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T-cytotoxic Expression of Leukocyte-Associated Immunoglobulin-like Receptor-1 (LAIR-1) in post-HCV Hepatocellular Carcinoma

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Abstract Background and Aim. Since virus-related hepatocellular carcinoma (HCC) pathogenesis involves liver inflammation, therefore, post-hepatitis C virus (HCV) infection would be a cause for liver cirrhosis that would progress to HCC. Cytotoxic T cells (Tc) are known to be involved in post-HCV complications and HCC pathogenesis. The inhibitory checkpoint Leukocyte-Associated Immunoglobulin-like Receptor-1 (LAIR-1) is expressed on Tc. Therefore, we aimed to determine whether the Tc expression level of LAIR-1 is associated with HCC progression post-HCV and moreover, to evaluate LAIR-1 expression as a non-invasive biomarker for HCC progression in the context of liver cirrhosis post-HCV genotype 4 (G4) in Egyptian patients' peripheral venous blood liquid biopsy. We studied LAIR-1 expression on Tc related to the progression of liver cirrhosis in a case-controlled study enrolled 64 patients with post-HCV G4-HCC and 37 patients with post-HCV G4-liver cirrhosis. Methods: LAIR-1 expression was analyzed by flow cytometry. Results: LAIR-1 expression on Tc and the percentage of Tc positive for LAIR-1 (LAIR-1+Tc %) were significantly higher in the post-HCV G4-HCC group compared to the post-HCV G4-liver cirrhosis

group. LAIR-1+Tc% was correlated with the HCC surrogate tumor marker AFP (r = 0.367, p= 0.001) as well as insulin resistance and inflammation prognostic ratios/indices. ROC curve revealed that adding LAIR-1+Tc % to AFP can distinguish HCC transformation in the Egyptian studied patients' cohort. Conclusion: Upregulated LAIR-1 expression on Tc could be a potential screening non-invasive molecular marker in the chronic inflammatory post-HCV G4 liver cirrhosis course. Moreover, LAIR-1 expression on Tc might be one of the players involved in the progression of liver cirrhosis to HCC post-HCV G4.

Key words: T cytotoxic cells; Leukocyte-associated Immunoglobulin-like Receptor-1; LAIR-1; Hepatitis C virus genotype 4; HCV G4; hepatocellular carcinoma; cirrhosis; immune inhibitory checkpoints; inflammation; prognosis; insulin resistance

1. Introduction

Background. Liver cancer ranks third in the world for lethality with HCC accounting for about or even more than 80% of primary liver cancers **[1]**. Despite significant advancements in HCC care, liver cancer diagnosis, surveillance, and the disease's death rate still unacceptably high **[2]**. Most of HCC cases happen in the milieu of chronic inflammatory disease(s) with cirrhosis being the strongest risk factor for HCC **[3].** HCV infection is among the main risk factors for HCC development **[4]**. Although, HCV effect risk has been considerably declined due to the recent invention of new directly acting antiviral (DAA) drugs [5]. However, patients with cirrhosis are still believed to have higher risk for HCC even after HCV clearance **[6]**. Since 90% of cases of HCC develop from persistent chronic HCV inflammation **[4]**.

Per HCC is thought to be an immunogenic tumor, therefore, the best therapy alternatives for these kinds of carcinomas would be immune therapeutics **[7]**. Cytotoxic T (Tc) cells have central role in the pathogenesis of HCC as well as control of HCV infection because these cells are able to recognize tumoral/infected cells to effect destroying them **[8]**.

For HCC, various studies have shown that shifting of the immune response toward an anti-tumor direction via cytotoxic T cells restoration together with modulation of the negative co-stimulatory signaling molecules expressed on these cells could have a substantial impact **[9, 10]**. A careful balance between the activating and inhibitory signals determines the immune system's proper response to damage. Maintaining this equilibrium is greatly aided by inhibitory receptors. One of the immune inhibitory receptors is Leukocyte-Associated Immunoglobulin-like Receptor-1 (LAIR-1), an immunological checkpoint widely expressed on immune cells, to deliver inhibitory signals **[11]**.

We have chosen LAIR-1/CD305 as the target of our research after filtration and discussion done previously in published literatures. LAIR-1 is a transmembrane type I glycoprotein of two tyrosine-based inhibitory motifs immunoreceptor in the cytoplasm and an external Ig-like domain C2-type **[12, 13]**. The expression of LAIR-1 has been described in cells of the immune system **[11, 14]**. It has been observed that LAIR-1 expression is raised, normally, on immune cells, where tumor cells hijack this immune regulatory system to avoid the "anti-cancer immune response" **[15]**. The decrease of the immune activity in the tumor microenvironment, along with a decline in T cell function is caused by LAIR-1 binding to its collagen or other ligands **[13]**. The attributed role of LAIR-1 in previous tested chronic inflammatory process as rheumatoid arthritis **[16]** and systemic lupus **[17]** suggests that this inhibitory receptor, might contribute to the pathophysiology of post-chronic inflammation seen post-HCV. Precisely, to anticipate the liver cirrhosis prognosis and/or progression from liver cirrhosis to HCC post-HCV G4, it is crucial to comprehend innate immune signaling and moreover, the immune regulators governing different signaling pathways.

We hypothesize that the level of the immunoinhibitory receptor LAIR-1 expression on circulating Tc is associated with post-HCV-liver cirrhosis progression to HCC.

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Unraveling the molecular events in HCC blood liquid biopsy underlying Tc cell function might provide guide for identifying potential drug targets.

Aim. To Figure out if the level of LAIR-1expression on Tc is associated with liver cirrhosis progression to HCC in the context of HCV infection. Second, to evaluate LAIR-1 expression as a sensitive, non-invasive prognostic biomarker in HCC Egyptian patients' peripheral blood. Being a mechanistic study, in other words, to explore, whether "Tc contributes to liver cirrhosis progression into HCC in Egyptian patient's HCV genotype 4 (G4), through LAIR-1 resistance, or not".

2. RESULTS

2.1. Participants' Demographic and Clinical Characteristics

This study included 101 HCC patients, divided into post-HCV G4-HCC (n= 64) and post-HCV G4-liver cirrhosis (n= 37) groups, compared to 20 control volunteers (Table 1A). Being case-controlled study no significant difference were found regarding the age and gender as well as the kidney function (s.creatinine results (data not shown)).

Table 1A: Study participants' demographic and clinical characteristics (unit) in all post-HCV patients' group (n= 101) compared to the control participants group (n= 20).

	Groups, n		
Characteristics (unit)	**post-HCV patients, 101**	**Control, 20**	***p* value**
Gender (M/F)	78/23	16/4	NS
Age (years)	61.0(55.0-67.0)	58.0(55.5 -60.0)	NS
BMI (Kg/m²)	29.3(27.2-32.0)	27.2(26.6- 27.8)	<0.001*
D.M (Yes/No)	43/58	0/20	<0.001*
s. Insulin (mIU/L)	20.0(10.13-33.0)	8.1(6.3-10.0)	<0.001*
Insulin resistance (Yes/No)	62/39	0/20	<0.001*
s. Albumin (mg/dl)	3.2(2.5-3.7)	3.25(3.1-3.5)	NS
AST (U/L)	58.0(43.0-81.0)	30.5(24.2-38.7)	<0.001*
ALT (U/L)	42.0(29.0-55.5)	27.0(23.0-36.5)	0.003*
Total Bilirubin (mg/dl)	1.6(1.0-3.4)	0.85(0.52-1.0)	<0.001*
Direct Bilirubin (mg/dl)	0.90(0.4-2.1)	0.30(0.20-0.4)	<0.001*
ALP (U/L)	112.0(78.0-151.0)	51.0(39.5-61.0)	<0.001*
GGT (U/L)	54.0(40.0-70.0)	19.0(17.2-23.7)	<0.001*
Total Cholesterol (mg/dl)	149.0(111.5-192.0)	155.5(151.2-161.7)	NS
TAG (mg/dl)	122.0(88.0-189.5)	114.5(98.2-122.7)	NS
HDL-C (mg/dl)	34.0(27.0-41.5)	46.5(42.2-50.7)	<0.001*
TAG/HDL-C ratio	3.8(2.4-6.1)	2.3(2.1-2.7)	<0.001*
TLC (/mm³)	7.5(4.5-11.8)	8.3(7.4-9.6)	NS
Hgb (gm/dl)	11.0(8.9-12.6)	13.0(12.0-13.8)	<0.001*
PLTs (× 10⁹/L)	126.0(90.0-231.0)	244.5(223.0-276.0)	<0.001*
INR	1.3(1.2-1.6)	1.1(1.0-1.3)	<0.001*
Lymphocyte %	24.8(19.0-32.0)	23.5(20.6-29.7)	NS
PLR	81.4(52.5-123.1)	117.7(93.1-172.0)	0.005*

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AFP (ng/mL)	15.3(5.8-163.0)	4.5(2.8-6.6)	<0.001*
Tc%	19.0(13.8-23.0)	17.4(11.9-28.0)	NS
LAIR-1 MFI on Tc	38.5(29.3-49.2)	22.0(17.5-31.2)	<0.001*
LAIR-1+Tc %	75.20(59.3-87.0)	13.6(8.3-19.0)	<0.001*
GLR	28.7(17.6-45.5)	9.1(7.6-11.0)	<0.001

Data shown are median (inter quartile range (1^{st}-3^{rd} quartile)), statistics were computed using SPSS software, Mann–Whitney test (non-parametric data),* statistical significance *p*-value < 0.05, NS, non-significant. [ALT, alanine aminotransferase; AST, aspartate aminotransferase, ALP, Alkaline phosphatase; AFP, alpha feto protein, BMI, Body mass index; HDL, high-density lipoprotein; INR, international normalized ratio; GGT, gamma glutamyl transferase; GLR Leukocyte-associated immunoglobulin-like receptor-1 LC, liver cirrhosis; PLT, platelet; Tc. T cytotoxic; TLC, total leukocytic count.]

Clinical characteristics of the studied post-HCV G4-HCC and post-HCV-liver cirrhosis cases are shown in (Table 1B) compared to each other and to the control group.

According to Table 1B, BMI, s. insulin and I.R as well as the inflammation prognostic ratios PLR decreased, TAG/HDL-C increased and the HCC prognostic ratio GLR increased, an increased liver function test results and AFP level increased were all significant between patients groups as well as between the patients and the control group. The percentage of total lymphocytes was significantly increased in the HCC group when being compared to the liver cirrhosis group, whereas the percentage of Tc % showed no significant difference when compared between groups. In addition, comparison of LAIR expression revealed its significant increase in the post-HCV G4 group when compared to the control. LAIR-1 MFI on Tc and LAIR-1+Tc % were 38.5(29.3-49.2) and 75.20(59.3-87.0) in the patients group vs their level in the control group 22.0(17.5-31.2) and 13.6(8.3-19.0), respectively.

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Table 1B: Study participants' demographic and clinical characteristics (unit) in post-HCV G4-HCC group (n= 64), post-HCV G4-liver cirrhosis group (n= 37) compared to each other and to the control participants group (n= 20)

Groups, n Characteristics (unit)	post-HCV G4- HCC, 64	liver cirrhosis, 37	Control, 20	*Significance*		
				p1	*p2*	*p3*
Gender (M/F)	50/14	28/9	16/4	NS	NS	NS
Age (years)	62.0(57.3-67.0)	60.0(54.5-66.0)	58.0(55.5-60.0)	NS	0.006*	NS
BMI (K.gm/m²)	29.0(27.0-31.0)	29.4(28.0-33.8)	27.2(26.6-27.8)	NS	NS	0.005*
D.M (Yes/No)	25/39	18/19	0/20	NS	0.001*	<0.001*
s. Insulin (mIU/L)	22.3(12.9-35.2)	14.4(5.4-24.9)	8.1(6.3-10.0)	0.002*	<0.001*	0.048*
Insulin resistance (Yes/No)	47/17	15/22	0/20	0.001*	0.001*	0.001*
s. Albumin (mg/dl)	3.4(2.9-3.7)	2.6(2.1-3.7)	3.2(3.1-3.5)	0.004*	NS	0.016*
AST (U/L)	63.0(52.0-94.2)	38.0(23.0-68.0)	30.5(24.2-38.7)	<0.001	<0.001	NS
ALT (U/L)	45.0(34.3-61.7)	30.0(19.0-40.0)	27.0(23.0-36.5)	<0.001*	<0.001*	NS
Total Bilirubin(mg/dl)	1.4(1.0-2.3)	2.3(1.0-6.45)	0.85(0.52-1.0)	NS	<0.001*	<0.001*
Direct Bilirubin(mg/dl)	0.80(0.40-1.5)	1.1(0.40-3.3)	0.30(0.20-0.40)	NS	<0.001*	<0.001*
ALP (U/L)	112.0(83.3-169.3)	102.0(73.0–139.5)	51.0(39.5-61.0)	NS	<0.001*	<0.001*
GGT (U/L)	56.0 (45.0–76.0)	39.0(25.0-63.0)	19.0(17.2-23.7)	<0.001	<0.001*	<0.001*
Total Cholesterol (mg/dl)	154.5(117.2-212.0)	130.0(105.0-171.0)	155.5(151.2-161.7)	NS	NS	NS
TAG (mg/dl)	134.5 (94.5-205.7)	105.0(65.0-140.5)	114.5(98.2-122.7)	0.006*	NS	NS
HDL-C (mg/dl)	34.0(26.0-39.7)	35.0(29.0-43.4)	46.5(42.2-50.7)	NS	<0.001*	<0.001*
TAG/HDL-C ratio	4.1(2.8-6.8)	3.1(2.0-5.8)	2.3(2.2-2.7)	0.02*	<0.001*	NS
TLC ×10³/mm³	7.3(4.2-11.1)	7.8(4.9-12.1)	8.3(7.4-9.6)	NS	NS	NS
Hgb (gm/dl)	11.3(9.9-12.8)	9.1(8.0-12.3)	13.0(12.0-13.8)	0.003*	0.001*	<0.001*
PLTs ×10³/mm³	162.5(115.2-234.0)	94.0(60.0-149.0)	244.5(223.0-276.0)	<0.001*	<0.001*	<0.001*

INR	1.2(1.1-1.40)	1.5(1.2-1.8)	1.1(1.0-1.3)	<0.001*	0.006*	<0.001*
Lymphocyte %	25.1(21.8-32.4)	21.0(15.0-30.0)	23.5(20.6-29.7)	0.032*	NS	NS
PLR	92.8(58.3-145.7)	62.4(38.2-94.8)	117.7(93.1-172.0)	0.004*	0.072	<0.001*
AFP (ng/mL)	101.7(17.6-386.2)	6.0(3.3-10.2)	4.5(2.8-6.6)	< 0.001*	<0.001*	NS
Tc%	19.4(13.4-25.7)	17.0(13.7-20.0)	17.4(11.9-28.0)	NS	NS	NS
LAIR-1 MFI on Tc	39.8(31.0-51.1)	32.0(26.0-42.0)	22.0(17.5-31.2)	0.012*	<0.001*	<0.001*
LAIR-1+Tc %	82.8(64.7-90.0)	65.0(38.5-77.4)	13.6(8.28-19.0)	<0.001*	<0.001*	<0.001*
GLR	34.4(21.4-56.9)	27.8(14.8-39.7)	9.1(7.6-11.0)	NS	<0.001*	<0.001*

Data shown are median(inter quartile range (1^{st}-3^{rd} quartile)), statistics were computed using SPSS software, Mann–Whitney test was used (non-parametric data), *p1* indicates comparison between number of populations in post-HCV G4-HCC & post-HCV G4-liver cirrhosis groups, *p2* indicates comparison between number of populations in post-HCV G4-HCC & control, *p3* denotes comparison between number of populations in post-HCV G4-liver cirrhosis & control, * statistical significance *p*-value < 0.05, NS, non-significant. [ALT, alanine aminotransferase; AST, aspartate aminotransferase, ALP, Alkaline phosphatase; AFP, alpha feto protein, BMI, Body mass index; DM, diabetes, GGT, gamma glutamyl transferase; GLR, GGT-to-lymphocytes ratio; Hgb; hemoglobin; HDL, high-density lipoprotein; INR, international normalized ratio; LAIR-1, Leukocyte-Associated Immunoglobulin-like Receptor-1, Leukocyte-associated immunoglobulin-like receptor-1 LC, liver cirrhosis; PLT, platelet; PLR, platelet /lymphocyte ratio; TAG, triacylglycerol; Tc. T cytotoxic; TLC, total leukocytic count.]

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2.2. ***Participants' pathological Characteristics*** are presented in Table 2.

Ascites, LN and lung findings were all significantly different between the liver cirrhotic and HCC groups.

Table 2: Pathological characteristics of the studied post-HCV G4-HCC cases (n=64) and post-HCV G4-liver cirrhosis cases (n= 37).

	post-HCV G4-		
Groups, n (%)	**HCC, 64(100%)**	**liver cirrhosis, 37 (100%)**	**Statistics test, *P*-value**
	Pathological characteristics		
Parameters	**Ascites**		X^2= 9.0, 0.03*
No	43 (67.2%)	18 (48.6%)	
Minimal	9 (14.1%)	2 (5.4%)	
Moderate	8 (12.5%)	12 (32.4%)	
Marked or Massive	4 (6.3%)	5 (13.5%)	
	Lung radiological findings		X^2 = 0 .48, 0.48
Normal	57 (89.1%)	35 (94.6%)	
Abnormal findings #	7 (10.9%)	2 (5.4%)	
	LN involvement		X^2= 6.4, 0.012*
N0	54 (84.4%)	37 (100.0%)	
N1/Yes	10 (15.6%)	0 (0.0%)	
Liver size[$]	16.3 ± 2.8	12.7 ± 2.0	*t*-test = 7, < 0.001
Splenomegaly[$]	16.5 ± 2.1	17.6 ± 4.4	*t*-test = 1.7, 0.8
Portal vein^dilatation up to 13	51.33(3285)	50.43(1866)	U-test = 1163, 0.88
	Liver pattern		N.A
Heterogenous mass	3 (4.7%)	0 (0.0%)	
Focal single lesion	40 (62.5%)	0 (0.0%)	
Multiple lesions	21 (32.8%)	0 (0.0%)	
Cirrhotic	0 (0.0%)	37 (100.0%)	
	Liver mass number		N.A
1.00	32 (50.0%)	0 (0.0%)	
2.00	7 (10.9%)	0 (0.0%)	
3.00	2 (3.1%)	0 (0.0%)	
≥ 4.00	23 (35.9%)	0 (0.0%)	
	Portal vein patency		N.A
Patent	45 (70.3%)	37 (100.0%)	
Partially occluded	4 (6.3%)	0 (0.0%)	
Thrombosed	15 (23.4%)	0 (0.0%)	

[$]Data shown as mean ± S.D for parametric data or ^ or n frequencies, % percentage for non-parametric data, statistics were computed using SPSS software, using Student's *t*-test (parametric data), Chi square test X^2 (dichotomous parameters), or Mann–Whitney U-test (non-parametric data), *

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statistically significant at $p< 0.05$, # 2 patients have effusion, 4 patients have nodules, 1 collapse, 1 consolidation. N.A.; not applicable/not available.

2.3. *LAIR-1 expression levels in post-HCV G4 groups*

Stratified analysis of disease susceptibility and LAIR-1 expression level was done in **Table 3.** A summary of the comparison between groups regarding all lab. analysis is shown in Table 3. Gender, age, BMI, s. insulin and liver function ALT, bilirubin, AFP, s. albumin, GLR, TAG/HDL-C and I.R. However, liver patency, liver size LN, ascites, lung findings, didn't differ between LAIR-1 expression level in the patients' groups.

Regarding LAIR-1 expression on Tc cells, the percentage of Tc positive for LAIR-1 (LAIR-1+Tc %) and LAIR-1 MFI on Tc were both significantly increased in the post-HCV G4-HCC group when compared to the post-HCV G4-liver cirrhosis group. FC results are presented in Figure 1.

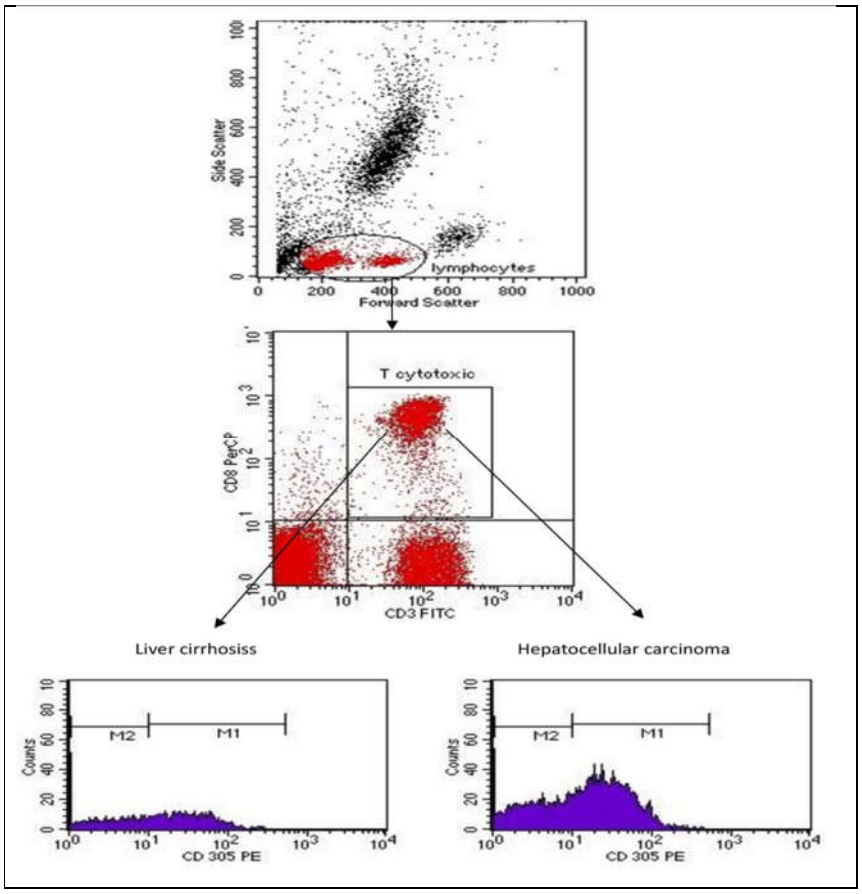

Figure 1. Percentage of Tc expressing LAIR-1/CD305 and LAIR-1 MFI on single histogram from CD3+CD8+ co-expressing population for HCC group (bottom right) and liver cirrhosis (bottom left) obtained by FC initial gating by typical forward and sideways scatter on mature lymphocytes expected area. [MFI; mean fluorescence intensity of positive population detected on area under M1 marker.]

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Table 3: Expression levels of LAIR-1+Tc % and LAIR-1 MFI on Tc in all post-HCV G4 cases (n= 101) stratified subgroups, when the cutoff values of the demographic or the clinicopathological parameters estimated/measured were considered.

			LAIR-1	
Groups n, % Characteristics	**post-HCV cases, 101, 100%**		**MFI on Tc**	**+Tc %**
Gender	**N**	**%**		
Male	78	77.3	38.75 (29.85- 49.10)	75.62 (60.00-86.25)
Female	23	22.7	38.00 (26.90- 54.00)	74.20 (45.5-90.50)
p-value			NS	NS
Age (years)				
< 60	41	40.6	38.00 (30.25-56.00)	76.00 (60.74-88.50)
≥ 60	60	59.4	39.00 (28.93-47.28)	74.10 (56.08-85.90)
p-value			NS	NS
BMI (kg/m²)				
Under weight > 25	8	7.9	41.50 (30.75-61.43)	76.00 (51.05-88.13)
Lean 25-29.9	45	44.6	37.00 (29.20-48.50)	75.23 (62.74-88.00)
Over weight 30-34.9	37	36.7	40.60 (30.62-56.00)	84.00 (62.00-87.48)
Obese 35-39.9	5	4.9	29.00 (23.73-39.50)	56.07 (48.97-80.23)
Morbid obesity ≥ 40	6	5.9	32.00 (22.75-33.35)	46.55 (35.38-66.45)
p-value			NS	NS
Diabetes Mellitus				
Yes	43	42.6	39.00 (28.9-50.00)	73.20 (56.00-86.00)
No	58	57.4	37.50 (30.23-49.10)	77.00 (61.20-87.81)
p-value			NS	NS
Insulin resistance				

Yes	62	61.4	40.30 (30.38-55.25)	82.80 (59.7-89.25)
No	39	38.6	33.00 (28.00-40.00)	73.2 (56.55-82.60)
p-value			0.007	0.038
AFP (ng/mL)				
< 20	48	47.5	34.00 (28.68-43.05)	70.69 (55.55-84.00)
≥ 20	53	52.5	40.00 (31.02-55.00)	83.00 (69.66-90.00)
p-value			0.023	<0.001
s. Albumin (gm/dL)				
< 2.5	20	19.8	31.35 (24.25-40.25)	73.60 (43.40-82.00)
≥ 2.5	81	58.2	39.40 (30.62-50.70)	77.00 (60.19-87.60)
p-value			0.013	0.05
AST (U/L)				
< 40	24	23.8	33.50 (25.00-46.28)	65.80 (41.25-80.50)
≥ 40	77	76.2	39.02 (30.37-50.70)	77.00 (60.92-88.13)
p-value			NS	0.010
ALT (U/L)				
< 40	49	48.5	38.00 (30.85-47.85)	76.00 (51.70-88.50)
≥ 40	52	51.5	39.20 (28.52-52.60)	74.88 (60.00-85.98)
p-value			0.000	0.013
Total bilirubin (mg/dL)				
< 1.4	41	40.6	38.50 (29.82-50.70)	74.00 (60.92-85.95)
≥ 1.4	60	59.4	38.90 (29.05-46.00)	75.62 (56.00-87.61)
p-value			NS	NS
Direct bilirubin (mg/dL)				

≤ 0.4	21	20.8	34.00 (28.65-43.55)	62.80 (52.74-79.23)
> 0.4	80	79.2	39.50 (30.29-51.05)	76.50 (61.00-87.94)
p-value			0.0182	0.048
ALP (U/L)				
< 145	27	26.7	37.09 (28.77-49.55)	74.00 (56.05-85.90)
≥ 145	74	73.3	40.00 (32.00-49.00)	83.02 (73.00-88.50)
p-value			NS	0.042
GGT (U/L)				
< 40	25	24.8	33.00 (28.01-47.00)	66.60 (46.55-82.00)
≥ 40	76	75.2	39.20 (30.06-49.85)	77.20 (60.09-87.94)
p-value			NS	0.040
Total cholesterol (mg/dL)				
< 200	79	78.2	38.00 (29.00-47.70)	74.20 (57.65-85.90)
≥ 200	22	21.8	39.80 (29.35-56.00)	82.80 (59.61-87.90)
p-value			NS	NS
TAG (mg/dL)				
< 150	64	63.4	37.59 (28.52-45.25)	74.26 (56.02-87.75)
≥ 150	37	36.6	40.02 (30.37-53.00)	77.01 (64.00-86.50)
p-value			NS	NS
HDL-C mg/Dl				
< 35	53	52.5	35.00 (29.20-42.50)	74.00 (57.98-85.90)
≥ 35	48	47.5	40.50 (29.65-52.75)	78.00 (59.47-87.15)
p-value			NS	NS
TAG/HDL-C ratio				

< 2.4	25	24.8	33.00 (26.29-39.70)	64.01 (40.00-80.03)
≥ 2.4	76	75.2	39.80 (30.78-52.00)	77.43 (61.28-87.94)
p-value			0.009	0.011
TLC ×10³/mm³				
< 4.5	24	23.8	35.50 (29.55-47.80)	74.00 (60.70-82.60)
≥ 4.5	77	76.2	39.00 (29.00-49.50)	77.40 (56.04-88.75)
p-value			NS	0.0228
PLT ×10³/mm³				
< 150	57	56.4	35.00 (28.50-48.00)	75.23 (57.55-86.45)
≥ 150	44	43.6	39.00 (30.42-49.30)	75.26 (59.91-87.75)
p-value			NS	NS
INR				
< 1.1	11	10.9	38.20 (29.20-56.00)	90.00 (59.88-91.00)
≥ 1.1	90	89.1	38.75 (29.30-49.1)	74.87 (58.51-85.90)
p-value			NS	NS
PLR				
< 81.4	50	49.5	33.50 (28.01-43.05)	72.50 (52.85-87.05)
≥ 81.4	51	50.5	40.03 (32.00-51.40)	78.00 (62.80-87.25)
p-value			0.023	NS
GLR				
< 20.5	30	29.7	32.00 (27.15-42.25)	64.50 (44.63-85.23)
≥ 20.5	71	70.3	39.60 (30.50-39.60)	77.00 (64.00-87.75)
p-value			0.030	0.040
Ascites				

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0, No	61	60.4	38.20 (28.95-49.20)	74.00 (59.94-86.45)
1, Minimal	11	10.9	46.00 (40.00-58.00)	84.90 (76.00-88.50)
2, Moderate	20	19.8	38.50 (29.38-45.30)	75.00 (48.13-88.90)
3, Marked	9	8.9	32.00 (26.00-30.09)	55.40 (41.80-88.38)
p-value			0.027 (Marked vs Minimal, p=0.020)	NS
LN				
N0/Yes	10	9.9	34.50 (28.15-48.25)	73.50 (56.21-88.73)
No	91	90.1	39.00 (29.40-49.40)	75.23 (59.88-787.20)
p-value			NS	NS
Liver pattern				
Heterogenous mass	3	3.0	50.00 (31.00-NA)	77.00 (73.00-NA)
Focal single lesion	40	39.6	39.70 (29.40-49.30)	83.00 (62.10-90.00)
Multiple lesions	21	20.8	39.60 (34.00 -54.00)	79.00 (66.59-88.38)
Cirrhotic	37	36.6	32.00 (26.00-42.00)	65.00 (38.50-77.43)
p-value			NS	*<0.001* (Cirrhotic vs focal, *p*<0.001) (Cirrhotic vs multiple, *p*=0.018)
Liver mass number (n=64)				
1	32	50.0	40.45 (30.42-50.00)	83.65 (60.00-90.08)
2	7	10.9	28.90 (26.90-46.00)	69.66 (62.8-83.00)
3	2	3.1	37.14 (22.88-NA)	69.28 (55.93-NA)
≥ 4	23	36.0	40.00 (36.00-56.00)	84.90 (74.20-90.50)
p-value			NS	NS
Lung findings				

Abnormal	9	8.9	33.00 (31.50-44.50)	79.00 (55.70-87.75)
Normal	92	91.1	38.75 (29.05-49.85)	74.36 (59.91-86.75)
p-value			NS	NS
Portal vein patency				
Patent	82	81.2	38.35 (28.98-49.55)	73.10 (56.05-85.42)
Partially occluded	4	3.9	35.70 (32.00-40.30)	82.10 (60.10-90.75)
Thrombosed	15	14.9	40.00 (32.00-54.00)	84.90 (76.00-90.00)
p-value			NS	0.018 (Patent vs thrombosed, p=0.018)

All data were expressed as median (quartiles) and comparison was assessed using Mann–Whitney test (U) for comparison of two non-parametric groups and Kruskal–Wallis one-way ANOVA (H) for more than two non-parametric groups on SPSS software, * significant correlation at $p<0.05$ level (2-tailed), NS; none-significant. [ALT, alanine aminotransferase; AST, aspartate aminotransferase, ALP, Alkaline phosphatase; AFP, alpha feto protein, BMI, Body mass index; DM, diabetes, GGT, gamma glutamyl transferase; GLR, GGT-to-lymphocytes ratio; Hgb; hemoglobin; HDL, high-density lipoprotein; INR, international normalized ratio; LAIR-1, Leukocyte-Associated Immunoglobulin-like Receptor-1, Leukocyte-associated immunoglobulin-like receptor-1 LC, liver cirrhosis; PLT, platelet; PLR, platelet /lymphocyte ratio; TAG, triacylglycerol; Tc. T cytotoxic; TLC, total leukocytic count.]

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2.4. *The Correlation between LAIR-1 expression level and post-HCV G4 status of HCC and liver cirrhosis patients*

Spearman correlation included all post-HCV patients (n=101) revealed that both the frequency of LAIR-1+Tc & LAIR-1 MFI on Tc positively correlated with AFP as depicted in Table 4. LAIR-1 MFI on Tc positively correlated with in I.R and GLR, moreover, LAIR-1+Tc positively correlated with liver size and PV patency.

Table 4: Spearman's correlation coefficient among investigated LAIR-1 expressions LAIR-1+Tc % and LAIR-1 MFI on Tc in all post-HCV patients (n= 101)

	post-HCV G4 patients (n= 101)			
	LIAR-1 MFI on Tc		LIAR-1+Tc%	
Characteristics	*r*	***p*-value**	*r*	***p*-value**
Age (years)	-0.063	NS	-0.063	NS
BMI (kg/m²)	-0.112	NS	-0.155	NS
s. Insulin	0.035	NS	0.044	NS
AFP (ng/mL)	0.218	0.028*	0.213	<0.001*
AST (U/L)	0.104	NS	0.178	NS
ALT (U/L)	0.038	NS	0.101	NS
ALP (U/L)	0.115	NS	0.096	NS
GGT (U/L)	0.106	NS	0.176	NS
TAG (mg/dL)	0.125	NS	0.127	NS
Total Cholesterol (mg/dL)	0.134	NS	0.178	NS
HDL-C (mg/dL)	0.061	NS	0.055	NS
TAG/HDL-C	0.111	NS	0.077	NS
TLC x10³ mm³	0.124	NS	0.076	NS
PLT x10³ mm³	0.101	NS	0.092	NS
PLR	0.108	NS	0.053	NS
GLR	0.196	0.049*	0.178	NS
Liver size	0.190	NS	0.401	<0.001*
Insulin resistance#	0.302	0.002*	0.188	NS
Portal vein patency#	0.050	NS	0.271	0.006*

Spearman correlation coefficient (*r*) was calculated using SPSS software, * significant correlation at p<0.05 level (2-tailed), NS; none significant, # analyzed by point-biserial correlation. [ALT, alanine aminotransferase; AST, aspartate aminotransferase, ALP, Alkaline phosphatase; AFP, alpha feto protein, BMI, Body mass index; DM, diabetes, GGT, gamma glutamyl transferase; GLR, GGT-to-lymphocytes ratio; Hgb; hemoglobin; HDL, high-density lipoprotein; INR, international normalized ratio; LAIR-1, Leukocyte-Associated Immunoglobulin-like Receptor-1, Leukocyte-associated immunoglobulin-like receptor-1 LC, liver cirrhosis; PLT, platelet; PLR, platelet /lymphocyte ratio; TAG, triacylglycerol; Tc. T cytotoxic; TLC, total leukocytic count.]

LAIR-1+Tc was significantly positively correlated LAIR-1 MFI on Tc (r= 0.734, p <0.001).

AFP levels were positively correlated with markers of inflammation (PLR and GLR) where (r= 0.201, p =0.040 and (r= 0.214, p = 0.032, respectively).

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2.5. *ROC curve for the discriminative ability of LAIR-1+Tc % and LAIR MFI on Tc to differentiate post-HCV-HCC from post-HCV-liver cirrhosis*

LAIR-1+Tc % at a cutoff ≥ 73.6 and an AUC 0.756 can distinguish post-HCV G4-HCC transformation from post-HCV G4-liver cirrhosis with 67.2 % sensitivity and 62.2% specificity (p < 0.001). LAIR-1 MFI on Tc had the same sensitivity and specificity at a cutoff > 34.5 and an AUC 0.651 (p= 0.012). AFP at cut off≥ 10.2 had 82.2 % sensitivity and 75.7% specificity with AUC =0.876 (p<0.001) (Table 5, Figure 2).

Table 5: ROC curve for the discriminative ability of LAIR-1+Tc % and LAIR-1 MFI on Tc to differentiate HCC from liver cirrhosis

		%					Asymptotic 95% CI	
Variables	**Cut-off point**	**Sensitivity**	**Specificity**	**AUC**	**S.E.**	***P*-value**	**Lower bound**	**Upper Bound**
LAIR-1+Tc %	73.6	67.2	62.2	0.756	0.049	<0.001*	0.661	0.851
LAIR-1 MFI on Tc	34.5	67.2	62.2	0.651	0.058	0.012*	0.538	0.764
AFP (ng/mL)	12.5	81.2	89.2	0.876	0.035	<0.001*	0.807	0.946
LAIR-1+Tc % + LAIR-1 MFI on Tc	--	20	60	0.753	0.049	<0.001*	0.656	0.850
LAIR-1+Tc % + AFP	--	83	70	0.912	0.027	<0.001*	0.858	0.965
LAIR-1 MFI on Tc + AFP	--	82	65	0.885	0.033	<0.001*	0.821	0.949
LAIR-1+Tc % + LAIR-1 MFI on Tc + AFP	--	85	73	0.918	0.027	<0.001*	0.865	0.970

Data calculated using SPSS software, * significant correlation at p<0.05 level (2-tailed). [AFP, alpha feto protein; LAIR-1, Leukocyte-Associated Immunoglobulin-like Receptor-1, MFI, Mean fluorescence intensity, Tc, T cytotoxic.]

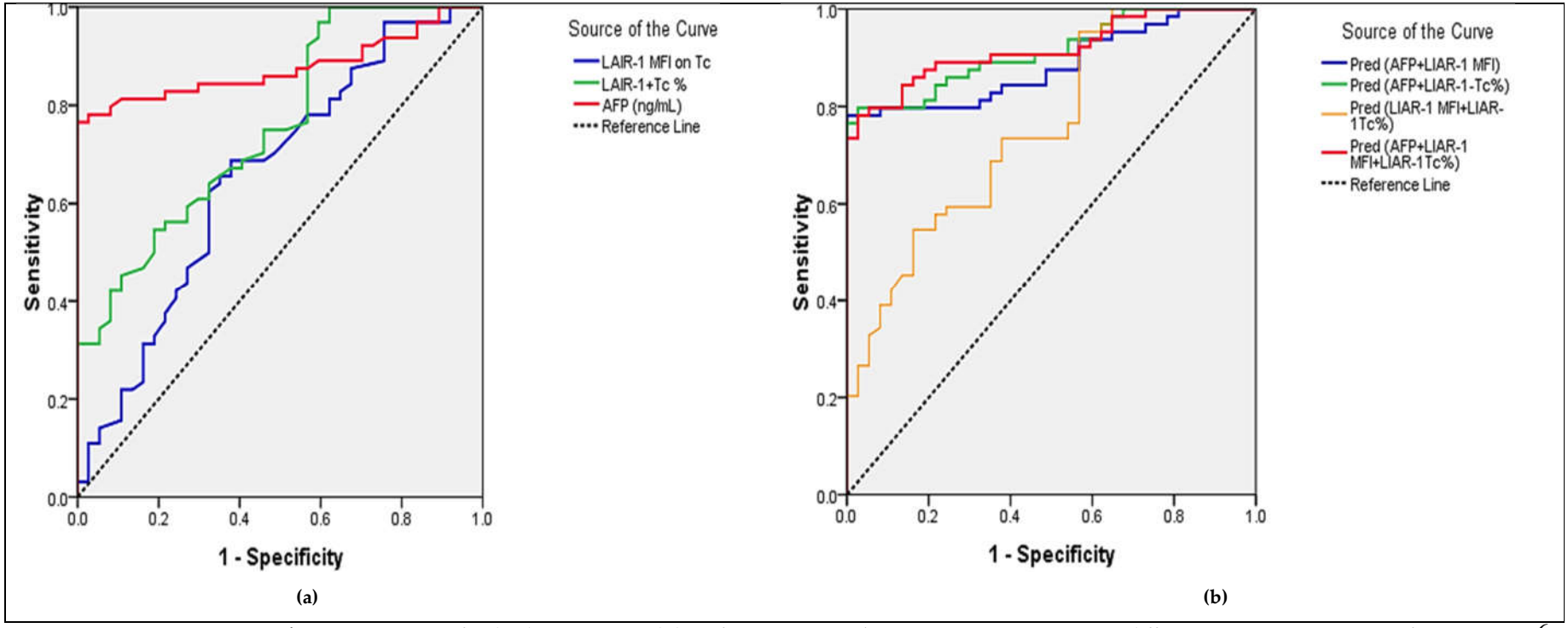

Figure 2. ROC curve for the discriminative ability of (a) LAIR-1+Tc %, LAIR-1 MFI on Tc or AFP to differentiate post-HCV G4-HCC from post-HCV G4-liver cirrhosis in comparison to (b) different combination of variables

2.6. *In Silico* Biology.

2.6.1. *LAIR-1* gene located on chromosome 19, exon 13. External Ids for *LAIR1* Gene; HGNC: 6477, NCBI Entrez Gene: 3903, Ensembl: ENSG00000167613, OMIM®: 602992, UniProtKB/Swiss-Prot: Q6GTX8.

https://www.genecards.org/Search/Keyword?queryString=LAIR-1 (date July, 2022) as presented in Figure 3 addressing *LAIR-1* gene characteristics, according to https://www.genecards.org/cgi-bin/carddisp.pl?gene=LAIR1&keywords=LAIR-1,

https://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000167613;r=19:54351384-54370558 (date July, 2022).

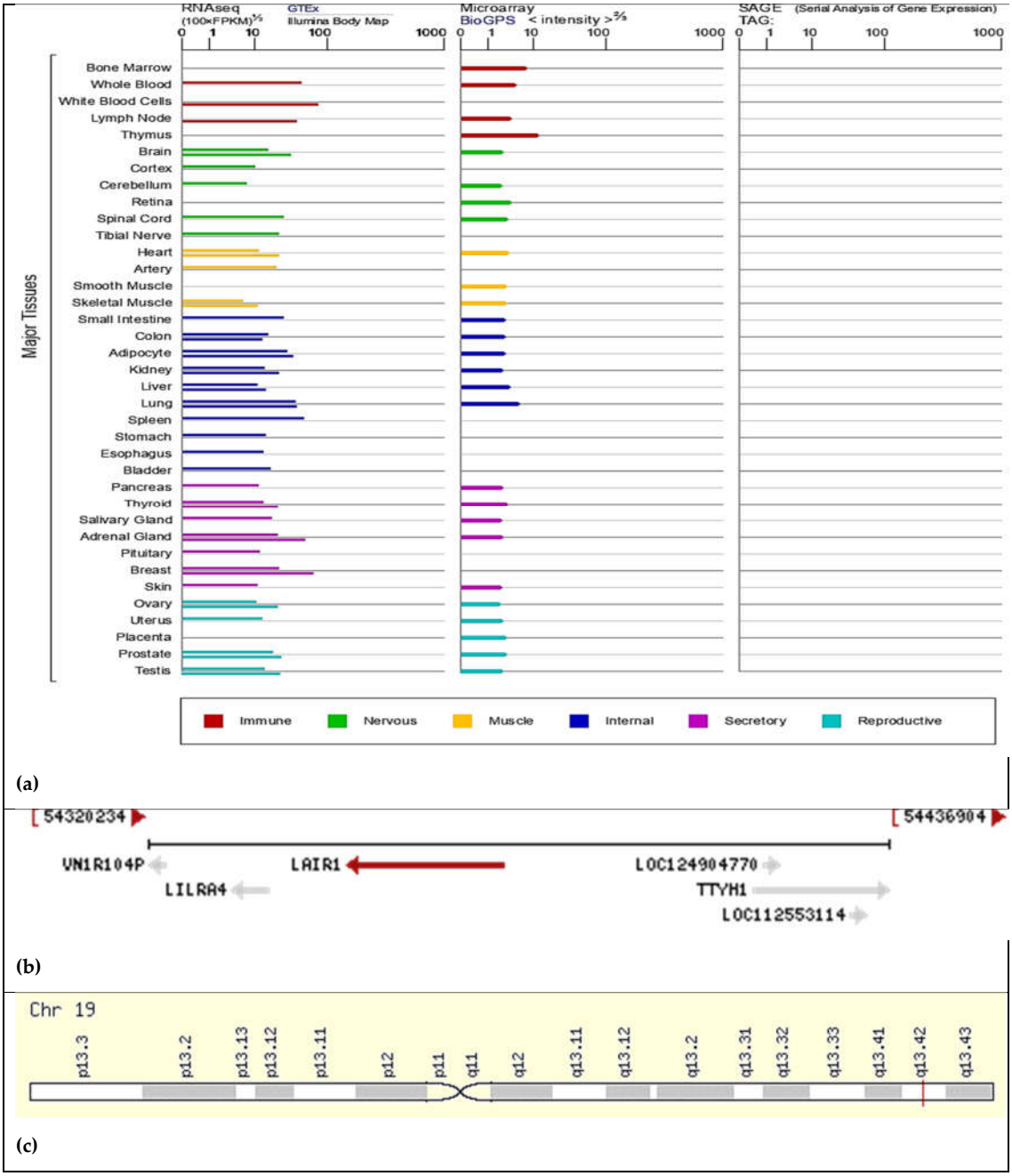

Figure 3. *LAIR-1*gene characteristics a) mRNA expression in normal human tissues from GTEx, Illumina, BioGPS, SAGE for *LAIR-1* gene

https://www.genecards.org/cgi-bin/carddisp.pl?gene=LAIR1&keywords=LAIR-1, b) genomic context https://www.ncbi.nlm.nih.gov/gene/3903

c) Cytogenetic band: genomic location: bands according to Ensembl, locations according to GeneLoc, latest assembly: chr19:54,351,384-54,376,088(GRCh38/hg38), Size: 24,705 bases, Orientation: Minus strand

https://www.genecards.org/cgi-bin/carddisp.pl?gene=LAIR1&keywords=LAIR-1

2.6.2. Functional enrichments in the current network (Figure 4) obtained via the local network cluster (STRING) mixed constitutive signaling using

https://string-db.org/cgi/network?taskId=bZ25wTH8KOIM&sessionId=bOdQ8mhP3zbB (date July, 2022).

Where the reactome pathways are cytokine signaling in the immune system and adaptive immune system. The biological process is cell activation, regulation of cytokine production as well as regulation of cytokine-mediated signaling pathways. The molecular function is protein tyrosine kinase and phosphatase binding, and insulin receptor binding.

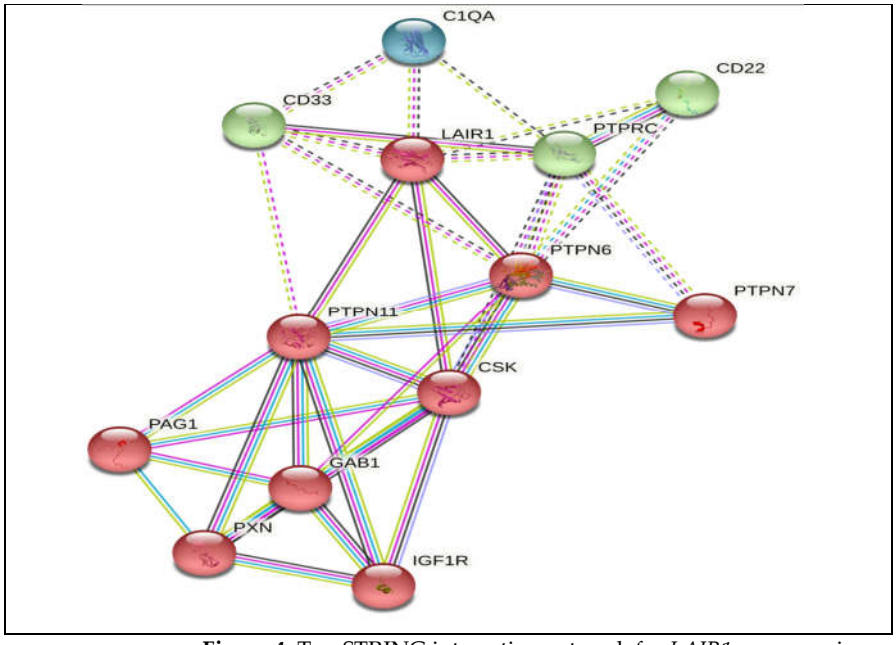

Figure 4. Top STRING interaction network for *LAIR1* gene preview

https://string-db.org/cgi/network?taskId=bZ25wTH8KOIM&sessionId=bOdQ8mhP3zbB

3. DISCUSSION

HCC's pathogenesis in Egypt involves liver inflammation that is post-HCV-related, and cirrhosis is an intermediate process **[18]**. Thence, understanding the molecular and cellular events underlying liver cirrhosis progression to HCC is essential for identifying potential therapeutic targets.

An important factor during HCC development is an inflamed and cirrhotic liver with significant immune infiltration, brought on by HCV G4 infection. With few available treatments, HCC is one of the main causes of cancer-related deaths globally **[19]** and nationally **[18]** From the clinically-inter-related risk factor(s) for HCC development is the progression of the scare-related cirrhosis disease to cancer, mostly the male gender, older age, being diabetic, obese patients with hyperinsulinemia, I.R, and dyslipidemia, inflammation prognostic ratios and indices (TAG/HDL-C and GLR) as well as having HCV infection history, as seen in our patients' cohort.

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Per the immune system recognizes tumor cells as non-self-cells via T-cytotoxic cells, the immune response is controlled by inhibitory receptors to stop self-cells lysis. One of the inhibitory receptors on immune cells is the cytotoxic T-lymphocyte–associated antigen 4 measured previously in post-HCV G4-HCC as well as its single nucleotide polymorphism in Egyptian HCV G4 patients **[20]**.

Moreover, ligands for inhibitory receptors, related to the immune system, are constitutively expressed on healthy cells but can be lost on malignant cells **[21]**. Loss of the inhibitory checks will allow activating signals to predominate that might be involved in liver cirrhosis progression to HCC post-HCV G4 infection.

The collagen-binding inhibitory receptor, *LAIR1*, is encoded on chromosome 19 by the leukocyte receptor complex **[22]**. *LAIR-1* gene produces an inhibitory receptor that is located on peripheral mononuclear cells such T-cells. The *LAIR-1* gene belongs to the leukocyte-associated inhibitory receptor family. The leukocyte receptor cluster, contains at least 29 genes encoding leukocyte-expressed receptors of the immunoglobulin superfamily domain containing (Figure 3B). *LAIR-1* gene encoded protein may cause cell death in myeloid leukemias' **[23]** and has been identified as an anchor for tyrosine phosphatase **[24]**.

LAIR-1 is expressed on immune cells including CD3+CD8+ cytotoxic T-cells in the tumor microenvironment and in the circulation, inhibiting immune cell activities **[25, 26]** explaining liver cirrhosis progression to HCC post-HCV G4 chronic infection.

The current study was undertaken to understand the interaction or the relationship of Tc LAIR-1 level expression in the primary liver cancer post-HCV G4.

Per the STRING network reactome pathways illustrated in Figure 4, cytokine signaling in the adaptive immune system, the molecular LAIR-1 function is protein tyrosine kinase and phosphatase binding, and insulin like growth factor receptor IGFR1-related. Where in our study s. insulin level were increased significantly in the patients' group (n=101) in comparison to the control group (n=20) as well as diabetes, I.R and its prognostic markers in the liver cirrhosis group (n=37) in comparison to either the control or the HCC (n=64) groups. This is in line that the primary liver cancer, HCC, mostly commonly affects people with cirrhosis with an underlying chronic liver inflammatory disease post-HCV **[1].**

LAIR-1 serves as an inhibitory receptor for T cells on a permanent basis, with tyrosine phosphorylation component to activate PTPN6 and PTPN11 phosphatases. However, without the aid of phosphatases, LAIR-1 reduces anti-inflammatory cytokine IL-2 and the interferon IFNG production, while increasing the release of transforming growth factor beta in CD4+ T cells (per the STRING pathway). **Barnabei et al.** in 2021 found LAIR-1 to inhibit nuclear translocation of nuclear factor NF-kappa-B p65 subunit as well as the phosphorylation of the inhibitory I-kappa-B alpha in myeloid leukemia cell lines as well as proliferation inhibition and induction of apoptosis in these cells **[27]**.

Accumulation of fatty acids, presented in our current study resulted as a consequence to an increased serum TAG, obesity, and dyslipidemia with diabetes, leading to generalized inflammation, triggering pro-inflammatory cytokine(s) milliard, presented clinically as significant prognostic inflammatory indices TAG/HDL-C ratio and GLR. This points to the non-alcoholic steatohepatitis (NASH) component of the current patients' cohort, a step toward liver fibrosis that precedes cirrhosis. This is in line with a Korean population-based study showing an altered lipid metabolism being linked to HCC development with TAG (mg/dL) significantly increased in the HCC group **[28].**

In the current study, we aimed to examine whether LAIR-1 influence HCC susceptibility by performing a case-controlled study in Egyptian patients' population, evaluating the associations between post-HCV G4 and liver cirrhosis risk or cirrhosis progression to HCC. Moreover, if LAIR-1 could predict HCC prognosis, with odds ratio and 95% confidence interval under credible statistical models.

In our current study we aimed to explore if the level of T cytotoxic (Tc) expression of LAIR-1 is associated with post-HCV G4-liver cirrhosis progression as compared to post-HCV G4- HCC for sake of proving whether measuring and quantifying Tc expression of LAIR-1 can serve as sensitive, non-invasive prognostic molecular marker in post-HCV G4 infection Egyptian patients' peripheral blood liquid biopsy.

Tc % showed no significant difference when compared between groups, whereas, the % of Tc positive for the immunoinhibitory LAIR-1 (LAIR-1+Tc %) was significantly increased in the HCC group when compared to liver cirrhosis group (p<0.001). Regarding the expression of LAIR-1 % on cytotoxic T +cells and LAIR-1 MFI on Tc was significantly increased in the HCC group when compared to the liver cirrhosis group (p=0.012). These findings supported the findings of **Ma et al.** who showed that Tc cells are essential for the anti-tumor immunity **[29]**. **Wu et al**. documented LAIR-1 over expression in HCC tissues significantly associated with a worse overall survival (OS) **[30]**. Per, LAIR-1 cross-linking with its ligand, inhibits the cytotoxic activity of CD8+ T cells and the T cell receptor/CD3 complex signaling **[31]** worsening the OS, therefore, currently we are working on collecting the patients OS (years) and LAIR's-1 ligand measurement in a coming complementary manuscript (future prospective).

Martínez-Esparza et al. reported that blood monocytes exhibited higher LAIR-1 expression levels in cirrhotic patients and emphasized that liver cirrhosis is characterized by a progressive replacement of the functional hepatic architecture by non-functional fibrotic tissue rich in collagen deposition the ligand of immunoinhibitory LAIR-1 **[11].** Progression toward liver cirrhosis is caused by a dysregulation of immune regulatory mechanisms that govern the balance between activation/homeostasis of the immune system in case of chronic viral infections **[32].** In our study LAIR-1+Tc expression level was higher in cirrhotic vs focal liver lesion at p<0.001 and cirrhotic vs multiple liver lesions significantly (Table 3) as well as portal vein patency. Moreover, LAIR-1 MFI on Tc expression level was higher in ascitic vs no ascitic cases (p=0.027) and marked vs minimal ascites (p=0.020). however, LAIR-1 expression showed a higher trend in larger liver size and LN involvement vs N0, proving LAIR-1 collusion during liver cirrhosis and HCC post-HCV G4 infection. This is in addition to the correlation of either LAIR-1+Tc or LAIR-1 MFI on Tc expression % with the tumor marker AFP (r= 0.367, p <0.001 and r=0.213, p =0.033, respectively), which points out the diagnostic utility of LAIR-1 expression in HCC transformation post-HCV being measured together with AFP, proved more by better sensitivity and specificity % when both are plotted by ROC curve (Figure 2 and Table 5).

Despite no significant correlation was reported between LAIR-1+Tc % or LAIR-1 MFI on Tc with lipids profile (as illustrated in Table 4), however, HDL-C level was decreased significantly in the patients' group and stepwise in both post-HCV groups with increased serum total cholesterol and TAG (dyslipidemia as documented in Tables 1A and 1B). It is hypothesized that a link may be found between LAIR-1 expression and HDL-C during HCC development post-HCV as a previous mendelian meta-analysis reported that a 1 mg/dL reduction in HDL-C level was associated with a 14% increased overall cancer risk **[33]**. **Tosi et al.** previously reported that the scavenger receptor class B type on LAIR-1 ligand Complement C1q, an HDL-C receptor, enhances the uptake of cholesteryl esters, leading to a reduction in serum HDL-C levels **[34].** LAIR-1 overexpression, during liver cirrhosis progression to HCC post-HCV infection, is associated with exhaustion of Tc and progression of inflammation and build-up of fatty acids **[34, 35]** and cholesterol crystals increase from dys-functioned clearance **[36]** in the HCC microenvironment **[37].**

According to the ROC curves, LAIR-1+Tc %, at a cutoff of ≥ 73.6 and an AUC of 0.756, can distinguish post-HCV HCC transformation from post-HCV LC, with 67.2 % sensitivity and 62.2% specificity (p< 0.001), while LAIR-1 MFI had 67.2 % sensitivity and 62.2 % specificity at a cutoff of > 34.5 and an AUC of 0.651 (p= 0.012). Still AFP at cut off ≥ 10.2 had 82.2 % sensitivity and 75.7% specificity with

AUC =0.876 (p<0.001) (Table 5, Figure 2).

This implies the clinical significance of utilizing either expression % of LAIR-1+Tc and/or LAIR-1 MFI on Tc mainly with AFP for better diagnosis, with accepted sensitivity and specificity. Therefore, % of LAIR-1+Tc and/or LAIR-1 MFI on Tc could be a good diagnostic choice in AFP-negative HCC cases **[38]** a recommendation worth further examination and proof in clinical practice.

Short-comings. (i) The capacity of the molecular bio-marker assay refining is limited by the absence of comparable formalin-fixed, paraffin-embedded (FFPE) tissue specimens to complement the liquid biopsy samples. Therefore, LAIR-1 and its ligand expression in liver tissue biopsy are recommended to be done in one future study.

Sustainability Plan. One ongoing research, by our team, addressing LAIR-1 SNPs variants haplotype role in HCC Egyptian patients' cohort. Therefore, continuing the team oncology research work, addressing the role of several tumor immune-related check-point effectors/down-stream target genes/proteins, addressed in the STRING pathway (Figure 4), in primary and/or metastatic HCC diagnosis and/or prognosis as well as unravelling their exact role in post-HCV G4-HCC tumorigenesis.

4. SUBJECTS and METHODS

4.1. Sample Size and Power Study. Based on the previous study by **Gu et al., 2021 [39]** presented results as a graph chart, from which the mean of expression for each group by using a graph reader tool http://www.graphreader.com/ (Nov., 2021) was used to calculate the S.D online. Then, the sample size estimation was performed by G power* sample size online calculator https://riskcalc.org/samplesize/# (Nov., 2021) depending on two-sided significance level 0.05 and power (1-beta) 0.95. According to ***Gu et al.*** gray zone group population standard deviation (5), expected means 74, 80, and the large effect size (1.2), hence, our current study group sample size is 40 patients and 12 control subjects. This is to be able to reject the null hypothesis that the population means of the studied groups are equal with a probability (power) of 0.9.

4.2. Study Design. Case-controlled study.

4.3. Study Participants.

Control subjects. Controls were randomly selected, being apparently healthy volunteers, not suffering from any disease or taking any medication. 20 control subjects with normal kidney and liver functions, absence of any clinical or laboratory evidence of steatosis or cirrhosis. Control subjects were recruited during routine checkup examinations for themselves or their relatives, with ages (55.5-60) years, 16/4 male to female. **Patients' groups.** The study enrolled 64 post-HCV G4-HCC patients recruited from Hepatology and Gastroenterology Department, National Liver Institute, Menoufia University, ages (57.3-67) years, 50/14 male to female. Post-HCV G4-HCC group to be compared to 37 patients with post-HCV G4-liver cirrhosis recruited from Faculty of Medicine, Alzahraa Hospital, Al-Azhar University, ages (54.5-66) years, 28/9 male to female. For all study patients (n=101) a full history was collected and recorded. Blood samples were collected at the time of diagnosis for those who met the inclusion criteria and signed the IC or were rejected for those who met the exclusion criteria. **Patients' inclusion's criteria** are adult over 18-years old and confirmed pathological examination of newly diagnosed HCC of no specific type, post HCV G4 (confirmed by serology). **Exclusion criteria** were attributes that prevent a person from being included in the study as blood diseases, patients with HBV (as determined by serology), schistosomiasis, HIV, thyroid dysfunction, inflammatory diseases, and cardiovascular disorders. Also, HCC patients those were not linked to HCV were excluded. Subjects receiving any chemotherapy or radiotherapy, or had undergone a gastrointestinal surgical operation, any cancer other than HCC, patients with neuronal diseases, uterine diseases, kidney diseases, prolonged use of corticosteroids or sex hormones. Additionally, patients with incomplete data or

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incomplete histopathology diagnosis report. **Patients' Clinical and Pathological Features.** Patients' tumor clinical assessment was done at the Pathology Unit, Faculty of Medicine. An abdominal computed tomography (CT) scan was done by an expert, unaware of the study, the CT data were CT scan liver size and mass number from 1 to 4, incidence of cirrhosis with heterogenous mass or focal or multiple lesions and the portal vein whether occluded or thrombosed **[40]**. The International Ascites Club **[41]** classifies the severity classification of ascites as mild ascites (grade 1): ascites only detectable by ultrasound, moderate ascites (grade 2): moderate abdominal distention, and massive ascites (grade 3): marked large abdominal distention. Lymph node (LN) involvement with enlargement incidence either as no (N0) or present (N1), were recorded from patients' files. HCV is the G4 endemic in Egypt, confirmed by serology (data not shown). HCV G4 was confirmed in liver cirrhosis patients more than six months ago. According to recent standards, HCC diagnosis was histologically verified or based on specified imaging criteria **[42]** and only early diagnosed treatment naïve HCC patients were included.

4.4. Blood Samples. Six mLs of peripheral venous blood were withdrawn under strict sterile conditions following standard biosecurity and international safety procedures, from each participant and divided into two aliquots. Blood was withdrawn at the time patients were first diagnosed clinically with HCC and before any medical therapy or surgical intervention. The first 3 mL of blood was placed onto EDTA anticoagulant vacutainers for complete blood count (CBC) using (Sysmex KX-21, Japan) and flow cytometry (FC) assay. The second part of blood was transferred into polymer serum gel separator tube with a clot activator (Greiner Bio-One GmbH, Australia), left for 15 min. at room temperature 24°C to clot, followed by a 10-min. centrifugation at 10,000g at 4°C. Sera obtained were aliquoted into Eppendorf tubes and stored at −80°C until biochemical assessment.

4.5. Research setting. Clinical Pathology Department, Faculty of Medicine, Al-Azhar University, Cairo and the Advanced Biochemistry Research Lab (ABRL), Biochemistry Dept., Faculty of Pharmacy, Ain Shams University, Cairo, Abassia.

4.5.1 Biochemical testing.

Routine Biochemical testing. Liver function tests; alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), total and direct bilirubin, serum albumin. **Lipids profile** measurement; triacylglycerol (TAG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C). Finally, serum creatinine as **kidney function indicator**. Serum glucose determination. All were done using Cobas Integra 400 Plus, Roche Diagnostics, Germany. **serum alpha fetoprotein** (AFP) was done by the electrochemiluminescence immunoassay (ECLIA) using Cobas 6000 (e601 module), Roche Diagnostics, Germany. **INR** Coagulation assay using the automated coagulation analyzer Stago, France.

Serum Insulin assay by enzyme immunoassay for the quantitative determination of human s. insulin concentrations **[43].** Based on an enzyme-linked immunosorbent test (ELISA) in solid phase. One anti-insulin antibody is used in the assay method for solid phase em-mobilization (microtiter wells) and an added manually, second anti-insulin antibody is used in the antibody-enzyme (horseradish peroxidase) conjugate solution (100 µL). The Insulin antibody coated microtiter wells are filled with the standards and test sample (serum) (50 µL). The addition of 100 µL an anti-insulin antibody conjugated with horseradish peroxidase follows. If there is human insulin in the sample, it will react with the antibody on the well and the enzyme conjugate, sandwiching the molecules of insulin between the solid phase and the enzyme-linked antibodies. The wells are rinsed at least 3 times to eliminate unbound tagged antibodies following a one-hour incubation at room temperature 24°C. A 100 µL trimethyl benzidine solution is added, and after 20 minutes, the mixture is incubated, and the blue developed color is stopped by addition of stop solution (100 µL). Therefore, the formed yellow is spectrophotometrically quantified, within 15 minutes, at 450 nm using the

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Hyprep automated ELISA system (Hyperion Inc, Miami, FL). The test sample's color intensity is directly inversely related to its insulin concentration. Insulin normal adult range levels are 0-25 mU/L.

Ratios and Indices.

Weight in kg. and height in meter recorded for all participants for body mass index (BMI in kg/m^2) calculation according to; https://www.nhlbi.nih.gov/health/educational/lose_wt/BMI/bmicalc.htm (date July, 2022) 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

TAG/HDL-C ratio with cutoff value more than healthy control group average to be set diagnostic for insulin resistance (I.R) **[44].**
PLTs-to-lymphocytes ratio (PLR) as systematic inflammation biomarker and immune response related indicator, superior to neutrophiles-to-lymphocytes ratio for HCV infections as well as HCC for correlation with disease severity **[45].**
GGT-to-lymphocytes ratio (GLR) as prognostic for HCC with size less than 5 cm **[46].**
Insulin resistance is considered positive in obese, diabetic, dyslipidemic patients as well as having insulin levels of 18 or more mU/mL after glucose/meal, with disturbed PLR **[47, 48]**.

4.5.2. Flow Cytometry assay. FC was done for Tc and lymphocytes % quantification as well as LAIR-1 expression determination. FC was conducted using four color FACS Calibur (Biosciences Becton, Dickinson and Company, San Jose, California, USA). One tube was prepared for each patient, using 50 µg of fresh peripheral venous blood sample after adjustment of peripheral blood mononuclear cells (PBMCs)cells count ($1x10^6$) in each sample. Blood was incubated with 5 µg of each fluorochrome-conjugated antibodies anti-human IgG, fluorescein isothiocyanate (FITC) conjugated anti-human CD3 (BD Biosciences, San Jose, USA. cat. no. 555332) phycoerythrin (PE)-conjugated anti-human CD LAIR-1 (BD Biosciences, San Jose, USA. cat. no. 550811, lot no. 5329747) and peridinin chlorophyll protein complex (PerCP)-conjugated anti-human CD8 (Analysis, Thermofisher USA, cat. no. MA1-19793) for identification of Tc cells expressing LAIR-1. Compensation setting was established before acquiring samples using color calibrate beads (BD, Biosciences, San Jose, USA, lot no. 5093879).

Gating strategy (Antibody testing strategy): Initial gating by typical forward and sideways scatter on mature lymphocytes expected area, then Tc cells will be evaluated as percentage of total lymphocytes, according to the surface marker expression as $CD3^+CD8^+$. Then percentage of Tc expressing LAIR-1 and LAIR-1 mean fluorescence intensity (MFI) were detected on single histogram from $CD3^+CD8^+$ co-expressing population. MFI of positive population was detected on area under M1 marker (Figure 2).

4.6. **Statistical analysis.** Data collected were coded and analyzed using the Statistical Package for Social Science software (SPSS, Version 17, Chicago, IL). Qualitative data are presented as frequencies (n) and percentages (%). Test for data normality using Shapiro-Wilk calculator https://www.statskingdom.com/shapiro-wilk-test-calculator.htmL (date July, 2022), where normally distributed variables are to be expressed as mean $\pm$ S.D and analyzed using two samples independent Students' *t*-test or ANOVA for comparison of 2 or more groups, respectively. Data to be presented as median (interquartile range as 1^{st}-3^{rd} quartiles or 25^{th}-75^{th} quartiles) if not normally distributed, then Mann-Whitney (U) or Kruskal-Wallis (H) will be conducted to compare between any two or more independent groups, respectively. Kruskal Wallis post hoc was conducted to determine which pairs of groups differ significantly. 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. Point-

biserial correlations was used to determine the correlation between parameters when one of them was dichotomous variable. Receiver operating characteristic (ROC) curve was performed to detect the best cutoff, 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 calculated values would give an idea of how well the discriminating ability of LAIR-1+Tc % and/or LAIR-1 MFI on Tc to differentiate post-HCV G4-HCC cases from post-HCV G4-liver cirrhosis cases. Level of significance was set at p-value < 0.05, confidence level or interval (CI) as 95% and 5%, respectively.

5. Conclusions

Expression of LAIR-1 is significantly upregulated on circulating T cytotoxic cells in post-HCV G4-HCC when compared to liver cirrhosis. LAIR-1 expression on Tc comes second to AFP sensitivity and specificity as a potential screening molecular marker for HCC post-HCV. However, sensitivity of LAIR-1 expression as a non-invasive molecular marker in liquid biopsy is accepted for detection of HCC transformation from liver cirrhosis post-HCV G4 infection which opens the door for better screening of AFP-negative-HCC transformation. LAIR-1 expression on Tc is linked to I.R and inflammation in the context of HCC prognosis post-HCV G4.

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

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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.

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