Alterations in microRNA-15a and "γ-synuclein"Expression Levels Among HCV-Related Hepatocellular Carcinoma Patients in Egypt.

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ABSTRACT

Background: The Egyptian health authorities consider hepatocellular carcinoma (HCC) as the most challenging health problem, where the number of HCC patients increased to two folds over a decade. Although the prognosis of patients with HCC is generally poor, the 5- year survival rate is >70% if patients are diagnosed at an early stage**.** Advanced molecular studies determined that "γ-synuclein" (SNCG) proved to be contributed in some cancers, and that microRNA-15a (miR-15a) directly targets SNCG. **Objective**: Our goal was to study the altered expression level of miR-15a and its target protein SNCG in chronic HCV (CHC)- related HCC among Egyptian patients. **Subjects & Methods:** 60 subjects were involved in this case-control study; 20 newly diagnosed HCC cases of different stages, 20 CHC, and 20 healthy controls. All of them were tested for circulating miR-15a using Real-time PCR technique and ELISA for the SNCG protein in serum. **Results:** upregulation in miR-15a expression level and reduced concentration of its target protein SNCG were demonstrated in serum of HCC patients than CHC group. miR-15a revealed ability to differentiate between presence of single and multiple focal lesions in HCC patients (Mean±SD= 0.40±0.10 *vs* 1.96±0.56), respectively. Efficacy to be used as a tumor marker was tested by ROC curve analysis, which showed that miR-15a was more accurate in diagnosing HCC among CHC group with sensitivity= 61% & specificity= 89% at cut-off value of ≥0.49 Fold Change, than the conventional tumor marker AFP (with 50% sensitivity and 84% specificity), respectively at cut off value ≥200 ng/mL. **Conclusion:** miR-15a has a role in pathogenesis through HCC, at least *via* SNCG, and it may represent a promising biomarker for early diagnosis of HCC in CHC population.

Keywords: Hepatocellular carcinoma, early diagnosis, microRNA-15a, SNCG.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of primary hepatic tumors and is the second leading cause of global cancer-related death, responsible for more than 745,000 deaths every year (Wong *et al.*, 2017). Late diagnosis, high postoperative recurrence rate, and lack of effective treatment for patients with advanced disease explain the poor

outcomes for most HCC patients (ClinicalTrials.gov., 2018).

Complications of HCV infection in Egypt are responsible for 67% of morbidity related to liver disease (Atta *et al.,* 2016). Expected changes of HCV- related diseases and related morbidity and mortality are estimated to increase by 2030 due to the aging of the population infected by HCV and currently, available treatment will be insufficient (Sibley *et al.,* 2015).

In HCC, several different kinds of genomic alterations have been reported (Hung and Wang, 2019). Emerging evidence showed that dysregulation of microRNAs, a class of short, noncoding RNA, contributed to activation of oncogenic signaling in HCC; for instance, (Meng *et al.,* 2007) showed that over-expression of miR-21 inhibits the expression of the phosphatase and tensin homolog (PTEN) tumorsuppressor.

Several studies had shown that miR-15a is altered in tumors such as non-small cell lung cancer, prostate cancer, pituitary adenoma, and pancreatic cancer, suggesting that it has 'hot spot' roles in cancer transformation (Lovata *et al.,* 2018). Simultaneously, gamma synuclein (γ-synuclein or SNCG) was found to be one of the target proteins of miR-15a, where it participates in the pathogenesis of several types of cancer (Liu *et al.,* 2018). So, the present work was designed to study the alteration in expression levels of both miR-15a and its target protein "SNCG" in serum of HCC patients- with background of chronic hepatitis C (CHC) infection.

1. SUBJECTS AND METHODS

1.1. Subjects & Study design

This case-control study was conducted on 60 Egyptian subjects; selected from outpatient's clinic of liver unit, Tropical Medicine Department, Kasr El-Ayni Hospital, Faculty of Medicine, Cairo University-Egypt during the period between October 2017 to December 2018. Controls were selected from healthy volunteers attending hospital blood bank after blood donation. A full history was taken from all patients and control, through clinical sheets of patients which include conventional laboratory investigations and physical examinations.

All the subjects were categorized into the following three groups:
-Group I: consisted of 20 apparently healthy individuals (control group).
-Group II: consisted of 20 patients previously diagnosed as chronic HCV (CHC group).
-Group III: consisted of 20 confirmed patients with HCC on top of chronic HCV infection (HCC group).

Exclusion criteria included: patients with malignancies other than HCC, coinfection with other than HCV, active schistosomiasis, end stage- renal disease, autoimmune disorders, and metabolic syndrome lead to liver injury as Wilson's disease, haemochromatosis, α-1 anti-trypsin deficiency. All patients were recruited after a written informed consent and the study protocol was approved by the ethics review committee of CairoUniversity Hospital.

1.2. Laboratory investigations

Full history was taken from all subjects through their clinical sheets which included conventionallaboratory investigations including complete blood picture, liver function tests, prothrombin time, international normalized ratio, AFP, anti-HCV antibody, quantitative HCV-RNA, HBVs-Ag using commercially available assays. Fasting venous blood samples were collected from all individuals for quantitation of miR-15a gene expression by Real-time PCR, and estimation of SNCG serum level by ELISA technique.

1.2.1 Assessment of miRNA-15a expression level by quantitative real-time reverse transcription PCR assay

It included RNA extraction, quantification of

extracted total RNA (including microRNAs), and reverse transcription of the extracted miRNA into complemen-tary DNA (cDNA) using appropriate primers, then relative quantitation of miRNA-15a to the control was applied. Data analysis was accomplished to get the expressed levels of miRNA-15agene.
Fresh blood samples were collected from all patients and controls, centrifuged, serum was separated, aliquoted and stored at −80°C for further investigations. Total RNAs extraction including microRNAs was performed using miRNeasy mini kit and protocol for purification of serum total RNA, including miRNA and LncRNA (QIAGEN, Qiagen GmbH, Germany). Using QIAzol lysis reagent according to the manufacturer's instructions. RNA quantitation and purity assess-ment using the NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA).

Reverse transcription (RT) was carried out on total RNA in a final volume of 20 uL RT reactions using the miScript II RT kit (QIAGEN, Qiagen GmbH, Germany) according to the manufacturer's instructions. Expression of mature miRNA-15a was evaluated by real time RT- PCR analysis using miScript miRNA PCR primer Assay in combination with miScript SYBR Green PCR kit (QIAGEN, Qiagen GmbH, Germany) according to the manufacturer's protocol. The housekeeping miScript PCR control- miRNA SNORD68- was used as internal control (El-Garem *et al.*, 2014; Motawi *et al.*, 2015).

Real-time PCR was performed using Rotorgene Q Real-Time PCR System (QIAGEN, Qiagen GmbH, Germany) with the following conditions: 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. Fold change (FC) of miRNA expression levels was calculated by using the equation $2^{-\Delta\Delta Ct}$ method: [ΔCt= (Ct miR-15a) – (Ct SNORD68)] (Livak and Schmittgen, 2001).

2.2.2. Quantitation of SNCG in serum

The serum level of gamma- synuclein (γ-SNC or SNCG) 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).

2.2.3. 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 variableswere done using the non-parametric Kruskal-Wallis and Mann-Whitney tests (Chan, 2003a).

For comparing categorical data, Chisquare (χ^2) 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 SNCG (Chan, 2004). *P*-values less than 0.05 were considered as statisticallysignificant.

2. RESULTS

2.1. Patients' characteristics

This study was conducted on 40 CHC patients-20 of them had progressed to HCC and 20 apparently healthy individuals from the blood bank donors, serving as control. Demographic data for all subjects involved in this study is represented in the following table (1).

Table (1): Demographic study for control, CHC and HCC 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 (%)	15(75%)	15(75%)	16(80%)	N.S.
Female (%)	5(25%)	5(25%)	4(20%)	

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

2.2. Clinical laboratory investigations

Results of all the hematological and bio-chemical parameters acted upon for all subjects are illustrated in table (2).

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

Parameter/Group	Control (n=20)	CHC (n=20)	HCC (n=20)	*P*
Hgb (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	6.10±1.90	6.03±2.70	N.S.
PLT ($x10^3/mm^3$)	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 (U/L)	25.15±5.68	56.05±30.28	110.35±68.80	<0.001
AST (U/L)	25.45± 6.30	50.75± 26.58	149.65±69.86	<0.001
ALP (U/L)	125.10±39.60	95.40±34.10	168.40±40.70	<0.001
GGT (U/L)	27.60±9.70	21.20±8.70	74.10±10.80	<0.01
T. Bil (mg/dL)	0.73±0.16	0.86±0.22	1.61±0.72	<0.001
Alb. (gm/dL)	4.36±0.36	4.13±0.45	3.00± 0.51	<0.001
AFP (ng/mL)	1.92±0.07	4.75±0.262	966.37±301.54	<0.001

Data were expressed as mean±SD. SD: standard deviation; Hgb: hemoglobin; TLC: total leucocytic count; PLT: platelets; INR: international normalized ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutmyltransferase; T. Bil: total bilirubin; Alb: albumin; AFP: alpha fetoprotein. $P< 0.05$ was considered statistically significant.

2.3. Serum expression level of miR-15a and SNCG

Assessment levels of miR-15a and " SNCG" in serum of the diseased groups are illustrated in the following table (3).

Table (3): Signature of the tumor markers investigated in the infected groups.

Biomarker/Group	Control (n=20)	CHC (n=20)	HCC (n=20)	*P*
miR-15a (FC):	1.01±0.006	0.17±0.09	1.25±0.30	<0.001
SNCG (pg/mL):	226.86±31.17	2436.44±55.37	254.20±10.38	<0.001

Data were expressed in form of mean± (SD); miR-15a: microRNA-15a; SNCG: γ-synuclein.

2.4. Correlation of miR-15a and SNCG with severity of hepaticdysfunction

Pearson's correlation was performed between miR-15a and SNCG in one-side and the laboratory investigated

parameters in the other-side along with the severity of the liver damage in the infected groups; serum miR-15a expression level showed significant correlation with AST (r=0.502, P<0.01), GGT (r=0.640, P<0.01), total bilirubin (r=0.520, P<0.01) and albumin (r=-0.517, P<0.01) through progression to HCC, while SNCG showed significant correlation with AST (r=-0.648, P<0.01), total bilirubin (r=-0.516, P<0.01), albumin (r=0.755, P<0.01), in addition to Hgb (r=0.660, P<0.01) and platelet count (r=0.682, P<0.01) along with progression to HCC too. Table (4) shows that there is a distinct difference in miR-15a expression level in blood of HCC patients due to change in number of focal lesions (one *versus*multiple).

Table (4): Altered expression level of miR-15a due to number of focal lesions.

Number of Focal Lesions	Number of Cases	miR-15a (Mean±SD)	*P*
One	10	0.40±0.10	
Multiple	8	1.96±0.56	<0.05

For discriminating HCC patients from the liver fibrotic diseased ones (CHC group) using the tested biomarkers, ROC analysis was applied, and results are illustrated in figure: (1) and table: (5).

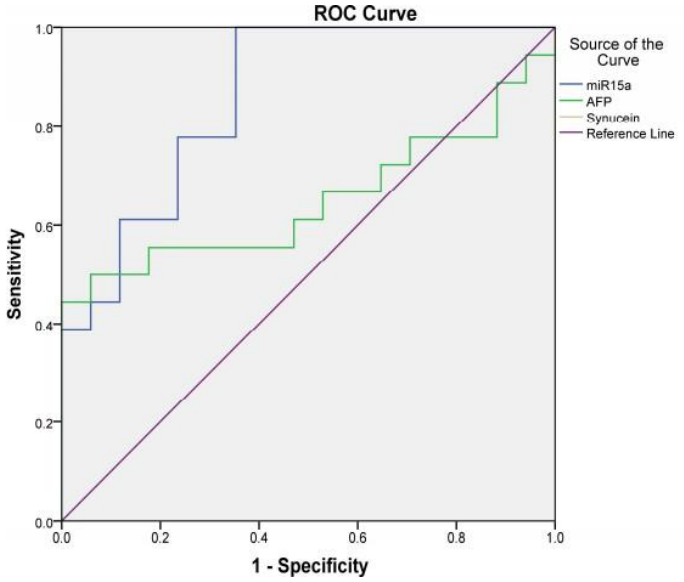

Fig. (1): ROC analysis demonstrates the diagnostic efficacy of miR-15a, SNCG and AFP for HCC.

Table (5): Diagnostic performance of miR-15a & SNCG for HCC cases compared to AFP.

Biomarker	AUC	Cut-off	Accuracy	Sensitivity	Specificity	95% CI	*P*
miR-15a (FC)	0.859	≥ 0.49	62.75	61.0%	89.0%	0.738- 0.981	0.05
SNCG (pg/mL)	1	≤253	89.5	98.8%	80.0%	1.000-1.000	0.07
AFP (ng/mL)	0.650	≥ 200	77.5	50.0%	84.0%	0.458– 0.843	0.04

3. DISCUSSION

Chronic HCV accounted for 94% of HCC cases in Egypt, with 6000- 7000 deaths/year due to HCC (Hatzakis *et al.*, 2013). Advances in genomics and proteomics platforms and biomarker assay techniques have resulted in the identification of numerous novel biomarkers and have improved the diagnosis of HCC (Chiou and Lee, 2016).

Nowadays, circulating microRNAs have been demonstrated to be highly stable in serum and plasma due to their protection from RNase activity, therefore representing a possible source of diagnostic and prognostic biomarkers to be explored, especially for detection of early stage, pre-asymptomaticdiseases (Keshk *et al.*, 2016). Thus, this work was designed to demonstrate the alteration of miR-15a and its target protein "SNCG" expression levels in serum of HCC patients- on background of CHC- and to examine their potentiality as non-invasive biomarkers for diagnosing HCC at its early stages.

Statistical analysis of this case- control study revealed a significant difference in age (P<0.001) between studied groups: HCCaffecting old patients than CHC while gender was non-significant (P=1.00) despite male preponderance in all studied groups. These findings were in agreement with other study which mentioned that the age-dependent patterns in the sex difference- concerning incidence of hepato- cellular carcinoma support the hypothesis of a protective role of estrogens. The underlying reasons for the sex and age difference in hepatocellular carcinoma remain to be further explored in analytic epidemiological studies (Liu *et al.*, 2017).

As expected, ALT, AST, ALP and GGT in addition to total Bilirubin showed significant increase with progression to HCC with (P<0.001). This is in concordance with preceding studies (Arisar and Hamid, 2018; Ren *et al.*, 2019; Xie *et al.*, 2017; Yang *et al.*, 2016), which could be attributed to cholestasis or hepatitis or related to cancer proliferation or promotion mechanisms (Axley *et al.*, 2018; Chan *et al.*, 2015; Hanigan *et al.*,1999; Kim *et al.*, 2008; Ma *et al.*, 2014; Wu *et al.*, 2016).Hepatic synthetic function was reduced dueto debilitating synthetic ability of theliver during liver diseaseprogression to hepatocarcinoma, with the least concentrations in the HCC group (Bağırsakç *et al.*, 2015; Hessien *et al.*, 2015).

Acute and chronic gastrointestinal blood loss, folate deficiency,hypersplenism, bone marrow suppression and the anemia of chronic disease could be the cause for decreased Hgb level through HCC (Youssef *et al.*, 2015); which could be the cause for our results in this context; hemoglobin showed significant decrease in the HCC group (M±SD= 10.25± 2.06) than the CHC and control groups (M±SD= 13.83± 1.32 and 14.20± 1.15), respectively.

Simultaneously, platelet count showed vigorous decrease in the HCC group too (M±SD= 109.80± 34.64), with a significant difference (P<0.001) than CHC group (M±SD= 229.30± 80.86), which is in harmony with other study (Hack *et al.*, 2016). This situationcan be referred to that most cases of HCC are accompanied by liver cirrhosis, which could ultimately lead to portal hypertension and hypersplenism and which cause a sub-sequent decrease in platelet count (Undell *et al.*, 2012).

The newly examined miR-15a revealed a significant upregulation in its expression level (P<0.001) in HCC group than the fibrotic (CHC) group (M±SD= 1.25± 0.30 *vs* 0.17± 0.09), respectively. This result is in concordance with many previous studies dealing with the role of miR-15a as an oncomir in HCC (Groszmann *et al.,* 2015; Liu *et al.*, 2019; Wang, 2017). This result contradicts with other studies found that miR-15a act as a tumor suppressor and decreased through HCC (Sumit., 2018; Wang *et al.,* 2013). Besides, our results concluded that miR-15a expression level distinctly (P<0.05) differed within HCC group due to the number of focal lesions, i.e., single *versus* multiple (M±SD= 0.40±0.10 *vs* 1.96±0.56), respectively.

The dual function of miR-15a could base on the heterogeneity of cancer and versatile nature of miRs; miR-15a's role as either an oncogenic miRNA or a tumor suppressive miRNA does not lie firmly within one category (Kontos *et al.*, 2017), which may be attributed to the HCV genotype itself, as (El Mahdy *et al*., 2019) reported that about 90% of Egyptian patients suffering from HCV belong to genotype-4 and this differs from other countries at which HCC patients suffering from other genotypes.

SNCG was associated with many different types of cancer as breast, ovarian, bladder, gastric cancer, pancreatic adenocarcinoma, glial tumors and medulloblastomas. Its expression was approved to be regulated on post-transcriptional level by micro-RNAs (Surguchov *et al.*, 2016). and it was proved to be acted upon by miR-15a *via* its mRNA transcript (Connolly *et al.*, 2008). So, SNCG serum level was estimated in the infected groups by ELISA, and results showed a great difference in its concentration between those groups; it was dramatically decreased in HCC group than the CHC one (M±SD= 254.20±0.38 *vs* 2436.44± 55.37), respectively.

This result was in agreement with other study which explained that miR-15a may directly interact with the 3'-untranslated regions (3'- UTR) of SNCG genes (mRNA) which contains a target sequence of miR-15a that was completely complementary to the 2-8 seed nucleotides of the miR-15a, down- regulating its mRNA and protein expression levels; SNCG proved to be contributed to the cell cycle and cell apoptosis in breast cancer, and that miR-15a directly targets the SNCG (Li *et al.*, 2014). This may be attributed specifically to the up-regulation of the long non-coding RNA (LncRNA-AK058003) that could increase the expression of miR-15a, which acts as an inhibitor for SNCG (He *et al.,* 2017). Conversely, another study disagreed with this result; stating that SNCG was abnormally expressed in a high percentage of tumor tissues of many cancer types including liver, esophagus, gastric, cervical, colon, prostate, lung, pancreatic ductal adenocarcinoma cancer patients (Zou *et al.*, 2012).

As a reference, Mayo clinic (USA) 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 level is ≥200 ng/mL (Khattab *et al.*, 2015). So, we examined the diagnostic performance of the newly tested biomarkers compared to AFP at 200 ng/mL for Egyptian patients (HCC group) *via* applying ROC curve analysis (figure: 1 and table: 5). It was concluded that miR-15a had bigger AUC= (0.859) compared to AFP (0.650). At cut off value of ≥200 ng/mL, the specificity and sensitivity for AFP were (84.0% and 50.0%), respectively. While miR-15a showed higher specificity and sensitivity (89.0% and 61%), respectively at cut off value ≥0.49 FC for diagnosing HCC.

4. Conclusion

Taken together, microRNA-15a could contribute to HCC growth and spread at least by affecting SNCG expression. Circulating microRNA-15a level could be beneficial as a biomarker for early diagnosis of HCC, as it showed higher accuracy in this fieldthan AFP.

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