African J. Biol. Sci., 20 (1): 1-16(2024)
ISSN 1687-4870 **e- ISSN 2314-5501 (online)**
www.ajbs.journals.ekb.eg **E.mail: aasdjournal@yahoo.com**

miR-203 and endocan as diagnostic markers for hepatocellular carcinoma related viral infections

Nouran M. Elkady[1], Eman A. El-maadawy[1]*, Mahmoud GadAllah[2], Alshimaa M. Elmalawany[3] and Mohamed F. Elshal[1]

[1]Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute [GEBRI], University of Sadat City, Egypt. Postal code 32897

[2]Molecular Diagnostics Department, Genetic Engineering and Biotechnology Research Institute [GEBRI], University of Sadat City, Egypt. Postal code 32897

[3]Department of Clinical Pathology, National Liver Institute, Menoufia University, Egypt. Postal code 32951

Corresponding author E-mail: Emananwr2020@gmail.com
eman.anwr@gebri.usc.edu.eg.

Corresponding Co-authors E-mail:
nm2600083@gmail.com
mohamed.gadallah@gebri.usc.edu.eg
smalawany@liver.menofia.eg.edu
mohamed.elshal@gebri.usc.edu.eg

Received: December 6 , 2023; Accepted: January 3, 2024; Available online : January 11, 2024
DOI 10.21608/AJBS.2024.253715.1064

ABSTRACT

This study aimed to evaluate the expression of miR-203 and serum endocan in Hepatocellular carcinoma (HCC) patients suffered from hepatitis C virus (HCV) or hepatitis B virus (HBV) to investigate their diagnostic value. This study was conducted on 70 patients divided into 29 HCC [20 on top of HCV and 9 on top of HBV], 41 non-HCC chronic liver disease [20 positive HCV and 21 positive HBV] patients. Ten healthy volunteers were recruited as controls. The expression of miR-203 was detected using quantitative real time (qRT-PCR). Serum endocan was assessed by Enzyme linked immunosorbent assay (ELISA). The results indicated that there was a significant reduction in miR-203 expression level in HCC-related HCV group compared to HCV ($p<0.001$) and control group ($p<0.001$) and in HCV group compared to controls ($p<0.001$). On the other hand, there was a significant increase ($p<0.001$) in miR-203 HCC-related HBV group compared to HCC-related HCV group, with a significant elevation ($p<0.01$) in HBV versus HCV group. At a cutoff value of 0.3276, miR-203 showed 100% sensitivity and 90% specificity [AUC=0.989] in HCC-related HCV group. Serum endocan was significantly elevated ($p<0.05$] in HCC patients versus controls. A significant increase ($p<0.05$] was found in HCC-related HCV compared to HCV group, with a significant diminution ($p<0.001$) in HBV group versus controls. HCV patients showed a significant increase ($p<0.05$) in endocan level compared to HBV ones. **In conclusion:** miR-203 and serum endocan could be used as diagnostic markers for HCC related different viral infection in the Egyptian patients. Further research is required to confirm our findings.

***Keywords*:** miR-203, Endocan, HCC, HCV, HBV.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth and the fourth most common primary liver cancer worldwide and in Egypt, respectively (Sharma, 2020, Rashed *et al.*, 2020). Egypt comes after Mongolia,

and the highest in Africa as regard the estimated age-standardized incidence rates [ASIRs] per 100,000 for liver cancer in the world in 2018 (Sharma, 2020). Worldwide, it ranks as the fourth leading cause of cancer-related mortality (Edwards & Macdonad, 2020) and by the next decade, the global incidence is expected to rise to one million cases per year (Sharma, 2020). In Egypt, HCC represented 70.48% of all liver tumors, acting as the most leading cause of cancer related mortality and morbidity (Elghazaly *et al*., 2018; Hamdy Elsisi *et al*., 2019). Numerous risk factors have been associated with the development and progression of HCC globally (Petruzziello, 2018), however chronic infection with hepatitis C virus (HCV) and to a lesser extent hepatitis B virus (HBV) account for approximately 75–80% of HCC cases (Ziada *et al.*, 2016; Edwards & Macdonad, 2020). Viral infection with liver viruses specially HCV and HBV is one of Egypt's most serious public health issues, leading to chronic hepatitis, liver cirrhosis, and ultimately HCC (Elbahrawy *et al*., 2021). It was reported that HCV prevalence in Egypt is 4.6% (Waked *et al.*, 2020). The high burden of chronic HCV infection in Egypt resulted in an increase in the number of HCC cases (Elghazaly *et al.*, 2018). That was supported by the finding that 74.0% of Egyptian patients with HCC are positive for HCV antibodies (Kouyoumjian *et al.*, 2018), supporting the role of HCV in causing liver cancer. Moreover, it was reported that about 3.3 million Egyptians are infected with HBV and that 15%–25% of them has increased risk of death from liver cancer or end-stage liver disease (Elbahrawy *et al.*, 2021).

MicroRNAs [miRNAs/miRs] are small non-coding RNAs that regulate gene expression at post-transcriptional levels (O'Brienal *et al.*, 2018). In recent years, miRNAs were reported to play a vital role in a wide range of important biologic processes including modulation of host response to viral infection, cellular growth, death, and differentiation (El-Maadawy *et al.*, 2020). Additionally, aberrant expression of miRNA has been implicated in tumorigenesis, progression, and invasion in several types of cancers, including liver tumors (Khairy *et al*., 2016; Mohamed *et al*., 2017). A number of miRNAs have been reported to be associated with HCC progression, in particular miR-203 which has been considered as tumor suppressor miRNA because it suppresses tumor growth through mechanisms involve PIK3CA, p38 MAPK, c-Jun, and GSK3 signaling pathways in liver cancer (Wei *et al.*, 2013; Zhang *et al*., 2018). Moreover, miR-203 is implicated in HCC progression and metastasis [Wan *et al.*, 2016). Upregulation of miR-203 suppressed HCC growth and metastasis by downregulating the expression of RAB22A (Zhang *et al.*, 2014; Cao *et al.*, 2019). In addition, downregulation of miR-203a was found to be associated with HCV core protein-induced angiogenesis and HCC cells invasion (Liu *et al.*, 2015). Overall, these studies suggest that miR-203 may play an important role in suppressing metastasis in HCC and targeting miR-203 or its downstream signaling pathways may have therapeutic potential for treating metastatic cancers.

HCC angiogenesis and invasion has also been linked to a wide range of proteins and their expression levels were found to have diagnostic and prognostic values (Mansouri *et al.*, 2020). Endocan, also called endothelial cell-specific molecule-1 [ESM1], is a soluble 50-kDa proteoglycan which is synthesized and produced by activated endothelial cells as well as tumor endothelial cells (Omar *et al.*, 2021). It has been identified as a key regulator of inflammatory diseases, vascular and endothelial injury, cell adhesion, angiogenesis and tumor progression (Yang

et al., 2015). It is aberrantly expressed in several types of cancers, including solid and liquid tumors, serving as a diagnostic and prognostic tumor marker (Yang *et al.*, 2020). Also, endocan over expression has been associated with viral infection (Ziada *et al*., 2016; Edwards & Macdonad, 2020; Domínguez-Alemán *et al*., 2021) and with viral-related cancers including HCC (Domínguez-Alemán *et al*., 2021). Endocan was also found to augment tumor invasiveness, metastasis, and recurrence (Liu *et al*., 2015). Therefore, endocan has been suggested not only as a diagnostic tumor marker but also as a target for cancer therapy (Yang *et al*., 2015; Zhang *et al*., 2021). Several studies suggested that endocan expression is associated with poor prognosis in HCC and targeting endocan expression may offer a potential therapeutic strategy for improving patient outcomes (Wu *et al.*, 2016; Mähringer-Kun *et al*., 2021). However, whether endocan might serve as a useful biomarker to monitor disease progression and correlation with miR-203 in patients with liver cancer is largely unknown. Therefore, this study was designed to investigate the potential benefit of assessing miR-203 expression and serum endocan as non-invasive biomarkers in the Egyptian patients suffering from HCC-related HCV and/or HBV infection. Also, to determine the association between miR-203 and serum endocan in HCC patients and their correlation with the clinical manifestations of the disease.

SUBJECTS AND METHODS

1. Subjects

This study was approved by the local ethics committee of the National Liver Institute at Menoufia University, and each subject provided signed informed consent. A total of 80 participants were recruited from October 2018 to December 2020, divided into 5 groups; A: a healthy control group [10 subjects] B: patient with HCV and has no evidence of HCC [20 patients], C: patient with HCC related HCV on a background of cirrhosis and fibrosis [20 patients], D: patient with HBV and has no evidence of HCC [21 patients] and E: patient with HCC related HBV on a background of cirrhosis and fibrosis [9 patients]. All participants were subjected to thorough history taking, full clinical examination and laboratory investigations. Abdominal ultrasound was done to assess liver size, coarseness of parenchyma, liver surface nodularity, lymph nodes enlargement and size, spleen size [which if enlarged can suggest portal hypertension], patency and flow of veins and arteries and focal lesions [which if present can suggest HCC].

All HCC patients were diagnosed by the characteristic vascular enhancement pattern detected by computed tomography (CT) scan or Magnetic resonance imaging (MRI) according to established diagnostic criteria (Liu *et al*., 2019). All patients with human immunodeficiency virus [HIV], immunosuppression, all other malignancies, and lastly, patients who were under chemotherapy and antiviral treatment were excluded. All protocol investigations were carried out with consideration for the human subject and in conformity with the Helsinki Declaration and the Human Ethical Clearance Committee rules for clinical research.

2. Blood sampling

For the study, 3 mL of peripheral venous blood was withdrawn into plain blood sterile collection tubes without any additives and allowed to clot before centrifugation [Zhang et al., 2018)1500 rpm for 10 minutes]. Serum was then separated, aliquoted [to be thawed only once] and

stored at −80 °C for RNA extraction and estimation of the serum levels of endocan.

a. **Evaluation of miR-203 by quantitative real time PCR [qRT-PCR]**

Total RNA was extracted from 200μl serum samples utilizing the miRNeasy Mini Kit [Qiagen, Valencia, CA, USA; Cat.No.217004] regarding to the manufacturer's guidelines. The quantity of extracted RNA was spectrophotometrically assessed by NanoDrop™ 2000/2000c [Thermo Fisher Scientific, Waltham, MA, USA]. RNA was reverse transcribed into cDNA using the miScriptII RT kit [Qiagen, Valencia, CA, United States, Cat. No. 218061] according to the constructor's instructions. The reverse transcription reaction was performed with 20µl final reaction volume consisting of 4µl 5X HiSpec buffer; 2µl 10X nucleic mix, 2 µl miScript RT enzyme and 200ng RNA. The reaction was incubated at 37°C for 60 min followed by an inactivation RT enzyme step at 95°C for 5 min and was done in a 2720 thermal cycler [Applied Biosystems]. The quantitative real-time PCR [qRT-PCR] for the detection of miR-203 was performed on the AriaMax Real-Time PCR System [Agilent Technologies]. The reaction was done in 25 µl PCR reactions using miScript Primer Assays and miScript SYBR Green PCR Kit [catalog number 218073] [Qiagen] along with the manufacturer's protocol. The PCR cycles were 95°C for 15 minutes, then 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. The post-amplification melting curve program was 95˚C for 30 seconds, 65˚C for 30 seconds, followed by 95˚C for 30 seconds. The data normalization of miR-203 expression level was assessed in comparison to the endogenous controls, U6B small nuclear RNA [RNU6B] in all samples and controls. The relative expression level of miR-203 was calculated using the $2^{-\Delta\Delta Ct}$ method and the results were expressed as relative fold change [RFC] [Zhang et al., 2020).

b. **Measurement of serum endocan levels**

Human endocan levels were evaluated in the serum samples of whole subjects using an Enzyme-linked immunosorbent assay [ELISA] kit [Catalog no:SG-10619, Sinogeneclon Co., Ltd., USA] in accordance with kit procedures. The measurement steps were performed according to manufacturer's instructions. The ELISA Microplate Reader was used to measure the intensity of color at an absorbance of 450 nm [Sunrise^{TM}, Tecan Group Ltd. Mannedorf, Switzerland]. The ELISA reader-controlling software processed the absorbance values into a standard curve from which the Endocan concentrations were derived [Softmax; Molecular Devices, Sunnyvale, CA, USA]. The results were expressed in pg/ml.

Statistical analysis

Results were analyzed utilizing the Statistical Package for Social Science [SPSS] version 21.0 [IBM Corporation, USA]. Data were presented as mean ± standard error [M ± SE] or frequencies and percentages when appropriate. Univariate analysis was calculated using the independent *t*-test, or Chi-square [X^2] test. The one-way analysis of variance [ANOVA] test was used to compare the different groups. The cut-off values for endocan and miR-203 were performed using the receiver operator characteristic curve [ROC]. For combined markers, a binary logistic regression was used to get the predicted probability of the combination that was used as the test variable in the ROC curve analysis. Sensitivity and specificity values were conducted according to this value. Pearson's correlation test was used to determine the relationship between continuous variables, whereas spearman's correlation was used ranked parameters. All P values were two-sided and statistical

significance was defined as a value of less than 0.05.

RESULTS

1. Patient's characteristics

The demographic and biochemical data of the studied groups were presented in Table (1 a & b). The clinical features of the patients' groups were summarized in Table (2). All subjects were age and sex matched. Most HCC patients did not suffer from ascites, except for the HCC-related HCV group, where 50% of cases endured ascites.

Table 1a. Demographical and biochemical characteristics of control, HCV, and HCC_HCV patients.

Parameter	Control [10]	HCV [20]	HCC_HCV[20]
Age [Year]	50.80 ± 3.68	53.30 ± 1.62	53.00 ± 0.88
Gender [M/F]	8/2	16/4	17/3
ALT [IU/L]	16.60 ± 1.5 ^{a***}	40.6 ± 3.02 ^{c**}	58.15 ± 5.04 ^{b***}
AST [IU/L]	17.80 ± 1.45 ^{a***}	52.75 ± 3.32	53.55 ± 2.85 ^{b***}
Albumin [g/L]	4.11 ± 0.12 ^{a**}	3.42 ± 0.13	3.31 ± 0.14 ^{b**}
TP [g/dL]	6.83 ± 0.13	7.06 ± 0.15^{c***}	6.02 ± 0.16 ^{b**}
T_BILI [mg/dL]	0.61 ± 0.08 ^{a***}	1.43 ± 0.12	1.49 ± 0.12 ^{b***}
D_BILI [mg/dL]	0.10 ± 0.04 ^{a**}	0.33 ± 0.03 ^{c**}	0.55 ± 0.06 ^{b***}
INR	1.04 ± 0.03	1.12 ± 0.02	1.64 ± 0.45
AFP [ng/ml]	2.51 ± 0.26 ^{a***}	13.09 ± 1.78 ^{c***}	2426.72 ± 566.23 ^{b**}
HCV_PCR	----.	3.7 x10$^{\wedge 5}$± 2.9 x10$^{\wedge 5}$	3.4x10$^{\wedge 6}$±1.9 x10$^{\wedge 6}$

All data are presented as mean ± standard Error [mean ± SE]. Aspartate aminotransferase [AST]; Alanine aminotransferase [ALT]; Total Bilirubin [T. Bili]; Direct Bilirubin [D.Bili]; Total protein [TP]; international normalized ratio [INR]; Alpha fetoprotein [AFP]; a:HCV from control, b:HCC_HCV from control, c: HCC_HCV from HCV,*:P<0.05 **: p<0.01, ***: p<0.001

Table 1b. Demographic and biochemical characteristics of control, HBV, and HCC_HBV patients.

Parameter	Control [10]	HBV [21]	HCC_HBV [9]
Age [Year]	50.80 ± 3.68	50.66 ± 1.98	52.55 ± 3.64
Gender [M/F]	8/2	16/5	7/2
ALT [IU/L]	16.60 ± 1.51 ^{a***}	41.80 ± 1.73 ^{c*}	224.66 ± 122.17
AST [IU/L]	17.80 ± 1.45 ^{a***}	40.33 ± 1.80 ^{c*}	344.55 ± 228.46
Albumin [g/L]	4.11 ± 0.12	4.18 ± 0.11 ^{c***}	2.82 ± 0.19 ^{b***}
TP [g/dL]	6.83 ± 0.13	6.73 ± 0.14	6.44 ± 0.41
T_BILI [mg/dL]	0.61 ± 0.08	0.73 ± 0.08 ^{c***}	3.11 ± 0.61 ^{b**}
D_BILI [mg/dL]	0.10 ± 0.04	0.12 ± 0.02 ^{c***}	1.63 ± 0.41 ^{b**}
INR	1.04 ± 0.03	1.02 ± 0.02 ^{c***}	1.36 ± 0.09 ^{b**}
AFP [ng/ml]	2.51 ± 0.26 ^{a*}	4.11 ± 0.42 ^{c**}	17546.6 ± 7847.5 ^{b*}
HBV_PCR	----	1.0 x10$^{\wedge 6}$± 3.9 x10$^{\wedge 5}$	1.5 x10$^{\wedge 8}$ ± 1.5x10$^{\wedge 8}$

All data are presented as mean ± standard Error [mean ± SE]. Aspartate aminotransferase [AST]; Alanine aminotransferase [ALT]; Total Billirubin [T. Bili]; Direct Billirubin [D.Bili]; Total protein [TP]; international normalized ratio [INR]; Alpha fetoprotein [AFP]; a:HCV from control, b:HCC_HCV from control, c: HCC_HCV from HCV,*:P<0.05 **: p<0.01, ***: p<0.001

Table 2. Clinical features of the patient groups.

Parameter		HCV [20]	HCC_HCV [20]	p-value	HBV [21]	HCC_HBV [9]	p-value
Ascites	Yes	0 [0%]	10 [50%] [a*]	**p<0.001**	0 [0%]	9 [100%]	**P<0.001**
	No	20 [100%]	10 [50%]		21 [100%]	0 [0%]	
Cirrhosis	Yes	0 [0%]	16 [80%]	**p<0.001**	3 [14.3%]	9 [100%]	**P<0.001**
	No	20 [100%]	4 [20%]		18 [85.7%]	0 [0%]	
Fibrosis	No	17 [85%]	0 [0%] [a*]	**p<0.001**	11 [52.4%]	0 [0%]	**P<0.001**
	F1	3 [15%]	2 [10%]		7 [33.3%]	0 [0%]	
	F2	0 [0%]	5 [25%]		1 [4.8%]	0 [0%]	
	F3	0 [0%]	6 [30%]		2 [9.5%]	0 [0%]	
	F4	0 [0%]	7 [35%]		0 [0%]	9 [100%]	

a: HCC_HCV from HCC_HBV, *:P<0.05 **: p<0.01, ***: p<0.001

2. The expression level of miR-203

The expression level of miR-203 in all studied groups was shown in Figure (1). All results were expressed as the mean of the RFC of the expression level. There was a statistically significant decrease in relative fold change (RFC) of miR-203 expression levels in HCC-related HCV (C group) compared with HCV (B and control A groups) (p<0.001, p<0.001 respectively). In addition, the HCV (B group) showed a significant decrease (p<0.001) in the miR-203 levels compared to the control (A group). On the other hand, there was no significant difference in the miR-203 levels between HBV and HCC-related HBV (D and E groups) groups compared to healthy subjects (A group). Focusing on HCC, the HCC-related HCV group (C group) showed a significant (p<0.001) lower miR-203 expression level compared to the HCC-related HBV group (E group). Regarding the viral infection only, the mean concentration of serum miR-203 was significantly lower (p<0.01) in the HCV group (C group) compared to the HBV group (D group).

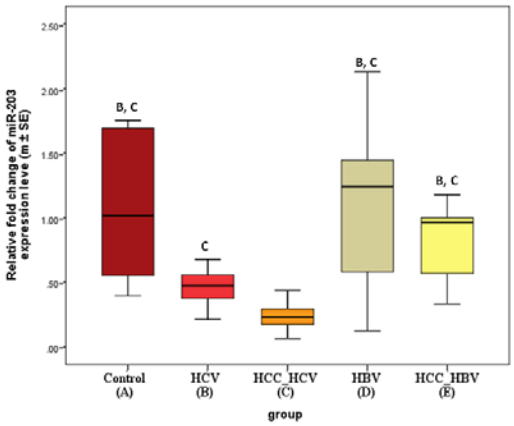

Fig. (1). Relative fold change of miR-203 expression level of the different groups. Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the significantly smaller category appears above the category with larger mean.

a. **Serum endocan secretion levels**

It was obvious from Figure (2) that level of serum endocan was found significantly higher ($p<0.05$) in HCC patients compared to healthy subjects. After segregating patient groups according to their existing type of viral infection, HCC-related HCV group showed significantly ($p<0.05$) higher serum endocan levels compared to the HCV group. Moreover, HCC-related HBV group (E group) demonstrated significantly higher ($p<0.001$) endocan levels than patients with HBV (D group). As regard to viral infection, serum endocan was substantially higher ($p<0.05$] in the HCV groups than the HBV groups.

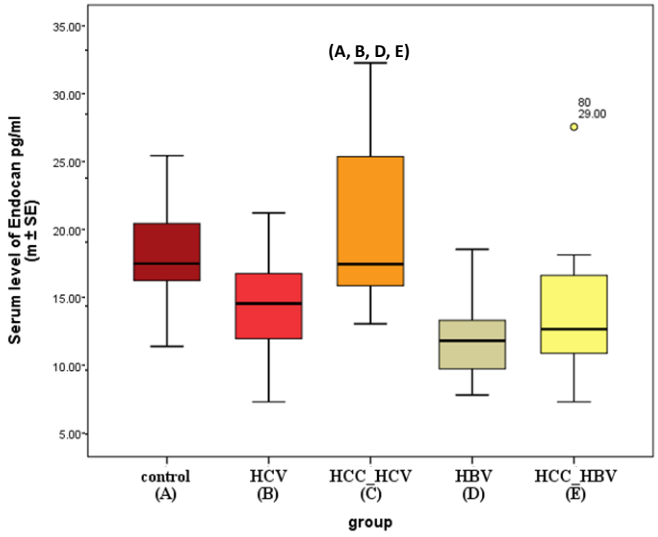

Fig. (2). **Mean expression level of serum endocan in different groups.** Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key to the significantly smaller category appears above the category with larger mean.

b. Correlation of miR-203 and serum endocan levels with the clinical and laboratory parameters

It was clear from data in Table (3) that cirrhotic patients and patients with ascites showed significantly lower miR-203 and higher endocan levels compared with non-cirrhotic and non-ascitic patients (both at $p<0.05$). Also**,** miR-203 was positively correlated with albumin ($r=0.336$, $p<0.01$), and negatively with fibrosis and cirrhosis ($p<0.05$ and $p<0.01$, respectively). There was no significant correlation found between miR-203 and any of the studied laboratory parameters. On the other side, serum endocan was positively correlated with age ($r= 0.286$; $p<0.05$), the stage of liver fibrosis ($r= 0.280$; $p<0.05$), cirrhosis ($r= 0.295$; $p<0.01$), and ascites ($r= 0.278$; $p<0.05$).

Table (3). Mean expression level of miR-203 and serum endocan with the clinical manifestations of the 29 HCC patients.

Parameter		miR-203		Endocan	
		Mean	S.E.	Mean	S.E.
Fibrosis	No-fibrosis	0.83	.08	14.33	.67
	F1	0.94	.25	15.96	2.08
	F2	0.26	.05	21.19	3.32
	F3	0.37	.13	17.76	1.59
	F4	0.57	.09	17.95	1.82
Cirrhosis	Non-cirrhotic	0.84	.08	14.78	.68
	Cirrhotic	0.46*	.06	18.28*	1.21
Ascites	Non-ascitic	0.74	.07	15.24	.62
	Ascites	0.52*	.08	19.01*	1.74

*:P<0.05

Table 4. Correlation between demographic, biochemical, clinical features, miR-203 and serum endocan in HCC group.

	miR-203	Endocan
Pearson Coefficient		
AGE	-0.181	٠.286*
ALT	0.023	-0.119
AST	0.033	-0.113
ALB	٠.336**	-0.116
TP	0.14	-0.198
T_BILI	-0.199	-0.012
D_BILI	-0.116	-0.027
INR	-0.146	0.233*
AFP	0.004	-0.092
HCV_PCR	-0.141	-0.059
HBV_PCR	-0.034	-0.089
Spearman Coefficient		
Sex	0.049	0.003
Fibrosis	-٠.282*	٠.280*
Cirrhosis	-٠.348**	٠.295**
Ascites	-0.181	٠.278*

*:P<0.05 **: p<0.01.

c. Diagnostic value of single/combined markers

Results of the ROC curve of single markers for HCC-related HCV and HCV patients (Fig. 3a, b) showed an area under the curve (AUC) value for RFC miR-203 of (0.989) and at a cutoff value of 0.3276, miR-203 showed 100% sensitivity and 90% specificity. For serum endocan, a cutoff value of 15.975, endocan showed 90% sensitivity and 65% specificity (Table 5). Logistic regression and ROC curve analyses were used to comprehensively analyze the differential diagnostic value of the combination mode, as shown in Figure (3c). It was found that the AUC results obtained for endocan and miR-203 markers combination with greater AUC than that found using each marker alone, and comparable to that of AFP.

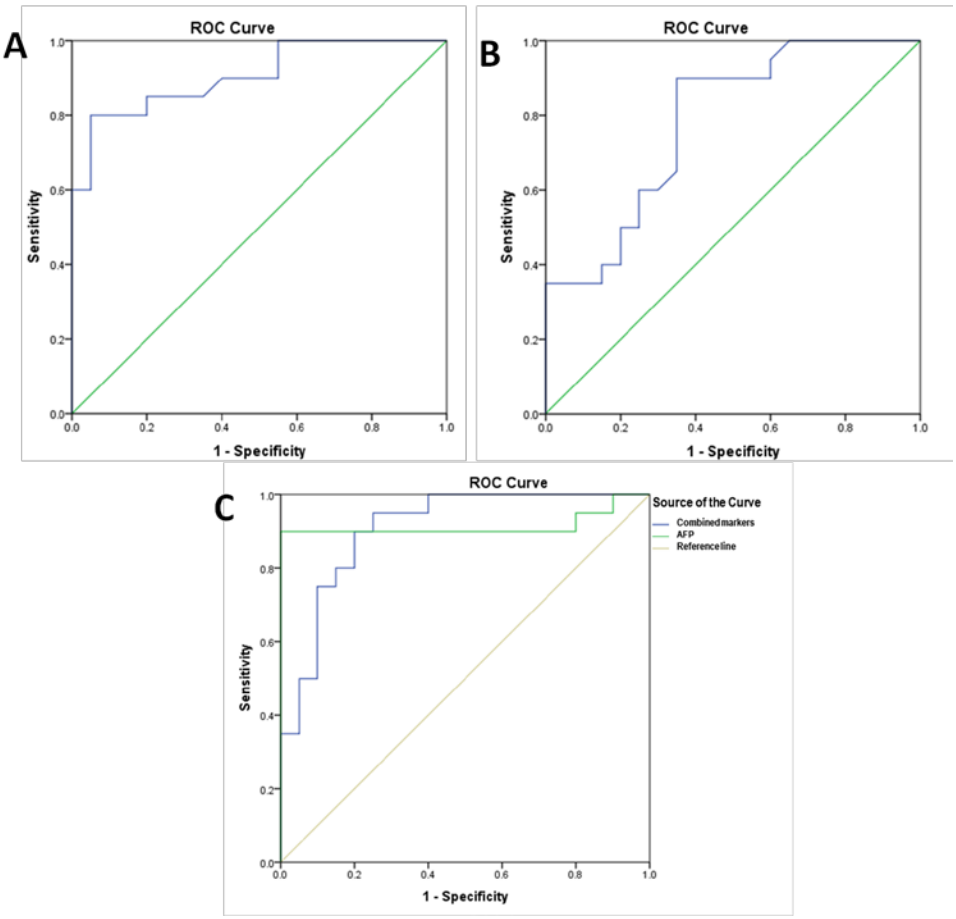

Fig. (3). ROC curves of A: miR-203, B: endocan and C: combined parameters between HCC-related HCV and HCV groups.

Table (5). ROC curve analysis of the parameters used in the diagnosis between HCC-related HCV and HCV patients.

Variable[s]	Area Under curve	Std. Error	Sig.	95%Confidence Interval		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
AFP	0.915	0.058	<0.001	0.802	1	41	90	100
miR-203	0.906	0.047	<0.001	0.814	0.998	0.355	80	95
Endocan	0.783	0.072	<0.01	0.64	0.925	15.975	90	65
Predicted probability	0.908	0.047	<0.001	0.816	0.999	0.215	95	65

DISCUSSION

HCC is a very aggressive solid tumor characterized by a very poor prognosis with around 80% of HCC patients will die within one year from diagnosis and with a 5-year survival rate of 18% (Janevska *et al*., 2014). Viral infection with HCV is considered the most important risk factor for HCC in Egypt (Ibrahim *et al*., 2014; Elbahrawy *et al*.,2021). Even after the introduction of the direct-acting antivirals [DAAs] drugs used in the eradication of HCV, HCC remains a

major cause of morbidity and mortality due to increased recurrence rates (El Kassasi *et al*., 2018). Besides, treatment of patients with HCC using these expensive targeted therapies is limited especially in Egypt due to the economic burden (Elsisi *et al*., 2019). Therefore, the development of novel biomarker (s) for the early cancer detection, diagnosis, and treatment is indispensable for decreasing HCC mortality rate. A number of proteins and signaling molecules are involved in the process of HCC progression, angiogenesis and metastasis, including endocan and miR-203 (Zhang *et al.*, 2018; Kurebayashi *et al*., 2021). Both endocan and miR-203 has been associated with viral infections (Melar-New & Laimins, 2010;Yu *et al*., 2012; Yu *et al*., 2013; Domínguez-Alemán *et al*., 2021). However, most of these studies did not examine the role of concomitant viral infection. Therefore, in the current study the circulating endocan levels (protein levels] was measured by ELISA and miR-203 expression levels have been analyzed using RT-PCR in blood of HCV- or HBV-related HCC and compared them with non-HCC subjects.

miR-203 has been demonstrated to be a potential tumor suppressor that often silenced in different malignancies including HCC (Chen *et al*., 2017; Zhang *et al*., 2018). The present data indicated a significant reduction in miR-203 expression of HCC-related HCV compared to HCV and control groups. Additionally, there was a significant decline in miR-203 expression in the HCV group compared to the healthy subjects. In accordance with the current results, Khairy *et al*. (2016) described a reduction of miR-203 in HCC-related HCV compared to the HCV-infected group. As well, HCV core protein has been reported to downregulate miR-203 levels in the immortalized human normal hepatocyte cell line (L02) and in a human hepatocellular carcinoma cell line (HepG2) (Liu *et al.*, 2015).

Analysis of the present results revealed that the HCC-related HBV group showed a significant elevation of miR-203 compared to the HCC-related HCV group. Getting closer to viral infection, the HBV group showed an increase in miR-203 expression than HBV-related HCC, however the difference did not reach statistical significance. These data are in agreement with Shang *et al*. (2015) who reported that HBV represses miR-203 expression in hepatoma cell lines. This finding may suggest that the impact of concomitant viral infection on miR-203 expression is more prominent in HCV-related HCC than in HBV-related ones.

It is worth mentioning that, as in current study, miR-203 expression has been altered via viral infection in several types of tumors. Epstein-Barr Virus (EBV) and human papillomavirus (HPV) were recorded to downregulate the expression of miR-203 in EBV-infected epithelial cells/EBV-associated nasopharyngeal carcinoma (Yu *et al*., 2012) and HPV-associated cervical cancer (Melar-New & Laimins, 2010). In contrast, overexpression of miR-203 was upregulated in squamous cell carcinoma, regardless of HPV status (Gocze *et al*., 2013). The current results report for the first time the variations in the levels of miR-203 expression between HCV- and HBV-related HCC.

Endocan was identified as an endothelial cell activation marker (Bechard *et al*., 2000), however subsequent studies demonstrated that endocan could stimulate the migration and angiogenesis ability of endothelial cells and this activity related to the tumor progression and metastasis (Stéphane *et al*., 2010; El Behery *et al*., 2013; Roudnicky *et al*., 2013). Endocan has been reported to have tumorigenic activities and its expression is associated with poor

prognosis in HCC (Huang *et al.*, 2009). In addition, it was demonstrated that HCC patients with endocan positivity in stromal cells had a higher rate of overall recurrence (Ziol *et al.,* 2018). Meanwhile, silencing endocan was shown to decrease cell survival, migration, and invasion and modulated cell cycle progression in HCC (Tok *et al*., 2014). These data propose that endocan can function as an oncogene and represents a prognostic / diagnostic biomarker in several types of cancer, including HCC (Liu *et al*., 2015; Youssef *et al*., 2018; Yang *et al*., 2020).

In the current work, data are in line with the above-mentioned studies as it revealed a significant elevation of serum endocan in HCC patients compared to healthy subjects. In addition, by segregating the studied patients into groups according to the type of infection, the HCV-related HCC patients showed a significantly higher concentration of serum endocan compared to HCV non-HCC patients. Meanwhile, serum endocan was found significantly lower in the HBV group and insignificantly in the HBV-related HCC group compared to the control group. Thus, it was seen that serum endocan might be upregulated in HCC patients due to HCV infection more eminently than in the case of HCC-related HBV infection. Ike current results agree with previous studies which have pointed out to the association between endocan levels with certain types of viruses-related tumors. Xing *et al*. (2016) indicated the role of cytomegalovirus infection in the upregulation of endocan in glioma disease. Moreover, Yu *et al*. (2013) pointed out that endocan expression can be upregulated by Epstein-Barr virus-encoded protein (Latent Membrane Protein 1) in nasopharyngeal carcinoma.

In the current non-HCC groups, it was found that serum endocan levels in patients with HBV or HCV infection were significantly lower than that in control subjects. These data are in agreement with previous work of Tok *et al*. (2014) who reported decreased endocan levels associated with HBV and HCV infection. In addition, endocan levels were found to be lower in Crimean-Congo hemorrhagic fever patients (caused mainly by Nairo virus) than in healthy controls (Doğan *et al.,* 2018).

Regarding clinical manifestations, it was found that serum endocan level was significantly decreased in HCC patients with the highest stage of liver fibrosis compared to the lowest one. This disagree with the results of Tok *et al.* (2014) who found no association between fibrosis stage and serum endocan levels. Interestingly, the current investigation indicated the presence of a significant negative correlation between the miR-203 expression and serum levels of endocan. In addition, there was a combination between serum concentration of endocan and the expression levels of miR-203 and this provided higher diagnostic efficacy than either marker alone or comparable to that of AFP.

One of the drawbacks of this study is the relatively small number of patients with HCC-related HBV group, as this group is very rare in Egypt, therefore it is recommended to carry out further investigations with a larger sample size to validate these findings and to investigate the molecular mechanisms between viral infection and host response during HCC development.

Conclusion

The current findings indicated the impact of concomitant HCV viral infection on miR-203 expression. Also, serum endocan was more prominent in HCV-related HCC than in HBV-related ones. Therefore, it is suggested that the diagnostic

significance of combined analyses of serum endocan and miR-203 can be helpful in diagnosing HCV-related HCC patients.

REFERENCES

Bechard, D.; Meignin, V.; Scherpereel, A.; Oudin, S.; Kervoaze, G.; Bertheau, P., *et al*. (2000). Characterization of the secreted form of endothelial-cell-specific molecule 1 by specific monoclonal antibodies. J. Vascular Res., 37(5):417–425.

Cao, Y.; Liu, X.; Lu, W.; Chen, J. and Zhang, Y. (2019). MiR-203 inhibits hepatocellular carcinoma cell proliferation and migration through targeting MAP3K2. European Review for Medical and Pharmacological Sciences, 23(14):6195-6202.

Chen, X,B.; Zheng, X.B.; Cai, Z.X.; Lin, X.J. and Xu, M.Q. (2017). MicroRNA-203 promotes liver regeneration after partial hepatectomy in cirrhotic rats. J. Surgical Res., 211: 53–63.

Doğan, H.O.; Büyüktuna, S.A.; Kapancik, S. and Bakir, S. (2018). Evaluation of the associations between endothelial dysfunction, inflammation and coagulation in Crimean-Congo hemorrhagic fever patients. Archives of Virology, 163(3):609–616.

Domínguez-Alemán, C.A.; Sánchez-Vargas, L.A.; Hernández-Flores, K.G.; Torres-Zugaide, A.I.; Reyes-Sandoval, A.; Cedillo-Barrón, L., *et al.* (2021). dengue virus induces the expression and release of endocan from endothelial cells by an NS1-TLR4-Dependent Mechanism. Micro-organisms, 9(6): 1305.

Edwards, J.T. and Macdonald, G.A. (2000). Hepatocellular carcinoma. Current Opinion in Gastroenterology, 16(3): 275–281.

El Behery, M.M.; Seksaka, M.A.; Ibrahiem, M.A.; Saleh, H.S. and El Alfy, Y. (2013). Clinicopathological correlation of endocan expression and survival in epithelial ovarian cancer. Archives of Gynecology and Obstetrics, 288(6): 1371–1376.

El Kassas, M.; Funk, A.L.; Salaheldin, M.; Shimakawa, Y.; Eltabbakh, M.; Jean, K., *et al*. (2018). Increased recurrence rates of hepatocellular carcinoma after DAA therapy in a hepatitis C-infected Egyptian cohort: A comparative analysis. J. Viral hepatitis, 25(6): 623–630.

Elbahrawy, A.; Ibrahim, M.K.; Eliwa, A.; Alboraie, M.; Madian, A. and Aly, H.H. (2021). Current situation of viral hepatitis in Egypt. Microbiol. Immunol., 65(9): 352–372.

Elghazaly, H.; Gaballah, A. and Eldin, N.B. (2018). Clinic-pathological pattern of hepatocellular carcinoma (HCC) in Egypt. Annals of Oncology, 29(v5-v6).

El-maadawy, E.A.; Elshal, M.F.; Bakry, R.M.; Moussa, M.M.; El-Naby, S.H. and Talaat, R.M. (2020). Regulation of CD4+CD25+FOXP3+ cells in pediatric acute lymphoblastic leukemia (ALL): Implication of cytokines and miRNAs. Molecular Immunology, 124:1–8.

Elsisi, G.H.; Nada, Y.; Rashad, N.; Carapinha, J., *et al*. (2019). Cost-effectiveness of sorafenib versus best supportive care in advanced hepatocellular carcinoma in Egypt. J. Medical Economics, 22(2):163–168.

Gocze, K.; Gombos, K.; Juhasz, K.; Kovacs, K.; Kajtar, B.; Benczik, M., *et al.* (2013). Unique microRNA expression profiles in cervical cancer. Anticancer Res., 33(6):2561–2567.

Huang, G.W.; Tao, Y.M. and Ding, X. (2009). Endocan expression correlated

with poor survival in human hepatocellular carcinoma. Digestive Diseases Sci., 54(2):389–394.

Ibrahim, A.S.; Khaled, H.M.; Mikhail, N.N.; Baraka, H. and Kamel, H. (2014). Cancer incidence in Egypt: results of the national population-based cancer registry program. J. Cancer Epidemiol., 2014: 437971.

Janevska, D.; Chaloska-Ivanova, V. and Janevski, V. (2015). Hepatocellular Carcinoma: Risk factors, diagnosis and treatment. Open access Macedonian J. Medical Sci., 3(4): 732–736.

Kang, Y.H.; Ji, N.Y.; Lee, C.I.; Lee, H.G.; Kim, J.W.; Yeom, Y.I.L., *et al*. (2011). ESM-1 silencing decreased cell survival, migration, and invasion and modulated cell cycle progression in hepatocellular carcinoma. Amino acids, 40(3):1003–1013.

Khairy, A.; Hamza, I.; Shaker, O. and Yosry, A. (2016). Serum miRNA Panel in Egyptian Patients with Chronic Hepatitis C Related Hepatocellular Carcinoma. Asian Pacific J. Cancer Prevention : APJCP, 17(5): 2699–2703.

Kouyoumjian, S.P.; Chemaitelly, H. and Abu-Raddad, L.J. (2018). Characterizing hepatitis C virus epidemiology in Egypt: systematic reviews, meta-analyses, and meta-regressions. Scientific reports, 8(1): 1661

Kurebayashi, Y.; Kubota, N. and Sakamoto, M. (2021). Immune microenvironment of hepatocellular carcinoma, intrahepatic cholangiocarcinoma and liver metastasis of colorectal adenocarcinoma: Relationship with histopathological and molecular classifications. Hepatology Research : the official journal of the Japan Society of Hepatology, 51(1):5–18.

Liu, D.; Wu, J.; Liu, M.; Yin, H.; He, J. and Zhang, B. (2015). Downregulation of miRNA-30c and miR-203a is associated with hepatitis C virus core protein-induced epithelial-mesenchymal transition in normal hepatocytes and hepatocellular carcinoma cells. Biochem. Biophys. Res. Commun., 464(4):1215–1221.

Liu, L.; Chen, X.; Wang, Y.; Qu, Z. and Lu, Q. (2019). Expression and clinical significance of endocan in hepatocellular carcinoma patients. Int. J. Clin. Exp. Pathol., 12(7): 2617–2624.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.), 25(4): 402–408.

Mähringer-Kunz, A.; Meyer, F.I.; Hahn, F.; Müller, L.; Düber, C.; Pinto Dos Santos, D., *et al*. (2021). Hepatic vein tumor thrombosis in patients with hepatocellular carcinoma: Prevalence and clinical significance. United Eur. Gastroenterol. J., 9(5): 590–597.

Mansouri, V; Razzaghi, M.; Nikzamir, A.; Ahmadzadeh, A.; Iranshahi, M.; Haghazali, M., *et al.* (2020). Assessment of liver cancer biomarkers. Gastroenterol. Hepatol. from bed to bench, 13(Suppl1): S29–S39.

Melar-New, M. and Laimins, L.A. (2010). Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J. Virol., 84(10):5212–5221.

Mohamed, A.A.; Ali-Eldin, Z.A.; Elbedewy, T.A.; El-Serafy, M.; Ali-Eldin, F.A.

and AbdelAziz, H. (2017). MicroRNAs and clinical implications in hepatocellular carcinoma. World J. Hepatol., 9(23):1001–1007.

O'Brien, J.; Hayder, H.; Zayed, Y. and Peng, C. (2018). Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. Frontiers in Endocrinology, 9:402.

Omar, S.A.; Attia, N.M.; Sheir, M.I.; Amer, A.S.; EL Shabrawy, M.M. and Hasan, B.B. (2021). Is serum endocan a sensitive biomarker for early recurrence of hepatocellular carcinoma after radiofrequency ablation? Eur. J. Gastroenterol. Hepatol., 33(7): 1015–1022.

Petruzziello, A. (2018). Epidemiology of Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) Related Hepatocellular Carcinoma. The open Virology J., 12: 26–32.

Rashed, W.M.; Kandeil, M.A.M.; Mahmoud, M.O. and Ezzat, S. (2020). Hepatocellular Carcinoma (HCC) in Egypt: A comprehensive overview. J. Egypt. Nat. Cancer Institute, 32(1): 5.

Roudnicky, F.; Poyet, C.; Wild, P.; Krampitz, S.; Negrini, F.; Huggenberger, R., *et al*. (2013). Endocan is upregulated on tumor vessels in invasive bladder cancer where it mediates VEGF-A-induced angiogenesis. Cancer Res., 73(3): 1097–1106.

Shang, J.; Zheng, Y.; Guo, X.; Mo, J.; Xie, X.; Xiong, Y., *et al*. (2015). Hepatitis B virus replication and sex-determining region Y box 4 production are tightly controlled by a novel positive feedback mechanism. Scientific Reports, 5:10066.

Sharma, R. (2020). Descriptive epidemiology of incidence and mortality of primary liver cancer in 185 countries: evidence from GLOBOCAN 2018. Jap. J. Clin. Oncol., 50(12):1370–1379.

Stéphane, S.; Claude-Alain, M.; Dominique, D.; Philippe, L. and Maryse, D. (2010). Endocan as a biomarker of endothelial dysfunction in cancer. J. Cancer Sci. Therapy, 02(02). DOI:10.4172/1948-5956.1000022

Tok, D.; Ekiz, F.; Basar, O. and Coban, S. (2014). Serum endocan levels in patients with chronic liver disease. Int. J. Clin. Exp. Med., 7(7):1802–1807.

Waked, I.; Esmat, G.; Elsharkawy, A.; El-Serafy, M.; Abdel-Razek, W.; Ghalab, R., *et al.* (2020). Screening and Treatment Program to Eliminate Hepatitis C in Egypt. The New England J. Medicine, 382(12):1166–1174.

Wan, D.; Shen, S.; Fu, S.; Preston, B.; Brandon, C.; He, S, *et al.* (2016). miR-203 suppresses the proliferation and metastasis of hepatocellular carcinoma by targeting oncogene ADAM9 and oncogenic long non-coding RNA HULC. Anti-cancer agents in Medicinal Chemistry, 16(4):414–423.

Wei, W.; Wanjun, L.; Hui, S.; Dongyue, C.; Xinjun, Y. and Jisheng, Z. (2013). miR-203 inhibits proliferation of HCC cells by targeting survivin. Cell Biochemistry and Function, 31(1):82–85.

Wu JX, Zhu Y, Zhu YY, Zhang SL, Wang Q, & Zuo L. 2016. Endocan expression is associated with poor prognosis in human hepatocellular carcinoma. Oncology Letters, 12(3), 2023–2028.

Xing, Y.; Wang, Y.; Wang, S.; Wang, X.; Fan, D.; Zhou, D., *et al*. (2016). Human cytomegalovirus infection contributes to glioma disease progression via upregulating endocan expression. Translational Research :

Journal of laboratory and clinical medicine, 177:113–126.

Yang, J.; Yang, Q.; Yu, S. and Zhang, X. (2015). Endocan: A new marker for cancer and a target for cancer therapy. Biomedical reports, 3(3):279–283.

Yang, Y.C.; Pan, K.F.; Lee, W.J.; Chang, J.H.; Tan, P.; Gu, C.C., *et al.* (2020). Circulating proteoglycan endocan mediates EGFR-Driven progression of non-small cell lung cancer. Cancer Res., 80(16):3292–3304.

Youssef, A.A.; Issa, H.A.; Omar, M.Z.; Behiry, E.G.; Elfallah, A.A.; Hasaneen, A. *et al*. (2018). Serum human endothelial cell-specific molecule-1 (endocan) and vascular endothelial growth factor in cirrhotic HCV patients with hepatocellular carcinoma as predictors of mortality. Clin. Exper. Gastroenterol., 11:431–438.

Yu, H.; Lu, J.; Zuo, L.; Yan, Q.; Yu, Z.; Li, X., *et al.* (2012). Epstein-Barr virus downregulates microRNA 203 through the oncoprotein latent membrane protein 1: a contribution to increased tumor incidence in epithelial cells. J. Virology, 86(6):3088–3099.

Yu, P.H.; Chou, S.F.; Chen, C.L.; Hung, H.; Lai, C.Y.; Yang, P.M., *et al.* (2013). Upregulation of endocan by Epstein-Barr virus latent membrane protein 1 and its clinical significance in nasopharyngeal carcinoma. PloS one, 8(12):e82254.

Zhang, A.; Lakshmanan, J.; Motameni, A. and Harbrecht, B.G. (2018). Micro RNA-203 suppresses proliferation in liver cancer associated with PIK3CA, p38 MAPK, c-Jun, and GSK3 signaling. Molecular and Cellular Biochemistry, 441(1-2):89–98.

Zhang, H.; Shen, Y.W.; Zhang, L.J.; Chen, J.J.; Bian, H.T.; Gu, W.J., *et al.* (2021). Targeting endothelial cell-specific molecule 1 protein in cancer: a promising therapeutic approach. Frontiers in Oncology, 11:687120.

Zhang, J.; Han, C.; Zhu, H.; Song, K.; Wu, T. and Wang, C. (2014). MiR-203 suppresses tumor growth and metastasis in human hepatocellular carcinoma by downregulating RAB22A. Clin. Cancer Res., 20(20):1-12.

Zhang, J.; Ma, M.; Yuan, X.; Shi, H. and Wu, Q. (2020). Inhibition of endocan expression enhances the radiosensitivity of hepatocellular carcinoma cells. Oncology Letters, 20(4):1–9.

Ziada, D.H.; El Sadany, S.; Soliman, H.; Abd-Elsalam, S.; Salama, M.; Hawash, N.; Selim, A.; Hamisa, M. and Elsabagh, H.M. (2016). Prevalence of hepatocellular carcinoma in chronic hepatitis C patients in Mid Delta, Egypt: A single center study. J. Egypt. Nat. Cancer Institute, 28(4): 257–262.

Ziol, M.; Sutton, A.; Calderaro, J.; Barget, N.; Aout, M.; Leroy, V., *et al.* (2013). ESM-1 expression in stromal cells is predictive of recurrence after radiofrequency ablation in early hepatocellular carcinoma. J. Hepatol., 59(6):1264–1270.

miR-203 وendocan كعلامات تشخيصية لسرطان الخلايا الكبدية المرتبط بالعدوى الفيروسية

نوران ممدوح القاضي[١]، إيمان انور المعداوي[١]*، محمود جاد الله[٢]، الشيماء محمد الملواني [٣] ، محمد فاروق الشال[١]

١ - قسم البيولوجيا الجزيئية، معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية، جامعة مدينة السادات، مصر. الرمز البريدي ٣٢٨٩٧

٢ - قسم المشخصات الجزيئية، معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية، جامعة مدينة السادات، مصر. الرمز البريدي ٣٢٨٩٧

٣- قسم الباثولوجيا الإكلينيكية، معهد الكبد القومي، جامعة المنوفية، مصر. الرمز البريدي ٣٢٩٥١

البريد الإلكتروني للمؤلف: Emananwr2020@gmail.com
.eman.anwr@gebri.usc.edu.eg
البريد الإلكتروني للمؤلفين المشاركين:
nm2600083@gmail.com
mohamed.gadallah@gebri.usc.edu.eg
smalawany@liver.menofia.eg.edu
mohamed.elshal@gebri.usc.edu.eg

المستخلص

تهدف هذه الدراسة لتقييم التعبير الجيني لكلا من miR-203 و endocan في مرضي سرطان الكبد الناتج من الاصابة بفيروس سي او فيروس بي لفحص امكانية استخدامهم كدلالات تشخيصية للمرض. تمت الدراسة علي عدد ٧٠ مريضاً مقسمين إلى ٢٩ مريضاً بسرطان الكبد (٢٠ مريض مصاب بفيروس سي و٩ مصابين بفيروس بي) في مقابل ٤١ مريض كبد مزمن غير سرطاني (٢٠ مصاباً بفيروس سي و٢١ مصاباً بفيروس بي) وعشرة متطوعين أصحاء كعناصر تحكم.اشارت النتائج الي ارتفاع ملحوظ في نسبة miR-203 في مرضي سرطان الكبد الناتج من الاصابة بفيروس بي مقارنةً بمرضي سرطان الكبد الناتج عن الاصابة بفيروس سي. كما اظهرت ارتفاع ملحوظ في نسبة miR-203 في المرضي المصابين بفيروس بي عن المصابين بفيروس سي. أوضحت النتائج ايضا وجود انخفاض كبير في مستوى miR-203 في مرضي سرطان الكبد الناتج من الاصابة بفيروس سي مقارنةً بمرضي فيروس سي غير المصابين بالسرطان والضوابط. اما بالنسبة لل Endocan فقد كان هناك زيادة ملحوظة في نسبته في مجموعة سرطان الكبد عامة مقارنة بالضوابط. كما وجدت زيادة ملحوظة في مرضي سرطان الكبد الناتج عن الاصابة بفيروس سي مقارنة بمجموعة فيروس سي غير المصابة بالسرطان مع انخفاض في نسبة ال endocan في المجموعة المصابة بفيروس بي مقارنة بالضوابط. كما لوحظ وجود زيادة في نسبة ال endocan في مرضي سرطان الكبد الناتج عن الاصابة بفيروس سي مقارنة بمرضي فيروس بي. وقد اشارت هذه الدراسة الي امكانية استخدام كلا من miR-203 و endocan كعلامات تشخيصية لمرضي سرطان الكبد المصابين بعدوي فيروسية مختلفة.