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EVALUATION OF MICRORNA-93, MICRORNA-210 IN EGYPTIAN PATIENT WITH POST HCV HEPATOCELLULAR CARCINOMA

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ABSTRACT

Background: Hepatocellular carcinoma is one of the most highly malignant and lethal cancers of the world. The most urgent needs are to find sensitive markers for early diagnosis for HCC. MicroRNAs are conserved non-coding RNAs which regulate gene expression at the posttranscriptional level. They have been recently identified as important regulators that affect carcinogenesis. This study was conducted to detect the serum *MiRNA 210 and MiRNA 93a to be applied as early detectors for HCC.* ***Methods:*** A total of 60 serum samples (30 samples from HCC patients,15 samples from chronic Hepatitis c virus patients and 15 healthy control) were collected. The levels of 2 mature miRNAs (miR-210 and miR-93 a) were detected by real time quantitative reverse-transcriptase PCR (RT-qPCR) in sera of the 60 subjects. ***Results:*** Results of the present study showed a higher mean baseline miR-210 level in patients with HCC compared with cirrhotic and control subjects (HCC, 4.25+ 6.18) *VS* (cirrhotic, 3.14+2.25) *VS* (control, 1.03+ 0.30),($P < 0.000$). ***Conclusion:*** In conclusion, The expression of miRNA 210 and miRNA 93a was significantly upregulated in serum of patient with HCC, As a result miRNA 210 and miRNA 93a could be as potential circulating promising noninvasive early detectors for HCC and useful for screening in high risk vulnerable subjects.

KEYWORDS: miRNA 210 and miRNA 93a.

INTRODUCTION

Hepatocellular carcinoma is one of the most highly malignant and lethal cancers of the world. Its pathogenesis has been reported to be multifactorial and the molecular carcinogenesis of HCC cannot be attributed to just few individual genes.[1]

In Egypt, HCC constitutes 13% of all cancers, and accounted for 12.7% of male cancers, 3.4% of female cancer.[2] And unfortunately according to a study published by *EL-Zayadi et al., 2005*.[3] They reported almost 2 folds increase in HCC among chronic liver disease patients over adecade. Chronic HCV accounted for 94% of HCC cases in Egypt in 2010, with 6000 - 7000 deaths/year due to HCC.[4]

Low survival of HCC patients is attributed to late diagnosis, tumor recurrence, and metastasis, with novel biomarkers for early diagnosis urgently needed. Prognosis and survival rates are improved significantly with early diagnosis.[5]

Current diagnostic methods for the diagnosis of HCC fall into two categories: imaging techniques and serological biomarkers, However the diagnostic performance of these modalities is insufficient for early detection of HCC. The widely used serological markers of HCC, α feto protein (AFP) and Des-γ-carboxyprothrombin (DCP), lack specificity and sensitivity. AFP specificity and sensitivity are 75% and 68% respectively. Whereas elevated DCP activity is only present in 44-77%of HCC.[6]

False negative rate with AFP level alone may reach to 40%for patients with early stage. Even in patients with advanced HCC, the AFP may remain normal in 15 – 30%of cases.[7]

New specific markers have been developed to improve the sensitivity, specificity, early detection and prediction of prognosis.[7]

MicroRNAs are conserved non-coding RNAs which regulate gene expression at the posttranscriptional level. They have been recently identified as important regulators that affect carcinogenesis.[8]

MicroRNAs (miRNAs) negatively regulate gene expression and may act as oncogenes, or tumor suppressors, or play dual roles in hepatocarcinogenesis regulating cell cycle, cell proliferation, differentiation, migration, and apoptosis.[9]

Circulating miRNAs are deregulated in HCC and are emerging as novel stable and easily detectable biomarkers for early diagnosis of HCC. Thereby, profiling of circulating HCC-related miRNAs may unravel new molecular biomarkers with high sensitivity and specificity for HCC.[10]

Yamamoto et al., 2009[11] first reported an increased amount of miR- 500 in the sera of HCC patients and its levels dropped to normal after the surgical treatment. MiRNAs has become a global concern for cancer research and extensive profiling studies over recent years have shown a variety of miRNAs are abnormally expressed in HCC.[12]

It is interesting to know that many miRNAs were found to be upregulated in HCC such as miR-15b, miR-21, miR-130b and miR-183. While others were found to be down regulated such as miR-122, miR-625.[10]

Among the upregulated MiRNAs in HCC are MiR-210 and MiR-93. *MiR-210* is highly expressed in hepatocellular carcinoma (HCC) and can drive the metastatic spread of HCC cells. In addition, miR-210 is induced by hypoxic conditions in HCC cells and can mediate hypoxia-induced HCC cell migration and invasion by directly targeting vacuole membrane protein 1 (VMP1). Moreover, MiR-210 upregulation under hypoxia not only helps tumor cells to adjust to hypoxic stress but also confers an aggressive phenotype to hypoxic tumor cells.[12]

MiR-93 promotes HCC cell proliferation, migration and invasion through activation of the oncogenic c-Met/PI3K/Akt pathway, and also inhibits apoptosis and drug- sensitivity by directly inhibiting PTEN and CDKN1A expression in HCC cells.[13]

Thus our aim in the current study is to clarify the significance of circulating microRNA- 210 and microRNA- 93 as novel biomarkers for early detection of hepatocellular carcinoma in post HCV hepatocellular carcinoma patients.

2. PATIENTS AND METHODS

2.1. Patients

This is a prospective case control study that was conducted on 60 individuals. They were divided into three groups selected from Tropical diseases Department, Ain Shams University Hospital in the period from December 2015 to August 2016. After taking the approval of research ethics committee of Faculty of medicine, Ain Shams University (FMASU 1559/2013), a written informed consent was obtained from each patient after informing him or her about the steps of the procedure and the expected effects. The studied groups are categorized into three subgroups as follow:

- **Hepatocellular Carcinoma (HCC) group:** included 30 HCC patients; they were diagnosed by ultrasonography and confirmed by tri-phasic CT (computed axial Tomographay).This group was sub-classified into BCLC A (n=15) patients, BCLC B (n=10) patients and BCLC (n=5) patients.The HCC stage was determined according to Barcelona Clinic Liver Cancer staging score.
- **Chronic Liver Disease (CLD) group:** This group consists of 15 patients previously diagnosed as liver cirrhosis based on clinical examination, laboratory investigation and ultrasonography. All cases belongs to this group were infected with hepatitis C virus.
- **Control group**: This group includes 15 normal healthy persons served as control group.

2.2. Methods

- **Sample collection:** Peripheral venous blood samples (with volume 3 ml) were collected into gel vacutainer containing vials. Samples were centrifuged at 10,000 rpm at room temperature for 10minutes; serum samples were stored at -80º for further analysis.
- **Routine Chemical and Hematological Tests:** Fasting venous blood samples were collected from all patients for routine workup, including complete blood picture, liver function tests, prothrombin concentration and prothrombin-international normalized ratio, AFP, anti-HCV titer, HBsAg, and HBc-Ab using commercially available assays.
- **Purification of total RNA, including miRNA from serum samples:** Total RNA was isolated from serum by using the"miRNeasySerum/Plasma Kit" (Qiagen, Hilden, Germany).
- **Reverse transcription:** Candidate miRNA (miR-125_b) were reversibly-transcribed using miScript II RT Kit. In a reverse transcription reaction with miScript HiSpec Buffer, mature miRNAs are polyadenylated by poly (A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT priming. The cDNA is then used for real-time PCR quantification of mature miRNA expression.
- **miRNA-$_{210}$ and miRNA-93a gene expression analysis by Real time PCR:** Relative miRNA expression levels for the candidate miRNA-125_b were analyzed by miScript SYBR Green PCR Kit (Qiagen, Germany) and targets specific primers (Hs_miR-125b_1 miScript Primer Assay (cat no: 218300) which targets mature miRNA: hsa-miR-125b-5p cat no: MS00006629 (Qiagen, Germany) and Hs_SNORD68_11 miScript Primer Assay cat no: 218300) as housekeeper gene (HK)which targets SNORD68 small nucleolar RNA, C/D box 68 cat no: (MS000337).

2.3. Statistical Data Management and Analysis

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 18.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Data was presented and suitable analysis was done according to the type of data obtained for each parameter.

- **Descriptive statistics**
- Mean, Standard deviation (± SD) and range for parametric numerical data, while Median and Interquartile range (IQR) for non-parametric numerical data.
- Frequency and percentage of non-numerical data.

- **Analytical statistics**
- **Student T** Test was used to assess the statistical significance of the difference between two study group means.
- **Mann Whitney Test (U test)** was used to assess the statistical significance of the difference of a non-parametric variable between two study groups.
- **ANOVA test** was used to assess the statistical significance of the difference between more than two study group means.
- **The Kruskal-Wallis** test is was used to assess the statistical significance of the difference between more than two study group non parametric variable.
- **P- value:** level of significance. -P > 0.05: Non significant (NS).

RESULTS

In this study the mean age of HCC group was 60.13 + 6.8; their ages ranged from 50 to 75 years. and HCV cirrhotic group had a mean age 54.93+ 9.2;their ages ranged from 39 to 68 years. The mean age of control group was 41.21 +1.1; their ages ranged from 17 to 57 years. subjects was subdivided according to their age into 2subgroups < 50 years (n=16, Hcc =1 and nonHcc =15),and >50 (n=44, Hcc=29 and nonHcc =15).

Our study involved 25 male (42%) and 35 female(58%). The male subjects were subdivided into Hcc group (n=19) and nonHcc group (n=6).The females also subdivided into Hcc (n=11)and nonHcc (n=24).The study involved 27 subjects from urban (Hcc=7 and non Hcc=20) and involved 33 rural ones (Hcc=23 and nonHcc=10).

In our study, we compared between *HCC and NON HCC groups (cirrhotic and Healthy control)* regarding demographic data and past history,It was found that the studied groups show a highly statistical significant difference regarding age (p = 0.000),gender (p=0.001) and residence (p=0.001).However, There is no any statistical difference regarding the past medical history.

Table 1: Comparison between HCC /non HCC subgroups regarding demographic data and patient past history.

Parameter	Groups	Total	Groups		Person's chi-square	P value
			HCC (n=30)	Non HCC (n=30		
Age (years)	*≤ 50*	16	1	15	16.7	0.000 (HS)
	>50	44	29	15		
Gender	*Male*	25	19	6	11.59	0.001 (HS)
	Female	35	11	24		
Residence	*Urban*	27	7	23	11.38	0.001 (HS)
	Rural	33	20	10		

As an attempt to find out if miRNA -93a and 210 expression can be used as predictable biomarker for HCC and can differentiate Hcc from Hcv cirrhotic and control, Our results revealed that there was a strong statistical correlation between the studied groups considering miRNA -93 a and miRNA-210 (P value =0.000).

Table 2: Comparison between the different studied groups regarding molecular data.

P- value	Test	Group	Mean ±SD	Mean Rank	Person's Chi-Square	
miRNA expression	miRNA-93a	HCC	17.6±3.87	44.43	38.27	**0.000***
		HCV	8.77±1.67	17.50		
		Control	0.86±0.22	15.63		
	miRNA-210	HCC	4.25±6.18	30.38	13.92	**0.000***
		HCV	3.14±2.25	37.00		
		Control	1.03±0.30	24.23		

DISCUSSION

Results of the present study showed a higher mean baseline miR- 210 level in patients with HCC compared with cirrhotic and control subjects (HCC, 4.25+ 6.18) *VS* (cirrhotic, 3.14+2.25) *VS* (control, 1.03+ 0.30),(P < 0.000).

Our results are in agreement with a study conducted by *Meixiao et al. (2014)*[14] that measured serum MiR-210 in 113 HCC patients and39 healthy control subjects, The results proved a higher mean baseline miR-210 level in HCC compared with control subjects (3.69 + 2.04 vs 1.08 + 0.45,p < 0.001).

Results of the present study showed that genetic expression of miRNA 210 was significantly increased in HCC (p<0.000) compared to cirrhosis and control by 1.4 and 4.2 folds respectively.

Weiqi et al. (2015),[15] In another study conducted on 21 pairs of HCC and the corresponding non-tumor liver samples reported that, The expression of miR-210 in the tumor liver samples was 3.4 fold increased over that of the corresponding non-tumor samples (P < 0.01), which

is similar to our results.

In the previous study, The expression of miR-210 was also determined for primary hepatocytes and HCC-derived HepG2 and HuH7 cells. In the hepatocytes, the relative miR-210 expression level was 0.13 ± 0.01 while that for HepG2 and HuH7 cells were 4.37 ± 1.48 and 2.39 ± 0.54 respectively.

Another study conducted by *Qiao et al. (2011),*[12] to determine the expression of miR-210 in HCC, the mature miR-210 was detected in 48 pairs of HCC and corresponding noncancerous liver samples using quantitative RT- PCR. The results showed that the expression of miR- 210 was significantly up-regulated in HCC when compared to the noncancerous liver samples ($P < 0.0001$).

In contrast to results of our study that showed rise level of MiR 210 in all samples *Qiao et al. (2011)*[12] found that, The overall expression level of miR-210 increased 5.2-fold in 29 HCC samples (60.42%) only, was unchanged in 10 samples and was down-regulated in nine samples, This difference can be due to that, their study was performed on pathologically confirmed HCC samples and our limited number of patients and controls.

Noteworthy, They have demonstrated that miR-210 is often up-regulated in hepatocellular carcinoma (HCC) and can drive the metastatic spread of HCC cells. In addition, MiRNA -210 is induced by hypoxic conditions in HCC cells and can mediate hypoxia-induced HCC cell migration and invasion by directly targeting vacuole membrane protein 1 (VMP1). However miR-210 upregulation under hypoxia not only helps tumor cells to adjust to hypoxic stress but also confers an aggressive phenotype to hypoxic tumor cells.[12]

MiR-93a is an oncogenic miRNA that stimulates tumorigenesis by inhibiting the PTEN (Tumor suppressor gene) and CDKN1A genes (main factor in cell cycle regulation), thereby controlling HCC cell apoptosis and is a potential therapeutic target in HCC.[13]

Results of the present study showed a higher mean baseline miR- 93a level in patients with HCC compared with cirrhotic and control subjects (HCC, 17.6+ 3.87) *VS* (cirrhotic, 8.77+ 1.67) *VS* (control, 0.86+ 0.22),($P = 0.000$).The expression level of MiR-93 in HCC shows 20.4 fold change than that of control group and increase 2 folds than HCV group.

Our study is in agreement with another study conducted by *Katsuya et al. (2014)*[13] They confirmed the significant increase of miR-93a expression in six HCC cells compared to normal hepatocyte cells by miR PCR assays. The expression levels of miR- 93 was stimulated 4.5-fold in a cohort of 47 HCC tumors compared to 40 normal liver and liver cirrhosis tissues.

Another study conducted by *Thomas et al. (2016)*[16] assessed the levels of 829 mature miRNAs, of which 32 were significantly differentially expressed. Statistical analysis indicates that six of these miRNAs regulate a significant proportion target mRNAs. Three of these miRNAs (miR-26a, miR-122, and miR-130a) were down-regulated in HCC, and their up-regulated gene targets are primarily associated with aberrant cell proliferation that involves DNA replication, transcription and nucleotide metabolism. The other three miRNAs (miR-21, *miR-93a*, and miR-221) were up-regulated in HCC, and their down-regulated gene targets are primarily involved in metabolism and immune system processes.

The expression of miRNA 210 and miRNA 93a was significantly upregulated in serum of patient with HCC, So miR-210 and 93 a could be as potential circuliting promising non invasive early detectors for HCC.

The present study has some limitations. Because of the retrospective nature of our study, serum samples of some patients were in storage for a prolonged period of time and might have been subject to degradation. Another limitation of this study is the limited number of patients and controls.

Finally, Our understanding of HCC pathology is still very much fragmented and little progress has been made to improve clinical outcome of HCC patients. While recently discovered miRNA deregulation in HCC has added to the complexity of our understanding of HCC, it has also presented promising novel approaches to understand, diagnose and treat HCC.[17]

CONCLUSION

In conclusion, The expression of miRNA 210 and miRNA 93a was significantly upregulated in serum of patient with HCC. As aresult, The miRNA 210 and miRNA 93a could be as potential circulating promising noninvasive early detectors for HCC and useful for screening in high risk vulnerable subjects.

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