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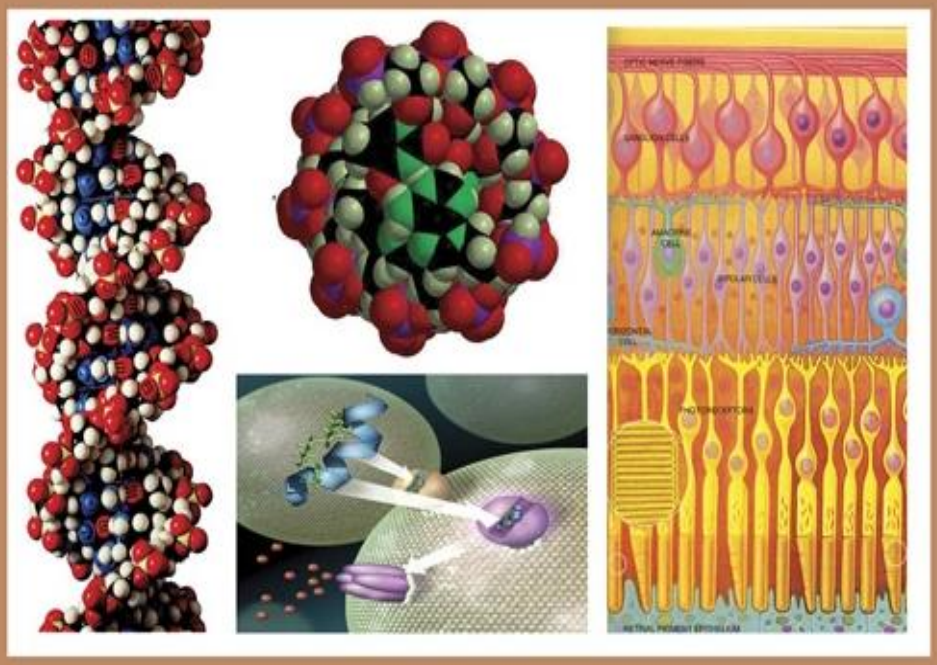

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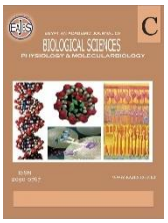

Significance of MicroRNA-200a and Long Noncoding RNA-HULC Expression in HCV-Associated Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related deaths worldwide. Hepatitis C virus (HCV) infection chronicity is a major risk factor for HCC in Egypt. Early diagnosis of HCC is of great clinical desirability since it promises a virtuous prognosis if the patient could get early specific treatment. Many microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) proved to be implicated in pathogenesis of several diseases, including HCC as they are known as key regulators of gene expression. In this work, we aimed to investigate the expression level of microRNA-200a, and lncRNA- HULC in sera of HCV-associated HCC Egyptian patients compared to chronic HCV patients and apparently healthy subjects. Ninety-nine subjects were included in this work, grouped in: HCC-on top of HCV- group, chronic HCV group and control group. Genetic expression levels of both miR-200a and lncRNA-HULC were assessed in sera of all subjects using real time PCR technique. Both miR-200a and HULC expression levels were significantly changed in chronic HCV and HCC groups compared to the control group ($P< 0.001$), with a significant change in both between the HCV and HCC groups ($P< 0.001$). Inverse significant correlation was found between miR-200a and HULC. Diagnosing potentiality of both for HCC was tested by receiver operating characteristic (ROC) curve analysis. miR-200a was more specific, while HULC was more sensitive in predicting HCC in chronic HCV cohort. Combining raised the sensitivity to 80.6% with 66.7% specificity and raised the significance of the test to the ultimate ($P< 0.001$). We conclude that miR-200a and HULC may play a role in the pathogenesis of HCC and seems to act as promising molecular biomarkers for early diagnosis of HCC in chronic HCV patients, especially if combined.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the world's second-leading cause of cancer-related deaths (De Toni *et al.*, 2020). Mortality due to HCC is higher, particularly in developing countries, where hepatitis B virus (HBV) and hepatitis C virus (HCV) have been related to HCC such as Asian and African countries (Shaker *et al*., 2020). It is worth noting that HCC accounts for 13% of all malignancies in Egypt and is the second most common disease in Egyptian men.

Egypt has a unique situation as one of the highest HCV-prevalence in the world, which has been attributed to previous mass use of tartar emetic to eradicate schistosomiasis, that lead to a twofold increase in HCC amongst chronic liver patients (Abdel-Aziz *et al*., 2016). Recently, Chronic HCV accounted for 94% of HCC cases in Egypt, with 6,000–7,000 fatalities per year owing to HCC (Abd-Elkader *et al*., 2020).

The commonly used biomarker for detecting primary HCC is alpha-fetoprotein (AFP), but it was proved that its sensitivity and specificity are not always sufficient for accurate diagnosis. Research is ongoing to identify and validate new biomarkers that can be used for non-invasive diagnosis, including imaging techniques and blood-based tests. These efforts could lead to earlier detection and better outcomes for patients with HCC (Moustafa *et al*., 2020).

Liver diseases such as HCV infection and cirrhosis- which are major risk factors for HCC- are associated with changes in the expression of various noncoding RNAs (ncRNAs) including microRNAs (miRs) and long noncoding RNAs (lncR) (Unfried and Fortes, 2020). miRNAs (18-22 nucleotides) are widely distributed in the human body and may be effective in the early detection of HCC (Abd-Elkader *et al*., 2020). LncRNAs are composed of more than 200 nucleotides and have different roles in gene regulation at the transcriptional and post-transcriptional levels in the cell (Fatica and Bozzoni, 2014).

HULC (highly upregulated in liver cancer) is a lncRNA, localized on chromosome 6p24.3, conserved in primates, and excessively expressed in HCC. It has been identified as an oncogenic factor and its overexpression has been detected in several other human cancers than HCC as well (Shaker *et al*., 2020). Transcription of HULC yields approximately 500 nucleotides in length and contains two exons, spliced and polyadenylated ncRNA localized in the cytoplasm. It has been reported to be associated with ribosomes. Its transcribed RNA lacks a considerable open-reading frame and does not give rise to any protein (Yu *et al*., 2017). HULC can competitively contribute in regulating the expression of miR-200 downstream target molecules and plays a key role in the progression of HCC.

Several reports have revealed the significant association of miR-200a with hepatocarcinogenesis (Becker *et al*., 2015; Mao *et al*., 2021). It is a complex molecule with potentially contrasting roles in HCV and HCC. On one hand, it may be necessary for efficient HCV replication and can be induced by HCV infection. On the other hand, it is believed to play a role in tumor suppression by inhibiting epithelial-mesenchymal transition (EMT), the initiating step of metastasis (Korpa*l et al*., 2008). It was suggested that HULC enhanced EMT by sequestering miR-200a-3p in HCC cells, where HULC depletion inhibited the growth and metastasis of HCC cell lines *in vitro* (Li *et al*., 2016).

Hence, we chose to investigate the expression level of "miR-200a" and lncRNA "HULC" in sera of HCC-on top of HCV-patients compared to chronic HCV patients and to examine their use as early biomolecular diagnostic tools for HCC.

MATERIALS AND METHODS

Subject Selection*:*

Ninety-nine Egyptian adult subjects were included in this case-control study, gathered from "The Tropical Department" at Kasr-Alainy Hospital and "Blood Bank" of the same hospital. They were segregated into: Group I: which included 30 healthy volunteers serving as control group, Group II: included 33 fibrotic-chronically infected HCV patients, and Group III: included 36 HCC patients on top of HCV chronically infected patients. The study was performed with the approval of Faculty of Medicine, Cairo University's local ethics committee and carried out in compliance with the Helsinki Declaration [2008].

Informed consent was obtained from

all the subjects enrolled in this study. Inclusion criteria included patients aged above 18 years and male and female genders. Exclusion criteria included any cancer other than HCC, patients who were to receive chemotherapy or radiation. Patients with cardiovascular disease, nervous system, pulmonary, renal, and endocrine or gastrointestinal disorders were also excluded.

2. Sample Collection*:*

Venous blood samples were withdrawn from subjects by professional laboratory technicians and then aliquoted. One aliquot was left to separate serum and used for routine laboratory investigations such as liver function test ~~tests~~ (LFT), alfa fetoprotein (AFP), and markers for hepatitis. Another aliquot was taken on ~~an~~ EDTA-vacutainer tube for ~~a~~ complete blood count (CBC). A third part was centrifuged, and serum was more aliquoted and stored at -80^0C until RNA extraction.

3. Total RNA Extraction, Quantitation and Purity Assessment of RNA:

miRNeasy mini kit was used for ~~the~~ purification of serum total RNA, including miRNA and long noncoding RNA (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Then, Nano Drop® (ND)-1000 spectrophotometer (Nano Drop Technologies, Inc. Wilmington, USA) was used to test the RNA quantitation and purity.

4. Reverse Transcription (RT) for RNA:

Complementary DNA (cDNA) synthesis of RNA was accomplished by RT reaction. A total RNA volume of 20 uL was used for the miScript II RT kit (Qiagen, Valencia, CA, USA) as instructed by the manufacturer's protocol.

5. Detecting miRNA-200a by Quantitative Real-Time PCR (qPCR):

miScript SYBR® Green PCR kit and protocol were used for detecting mature miRNAs quantitative (Qiagen, Valencia, CA- USA), and small nucleolar RNA C/D box 68 (SNORD 68) was used to normalize the expression pattern and for relative quantification of the target miRNAs (Moustafa *et al*., 2020). Reaction mixture of 25 µL ~~reaction~~ volume/well was prepared as follows: 2× QuantiTect SYBR Green PCR Master Mix (12.5 µL), 10× miScript Universal Primer (2.5 µL), 10× miScript Primer Assay for miR-200a or SNORD68 (2.5 µL), RNase-free water (≤5 µL), and template cDNA (2.5 µL).

Rotor-gene thermocycler (Qiagen-USA) was programmed as follows: initial activation step for 15 minutes (min.) at 95°C, then for 40 cycles, a three-step cycling: denaturation step for 15 seconds (s) at 94°C, annealing step for 30s at 55°C and finally an extension step for 30s at 70°C. Afterwards, melting curve analysis was performed to validate the specific generation of the expected PCR product: [95°C for 15s, 60°C for 1 min. and 95°C for 15s].

6. qPCR for Detection of Long Non-Coding HULC*:*

Serum expression level of HULC was evaluated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control using ready-made primer for HULC and customized primer for GAPDH. SYBR Green PCR kit (Thermo, USA) was used according to the manufacturer's protocol. The primer sequences for GAPDH were as follows: GAPDH-forward is 5′-CCCTTCATTGA CCTCAACTA-3′, and GAPDH-reverse is 5′-TGGAAGATGGTGATGGGATT-3′. Real-time PCR was done on 25 µl reaction mixture as instructed by the manufacturer, using Rotor gene Q System (Qiagen) with the following conditions: 95°C for 15 min as initial activation step, followed by 40 cycles: at 94°C for 15s, 55°C for 30s, and 70°C for 30s. Melting curve analysis was performed after that too, as mentioned previously.

7. Statistical Analysis*:*

After coding, data were entered using the statistical package SPSS (Statistical Package for the Social Sciences) *version* 17. Data were summarized using mean± standard deviation (M±SD) in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-

Wallis and Mann-Whitney tests. For comparing categorical data, the Chi square (X^2) test was performed.

Correlations between quantitative variables were done using Pearson correlation coefficient. Regression analysis was done to detect the predictor of HCC. Finally, Receiver operating characteristic (ROC) curve analysis was performed to detect the best cutoff values of miR-200a and HULC, specificity and sensitivity for detecting HCC. *P*-values <0.05 was considered significant.

RESULTS

Demographic and Disease History of the Studied Groups: The following Table (1) illustrates the demographic data; the mean age of HCC patients is significantly higher than control and HCV groups ($P< 0.001$). There was no significant change between the three groups regarding gender.

Table 1: Demographic data and disease history of the studied groups.

Clinical Data	Control (n=30)	HCV (n=33)	HCC (n=36)	*P*-value
Age (years)	27.07±7.3	40.48±8.27	60.42±6.99	<0.001[a] <0.001[b] <0.001[c]
Gender: Female Male	 11(36.67%) 19(63.33%)	 10(30.30%) 23(69.70%)	 5(13.89%) 31(86.11%)	N.S.
DM in HCV group: Yes No	 ----------	 15(45.45%) 18(54.55)	 ---------	N.S.
HTN in HCV group: Yes No	----------	6(18.18%) 27(81.82%)	---------	<0.01
Quant. HCV-RNA in HCV group:	----------	103005.53±714.25	----------	--------
DM in HCC group: Yes No	 ---------	 ----------	8 (22.22%) 28 (77.78%)	<0.01
HTN in HCC group: Yes No	 ---------	 ----------	19 (52.78%) 17 (47.22%)	N.S.

Age is expressed as M±SD. Gender is presented by number (percentage); DM= diabetes mellites; HTN= hypertension; Quant.= quantitative; N.S.= nonsignificant; [a]= significance between HCV and HCC, [b]= significance between HCV and control, [c]= significance between HCC and control.

Clinicopathological Characteristics of the Diseased Groups: Clinicopathological characteristics of our HCV studied group are illustrated in the following Table (2) as frequencies and percentages. Out of the 33 HCV patients, 3 of them had an A3 stage of liver activity, and 3 of them suffered from F3 fibrotic stage.

Table 2: Clinicopathological features of the HCV studied group.

Parameter	n (%)	*P*
Liver Activity		
A1	28(84.84%)	<0.001[a]
A2	2(6.0%)	<0.05[b]
A3	3(9.16%)	<0.001[c]
Liver Fibrosis		
F1	25(75.75%)	<0.001[a]
F2	5(15.09%)	<0.05[b]
F3	3(9.16%)	<0.001[c]

N.S.= non-significant; [a]= significance between A1 and A2, [b]= significance between A2 and A3, [c]= significance between A1 and A3.

Clinicopathological features of our HCC studied group are illustrated in the following Table (3) as frequencies and percentages too. Out of the 36 HCC patients, 11 of them had ascites, 7 had thrombosed portal vein, 15 had multiple focal lesions, and 29 had >5 cm^3 -focal size lesions.

Table 3: Clinicopathological features of the HCC studied group.

Parameter	n (%)	*P*
Ascites:		
Yes	11 (30.55%)	<0.01
No	25 (69.44%)	
Portal vein:		
Patent	24(66.67%)	<0.01
Thrombosed	7(19.44%)	
Nº of focal lesions:		
Single	21 (58.33%)	<0.05
Multiple	15 (41.66%)	
Focal size:		
<5cm^3	7 (19.44%)	<0.01
>5cm^3	29 (52.77%)	

Clinical Analyses of The Studied Groups:

Biochemical and haematological investigations of the three studied groups are illustrated in Table (4). A significant difference in serum albumin concentration was noticed between HCC and HCV groups (*P*<0.05). Meanwhile, haemoglobin (Hgb) and platelets were significantly decreased in HCC group than HCV group (*P*<0.05 and *P*<0.001), respectively. There was also a significant decrease in international normalized ratio (INR) in the HCC group than both control and HCV groups (*P*<0.05.

Table 4: Clinical laboratory analyses of all the studied groups.

Clinical Data		Control (n=30)	HCV (n=33)	HCC (n=36)	*P*-value
ALT	(U/L)	12.82±4.32	60.42±41.02	75.83±44.39	<0.05[a] <0.001[b] <0.001[c]
AST	(U/L)	15.27±5.54	47.12±34.28	108.78±62.95	<0.001[a] <0.01[b] <0.001[c]
Total Bil.	(mg/dL)	0.49±0.90	0.82±0.19	1.82±0.30	<0.01[a] <0.05[b] <0.001[c]
Direct Bil.	(mg/dL)	0.21±0.09	0.63±0.16	0.99±0.21	<0.01[a] <0.05[b] <0.001[c]
Albumin	(gm/dL)	4.58±0.26	4.09±0.36	3.52±1.11	<0.01[a] N.S.[b] <0.01[c]
AFP	(ng/dL)	4.37±2.38	15.18±8.70	719.10±859.26	<0.001[a] <0.001[b] <0.001[c]
Hgb	(gm/dL)	12.48±1.33	14.21±1.35	11.34±2.35	<0.05[a] <0.05[b] <0.05[c]
TLC	($x10^3$)/mm^3	6.22±1.41	6.59±1.90	5.42±2.11	N.S.[a] N.S.[b] N.S.[c]
Plts	($x10^3$)/mm^3	296.67±61.85	230.58±63.24	119.86±76.32	<0.001[a] <0.001[b] <0.001[c]
INR		1.003±0.03	1.104±0.09	1.38±0.41	<0.001[a] N.S.[b] <0.001[c]

Data are expressed as M±SD, ALT= alanine transaminase, AST= aspartate transaminase, Bil.= bilirubin, AFP= alfa fetoprotein; Hgb= haemoglobin; TLC= total leucocytic count; Plts= platelets; INR= international normalized ratio; N.S.= nonsignificant; *P*<0.05= significant; *P*<0.01= highly significant; *P*<0.001= very highly significant; [a]= significance between HCV and HCC, [b]= significance between HCV and control, [c]= significance between HCC and control.

Expression Levels miR-200a and HULC in the Studied Groups:

miR-200a expression levels were significantly decreased in HCC and HCV groups compared to control group, and there was a significant fold change decrease in its levels between HCC and HCV groups too. HULC showed a highly significant increase in its fold change (FC) from control to HCC group, with its highest expression level in the HCC group as illustrated in Table (5).

Table 5: Expression level of miR-200a and HULC in the studied groups.

Parameter	Control (n=30)	HCV (n=33)	HCC (n=36)	*P*
miR-200a (FC)	1.008±0.15	0.71±0.06	0.46±0.06	<0.01[a] <0.001[b] <0.001[c]
HULC (FC)	1.005±0.05	7.14±1.55	29.01±5.27	<0.001[a] <0.001[b] <0.001[c]

Data are expressed as M±SD; *P*< 0.01= highly significant; *P*< 0.001= very highly significant; [a]= significance between HCV and HCC, [b]= significance between HCV and control, [c]= significance between HCC and control.

Implication of Liver Activity Status on miR-200a and HULC Expression Levels in the HCV Group: expression level of miR-200a, and HULC were compared in different stages of liver activity as shown in the following Table (6). It seems that liver activity of the HCV patient had effect only on the expression level of HULC.

Table 6: Expression level of serum miR-200a, HULC in different liver activity stages of the HCV group.

Biomarkers	A1 (n=28)	A2 (n=2)	A3 (n=3)	*P*
miR-200a	0.69±0.07	0.79±0.015	0.86±0.09	N.S.[a] N.S.[b] N.S.[c]
HULC	7.48±1.78	1.35±1.24	7.32±1.08	<0.05[a] N.S.[b] <0.05[c]

Values are expressed as M±SD; N.S.= nonsignificant; [a]= significance between A1 and A2; [b]= significance between A1 and A3; [c]= significance between A2 and A3.

Implication of fibrotic status on miR-200a and HULC expression levels in the HCV Group: Our HCV patients were fibrotic, who had F1, F2, or F3 liver fibrosis, but not F4. Only HULC had responded to the liver fibrotic stage in HCV group as shown in Table (7). As there was a significant difference in expression level of HULC between F1 and F2, and between F3 and F2. The expression level of HULC in each of F1 and F3 was almost the same.

Table (7): Expression level of serum miR-200a, HULC in various liver fibrosis stages in HCV group.

Biomarkers	F1 (n=25)	F2 (n=5)	F3 (n=3)	*P*
miR-200a	0.722±0.07	0.576±0.11	0.86±0.09	N.S.[a] N.S.[b] N.S.[c]
HULC	7.94±1.97	2.75±9.81	7.78±3.72	<0.01[a] N.S.[b] <0.05[c]

Values are M±SD; N.S.= nonsignificant; [a]= significance between F1 and F2; [b]= significance between F1 and F3; [c]= significance between F2 and F3.

Correlations of miR-200a, and HULC with the Clinical Parameters: Pearson's correlation was performed to examine correlations between each of miR-200a, HULC and various estimated biochemical and hematological laboratory parameters, as shown in Table (8). As expected, miR-200a and HULC were inversely correlated with each other. miR-200a and HULC were not correlated with AFP. Additionally, there was non-significant correlation between miR-200a and HCV-viral load in the non-cancerous HCV group.

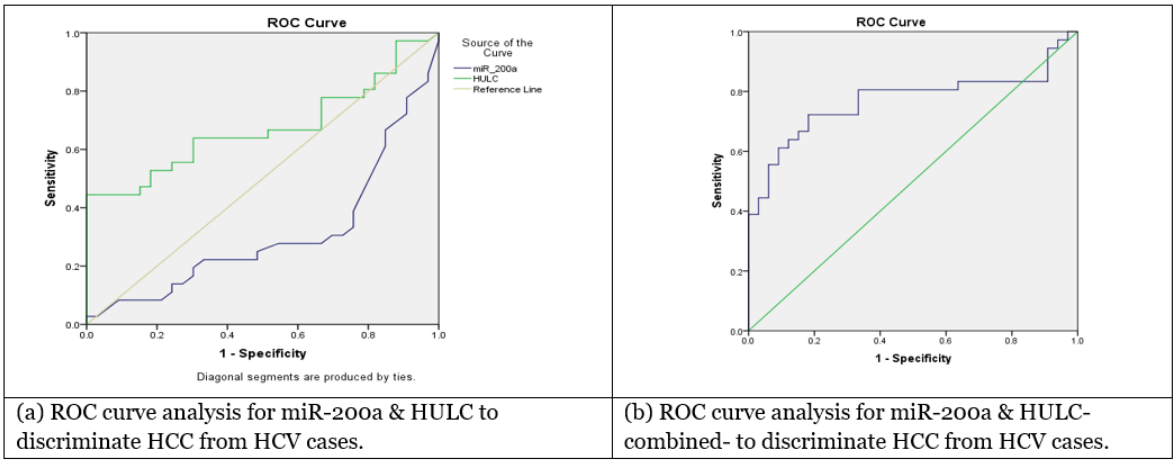
Table (8): Pearson's correlation of miRNA-200a, HULC with clinical parameters through HCC group.

Parameter	miR-200a		HULC	
	r	*P*	*r*	*P*
ALT	-0.372	<0.01	0.173	N.S.
AST	-0.444	<0.01	0.263	<0.01
Alb.	0.260	<0.01	-0.057	N.S.
Total Bil.	-0.352	<0.01	0.630	N.S.
TLC	-0.147	N.S.	0.068	N.S.
Hgb	0.160	N.S.	0.047	N.S.
Platelets	0.424	<0.01	-0.477	<0.01
INR	-0.413	<0.01	0.223	<0.05
AFP	-0.181	N.S.	0.098	N.S.
miR-200a	1	-------	-0.375	<0.01

r= correlation; *P* = significance; N.S.= non-significant, *P*< 0.05= significant; *P*< 0.01= highly significant.

Correlations of miR-200a, and HULC with the Clinicopathological Features in HCC Group: Spearman's correlation was conducted to examine correlation between each of the HULC, miR-200a and clinicopathological features of the HCC group. HULC expression is non-significantly correlated to neither ascites, number of focal lesions, size of focal lesion, or else status of the portal vein. Meanwhile, miR-200a expression level was inversely correlated with both ascites presence (*r*=-0.285, *P*< 0.01) and size of focal lesion >5cm (*r*=-0.381, *P*< 0.05).

Diagnostic Performance of miR-200a and HULC for HCC: Diagnostic potentialities of miR-200a and HULC to diagnose HCC- in chronic HCV cohort- were evaluated by ROC curve analysis (Fig. 1a and Table 9). Combining both molecular biomarkers raised AUC to be 0.769 as shown in Figure (1b) and Table (9), with increase in the significance of the test to the ultimate.



(a) ROC curve analysis for miR-200a & HULC to discriminate HCC from HCV cases.

(b) ROC curve analysis for miR-200a & HULC-combined- to discriminate HCC from HCV cases.

Fig. 1: ROC curve for miR-200a and HULC to diagnose HCC (a) segregated, and (b) combined.

Table (9): Diagnostic performances of miR-200a and HULC for HCC in chronic HCV cohort.

Biomarker	AUC	Cut-off value	Sensitivity	Specificity	95% C.I.	*P*
miR-200a (FC)	0.31	<0.825	22.2%	66.7%	.180 to .435	<0.01
HULC (FC)	0.67	>2.58	63.9%	48.5%	.542 to .804	<0.05
Combined (FC)	0.77	0.432	80.6%	66.7%	.650-.888	<0.001

FC= fold change; AUC= area under curve; C.I.= confidence interval; *P*<0.05= significant; *P*<0.01= highly significant; *P* <0.001= very highly significant

DISCUSSION

With lacking specific biomarkers, early diagnosis of HCC is still difficult although breakthrough progress has been gotten in the pathogenesis of HCC in the last few years (Mao *et al*., 2021). The goal of many researchers is to find non-invasive tools for the diagnosis of HCC which is the most common type of liver malignancy (Piñero *et al*., 2020). So, we chose to investigate these two noncoding RNA members: miR-200a and HULC in HCC and chronic HCV patients (noncancerous yet) to examine their use as molecular biomarkers for early diagnosis of HCV- associated HCC in chronic HCV society.

Statistical analysis of this case-control study revealed a significant difference in age (*P*<0.001) between studied groups, as HCC distresses old patients over chronic HCV while gender was non-significant (*P*>0.05) despite male preponderance in all studied groups. These findings agreed with another study mentioned that the age-dependent patterns in the sex difference- concerning the prevalence of hepatocellular carcinoma- support the hypothesis of a protective role of estrogen. The underlying reasons for the sex and age difference in hepatocellular carcinoma remain to be further explored in analytic epidemiological studies (Abd-Elkader *et al*., 2020).

As expected, ALT, AST, total and direct bilirubin, were significantly elevated in the HCC group than the HCV group and the control group. There was also a significant increase in them in the chronic HCV than control group. Serum albumin showed the most decreased level in the HCC group due to the devastating synthetic ability of the liver during liver disease progression to hepatocarcinoma, while there was no significant change in it between HCV and control group. These results were in concordance with other recent study (Moustafa *et al*., 2020).

Dramatic elevation in AFP was

revealed in the HCC group compared to the HCV group with marked significant variances (Gopal *et al*., 2014). Simultaneously, no significant change was noticed between chronic HCV group and control group concerning AFP as Isac and colleagues mentioned previously (2021). Anaemia and thrombocytopenia were two characteristics of our HCC group; Hgb concentration and platelet count were significantly decreased than the HCV group. These results were in harmony with other studies too (Knight *et al*., 2004; Axley *et al*., 2018).

Our results revealed that the expression level of miR-200a is significantly decreased in both HCV and HCC patients compared to the control group. Moreover, the expression of it is lower in the HCC group than that in the HCV group. These results are consistent with previous studies (Lin *et al*., 2014; Chen *et al*., 2017), which informed reduced miR-200a expression in HCC tissues compared to adjacent non-cancerous tissues.

The downregulation of miR-200a in HCC could be attributed to its role as a tumor suppressor gene, where it has been shown to inhibit cancer cell proliferation and migration by targeting genes involved in these processes. The reduced expression of miRNA-200a in HCC could therefore contribute to the development and progression of the disease. Moreover, it was reported that patients with low expression of miR-200a have a worse prognosis than those with high expression of miR-200a, as there remained increased cumulative recurrence rates and reduced survival rates at 1, 3 and 5 years (Feng *et al*., 2015). These findings support the idea that miR-200a may be a promising target for the development of new diagnostic and therapeutic strategies for HCC. In contrast, this result differs from that of (Banaudha *et al*., 2011), who found that miR-200a expression was increased with HCV infection. This discrepancy may be due to differences in the HCV genotype studied, as they used a different genotype of HCV than that predominant in Egypt (HCV genotype 4a).

In the present work, there was also a significant increased HULC expression level in HCC group than chronic HCV group. This finding is consistent with the results reported by (Yu *et al*., 2017). The former study also measured HULC expression level in HCC tissues and adjacent non-cancerous tissues using real-time PCR and found that HULC expression was higher in cancerous tissues (Heffernan-Stroud and Obeid, 2013).

Abnormal results of lipid metabolism in HCC cells have been linked to increased HULC expression, that may be an explanation for our results; accumulating evidence suggested that regulation of sphingolipid levels by sphingosine kinase 1 (SK1) played a crucial role in carcinogenesis; sphingolipids are important bioactive molecules that signal cell proliferation (Cui *et al*., 2015). Lu and colleagues (2016) improved this theory, as they found that HULC levels were positively correlated with levels of SK1 and its product, sphingosine-1-phosphate, in HCC. Importantly, knockdown of SK1 was found to retract HULC-enhanced angiogenesis (Yu *et al*., 2017). Additionally, HULC has been shown to downregulate p18, which accelerates the growth of hepatoma cells (Du *et al*., 2012).

HULC was found to interact with miR-200 members and plays a key role in the progression of HCC (Li *et al*., 2016). It was shown to affect miR-200a targets and interfere with miR-200a mediated inhibition of Zinc finger E-box binding homeobox 1 (ZEB1)- which is a prime element of a network of transcription factors controlling EMT and has been identified as an important molecule in the regulation of DNA damage, cancer cell differentiation, and metastasis- leading to differentiation from an epithelial to mesenchymal transition (Ashrafizadeh *et al*., 2020; Drápela *et al*., 2020). This interaction between HULC and miR-200 members may play a key role in the progression of HCC.

We examined the effect of various liver activity of the chronic HCV group- on

the expression levels of miR-200a and HULC, we found that HULC is the only parameter response to liver activity (P<0.05). HULC also seemed to respond to the change in liver fibrosis stage. Concerning chronic HCV group, there was nonsignificant correlation between miR-200a and HCV-RNA quantity. This particular finding was consistent with a result of ~~a~~ previous study conducted by Joshi *et al*. (2013), who found a nonsignificant positive correlation between estimated HCV viral load in HCV patients and miR-200a.

Based on our results, it appears that there was an inverse correlation was observed between miR-200a and HULC (P<0.01), which is consistent with previous research cited by (Li *et al*., 2016). It seems that miR-200a can negatively regulate HULC expression through silencing, which could explain the observed inverse correlation (Mao *et al*., 2021).

Interestingly, we found that miR-200a had stronger correlations with most of analysed clinical parameters- as ALT, AST, Alb., bilirubin, platelets, and INR- than HULC. This suggests that miR-200a and HULC may serve as complementary biomarkers to reflect the progression or deterioration of HCC in patients, with miRNA-200a potentially providing more information about the patient's overall health status. A preceding conducted study (Shaker *et al*. 2020) reported that there was a significant negative correlation between the level of HULC and haemoglobin, PLT, and albumin levels, while a significant positive correlation with AST and ALT levels was found, confirming that HULC can be used as a marker for diagnosing HCC.

Surprisingly, the present study found that both miR-200a and HULC were not correlated with AFP, which may be attributed to the shortage of number of samples. Meanwhile, miR-200a was inversely correlated with the presence of ascites and the size of focal lesions, while HULC did not show such correlation with any of the associated clinicopathological features. These findings are consistent with previous study made by (Mongroo and Rustgi, 2010), who reported a significant association between miR-200a expression and tumor size in HCC. However, ~~these are~~ in contrary to this result, Jeddi *et al*. (2019) found no association between miR-200a expression and tumor size in gastric cancer, but this discrepancy may be attributed to change in tumor type.

The diagnostic performance of a test is measured by the area under the receiver operating characteristic (ROC) curve. Area under the curve (AUC) ~~of~~ more than 50% gives an acceptable performance, and AUC of about 100% is the best performance for the test [~~???~~]. HULC was more sensitive than miR-200a and the latter was more specific than the HULC in this context (table: 10). A preceding study (Xie *et al*., 2013) suggested that HULC has adequate sensitivity and specificity to discriminate between HCC and control samples. This differs than our results as we discriminate HCC from chronic HCV cohort. When we combined both, their augmentation effect increased the potentiality of the test; AUC raised to 0.77 with raised sensitivity to reach 80.60%, at 66.7% specificity, and increased significance to the ultimate (P<0.001).

Overall, the expression of miR-200a and HULC in HCC and HCV patients is an active area of research, and further studies are needed to fully understand their roles in the pathogenesis and progression of these diseases, especially with respect to different HCV genotypes. Additionally, these findings suggest that HULC may play a complex role in the development and progression of HCC, potentially through its interactions with miR-200a and its effects on various cellular pathways.

CONCLUSION

Regardless of limitations in the present work, it demonstrates that changes in expression level of miR-200a and HULC may contribute to the pathogenesis of HCC on top of chronic HCV infection. These molecular biomarkers may be nominated as complementary tools with AFP for early HCC diagnosis. Further studies are needed to

validate the clinical utility of miR-200a and HULC as diagnostic markers for HCC in larger HCV patients' cohort.

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