MicroRNA-15a expression level and its target "c-Myb" expression in Hepatocellular carcinoma Among Chronic HCV Egyptian Patients.

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ABSTRACT

Egypt is experiencing an epidemic hepatocellular carcinoma (HCC) due to widespread of hepatitis C virus (HCV) transmission. Aim: To explore the expression level of microRNA-15a (miR-15a) and its target "c-Myb" in Egyptian HCV- related HCC patients. 20 newly diagnosed HCC cases of different stages, 20 chronic HCV cases, and 20 apparently healthy subjects acted as controls were tested for circulating miR-15a and c-Myb level in serum. Real-time PCR was performed to assess miR-15a expression level in serum of all samples along with liver function tests. ELISA technique was applied for quantification of AFP and c-Myb. miR-15a was upregulated with reduced expression level of its target "c-Myb" through progression to HCC. Receiver operating characteristic (ROC) curve study showed that the best cutoff value of miR-15a for HCC diagnosis was ≥0.49-fold change (FC) with a sensitivity of 61% and specificity of 89%, which surplus AFP at cut-off value of ≥200 ng/mL, with 50% sensitivity and 84% specificity, respectively. Increased expression level of miR-15a in HCC cases implicate its contribution to HCC growth and spread by at least affecting c-Myb pathway, and hence may be used as a diagnostic biomarker for early diagnosis of HCC.

Key words: Hepatocellular carcinoma, miRNA-15 a, c-Myb, early diagnosis.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) represents about 90% of primary liver cancers and constitutes a major global health problem [European Association, 2018]. The burden of HCC has been increasing in Egypt, with a doubling in the incidence rate in the past 10 years [Ibrahim et al., 2018]. This may be attributed to high prevalence of hepatitis C virus (HCV) [Atti, 2015], which induces cirrhosis and thereby HCC risk [Ma et al., 2018]. Increasing the survival rate of cirrhotic patients that increases the chance of developing HCC [Tarao et al., 2019]. On the other hand, low survival rate of HCC patients is attributed to late diagnosis, tumor recurrence and metastasis [Zeeneldin et al., 2015].

Development of HCC is very complex and occurs through a multistep biological process of malignant transformation of normal hepatocytes in which various factors, including genetic and epigenetic alterations [Yoon, 2018]. Recently, it has been demonstrated that microRNAs

(miRNAs) exhibit tissue- and disease-specific patterns in human cancers [Mao and Wang, 2015]. Even, some miRNAs have been recognized to participate in tumor invasion, metastasis, and angiogenesis [Mourad et al., 2018]. MiRNAs are kind of small, non-coding RNAs that regulate specific mRNAs expression by inhibiting the translation or degradation of mRNA. According to Calin's report, more than 50% of miRNAs are located in cancer-associated genomic act points. These miRNAs can function act as tumor suppressor actors or promoter factors depending on how these target genes are regulated by the miRNA. MiRNA-15a (miR-15a) has been demonstrated suppressor factor to act as a cancer suppress actor in multiple kinds of cancer. It was hypothesized that co-transcription factor c-Myb expression may be downregulated by miRs such as miR-15a in HCC [Liu et al., 2017]. So, this case-control study aimed to evaluate the circulating miR-15a expression level and its target protein "c-Myb" in Egyptian patients with CHC- related HCC, and to explore their potential use as novel non-invasive biomarkers for early diagnosis of HCC.

2. SUBJECTS & METHODS

During the period between October 2017 and December 2018 serum samples were collected from the patients attending outpatient's clinic of the "Liver Unit", Tropical Medicine Department at Kasr-Eleiny Hospital, Faculty of Medicine, Cairo University, Egypt: 20 patients affected with chronic HCV alone (CHC) and proved to be only fibrotic, and 20 patients affected with HCV-related HCC. Serum samples were also collected from 20 age and gender-matched healthy volunteers (normal biochemical and virologic investigations) from blood bank attendee in the same hospital. All subjects were recruited after a written informed consent and the study protocol was approved by the ethics review committee of Cairo University hospital. Exclusion criteria included: patients with

chronic HBV infection or any other identifiable cause for chronic hepatitis other than HCV, co-infection with other than HCV, renal diseases and any associated malignancies other than HCC.

2.1 RNA Extraction

For the real-time PCR, RNA was extracted from serum using TRIzol according to the manufacturer's instruction. The RNA purity was assessed by the RNA concentration and quantified by NanoDrop ND-1000 (Nanodrop, United States). Single-stranded cDNA was generated using the RT kit (Qiagen, Valencia, CA, United States)according to the manufacturer's directions, miScript miRNA PCR system: miRNeasy mini kit for miRNA extraction, miScript RT II for miRNA reverse transcription, miScript Primer Assay and miScript SYBR Green PCR Kit for PCR amplification.

2.2 RNA quantification

PCR quantification experiment was performed with PCR (Applied Biosystems; Foster City, CA) using the SYBR Green PCR Master Mix according to the manufacturer's protocol. The housekeeping miRNA SNORD68 was used as an endogenous control. The primer for miR-15a and housekeeping gene were supplied by Qiagen, Germany. Fluorescence measurement was made in every cycle and the cycling conditions used were: 95°C for 15 min as initial activation, followed by 40 cycles of: 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. Expression of miRNA was reported as ΔCt value. The ΔCt was calculated by subtracting the Ct values of miRNA SNORD68 from the Ct values of the target miRNAs. As there is an inverse correlation between ΔCt and miRNA expression level, lower ΔCt values were associated with increased miRNA. The resulting normalized ΔCt values were used in calculating relative expression values by using 2-ΔCt, these values are directly related to the miRNA expression levels. The 2-$\Delta\Delta Ct$ method was used to determine relative-quantitative levels of individual miRNAs, where $\Delta\Delta Ct$ = [Ct (target, test) -

Ct (reference, test)]- [Ct (target, calibrator)- Ct (reference, calibrator)].

2.3 Quantitation of Human Transcriptional activator c-MyB

The serum level of Human Transcriptional activator MyB (c-Myb) was measured using ELISA kit that was provided by (NOVA, Beijing, China) according to the manufacturer's instructions. The absorbance (O.D.) at 450 nm was read using a Microtiter Plate Reader (STAT FAX 2100, Awareness Technology, INC-USA).

2.4 Statistical analysis

Data was coded and entered using the statistical package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). Data was summarized using mean, standard deviation, median, minimum and maximum 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 [Chan, 2003a]. For comparing categorical data, Chi square (χ2) test was performed. Exact test was used instead when the expected frequency is less than 5 [Chan, 2003b]. Correlations between quantitative variables were done using Pearson's correlation coefficient [Chan, 2003c]. ROC curve was constructed with area under curve analysis performed to detect best cutoff value of miRNA for detection of HCC. Linear regression analysis was done to detect independent predictors of target protein c-Myb [Chan, 2004]. P-values less than 0.05 were considered as statistically significant.

3. RESULTS

Data analysis of the results are summarized and presented in the following tables and figure:

Table (1) show that:
a) There was a statistically significant difference between the diseased groups regarding age (*P*<0.001).
b) Considering gender; males were predominant in HCV related liver disease patients in the two diseased groups (CHC and HCC) and they represented 75 % and 80 %, respectively. There was no statistically significant differ-ence between them in the studied groups (*P*=1.00).

Table (1): Demographic analysis for the studied groups.

Parameter/Group	Control (n=20)	CHC (n=20)	HCC (n=20)	*P*
Age (year)	48.50 ± 15.05	43.20 ± 8.67	60.10 ± 7.88	*<0.001*
Gender: Male (%) Female (%)	15(75%) 5(25%)	15(75%) 5(25%)	16(80%) 4(20%)	N.S.

Data are expressed in form of mean± (SD) and frequency (percentage).

Table (2) shows that:
a) There was a significant change in blood level of Hb and PLT, citrated plasma level of INR, serum levels of ALT, AST, Alb and T. Bil (*P*<0.001).
b) There was a significant increase (*P*<0.001) in serum levels of both ALP and GGT among the studied groups too.
c) There was non-significant (N.S.) change in the mean value of TLC along the three groups. Data are expressed in form of Mean±SD. ALT: alanine aminotransferase; AST: aspartate aminotransferase; AFP: alfa-fetoprotein; SD: standard deviation; TLC: total leukocytic count; INR: prothrombin-international normalized ratio. A value of *P*<0.05 was considered statistically significant (derived from two-tailed test).

Table (2): Hematological and biochemical study for the studied groups.

Parameter/Group	Control (n=20)	CHC (n=20)	HCC (n=20)	*P*
Hb (gm/dL)	14.20± 1.15	13.83±1.32	10.25±2.06	<0.001
TLC (x10^3/mm^3)	7.23±1.56	6.1±1.9	6.03±2.7	N.S.
PLT (150-450 x103/mm3)	225.20±64.05	229.30±80.86	109.80± 34.64	<0.001
INR	1.02±0.05	1.10±0.09	1.32± 0.29	<0.001
ALT (up to 42 U/L)	25.15±5.68	56.05±30.28	110.35±68.80	<0.001
AST (up to 42 U/L)	25.45± 6.30	50.75± 26.58	149.65±69.86	<0.001
ALP (53-128 U/L)	125.1±39.6	95.4±34.1	168.4±40.7	<0.001
GGT (0-52U/L)	27.6±9.7	21.2±8.7	74.1±10.8	<0.001
T. Bil (0.1-1.2 mg/dL)	0.73±0.16	0.86±0.22	1.61±0.72	<0.001
Alb. (3.5-5.5 gm/dL)	4.36±0.36	4.13±0.45	3.00± 0.51	<0.001
AFP (up to 10.5 ng/mL)	1.92±0.07	4.75± 2.62	966.37±301.54	<0.001

Figure (1) shows that:

a) There was a significant increase in the expression level of miR-15a fold change (FC) in the HCC group compared with the CHC group.

b) The mean value of c-Myb serum level was significantly decreased in the HCC group compared with the CHC.
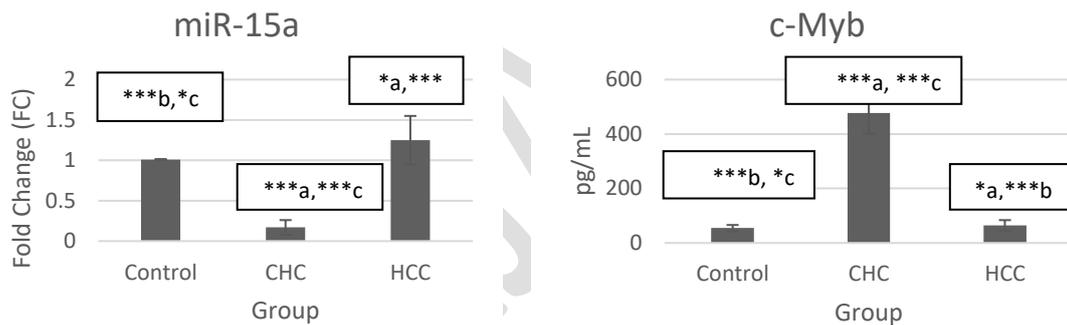

Figure (1): Significance of c-Myb and miR-15a among studied groups. Results are expressed as "Mean±SE". a: donates group is statistically significant from control group, b: donates group is statistically significant from HCV group, c: donates group is statistically significant from HCC group. *: *P*< 0.05, **: *P*<0.01, ***: *P*<0.001.

The following table (3) shows that the number of focal lesions (one *versus* multiple) is significantly reflected by the concentration of both miR-15a and c-Myb in comparison to AFP.

Table (3): Discriminating number of focal lesions by investigated markers in the HCC group.

Marker	Focal lesion		*P*
	One	Multiple	
miR-15a	0.40±0.44	1.96±0.56	<0.05
c-Myb	71.50±18.73	45.32±2.73	<0.05
AFP	1311±593	1163±523	N.S.

Significance between number of focal lesions and c-Myb within the HCC group. Data are represented as (Mean±SD).

Table (4) shows Pearson's correlation between miR-15a, c-Myb and the different tested hematological and biochemical parameters done.

Table (4): Pearson's Correlation between laboratory investigated parameters and (miR-15a & c-Myb).

Parameter	*r*	
	miR-15a	c-Myb
miR-15a	1	-0.423**
AFP	0.046	-0.399*
c-Myb	-0.423 **	1
Hb	-0.495**	0.658**
Plat	-0.372*	0.686**
INR	0.105	-0.399*
ALT	0.188	-0.421**
AST	0.502**	-0.626**
ALP	0.335**	-0.422**
GGT	0.640**	-0.251
T. Bilirubin	0.520**	-0.506**
Albumin	-0.517**	0.756**

r= correlation, *= Significant ($P<0.05$), **= highly significant ($P<0.01$).

Distinct alterations in expression level of the tested biomarkers (miR-15a and c-Myb) lead us to examine their efficacy to be used as tumor markers for early diagnosis of HCC, comparing to the traditionally used one "AFP" as illustrated in figure (2) and table (5).
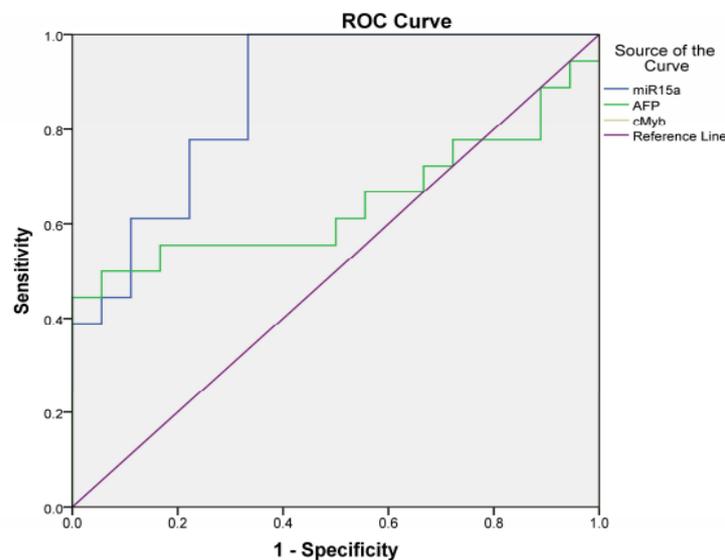

Figure (2): The diagnostic performance of miR-15a and c-Myb for HCC.

Table (5): Validity of using miR-15a, cMyb and AFP for predicting HCC.

Biomarker	AUC	Cut-off Value	Accuracy	Sensitivity	Specificity	95% CI	P
miR-15a (FC)	0.867	≥ 0.49	62.75	61.0%	89.0%	0.751- 0.983	0.05
c-Myb (pg/mL)	1.00	≤129.34	92	98.8%	98.0%	1.000-1.000	0.07
AFP (ng/mL)	0.645	≥ 200	77.5	50.0%	84.0%	0.452– 0.839	0.04

4. DISCUSSION

HCC is one of the most common and aggressive type of cancers worldwide [**Elkhodiry** *et al*.**, 2018**], accounting for 80–90%, with major geographical differences in prevalence [**Ringelhan** *et al*.**, 2018**]. Although the function of miR-15a has been demonstrated necessary in many studies, it remains to be reported in HCC. Targeted genes by miR-15a were predicted in http://www.mirbase.org/ and http://www.targetscan.org/vert_61/, and c-Myb was identified as a potential target gene [**Liu** *et al*.**, 2017**]. Firstly, we examined the 60 subjects that involved in the present work for hematological and biochemical parameters as liver profile, in addition to the demographic study.

Our results revealed a significant difference in age (*P*<0.001) among the studied groups, HCC groups (Mean±SD= 60.10± 7.88 years) *vs* (43.20 ± 8.67 years) for CHC, compared to (48.50 ± 15.05 years) for the control group. Gender was non-significant (*P*=1.00) among the studied groups in spite of male preponderance in all studied groups. These findings were in agreement with those of **Liu and coauthors** (**2017**) who mentioned the age-dependent patterns and the sex difference in the incidence of hepatocellular carcinoma. This findings supports the hypothesis of a protective role of estrogens.

As expected, liver enzymes (AST, ALT, ALP and GGT), in addition to total bilirubin found to be significantly elevated in HCC group in comparison to CHC and control groups (*P*<0.001), which is in concordance with other studies [**El-serag** *et al*.**, 2014;**

Xie *et al*.**, 2017**]. Hepatic synthetic function (albumin and prothrombin) was reduced through progression to HCC. This is explained by the debilitating power of the liver to synthesize proteins in these cases [**Bagirsakc** *et al*.**, 2017; Hessien** *et al*.**, 2015; Yang** *et al*.**, 2016**]. Besides, hematological parameters were analyzed too, there were a significant decrease in Hb level (10.25±2.06) and dramatic decrease in platelet count (109.80± 34.64) in HCC group compared to the CHC group (13.83±1.32) and (229.30±80.86), respectively as shown in table (2). Our results findings in this context are in the same way with other studies, as they found that HCC patients had other studies demonstrated the correlation between the degree level of anemia and the severity of liver cirrhosis, while the PLT level has been proven to be associated with the prognosis of various solid tumors [**Finkelmeier** *et al*.**, 2014; Gkamprela** *et al*.**, 2017; Pang** *et al*.**, 2015**].

A new role for miR-15a was identified as an oncogenic miRNA in HCC [**Zhao** *et al*.**, 2009**]. So, we estimated the expression of miR-15a and "c-Myb" as a potential target of it in liver cancer cells. This study reported significant (*P*<0.001) up-regulation of miR-15a serum expression level (M±SD=1.25±0.30 *vs* 0.17±0.09), respectively. Simultaneously, there was a repressing of c-Myb serum-concentration in HCC cases comparing to CHC (M±SD= 64.38±19.51 *vs* 476.79± 76.16), respectively as shown in figure (1). This is in harmony with other studies that identified in regulating the role of miR-15a in targeting the c-Myb pathway [**Kerenyi and**

Mullner, 2009; Zhao *et al*., 2009]. Also, regarding the mechanisms of the downregulation of c-Myb protein in HCC, a previous report has shown that miR-15a inhibits c-Myb gene transcription **[Feng *et al*., 2014]**.

The physiologic functions of Myb in its genuine role as transcription factor had received a lot of attention, since this protein is involved in multiple processes, such as stem cell self-renewal and lineage decisions **[Huilgol *et al*., 2019]**. **Liu and coauthors (2017)**, explained this result as the seed region (core sequences that encompass the first 2-8 bases of mature miRNA) of miR-15a was perfectly base-paired with the 3'-UTR of c-Myb mRNA. Furthermore, miR-15a binding sequences in the 3'-UTR of c-Myb mRNA are highly conserved across species **[Liu *et al*., 2017]**. Overexpressing miR-15a with the progression of the disease to HCC in our work was also augmented by **Sankaran and co-authors (2011)** who found that MYB protein levels were reduced with even a modest (two- to three-fold) increase in miR-15a/16-1 expression in erythroid cell lines **[Sankaran *et al*., 2011]**.

Inverse significant correlation was revealed between miR-15a and c-Myb (r=-0.423, $P<0.001$), which was in harmony with other study that established a negative correlation between the expression of miR-15a and c-Myb. This result explained as miR-15a may play a biologically relevant role in the inhibition of c-Myb expression during hematopoietic cell differentiation; suggesting that miR-15a might function a natural c-Myb inhibitor in hematopoietic cells. It was demonstrated that under physiologic conditions, the relationship between miR-15a and c-Myb protein is likely complex and may well be dependent on the stage of development. As a target for miR-15a, c-Myb mRNA declines with increase in miR-15a levels **[Zhao *et al*., 2009]**. Co-transcription factor "c-Myb" had also a negative significance correlation with

AFP (r=-0.399, $P<0.05$) as illustrated in table (4).

Correlations with the other investigated lab parameters were considered too, Pearson's correlation revealed that c-Myb was significantly correlated with hemoglobin (r=0.658, $P<0.01$), platelet (r=0.686, $P<0.01$) and the strongest one was with albumin (r=0.756, $P<0.01$), while it had a negative significant correlation with AST (r=-0.626, $P<0.01$). At the same time, miR-15a was significantly correlated with AST (r=0.502, $P<0.01$), GGT (r=0.640, $P<0.01$), total bilirubin (r=0.520, $P<0.01$) and inversely with albumin (r=-0.517, $P<0.01$). Besides, both (miR-15a and c-Myb) showed distinct differences in serum magnitude in relation to the number of focal lesions (*i.e.*, single *vs* multiple focal lesions) within HCC group (Table: 3), while AFP did not show this sensitivity in this context.

The preceding results lead us to compare them with conventionally HCC-tumor marker "AFP", as the high stability of miRNAs in human formalin-fixed tissue, serum, and plasma, in addition to their tissue-specific expression pattern were observed, where AFP is widely used to detect primary HCC, but its sensitivity and specificity are not satisfying **[Keshk *et al*., 2016]** and HCC novel biomarkers are needed **[Schütte *et al.*, 2015]**. Mayo clinic (USA)- in a recent study- proved that a high percentage of AFP-L3 seems to differentiate HCC from chronic liver diseases and may be an indicator of HCC when the total serum AFP levels are ≥ 200 ng/mL **[khattab *et al*., 2015]**. So, we examined the HCC diagnostic performance of AFP at this threshold value (≥ 200 ng/mL), compared to miR-15a and c-Myb by applying the receiver operating characteristic curve (ROC) which was plotted in figure: (2) to compare sensitivities, specificities, and cutoff values. The area under the curve for AFP was 0.645, with sensitivity and specificity (50% and 84%), respectively. While, the smallest cutoff point for the circulating miR-15a as a predictor marker for HCC was ≥ 0.49 FC

and the area under the curve was higher (0.867) than that of AFP. Sensitivity and specificity for miR-15a were 61% and 89%, respectively. This result potentiates the idea of using miR-15a as a biomarker for early HCC diagnosis.

5. Conclusion

Our results demonstrated that miR-15a is involved in tumorigenesis of HCC in part by suppression of the co-transcription factor "c-Myb". miR-15a also may act as a promising early diagnostic biomarker for HCC.

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