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Expression of microRNAs-21 and-223 in hepatocellular carcinoma in hepatitis C virus infected Egyptian population

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ABSTRACT

Background: The prognosis of hepatocellular carcinoma (HCC) in Egypt is poor due to high prevalence of hepatitis C virus (HCV). Moreover, the performance of laboratory markers for diagnosis of HCC at early stages has not been optimal.
Objective: We aim to assess expression of miR -21 and miR-223 in HCC patients in the presence of HCV infection and correlate this expression with other clinical, laboratory features of HCC and other markers such as cyclase-associated protein2 (CAP2) and macrophage migration inhibitory factor (MIF).
Subjects and Methods: The study included 40 cirrhotic, 40 HCC patients and 40 healthy subjects. Assessment of miRNAs was done using RT-PCR (Applied Biosystems).
Results: Levels of miR-21 were higher in patient groups compared to control group and in HCC patients compared to other patients (p=0.001, 0.002 respectively). Serum levels of miR-223 showed no significant differences between cirrhotic patients and control group (p=0.2), while marked decrease in miR-223 expression levels was found in HCC patients compared to cirrhotic patients and control group (p=0.004,0.001 respectively).
Conclusion: Altered serum miR-21 and -223 may play role in pathogenesis of HCC in the presence of HCV infection and might serve as a potential biomarker for detection of early HCC.

INTRODUCTION

Egypt has the highest hepatitis C virus (HCV) prevalence worldwide (Elgharably *et al.*, 2017). Thus, HCV is considered a major public health problem in Egypt. Complications of HCV infection in Egypt are responsible for 67% of morbidity related to liver disease (Atta *et al.,* 2016). Patients with chronic HCV are at risk of developing cirrhosis which may be followed by hepatocellular carcinoma (HCC),

previous studies reports that about94% of HCC patients are exposed with chronic HCV infection.HCC represents 13% of all cancers in Egypt (Schütte, 2015; Ziada, 2016). HCC is one of the most common neoplastic disorders worldwide (Forner and Bruix, 2012). In Egypt, it is the second common cancer among men and the sixth common cancer among women and represents the second cause of cancer related death (Bader El Din *et al.,* 2016; Fiorino *et al.,* 2016). It has very poor outcome because it is usually detected in advanced stage due to mild deviation of its clinical and pathological features from chronic liver disease (Sakamoto *et al.,* 2010).

To date, serum biomarkers to diagnose early HCC have not been satisfactorily established. Alpha-fetoprotein (AFP) is widely used to detect primary HCC but its sensitivity and specificity are sub-optimal as in almost 80% of early HCC patients AFP levels is not elevated (Fiorino *et al.,* 2016).

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Imaging techniques, including ultrasonography, Computed Tomography (CT) and Magnetic Resonance (MRI), have potentially limiting factors, including their availability, costs, and reproducibility (Khairy *et al.,* 2016). Thus, early detection of HCC is still challenging and requires novel, sensitive and specific biomarkers that could detect tumor at an early stage where curative therapy can be applied (Sakamoto *et al.,* 2010).

Different biological markers have been demonstrated to improve diagnosis of early HCC (Sakamoto *et al.,* 2010). These markers could indicate development of HCC such as cyclase-associated protein2 (*CAP2*) and macrophage migration inhibitory factor (MIF) (Sakamoto *et al.,* 2010 and Zhao *et al.,* 2011).In HCC, several studies demonstrated over-expression of CAP2 (Effendi *et al.,* 2013 and Shibata *et al.,* 2006 and Sakamoto *et al.,* 2010).

However, the prognostic role of CAP2 in HCC remains elusive (Fu *et al.,* 2015). As regard MIF different studies showed increased concentration of MIF in HCC and it also related to tumor size, tumor, node, and metastasis (TNM) staging and vascular invasion (Wang *et al.,* 2014,and Zhao *et al.,* 2011). However, results regarding the role of these factors in HCC are controversial.

MicroRNAs (miRNAs) are small non-coding RNAs that play key role in regulation of gene expression. Moreover, miRNAs are characterized by their remarkable stability in serum. Recent studies have reported that miRNAs regulate a variety of biological processes, including cell proliferation, differentiation and apoptosis (Huang *et al.,* 2015). In addition, altered expression of miRNAs including miR -21 and miR-223 has been linked to the development of different human cancer but their role in HCC remains unclear (Khairy *et al.,* 2016).

We hypothesized that levels of miR -21 and miR-223 in circulation could be altered in association with pathological changes in HCV patients'liver leading to HCC. If this hypothesis is established, major progress in HCC management could be achieved. The primary aim of this study was to assess expression of miR -21 and miR-223 in serum of HCC patients with HCV infection and correlate this expression with other clinical, laboratory features of HCC and other markers denoting stromal invasion and inflammation such as CAP-2 and MIF respectively.

SUBJECTS AND METHODS

Subjects

A total of 80 patients were recruited from the outpatient clinic of both the National Research Centre and the National Cancer Institute in Cairo from June 2015 to March 2016. All Patients were positive for HCV RNA testing. They were subdivided into 40 patients with newly diagnosed HCC at early stages (hepatic lesion mass of 2 cm or smaller as detected with four-phase CT and MRI) and 40 patients with Liver cirrhosis. HCC was diagnosed by presence of typical histological findings or imaging characteristics according to the Korean Liver Cancer Study Group guidelines and American Association for the Study

of Liver Diseases (AASLD) guidelines (Lee *et al.,* 2014). HCC staging was determined using the Barcelona Clinic Liver Cancer (BCLC) staging system (EASL, 2012). Liver cirrhosis was diagnosed by histological examination or clinical findings of portal hypertension (Marrero *et al.,* 2009), and adequate imaging studies were repeatedly conducted on patients with liver cirrhosis to exclude HCC within 6 months of enrolment. Data of all subjects were obtained from medical records, pathology reports and personal interviews with the subjects. Exclusion criteria included: patients with positive HBsAg or with history of other causes of congenital liver diseases or chronic liver disease other than HCV, previous treatment for HCC or antiviral therapy for HCV, extrahepatic dissemination, any associated malignancies other than HCC and any other systemic or chronic disease. The 40 control subjects enrolled in the study were seeking a routine health check-up in the National Research Centre clinics. These healthy subjects have met the following criteria: 1) seronegative for AFP; and 2) no evidence of any liver disease; 3) No evidence of associated malignancy, systemic or chronic disease. The protocol for this study was approved by National Research Centre Ethics Committee(ethical clearance number is 13167) and according to the ethical guidelines of the declaration of Helsinki with informed written consent taken from all participated individuals.

Methodology

All patients and controls were subjected to full clinical assessment and laboratory investigations including:

Clinical Examination: A detailed history taking including history of smoking, alcohol intake, occupational exposure to chemicals, drug intake, previous diseases etc. A thorough clinical assessment and total body examination with abdominal ultrasound and/or CT scan were done when indicated to detect tumor size and extension.

Laboratory Investigations: A blood sample was withdrawn from each subject and was divided, processed and stored according to the requirements and precautions of each test. The following investigations were done for all subjects of the study:

1. Conventional liver evaluation tests which include: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, serum albumin and prothrombin time.
2. Estimation of AFP,was assessed by enzyme-linked immunosorbent assay technique (ELISA) following instructions on kits purchased from Immunospec Co.
3. Assessment of MIF and CAP2 was done using ELISA kit supplied by Glory Science Co., Ltd, USA (catalog number: 10078 and 15048 respectively).

RNA analysis

Extraction of miRNAs serum was done using miRNA assay Serum/Plasma Kit (Qiagen, Hilden, Germany). All serum RNA preparations were quantified by NanoDrop 1000 (Nanodrop, Wilmingtion, Delaware, USA). cDNA was done using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems,

Carlsbad, CA, USA) and microRNA-specific stem-loop primers (part of the TaqMan microRNA Assay Kit; Applied Biosystems). Real-time Quantitative PCR was performed using the Quantistudio12K flex Real-Time PCR System (Applied Biosystems), and the results were analyzed using the RQ manager software (Applied Biosystems).

The following formula 2−⍙Ct was used to calculate the miRNA levels in serum:

⍙Ct =mean (Ct of internal references) − Ct of target miRNA.

The relative expression levels of miR-21 and miR-223 were calculated and normalized to miR-16 (Applied Biosystems, Foster City, CA) (Yu *et al.*, 2015) using the comparative ⍙Ct method and the equation 2−⍙⍙CT, as described previously.

Statistical analysis

Analysis was performed using SPSS version 17 software for Windows. Data are reported as means ± standard deviation (SD) or number (percentage). One-Way ANOVA analysis was used for multiple group comparisons. The least significant difference (LSD)-t test was used in pairwise comparison of averages among groups. Pearson's and Spearman's correlation tests (r= correlation coefficient) were used for correlating normal and non-parametric variables, respectively and linear regression analysis was done to confirm association of altered miRNA expression with HCC after adjustment of age and sex. A p-value of < 0.05 was considered statistically significant. All tests were two-sided, and P < 0.05 was considered statistically significant.
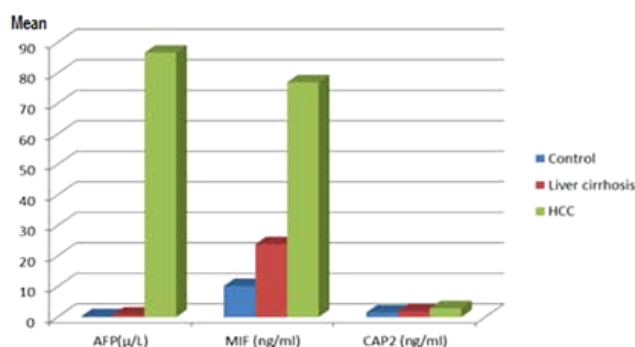
Clinical, demographic and laboratory data of the studied groups are shown in table 1. Our results showed increase of CAP2 serum levels in HCC compared to non HCC hepatic patients and controls (0=0.001), while no significant differences were detected between HCC free hepatic patients and control group (p=0.2). Serum levels of MIF increased in hepatic patients compared to controls (p=0.001) and in patients with HCC compared to cirrhotic patients without HCC (p=0.001) (Figure 1).

Levels of miR-21 were higher in patient groups compared to control groups (p<0.001) and in patients with HCC compared to HCC-free hepatic patients (p=0.002) (Table 1, Figure 2).

Serum levels of miR-223 showed no significant differences between HCC-free hepatic patients and control group (p=0.2), while marked decrease in miR-223 expression levels was found in HCC patients compared to HCC-free hepatic patients and control group (p=0.004,0.001 respectively) (Table 1, Figure 2).

Linear regression analysis confirmed altered expression of miR-21 after adjustment of age and sex (p=0.01). No significant correlation was detected between miR-21 and age, sex, MIF, CAP2 and miR-223 (p=0.2, 0.9, 0.9, 0.8, 0.8 respectively).

Linear regression analysis confirmed altered expression of miR-223 in HCC patients compared to other patients after adjustment of age and sex (p=0.001). No significant correlation was detected between miR-223 and age, sex and CAP2 (p=0.2, 0.4, 0.3 respectively). Weak negative linear relationship was detected between miR-223 and MIF (r=-0.25, p=0.02).

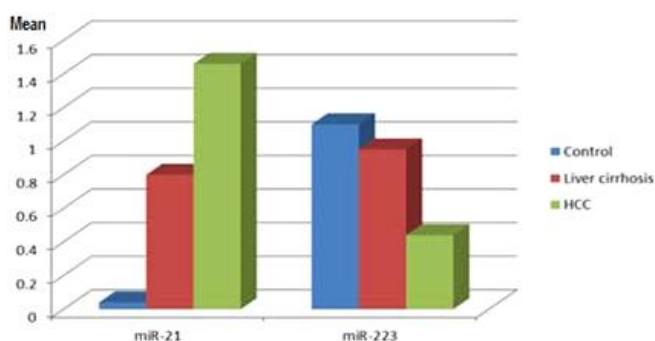**Fig. 1**: Results of AFP, MIF and cap2 among studied groups.
AFP = apha fetoprotein (represented in the chart as 10% of its real value)
HCC = hepatocellular.

Fig. 2: Expression of miR -21 and miR- 223 among studied groups.

Table 1: Demographic clinical and laboratory data of studied groups.

Variable	Control (N=40) Mean ± SD	Cirrhosis (N=40) Mean ± SD	HCC (N=40) Mean ± SD	p value
Age (years)	55.8± 4	58.3±7.3	58.6± 5.1	0.3
Sex				
Male	23(57.5%)	22(55%)	29(72.5%)	0.2
Female	17 (42.5%)	18(45%)	11(27.5%)	
AST (U/L)	21± 2.6	62.8± 31.1	1673.8 ± 1923	0.001
ALT (U/L)	22.9± 3.1	57.7± 24.6	1614.2 ± 1760	0.001
Albumin (g/L)	3.6 ±1.6	3.4± 0.5	2.870± 0.5	0.007
Bilirubin (μmol/L)	0.5± 0.18	1.1± 0.2	1.5710 ± 0.57	0.001
AFP (μg/L)	2.6± 0.32	8.4± 8.8	867.3 ± 1618	0.001
MIF (ng/ml)	10.2± 2.8	23.9± 4.5	77± 27.4	0.001
CAP2(ng/ml)	1.5 ±0.2	1.8± 0.8	2.91±0.9	0.001
miR-223	1.1± 1.06	0.95 ± 0.6	0.44± 0.4	0.001
miR-21	0.04 ±0.05	0.8± 0.7	1.46± 1.3	0.001

DISCUSSION

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers worldwide, and it is predominant in Asia and Africa (Fathy *et al*., 2016). Most HCC develops in patients with history of cirrhosis and chronic hepatitis as there is continuous inflammation and regeneration of hepatocytes (Gao *et al*., 2012).

Our results show deterioration in liver functions in patients groups when compared to control group. Our results agreed with Fathy and coworker (2016) who recorded that all measured liver functions as well as AFP showed highly significant difference between HCC patients and control group (Fathy *et al*., 2016). However, AFP is used in diagnosis of HCC with imaging techniques as ultrasonography, CT and MRI, whileFilmus and colleges (2004) found that the sensitivity of this marker is limited (41-65%) (Filmus and Capurro, 2004).

Published data regarding human cancers reported differential expression of CAP-2. For example, CAP2 was up-regulated in bladder and kidney human cancers and down-regulated in breast fibroadenoma (Peche *et al*., 2007). Different studies reported over-expression of CAP-2 in HCC. Current published data suggest that CAP-2 is a potential diagnostic marker for stromal invasion in early HCC (Sakamoto, 2009 and Fu etal.,2015). Sakamoto demonstrated that CAP-2 was expressed in 70% to 100% of early HCC (Sakamoto, 2009). Our results showed increase of CAP-2 serum levels in HCC compared to non HCC hepatic patients, while no significant differences were detected between HCC free hepatic patients and control group, suggesting role of CAP2 in HCC pathogenesis and its role as potential diagnostic marker for early HCC. Consistent with our study, Shibata *et al* reported that CAP-2 expression was increased in early HCC and frequently increased with progression (Shibata *et al*., 2006).

Accumulating evidences indicate that MIF represents a cytokine linking inflammation and cancer, due to its key role in inflammation and cell proliferation (Bucala and Donnelly, 2007). With regard to HCC, most hepatic lesions produced by hepatitis B or C viruses develop chronic inflammation as well as abnormal cell proliferation due to cirrhosis. In our study, we found increased serum MIF levels in patients with HCC compared to cirrhotic patients without HCC. Our results suggest that circulating MIF is associated with the development of HCC and could be considered as a potential diagnostic marker for it. In agreement with our study, different studies reported increased levels of MIF in HCC (Zhao *et al*., 2011andSad *et al*., 2014). Zhao *et al.* reported that serum MIF may differentiate patients with HCC from individuals with other liver diseases, such as cirrhosis (Zhao *et al*., 2011). However, Ren *et al*. mentioned that MIF acts as a local autocrine factor rather than a systemic mediator in cancer, indicating that serum MIF alone was not a useful marker in clinical practice (Ren *et al*., 2005).

MiR-21 is described as one of the most important miRNAs contributed in cancer pathogenesis and progression.

Altered level of miR21 has been reported in different types of human cancer (Qi *et al*., 2011). This concept agrees with results of this work that showed up-regulation of miR-21 in hepatic patients compared to controls and in HCC patients compared to HCV patients. Our results agrees with other studies that reported over-expression of miR 21 in HCC and liver diseases (Connolly *et al*., 2008; Ladeiro *et al*., 2008). Connolly *et al.* demonstrated that up-regulation of miR-21 occurs in precancerous stages of liver disease and in HCC in comparison with normal subjects (Connolly *et al*., 2008). Ladeiro *et al.* found that miR-21, were significantly over-expressed in patients with HCCs, associated with both viral- and non-viral risk factors (Ladeiro *et al*., 2008). Yan *et al.* reported in their meta- analysis, included five studies published from 2011 to 2014, that miR-21 over-expression in HCC correlated with poor outcome, and higher miR-21 expression was associated with metastases (Yan *et al*., 2015). However, published results regarding miR-21 showed high heterogeneity between studies. El-Guendy *et al.* reported downregulation of miR21 in liver biopsy of Egyptian patients with HCV Infection (El-Guendy *et al*., 2016). Other authors found lower levels of serum miR-21 in HBV positive patients with or without HCC than in healthy subjects (Qi *et al*., 2011). They mentioned that the detected circulating miRNAs might be derived from dying or active tumor cells. They added that miR-21 can act as either tumor suppressor or oncogene, depending on the targets they regulate and the associated epigenetic factors (Qi *et al*., 2011). The exact role of miR-21 in cancer is still under research and needs further studies in the future. This heterogeneity among studies is also mainly due to differences in reference control group (healthy subjects or patients with HBV or HCV -related liver disease), sample size and type, molecular techniques and the lack of common miRNA internal control.

MiR-223 is described as a bone marrow enriched miRNA that functions as regulator of cellular differentiation (Qi *et al*., 2011). Moreover, recent studies observed altered miR-223 expression in HCC, suggesting a potential role of this miRNA in pathology of liver disease (Roy *et al*., 2015; Roderburg and Luedd, 2014). In the current work, miR-223 was down regulated in hepatic patient group compared to control subjects and in patients with HCC compared to HCV positive patients. Our results agree with other studies reported decreased miR-223 expression in HCC. Khairy *et al* reported significant decrease in expression level in HCC group compared to non HCC groups (Khairy *et al*., 2016). Shi *et al* mentioned that miR-223 down regulated in HCC samples compared to matched non-tumor liver tissue (Shi *et al*., 2015). Bhattacharya *et al* found that miR-223, were expressed at significantly lower levels in the serum of HCC patients compared with patients with chronic liver disease and healthy subjects (Bhattacharya *et al*., 2016). On the other side, our results disagree with Xu *et al* who reported up regulation of miR-223 in HCC patients compared to controls and no significant difference was detected between HCC patients and patients with chronic hepatitis (Xu *et al*., 2011). Qi *et al* found that levels of miR-223 were significantly increased in HCC patients than in controls, while no

significant differential expression was reported for this miRNA between HBV patients with and without HCC. Moreover, serum levels of miR-223 in HBV subjects without HCC were significantly higher than those in HCC patients or healthy subjects (Qi *et al.*, 2011). Li *et al* also reported up regulation of miR-223 in HBV patients, while down regulation was reported in HBV negative HCC compared to HBV positive HCC (Li *et al.*,2010). The previous findings point out that elevated serum miR-223 in liver disease may come from tissue injury such as hepatitis. Thus, higher levels of miR-223 were reported in chronic hepatitis patients because chronic hepatitis may have more serious injury of hepatocytes than HCC patients (Qi *et al.*, 2011). Discrepancy in published results regarding miR-233 in HCC may be explained by differences in pathology of associated liver diseases, sample type and criteria of selection, sample size, associated epigenetic factors.

Scientists identified a miRNA panel (miR-192, miR-21,miR-36a, miR-223, miR-27a, miR-122 and miR-801) to discriminate healthy populations from patients with HCC as it provided high diagnostic accuracy (Zhu *et al.*,2013). This panel had considerable clinical value for the early diagnosis of HCC and could help patients who might have otherwise missed the curative treatment window benefit from optimal therapy (Giordano ,and Columbano, 2013). There are still some limitations in our study including small sample size and the fact that diagnosis of early stage HCC could be missed in some of cirrhosis patients which may partly lead to the false-negative of HCC diagnosis and bias in our results. In conclusion, altered serum miR-21 and -223 may play role in pathogenesis of HCC on top of HCV and liver cirrhosis. These miRNAs might serve as a novel and potential biomarker for detection of early HCC in HCV positive patients with liver cirrhosis. Large prospective studies are recommended before miR-21 and -223 can be used as a noninvasive tool for HCC screening in routine follow up of HCV patients.

Conflict of interest: The authors declare no conflict of interest.

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