCC participants' (n= 39) sub-classification according to BCLC; A= early stage, B= intermediate stage, C= advanced stage.

	HCC group BCLC staging		
Characteristics (unit)	**Stage A**	**Stages B, C and D**	***P* value**
AFP (ng/mL)	42.5(4.18-148.25)	106(17.6-400)	NS
hsa-miR-21-5p-fold changes	24.42(15.69-47.6	29.04(6.54-108.38)	NS
hsa-miR-155-5p-fold changes	4.46(1.81-8)	2.79(1.54-8.12)	NS
hsa-miR-192-5p-fold changes	0.91(0.15-1.88)	0.91(0.48-1.48)	NS
hsa-miR-199a-5p fold changes	0.25(0.1-0.92)	0.12(0.03-0.42)	NS
hsa-miR-21-5p/hsa-miR-199a-5p	84.5(20.9-161.8)	113.7(27.2-3125.7)	NS
hsa-miR-155-5p/hsa-miR-199a-5p	7.4(3.2-30.6)	17.3(5.6-179.7)	NS

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Data are median (inter quartile range (1st-3rd quartile)), statistics were computed using SPSS software, NS, non-significant. [HCC, hepatocellular carcinoma; LC, liver cirrhosis; AFP, alpha fetoprotein.].

Table 6: AFP level, investigated miRs expression level and monocytes subsets frequencies% in HCC study participants' (n= 39) sub-classification according to AFP-positivity (ng/mL)

	AFP -/+ HCC, n		
Characteristics (unit)	**AFP-ve HCC, 12**	**AFP+ve HCC, 27**	***P* value**
hsa-miR-21-5p-fold changes			
Min -Max	1.92-69.5	3.29 - 407.31	**0.015***
median (inter-quartiles)	14.8(3.0 - 34.8)	32.6(21.3-116.9)	
hsa-miR-155-5p-fold changes			
Min -Max	0.17 -247.3	0.80 - 324.03	NS
median (inter-quartiles)	1.7(0.46 - 6.6)	3.8(1.9 -11.2)	
hsa-miR-192-5p-fold changes			
Min -Max	0.14 -23.2	0.09 - 3.86	NS
median (inter-quartiles)	0.97(0.45 - 3.5)	0.90(0.36 -1.4)	
hsa-miR-199a-5p fold changes			
Min -Max	0.01 - 2.38	0.00 - 5.39	NS
median (inter-quartiles)	0.16(0.035 - 0.38)	0.23(0.04 -0.63)	
hsa-miR-21-5p/hsa-miR-199a-5p			
Min -Max	12.13 – 8364.1	1.06 - 121449.75	NS
median (inter-quartiles)	52.7(16.8- 311.3)	87.4(32.2 - 3468.3)	
hsa-miR-155-5p/hsa-miR-199a-5p			
Min -Max	2.7 - 29737.5	0.15 - 41764.80	NS
median (inter-quartiles)	12.3(3.8- 29.9)	17.4(6.9 - 207.9)	

Data are median (inter quartile range (1st-3rd quartile)), statistics were computed using SPSS software. *statistical significance *p*-value<0.05, NS, non-significant for comparison of AFP- HCC and AFP+ HCC sub-classification. [HCC, hepatocellular carcinoma; LC, liver cirrhosis; AFP, alpha fetoprotein.].

2.4. ***Correlation Coefficient between the Investigated miRs and various biomarkers in all cases (n=79):*** *presented in Table 7, highlights hsa-miR-21-5p expression being positively correlated with AFP, serum insulin, and insulin resistance condition (r=0.5, p<0.001, r=0.334, p=0.01 and r=0.303, p=0.02, respectively). Also, positive correlation was observed between AFP and hsa-miR-155-5p levels (r=0.371, p=0.001). In addition, hsa-miR-21-5p was positively correlated with blood dyslipidemia, presented as high TC and TAG (r=0.241, p=0.033 and r=0.235, p=0.037, respectively).*

Table 7: Spearman's correlation coefficient (*r*) among the investigated miRs expression level-fold change in all liver cirrhosis patients post-HCV (n= 79)

	CHCV-G4 patients (n= 79) miRs-fold change							
	hsa-miR-21-5p		**hsa-miR-155-5p**		**hsa-miR-192-5p**		**hsa-miR-199a-5p**	
Characteristics (unit)	*r*	***p*-value**	*r*	***p*-value**	*r*	***p*-value**	*r*	***p*-value**
Age (years)	0.004	NS	-0.004	NS	-0.060	NS	-0.054	NS
BMI (kg/m²)	0.103	NS	0.059	NS	-0.124	NS	**-0.222**	**0.050***
s. Insulin (mIU/L)	**0.334**	**0.01***	0.203	NS	-0.001	NS	-0.125	NS
AFP (ng/mL)	**0.534**	**<0.001***	**0.371**	**0.001***	-0.092	NS	-0.023	NS
AST (U/L)	0.104	NS	-0.001	NS	0.070	NS	0.03	NS
ALT (U/L)	-0.172	NS	-0.138	NS	0.114	NS	-0.068	NS
ALP (U/L)	**-0.263**	**0.019***	-0.163	NS	-0.043	NS	0.069	NS
GGT (U/L)	-0.144	NS	-0.120	NS	0.106	NS	0.145	NS
TAG (mg/dL)	**0.241**	**0.033***	0.180	NS	-0.144	NS	0.133	NS
TC (mg/dL)	**0.235**	**0.037***	0.120	NS	**-0.230**	**0.042***	0.075	NS
HDL-C (mg/dL)	-0.110	NS	-0.064	NS	-0.062	NS	-0.064	NS
TAG/HDL-C	0.195	NS	0.115	NS	-0.085	NS	0.111	NS
NLR	0.139	NS	0.079	NS	-0.185	NS	0.218	NS
PLR	-0.045	NS	0.137	NS	-0.119	NS	**0.298**	**0.008***
LMR	0.047	NS	0.024	NS	0.073	NS	-0.029	NS
Number of liver masses	-0.027	NS	-0.038	NS	0.010	NS	-0.018	NS
Insulin resistance	**0.303**	**0.02***	0.103	NS	0.140	NS	-0.090	NS

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Spearman correlation coefficient (*r*) was calculated using SPSS software, * significant correlation at $p<0.05$ level (2-tailed), NS; non-significant. [LC, liver cirrhosis; ALT, alanine aminotransferase; AST, aspartate aminotransferase, AFP, alpha feto protein, BMI, Body mass index; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; GGT, gamma glutamyl transferase; LC, liver cirrhosis; PLR, platelet lymphocyte ratio; NLR, neutrophil lymphocyte ratio; LMR, lymphocyte monocyte ratio; TC, total cholesterol; TAG, triacyl glycerol.].

2.5. ***ROC Curve Analysis. As depicted in Table 8 and Figure 1,*** *showed that hsa-miR-21-5p upregulation distinguished HCC from LC groups at AUC=0.8, with 74% sensitivity and 45% specificity at cut-off >7.3-fold increase from average expression. Whereas, hsa-miR-155-5p distinguished the two groups at AUC=0.7, 72% sensitivity and 48% specificity at cut-off >1.8-fold change. Hsa-miR-199a-5p distinguished the HCC group from LC group at AUC=0.68, 87% sensitivity and 48% specificity at cut-off <0.45-fold change. Moreover, the hsa-miR-21-5p/hsa-miR-199a-5p ratio could discriminate the HCC from LC group at AUC=0.76, 94% sensitivity and 48% specificity at cut-off >11.45. While, hsa-miR-155-5p/hsa-miR-199a-5p ratio distinguished the two groups at AUC=0.71, 92% sensitivity and 53% specificity at cut-off >2.89.*

Table 8: ROC curve for the discriminative ability of the studied miRs to differentiate HCC from liver cirrhosis either individually or added to AFP or as ratios

			%		
Variables	**Cut-off point**	**AUC**	**Sensitivity**	**Specificity**	***P*-value***
AFP (ng/mL)	>23.3	0.85	69	100	<0.001
hsa-miR-21-5p-fold changes	>7.3	0.8	74	45	<0.001
hsa-miR-155-5p-fold changes	>1.8	0.7	72	48	<0.01
hsa-miR-199a-5p fold changes	<0.44	0.63	79	45	<0.05
hsa-miR-21-5p-fold changes &AFP	-	0.888	87	77.5	<0.001
hsa-miR-155-5p-fold changes & AFP	-	0.847	82	77.5	<0.001
hsa-miR-199a-5p fold changes &AFP	-	0.904	84	80	<0.001
hsa-miR-21-5p/hsa-miR-199a-5p	11.45	0.76	95	48	<0.001
hsa-miR-155-5p/hsa-miR-199a-5p	2.89	0.71	92	53	<0.01

* significance at $p<0.05$ level (2-tailed). [AFP, alpha feto protein; AUC, area under the curve.].

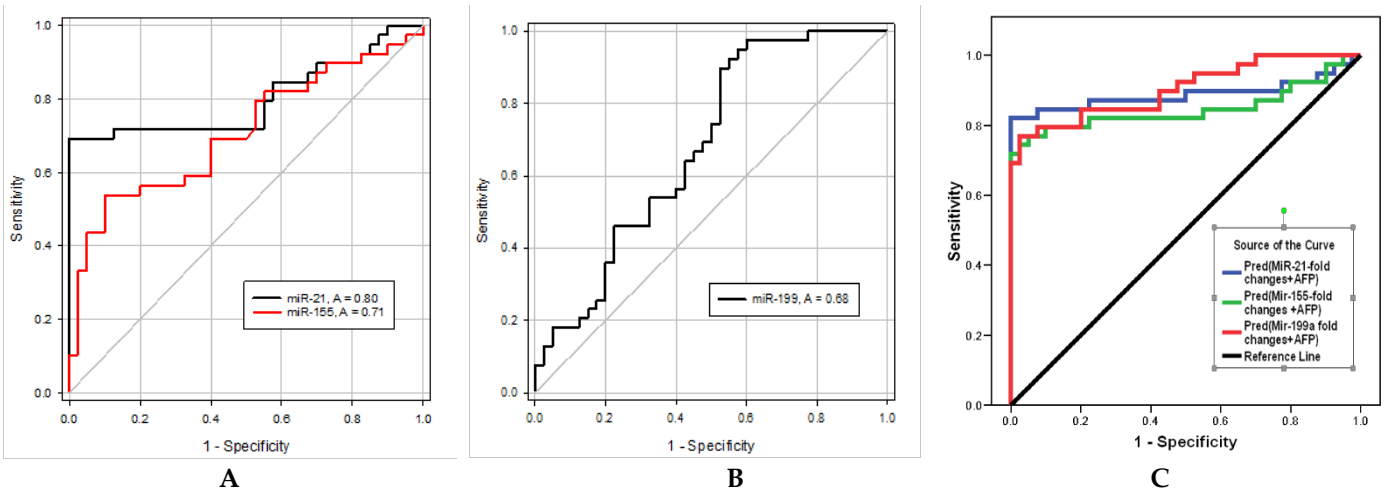

Figure 1: ROC curve for the discriminative ability of the investigated miRs expression level to detect CHCV-G4 mediated HCC development from liver cirrhosis; A) hsa-miR-21-5p & hsa-miR-155-5p, B) hsa-miR-199a-5p, C) hsa-miR-21-5p/hsa-miR-199a-5p & hsa-miR-155-5p/hsa-miR-199a-5p

2.6. Logistic Regression Analysis.

In order to evaluate if the altered expression panel of hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p can act as an independent risk factor for HCC progression from liver cirrhosis, a logistic regression analysis was conducted with adjustment of co-founders (age, BMI, RBS, TAG/HDL), as shown in **Table 9**. Now, we can say

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that hsa-miR-21-5p can be considered as an independent risk factor for HCC development [OR=1.198 (1.063–1.329, *P*=0.002].

Table 9: Logistic regression analysis using hsa-miR-21-5p, has-miR-155-5p, hsa-miR-192-5p, and hsa-miR199a-5p expression level-fold change and their ratios, as predictors of HCV-mediated HCC development from liver cirrhosis (n=79) after adjustment for confounders (age, BMI, RBS, lipids)

			95% C.I	
	***P* value**	**OR**	**Lower**	**Upper**
hsa-miR-21-5p	**0.002***	1.189	1.063	1.329
hsa-miR-155-5p	NS	0.984	0.881	1.100
hsa-miR-192-5p	NS	1.009	0.851	1.196
hsa-miR-199a-5p	NS	0.836	0.594	1.177
hsa-miR-21-5p/hsa-miR-199a-5p	NS	1.0	1.0	1.0
hsa-miR-155-5p/hsa-miR-199a-5p	NS	1.0	0.99	1.0
Age (years)	**0.027***	1.132	1.014	1.263
BMI (Kg/m²)	**0.02***	0.746	0.583	0.955
TAG/HDL-C	NS	1.180	0.927	1.501
TAG	NS	1.002	0.988	1.016
TC	NS	1.004	0.992	1.015
RBS	NS	0.993	0.981	1.005

*significant *p*-value <0.05. [BMI, Body mass index; C.I., confidence interval; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; OR, odds ratio; RBS, random blood sugar; TAG, triacyl glycerol; TC, total cholesterol.]

2.7. Potential Target Genes of Individual miRs and hsa-miRs Panel Predicted in silico using Online Algorithms

2.7.1. Gene-Gene Interactions and Pathways from Curated Databases and Text-mining (Figure 2)

Via gene-interaction on http://genome.ucsc.edu/index.html University of California Santa Cruiz (UCSC) Genomics institute (accessed on 28th of October, 2022). Pathways manually collected, often from reviews: OpenBEL

http://genome.ucsc.edu/cgi-bin/hgGeneGraph?gene=MIR21&supportLevel=ppi&geneCount=25&geneCount=25&geneAnnot=gnf2&1=OK&lastGene=MIR21 Where, MIR21 —phosphatase and tensin homolog tumor suppressor (PTEN) in figure 3, PTEN → MIR21 (directly decreases miR-21) and ribosomal protein S7 (RPS7)

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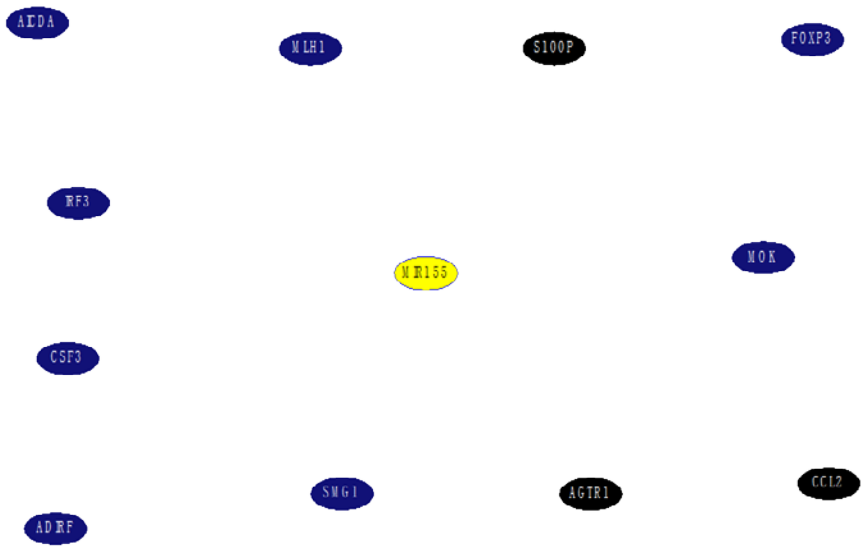

Figure 2: Gene interactions and pathways from curated databases and text-mining for miR-21 and miR-155 [black colored genes are being treatment hits by DrugBank.]

miR-155 top interacting genes are S100P; Calcium binding protein P calmodulin, targeted by (DrugBank) cromoglicic acid, AGTR1; angiotensin II receptor type 1 G-protein coupled receptor, targeted by Sartan drug group, CCL2; chemokine (C-C motif) ligand 2, targeted by Danazol, Mimosine.

http://genome.ucsc.edu/cgi-bin/hgGeneGraph?gene=MIR155&supportLevel=text&hideIndirect=on&geneCount=25&geneAnnot=none&1=OK&geneCount=25

However, hsa-miR-192-5p and hsa-miR-199a-5p are not present in this current gene interaction database.

2.7.2. hsa-miRs Target Analysis

Through the analysis of hundreds of miRNA-target interactions from high-throughput sequencing experiments, the bioinformatics tool MirTarget was able to create miR database (miRDB), an online library for miR target prediction and functional annotations https://mirdb.org/custom.html For hsa-miR-199a-5p target expression analysis, the expression levels of predicted hsa-miRs targets retrieved in miRDB (accessed on 28th of October, 2022) are cadherin epidermal growth factor (EGF) LAG 7 pass G-type receptor 1, mitogen-activated protein 3 kinase 11 (MAP3K11), cell division cycle associated 7 like, and zinc finger protein.

2.7.3.. KEGG Targeted Pathways Clusters/Heatmap Using DIANA TOOLS (accessed on 18th of November, 2022) Mirpath using reverse search to search for miRs involved in KEGG pathways, using DIANA-TarBase v7.0 method https://dianalab.e-ce.uth.gr/html/universe/index.php?r=mirpath/reverse and through search for KEGG targeted pathways clusters/heatmap results of the investigated miRs in Figure 3.

https://dianalab.e-ce.uth.gr/html/universe/index.php?r=mirpath#mirnas=hsa-miR-21-5p;hsa-miR-155-5p;hsa-miR-192-5p;hsa-miR-199a-5p&methods=Tarbase;Tarbase;Tarbase;Tarbase&selection=2 p value threshold set at 0.05 and MicroT threshold set at 0.8.

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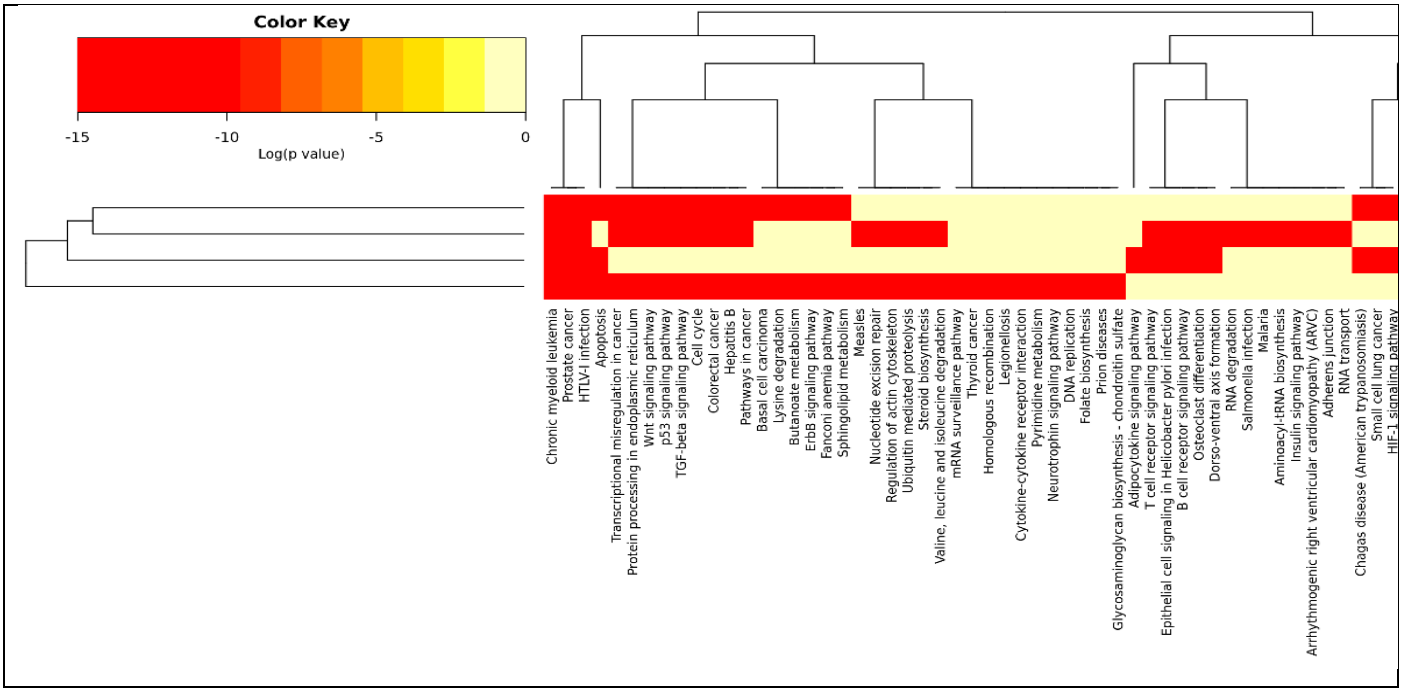

Figure 3: HeatMap showing KEGG pathways intersection/union with all the investigated miRs, miRs cluster dendrogram at the left side of the figure, and KEGG pathways cluster dendrogram at the figure upper part. [Targeted KEGG pathways with statistically significant results for all the investigated miRs in red by Tarbase (A posteriori analysis method after an enrichment analysis being done).]

Results are visualized as KEGG pathways union or intersected. KEGG pathways intersection of the 4 investigated miRs are those for chronic myeloid leukemia, several types of cancer. For HCV two miRs hsa-miR-21-5p and hsa-miR-155-5p are involved. However, KEGG pathways union involving the three miRs hsa-miR-21-5p, hsa-miR-155-5p, and hsa-miR-192-5p are those for cancer, cell cycle, HBV, TGF-B, Wnt signalling pathway, and p53 signaling. For mTOR signaling union pathways hsa-miR-21-5p, hsa-miR-155-5p, and hsa-miR-199a-5p are involved.

2.7.4. Genes that Share Domain with the Investigated MIRs

Determined via GenesLikeMe https://glm.genecards.org/#results (accessed on 15th of November, 2022). Best inferred functional partners found for MIR155 are NF-κB, TP53, STAT3, IL6, TNF, MIR199a, VEGFA, MIR21, MAPK8, TLR4. However, best inferred functional partners found for MIR192 are as follows, in a descending order, IL-6, TP53, INS, PPARG, STAT3, TLR4, VEGFA, BCL2, EGFR, ADIPQ, HIF1A, KRAS, MIR21, IGF1, PTEN. Interestingly, from the best inferred functional partners found for MIR199A1 are MIR21 and MIR155.

3. Discussion

In the current study, plasma hsa-miR-21-5p expression was significantly upregulated in HCC patients compared to cirrhotic patients, being an independent risk factor for HCC development tested by logistic regression analysis. These findings are in line with Tian et al. [18] findings who reported exosomal hsa-miR-21-5p being up-regulated in HCC due to the acidic microenvironment and that hsa-miR-21-5p activates HIF-1 and HIF-2, promoting growth and spread of HCC cells. Additionally, Cao et al. [19] found that hsa-miR-21-5p stimulates HCC development via controlling the tumor suppressor gene PTEN expression, which prevents tumor cells apoptosis [20]. This is witnessed via the bioinformatics analysis in figure 2 pointing to the straight relation of miR-21 to PTEN.

The HCC group (n=39) demonstrated significant up-regulation of plasma hsa-miR-155-5p expression level. This is consistent with the findings of Matsuura et al. [21] where

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hsa-miR-155-5p significantly increased angiogenesis in the hypoxic condition generated by HCC. Hsa-miR-155-5p was proved to facilitate HCC cells' invasion and migration, being a moderator for epithelial-mesenchymal transition (EMT) [22]. It is worth mentioning the RefSeq MIR155HG gene represents a miR host gene, where the long RNA transcribed from this gene is expressed at high levels in lymphoma and may function as an oncogene, as provided by RefSeq, Dec 2017 http://genome.ucsc.edu/cgi-bin/hgGene?db=hg19&hgg_gene=MIR155

AFP was found when detectable to be positively correlated with both hsa-miR-21-5p and hsa-miR-155-5p levels, pointing to the importance of both miRs as molecular markers in the context of HCC diagnosis [23], being related, as a panel, in-part to HCC development and progression.

No significant difference in hsa-miR-192-5p levels was detected between the HCC group and the other studied groups. However, Fründt et al. [14] claimed hsa-miR-192-5p might distinguish patients with HCC and those who have LC, and the healthy subjects, being downregulated in tumor tissues, thought to have an anti-cancer effect. Yin et al. [24] found hsa-miR-192-5p has an anti-HCC effect, with the ability to induce HCC cell apoptosis and autophagy via the axis hsa-miR-192-5p/CYR61/Akt signaling pathway.

On the other hand, our study showed significant downregulation in plasma hsa-miR-199a-5p expression level in the HCC group only. Lou et al. [25] studied the relationship between hsa-miR-199a-5p and the X-box binding protein 1 (XBP1) and cyclin D axes. They reported hsa-miR-199a-5p to be decreased in HCC tissue, resulting in an increased expression of XBP1 and cyclin D, impacting the cell cycle regulation, suggesting that hsa-miR-199a-5p has an antitumor effect. Moreover, the current bioinformatics analysis for "hsa-miR-199a-5p target expression analysis" retrieved in miRDB, are cadherin EGF G-receptor, MAP3K11, and zinc finger proteins, which are all related to tumorigenesis, therefore, hsa-miR-199a-5p would have an antitumor effect per downregulation in its plasma expression level, will affect its target protein expression (involved in tumor development). That is why its expression level decrease is considered as a potential good HCC diagnostic molecular marker.

Correlation studies in all patients (n=79) revealed that plasma hsa-miR-21-5p expression level was positively correlated with serum insulin and the presence of insulin resistance, dyslipidemia (increased TC and TAG). Lin et al. [26] suggested that altered plasma miRs might reflect liver lipid metabolism and stated that hepatic miRs expression contributes to the development of insulin resistance. This is per both hsa-miR-21-5p and hsa-miR-155-5p would control the expression of genes involved in hepatic TAG and cholesterol metabolism, evidenced by silencing hepatic MIR21 gene, where reduced hepatic inflammation and an enhanced fibrosis was achieved [27, 28]. Also, evidenced by our bioinformatics analysis in figure 3 showing the KEGG pathways heatmap in which the investigated miRs are involved in the inflammatory processes, diseases, adipogenesis, and fibrotic diseases.

At an AUC=0.85, AFP was able to distinguish between the HCC and LC groups with only 69% SN and 100% SP (cut-off >23.3). Despite of the high SP, but, the low SN threatens AFP utility as the sole HCC screening biomarker. Moreover, AFP would be occasionally high in liver cirrhosis. AFP measurements if combined with ultrasound for HCC screening, offers additional detection to 6%-8% of cases not previously identified by ultrasound alone [29].

Combining each of the investigated miRs individually to AFP ROC curve analysis, yielded an improved AUC in each time than either alone. Combination of hsa-miR-21-5p or hsa-miR-155-5p or hsa-miR-199a-5p to AFP, yielded an improved AUC than for AFP alone (0.89, 0.85, and 0.90, respectively vs. 0.85 for AFP alone). Also, an improved sensitivity (87%, 82%, and 84%, respectively vs 69% SN for AFP alone) with an accepted specificity (77.5%, 77.5%, and 80%, respectively) were obtained from such combinations. During the ROC curve analysis, we decided to increase AFP cut-off to 23.3 ng/mL to ensure the inclusion of AFP-negative HCC cases (12/39) [23].

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Moreover, we thought to investigate miRs fold-changes ratios. Where, hsa-miR-21-5p/hsa-miR-199a-5p ratio would detect early HCC development at cut-off >11.45, while hsa-miR-155-5p/hsa-miR-199a-5p ratio distinguished the two groups at cut-off >2.89. Both ratios provided a higher SN for early HCC detection reaching 95% and 92%, respectively, however, SPs did not get much better. Using these suggested ratios cut-offs, revealed a 100% detection rate of HCC in AFP-negative HCC patients (12/39) if using hsa-miR-21-5p/hsa-miR-199a-5p or 91.6% detection rate of HCC in AFP-negative HCC cases if using hsa-miR-155-5p/hsa-miR-199a-5p ratio as screening molecular markers, being confirmed with CT scan. This point addresses well one of our initial objectives in the current research to find sensitive detection biomarkers for HCC development, even AFP-negative HCC patients.

Logistic regression was performed to ensure the utility of using hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR199a-5p expression level-fold change, as panel or ratios, as predictors of CHCV-mediated HCC development from liver cirrhosis, after adjustment for confounders (age, BMI, blood sugar, lipids). Logistic regression revealed that hsa-miR-21-5p (p=0.002, OR=1.18, 95% CI 1.063-1.329) and age (years) (p=0.027, OR=1.132, 95% CI 1.014 - 1.263) are significant predictors of CHCV-mediated HCC development from liver cirrhosis.

Now, via bioinformatics analysis using DIANA TOOLS mirPath (Multiple microRNA Analysis), a web-based miR pathway analysis application compiling the pivotal role of miRs hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p panel from KEGG pathway intersection of the 4 investigated miRs regarding cancer (figure 3). Hence, during HCC development and progression from CHCV G4 infection, cell cycle and signaling pathways, apoptosis, TGF-B, Wnt signaling, and p53 signaling and mTOR signaling union pathways "all miRs" are involved as clusters.

Moreover, via GenesLikeMe inferred functional partners for MIR155 gene are those involved in tumorigenesis NF-κB, TP53, STAT3, IL-6, TNF, VEGFA, MIR21, MAPK8, and TLR4. Moreover, the following genes IL-6, TP53, INS, PPARG, STAT3, TLR4, VEGFA, BCL2, EGFR, ADIPQ, HIF1A, KRAS, MIR21, IGF1, and PTEN, share domain with MIR192.

Interestingly, MIR199A1 gene share domain with both MIR21 and MIR155 genes, supporting the miRs cluster dendrogram at the left side of figure 3, ***confirming the utility of ncRNA panel as a step toward precision health.***

Therefore, DrugBank targeting http://genome.ucsc.edu/cgi-bin/hgGeneGraph?gene=MIR155&supportLevel=text&hideIndirect=on&geneCount=25&geneAnnot=none&1=OK&geneCount=25 MIR155 top interacting genes are the anti-inflammatory drugs for the calcium binding protein P calmodulin S100P, or Sartans for angiotensin II receptor type 1 G-protein coupled receptor, or targeted by the synthetic steroid derivatives to inhibit chemokine (C-C motif) ligand 2, and drugs that will arrest the cell cycle in the G(1) phase before entry into S phase. All these drugs will affect MIR155 and MIR21, and therefore, the investigated panel step wise as clusters.

Limitations. The current study did not include the predictive survival role of the investigated miRs panel, hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p, in patients with CHCV G4-linked to HCC (a prospective study).

Strengths related to the current research. As far as we know, this study is the first to describe the diagnostic utility of the investigated miRs, hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p as panel, in combination with AFP, for an enhanced and, hopefully, early diagnosis of clinical CHCV G4-related HCC and liver cirrhosis. Ratios of hsa-miR-21-5p/hsa-miR-199a-5p and hsa-miR-155-5p/hsa-miR-199a-5p showed great diagnostic utility in AFP-negative HCC cases.

Recommendations. Considering hsa-miR-21-5p and/or hsa-miR-155-5p as potential precision therapeutic target(s) for CHCV-G4 related HCC and liver cirrhosis treatment, based on the examined sub-classification, via repurposed drugs (potentially to be obtained via miRDB), relying on targeting MIR21 and MIR155 genes, and after proofing the mechanism experimentally.

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Sustainability Plan. Blocking hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p target genes, based on the findings from gene-gene interaction network and KEGG pathways obtained from curated databases and text-mining, as potential treatment option, based on ncRNA, a step toward precision health.

4. Patients and Methods

4.1. miRs selection: either based on literature search as well as some miRs were examined by our research group, and bioinformatics identification of the investigated miRs panel (accessed on April 28th, 2022) retrieved from miRDB and human ncRNA gene database GeneCaRNA as well as the miRPathDB v2.0 and KEGG pathways, and more.

4.2. Study design: Cross-sectional case-controlled study.

4.3. Sample Size and The Study Power:

Based on the previous study by Hammad et al. [30], sample size estimation was performed using the G power* sample size online calculator https://riskcalc.org/samplesize/# depending on a two-sided significance level of 0.05 and power (1-beta) of 0.95. The current study group sample size is 40 patients' vs 12 controls to reject the null hypothesis that the population means of the studied groups are equal with a probability (power) of 0.9.

4.4. Study participants:

This study enrolled 79 Egyptian patients with chronic hepatitis C virus (CHCV) genotype 4 (G4) (serology confirmed) infection with liver cirrhosis (LC) divided into Group 1; LC patients with early HCC (n=39) and Group 2; LC without HCC (n=40). In addition to Group 3; apparently healthy control (n=15).

Patients were recruited from the National Liver Institute, Menoufia University and Al-Zahraa University Hospital. Patients Inclusion criteria: imaging criteria in accordance to the recent published recommendations guidelines [31] were used to confirm HCC diagnosis at the Pathology Unit. A blind abdominal computed tomography (CT) scan was performed using Siemens 128, Germany, using the following logged information: ascites severity, presence of lymph node (LN) enlargement, cirrhosis or growth pattern, and portal vein (PV) patency.

Child Pugh scores were used to categorize cirrhotic patients [32]. The Barcelona Clinic Liver Cancer (BCLC) classification system was used to staging HCC patients [33] into 0 =very early stage, A=early stage, B =intermediate stage, C =advanced stage, and D =terminal stage. Patients Exclusion criteria: patients with a history of alcoholism or autoimmune disease; acute or chronic HBV (as determined by serology); HCC not mediated by CHCV; and patients who were undergoing any type of radiation or chemotherapy for a malignancy other than HCC.

The control group included apparently healthy volunteers. Controls were asked to join the study during blood donation in Al-Zahraa University Hospital Blood Bank. The healthy volunteers' group were enrolled only after negative viral hepatitis G4 screening and normal laboratory results being reported.

4.5. Patient's Data:

Demographic data including age, gender, and patient's full history was retrieved from the hospital medical records. All patients were asked about family cancer history for recording as well as general clinical examination. For calculating body mass index (BMI) (in kg/m^2) calculation was done according to

https://www.nhlbi.nih.gov/health/educational/lose_wt/BMI/bmicalc.htm with normal weight = 18.5–24.9 kg/m^2, overweight = 25–29.9 kg/m^2, and obesity = BMI of 30 kg/m^2 or greater morbid obesity.

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4.6. Blood Sampling

Peripheral venous blood (3 mL) was withdrawn from each participant under strict sterile conditions following standard biosecurity and international safety procedures. Fresh EDTA blood sample 0.5 mL blood was placed onto EDTA tubes one for CBC. 2 mL blood onto EDTA tubes were centrifuged for 10 min at 1900g, after which the plasma was carefully withdrawn and centrifuged again for 10 min at 16,000xg at 4°C to remove additional cellular nucleic acids attached to cell debris. The supernatant was then transferred to microcentrifuge vials and stored at -80oC for RNA extraction and quantitative real-time PCR (qRT-PCR) for hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p. The rest of the blood (3 mL) was transferred into a polymer serum gel separator tube with a clot activator (Kremsmünster, Upper Austria, Greiner Bio-One GmbH, Australia) left for 15 min at room temperature (24°C) to clot, followed by 10-min centrifugation at 10,000× g at 4°C. Sera obtained were aliquoted into Eppendorf tubes and stored at −80°C until biochemical assessment.

4.6.1. RNA extraction and qRT-PCR

Plasma miRs were extracted from 200 µl plasma using miRNeasy commercial kit (Cat. NO. 217004, Qiagen, Germany) according to the manufacturer's protocol. Purity of the extracted RNA was tested spectrophotometrically at 260/280 nm NanoDrop 2000 (Thermo Fisher Scientific, UK).

Synthesis of complementary DNA (cDNA) was carried out using miRCURY LNA RT Kit (Cat. No. 339340, Qiagen, Germany) according to the manufacturer's instructions. Hsa-miR-21-5p, hsa-miR-155-5p, hsa-miR-192-5p, and hsa-miR-199a-5p expression was determined using miRCURY LNA SYBR® Green PCR Kit (Cat. No. 339345, Qiagen, Germany), following manufacturer's protocol, using a RT-PCR quaint studio 5 system (Applied Biosystem, USA). The levels of miRs were normalized using reference internal housekeeping endogenous control miR SNORD68. qRT-PCR analyses of the miRs were carried out in triplicate. miR-21-5p forward primer sequence (5′-3′) is 5′-ACGTGTTAGCTTATCAGACTG-3′, 5′-CCGTTAATGCTAATCGTG-3′ for miR-155-5p, 5′-CTGACCTATGAATTGACAGCCGT-3′ for miR-192-5p, and 5′-GGGCCCAGTGTTCAGACTAC-3′ for miR-199a-5p, however, SNORD68 forward primer sequence was 5′-ATCACTGTAAAACCGTTCCA-3′.

The qRT-PCR cycling conditions were as follows:

95°C for two min, then 40 cycles, each of 10 seconds at 95°C, 60 seconds at 56°C, and 30 seconds at 70°C.

Δ cycle threshold (Ct) was calculated by subtracting the Ct values of SNORD68 from the Ct values of the target miRs in all samples.

Fold change was calculated using 2-ΔΔCt for relative quantification.

4.6.2. Laboratory testing:

A full automated hematology analyzer (Sysmex, KX21N, Kobe, Japan) was used to perform a complete blood count (CBC) using fresh EDTA blood samples, in accordance with the manufacturer's recommendations.

INR coagulation assay using an automated coagulation analyzer (STA Compact Max, Asnières sur Seine Cedex, Stago, France).

Routine biochemical tests were conducted using a chemistry autoanalyzer device (Cobas Integra 400 Plus, Roche Diagnostics, Germany) following the manufacturer's instructions.

Biochemical analysis included blood albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, direct bilirubin, alkaline phosphatase (ALP), gamma GT (GGT), total cholesterol (TC), triacylglycerol (TAG), and high-density lipoprotein (HDL). The electro-chemiluminescence immunoassay (ECLIA) was used to measure the serum alpha feptoprotein (AFP) using a Cobas 6000, e601 module (Roche Diagnostics, Germany).

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Based on an enzyme-linked immunosorbent test (ELISA) in solid phase, using a HyPrep automated ELISA system (Hyperion Inc., Miami, FL, USA), insulin was detected in the test samples, where color intensity formed during ELISA is directly inversely related to its insulin concentration. The normal adult insulin range level is 0–25 mU/L.

4.6.3. Ratios:Indices

Neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), and platelets-to-lymphocytes ratio (PLR). PLR is a biomarker for systematic inflammation and is considered indicative to immune-related responses. For CHCV infections PLR is considered superior to NLR and is used in HCC cases for correlation with disease severity [34].

TAG/HDL-C ratio with a cut-off value of more than the apparently healthy control group is set diagnostic for insulin resistance (IR) [35]. Insulin Resistance to be considered positive in obese, diabetic, and dyslipidemic patients, and those with insulin levels of 18 mU/mL or more after glucose/meal, with disturbed PLR [36, 37].

4.7. Statistical Analysis

Data collected were coded and analysed using the Statistical Package for Social Science software (SPSS, Version 17, Chicago, IL, USA). Qualitative data are presented as frequencies (n) and percentages (%). Data was tested for normality using Shapiro-Wilk calculator https://www.statskingdom.com/shapiro-wilk-test-calculator.htmL Normally distributed variables are presented as mean ± S.D and analyzed using two samples independent Students' t-test for comparison of two, respectively. For non-normally distributed variables, data is presented as median (interquartile range as 1st-3rd quartiles or 25th-75th quartiles), then Mann-Whitney (U) was conducted to compare between any two independent groups, respectively.

Student's *t*-test and the Chi-square $\chi 2$ test were used to compare quantitative and qualitative normally distributed variables between the patients and control groups, respectively. Spearman's rho correlation test was used to assess the association between quantitative non-parametric variables. Receiver operating characteristic (ROC) curve was performed to detect the best cut-off, sensitivities (SNs), specificities (SPs), with an area under the curve (AUC) calculated range from 0 to 1. The higher the AUC, the better the parameter in classifying the outcomes correctly.

ROC curve analysis was used to determines the discriminative potential of the studied miRs to differentiate HCC cases from liver cirrhosis cases. A logistic regression analysis was performed to determine the independent factors association of the altered expression of the studied miRs with HCC progression. The level of significance was set at *p*-value <0.05, confidence level or interval (C.I) as 95% and 5%, respectively.

5. Conclusion.

In conclusion, plasma hsa-miR-21-5p and hsa-miR-155-5p demonstrated significant upregulation, while, hsa-miR-199a-5p was significantly downregulated in the HCC CHCV-G4 infection related group (n=39) when compared to LC group (n=40) with no HCC.

Combining each of hsa-miR-21-5p, hsa-miR-155-5p, or hsa-miR-199a-5p to AFP as HCC diagnostic markers, yielded an improved SNs than using AFP alone.

Regarding, AFP-negative HCC cases, hsa-miR-21-5p/hsa-miR-199a-5p and has-miR-155-5p/hsa-miR-199a-5p ratios can be used to better identify HCC development in LC patients with CHCV-G4 infection, with higher sensitivities.

Hsa-miR-21-5p play role in lipid and insulin metabolism in HCC-related to CHCV-G4 infected cases. Hsa-miR-21-5p upregulation is an independent risk factor for the emergence of HCC from liver cirrhosis in CHCV-G4 patients' cohort.

In the current study hsa-miR-192-5p was not shown to have any clinical significance per HCC development in LC patients.

These findings might encourage the use of the aforementioned epigenetic ncRNA markers in panel or ratios as prospective blood-based molecular markers of benefit, during liver cirrhosis early identification and/or CHCV-G4 infection follow-up, for possible HCC development as well as for ensuring all AFP-negative HCC cases identification.

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Institutional Review Board Statement: The study was conducted between November 2021 and July 2022. The study was performed following approval from the Research Ethics Committee of both Faculty of Medicine for Girls, Al-Azhar University, Cairo, Egypt (approval # 2022081485)**.** Participants' were informed about the aim of the study and provided their written informed consent (I.C) before enrolment in the study.

Informed Consent Statement: The study was carried out in adherence to the Declaration of Helsinki Guidelines (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, JAMA. 2013; 310: 2191-2194) stating the ethical principles for medical research involving human subjects.

Data Availability Statement: The original contributions presented in the study are included in the manuscript. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

List of Abbreviations. ADIPQ, Adiponectin gene; AFP, alpha-fetoprotein; AGTR1, AGTR1 angiotensin II receptor type 1; Akt, Protein kinase B; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BCL2, B-cell lymphoma 2; BCLC, Barcelona Clinic Liver Cancer; BMI, body mass index; C.I, confidence level or interval; CBC, Complete blood count; CCL2, chemokine (C-C motif) ligand 2; CD, cluster of differentiation; cDNA, complementary DNA; CHCV, chronic hepatitis C virus; CT, computed tomography; CYR61, Cysteine-rich angiogenic inducer 61; D.M, Diabetes Mellitus; ECLIA, electro-chemiluminescence immunoassay; EDTA, Ethylenediaminetetraacetic acid; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent test; FC, flow-cytometry; FS, forward scatter; GGT, gamma glutamyl transferase; HBV, Hepatitis B virus; HCC, Hepatocellular carcinoma; HCV, Hepatitis C virus; HDL, high-density lipoprotein; HIF-1, hypoxia-induced factor-1; hsa, homosapien; I.C, informed consent; IGF1, Insulin-like growth factor 1; IL-6, InterLeukin-6; INR, International normalized ratio; INS, Insulin; IR, insulin resistance; KEGG, Kyoto Encyclopedia of Genes and Genomes; KRAS, Kirsten rat sarcoma virus; LC, liver cirrhosis; LMR, lymphocyte-to-monocyte ratio; LN, lymph node; MAP3K4, mitogen-activated protein kinase kinase kinase 4; MAPK8, Mitogen-activated protein kinase 8; miRs, MicroRNAs; mRNA, messenger RNAs; mTOR, mammalian target of rapamycin; nc, non-protein coding; NF-κB, nuclear factor kappa B cell, NLR, Neutrophil-to-lymphocyte ratio; OR, odds ratio; PI3K, phosphatidyl inositol 3kinases; PKB:Akt, Protein kinase B;

PLR, platelets-to-lymphocytes ratio; PPARG, Peroxisome Proliferator Activated Receptor Gamma; PTEN, phosphatase and tensin homolog tumor suppressor; PV, portal vein; qRT-PCR, quantitative real-time PCR; RBS, Random blood sugar; ROC, Receiver operating characteristic; SMAD2, Suppressor of Mothers Against Decapentaplegic 2; SN, sensitivities; SNORD68, Small Nucleolar RNA, C/D Box 68; SP, specificities; SPSS, Statistical Package for Social Science software; STAT3, Signal transducer and activator of transcription 3; TAG, triacylglycerol; TC, total cholesterol; TGF-B, transforming growth factor-beta; TLR4, Toll like receptor 4; TP, Tumor protein; UCSC, University of California Santa Cruiz; VEGFA, Vascular endothelial growth factor A; XBP1, X-box binding protein 1.

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