International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG (USA) Volume 8, Issue 11 -2021

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2021.08.11.008

Serum Micro RNAs 122,221 and Cyclin G1 and Their Combinations as Non-Invasive Diagnostic Biomarkers for Early-Stage Hepatocellular Carcinoma

Hanan H. A. Zedan[1,*], Amina M. Medhat[1], Laila A. Rashed[2], Mahmoud M. Said[1]

[1]Biochemistry Department, Faculty of Science, Ain Shams University, Egypt
[2]Medical Biochemistry Department, Faculty of Medicine, Cairo University, Egypt
[*]Corresponding Author: *hanan_hanafy@hotmail.com*

Abstract

The present study aimed at investigating the expression levels of microRNA-122 (miR-122), miR-221 and cyclin G1 in hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) patients to identify whether they could be used as sensitive biomarkers for HCC development and its different stages as surrogate biomarkers for α-fetoprotein (AFP).The study included 28 HCV patients, 36 patients with stage I, II, III HCC and 13 healthy individuals.MiR-122, miR-221 and cyclin G1 gene expression levels were determined by quantitative real time polymerase chain reaction (qRT-PCR). HCV patients demonstrated a significant increase in AFP expression level, compared to healthy subjects while HCC patients manifested a significant elevation compared to healthy subjects and HCV patients. HCV patients demonstrated a significant increase in miR-221 and cyclin G1 levels, while HCC patients manifested a significant elevation in miR-122, miR-221 and cyclin G1 compared to healthy subjects. MiR-122 and cyclin G1 were able to discriminate between HCV and HCC patients. In conclusion, miR-122 and miR-221 may serve as non-invasive diagnostic biomarkers for HCC better than AFP in the diagnosis of stage I HCC. The combination between miR-122, miR-221 and cyclin G1 could be a useful diagnostic tool for HCC, and as a substitute for AFP.

Keywords: Micro RNA 122; Micro RNA 221; Cyclin G1; Hepatocellular Carcinoma; Hepatitis C virus, α-fetoprotein

1. Introduction

Hepatocellular carcinoma (HCC) is a common solid organ malignancy worldwide, with about 600,000 new cases diagnosed each year. The World Health Organization (WHO) identified HCC as the fifth most frequent human cancer and a fatal disease ***(Awwad et al., 2021)***. Hepatocellular carcinoma is a highly prevalent, treatment-resistant malignancy with a multifaceted molecular pathogenesis ***(Le et al., 2019)***.Current evidence indicates that during hepatocarcinogenesis, two main pathogenic mechanisms prevail; cirrhosis associated with hepatic regeneration after tissue damage caused by hepatitis infection, toxins (for example, alcohol or aflatoxin) or metabolic influences, and mutations occurring in single or multiple oncogenes or tumor suppressor genes **(*Whittaker et al., 2010*).**The highest frequency rate of HCC occurs in Eastern and Southeast Asia and Central and Western Africa. In Egypt, liver cancer counts for 11.75% of the malignancies of the digestive organs and 1.68% of the total malignancies. HCC

forms 70.48% of all liver tumors among Egyptians and it is considered the main complication of cirrhosis, and represents a growing incidence in Egypt, which may be due to a shift in the relative importance of hepatitis B virus(HBV) and hepatitis C virus(HCV) as primary risk factors, and advancements in screening programs and diagnostic materials**(*Wei et al., 2020*).**

The HCV infections are the most common reasons for most liver diseases. HCV, a hepatotropic RNA virus of the genus *Hepacivirus* in the *Flaviviridae* family, exists as an enveloped, positive-stranded RNA virus which is ~50 nm in size. HCV infection is largely asymptomatic with little visible symptoms in its acute infection stage. It is a substantial health problem worldwide **(*Wei et al., 2020*)**. The WHO reported that about 71 million individuals worldwide chronically infected by HCV, and 700,000 individuals die annually due to complications of HCV, such as liver cirrhosis and HCC. The highest HCV burden is in Africa, followed by Asia, where the HCV seroprevalence is 2.8% and 2.7%, respectively **(*Le et al., 2019*).** Research in Egypt suggested raising the association between the HCC and HCV infection (40-50% of cases), making this the highest prevalence in any population in the world **(*Kandeel et al., 2017*)**.

MicroRNAs (miRs) are small non coding single-stranded RNA segments of nearly 22 nucleotides that play an important role in all biological processes like cellular proliferation, differentiation, apoptosis, as well as carcinogenesis processes by post-transcriptional regulation of protein-coding genes**(*Yasser et al., 2021*)**. They constitute a large class of phylogenetically conserved genes, with more than 2000 miRs discovered in humans. It has been reported that many miRs are involved in human cancers, such as lung, breast, brain, liver, colorectal cancer, and leukemia. By targeting different genes in tumor development, miRs function as oncogenes or tumor suppressor genes **(*Uccello et al., 2012*)**. The clinical applications of miRs as biomarkers in cancer include early detection and identification of the tissue of origin of tumor cells, sub classification of tumors, and predictive markers for the disease's course and response to treatment **(*Kashyap et al., 2018*)**.

MiR-122 is unique among the deregulated miRs, as it is almost exclusively expressed physiologically in the adult liver, where it appears to act as a key regulator of the differentiation of adult hepatocytes. It represents about 50-70% of all miRs in the liver ***(Michael et al., 2020).***MiR-122 is located on the positive strand of chromosome 18 (18q21.31) between base pairs 56118306 and 56118390, and it is composed of 85 nucleotides encoding a single exon. The binding of miR-122 to the 5$^{'}$-UTR of HCV genomic RNA is critical for viral replication.Serum miR-122 level in patients with HCC was correlated with known risk factors for HCC **(*Wei et al., 2020*)**.

MiR-221 is upregulated in several types of human tumors, andact as an oncogene or tumor suppressor, depending on tumor system ***(Awwad et al., 2021).*** HCC cells overexpressing miR-221 show increased growth, proliferation, migration, and invasion capability. MiR-221 is encoded from a gene cluster located on chromosome X (Xp11.3) and has identical 5$^{'}$ regions that enable it to target the same genes **(Dietrich *et al.*, 2018).**There is a strong relationship between the high expression of miR-221 and tumor progression and patient survival. Overexpression of miR-221 confers cell migratory advantages in HCC through enhancing protein kinase B signaling **(*Michaelet al., 2020*).**Angiogenesis and metastasis play important roles in the progression and recurrence of HCC. MiR-221 is known to modulate the angiogenic properties of human umbilical vein endothelial cells through directly regulating downstream targets, such as c-kit, p27Kip1, p57Kip2, and cyclin G1, miR-221 impact migration and proliferation of endothelial cells **(*Wei et al., 2020*).**

The current study aimed at investigating the expression levels of serum miR-122, miR-221 and cyclin G1 in HCV and HCC patients to identify whether they could be used as single or combined sensitive biomarkers for HCC development, and to further evaluate their levels in the different HCC stages as surrogate biomarkers for α-fetoprotein(AFP).

2. Subjects and Methods

2.1 Subjects: In the present study, a total of 77 male subjects were recruited from Gastroenterology and Hepatology Department, Faculty of Medicine, Cairo University Hospitals. The subjects were divided into 2 groups; patients with HCV infection (n=28, aged from 21-58 years old) and patients with HCC. The later group was subdivided into 3 subgroups; patients with early-stage HCC infection (stage I, n=10, aged from 48-68 years old), patients with intermediate stage HCC infection (stage II, n=14, aged from 28-61 years old) and patients with advanced stage of HCC infection (stage III, n=12, aged from 46-70 years old).

A normal control group of 13 healthy subjects aged from 32-76 years old was also included. Patients' characteristics are shown in **Table 1.**

Table (1): Baseline characteristics of control subjects, HCV and all HCC patients.

Groups Parameters		Control (n=13)	HCV (n=28)	HCC		
				Stage I (n=10)	Stage II (n=14)	Stage III (n=12)
Age (years)		32-76	21-58	46-68	28-61	46-70
HCV-RNA viremia		--	8,959-1,100,000	26,000-432,000	14,000-480,000	37,000-521,000
Lymph nodes number	1	--	--	10	--	7
	2	--	--	--	4	--
	Multiple	--	--	--	10	5

A written informed consent was obtained from each subject. Clinical data of study subjects were obtained, including routine laboratory investigation as serum creatinine and liver function tests (AST, ALT, ALP, serum albumin, serum direct and total bilirubin).Imaging techniques by abdominal ultrasound and abdominal triphasic spiral computer tomography (CT). Inclusion criteria include all patients with chronic HCV infection for > 6 months and high level of serum AFP. Patients excluded from the study were those associated with chronic HBV infection, other recognizable causes for chronic hepatitis than HCV, renal diseases, parathyroid diseases, and other types of cancer than HCC were excluded.

2.2. Study design: MiR-122 and -221 gene expression levels were quantitatively determined in serum samples of HCC & HCV patients and healthy subjects by quantitative real time polymerase chain reaction (qRT-PCR). Cyclin G1 gene expression level was quantitatively determined in whole peripheral blood of HCC & HCV patients and healthy subjects by qRT-PCR.

2.3. Blood sample collection and storage: Peripheral blood was collected into either ethylene diamine tetraacetic acid (EDTA) containing vials for further determination of cyclin G1 expression or separated by centrifugation as sera and stored at - 80 ° C until miRs' extraction and AFP determination.

2.4. Methods

2.5. Serum AFP analysis: AFP level was determined by enzyme linked immunosorbent assay (ELISA) using a solid phase enzyme-linked immunosorbent assay kit (DRG International Inc., USA) according to the method of ***Uotila et al. (1981)*.**

2.6. Molecular techniques:

2.6. a. RNA extraction: Total RNA, including miRs, was isolated from serum samples using mirVana™ PARIS™ Extraction Kit (Ambion, USA),and from whole blood samples (for investigation of cyclin G1 gene expression level) using QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany). The concentration of extracted RNA was estimated by measuring the absorbance at 260 nm using Q-5000 NanoDrop Spectrophotometer (Quawell Technology, Inc., USA). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the extracted RNA, where a ratio of ~2.0 is generally accepted as "pure" for RNA.

2.6.b. Complementary DNA (cDNA) synthesis: Mature miRs were reverse transcribed to cDNA by using TaqMan ®MiRNA Reverse Transcription Kit with miR-specific primers (Applied Biosystems, USA), and using AMV Reverse Transcriptase kit (Promega, Madison, WI, USA) forRNA reverse transcription for cyclinG1.The primers sequences were as follows;
miR-221:5̊-GAAACCCAGCAGACAATGTAGCT-3̊,
miR-122: 5̊-ACAAACACCATTGTCACACTCCA-3̊,
cyclin G1:
5̊-ATAAGCTTTTTGGCACAGTAAGGGCATC-3̊,
RNU6B:
5̊-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3̊and GAPDH:
5̊-AGAACATCATCCCTGCATCC-3̊.

The relative expression of the candidate miRs (miR-122 and miR-221) was analyzed by qRT-PCR using Applied Biosystems StepOne™ Real-Time PCR System using TaqMan® MiRNA-122,221 and RNU6B Assay, TaqMan® 2× Universal PCR Master Mix II. Reagents purchased from Applied Biosystems (Life Technologies, Carlsbad, CA, USA), and Power SYBR® Green PCR Master Mix(Applied Biosystems, USA) were used for relative expression of cyclinG1 by qRT-PCR using the endogenous control GAPDH.

2.6.c.Quantitative real-time PCR: The amplification program was adjusted as follows; initial activation step for 15 min at 95°C to activate HotStar Taq DNA polymerase, and 40 cycles (denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 70°C for 30 sec). Melting curves were performed by rapid heating to 95°C for 15 sec to denature the DNA, followed by cooling to 60°C to assure the purity and specificity of amplified products. The relative expressions of selected miRs and cyclinG1 were normalized to an endogenous control (RENUB6) and (GAPDH), respectively. The relative quantification of miRNA-122,221 and cyclinG1was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), where ΔΔCt is the difference of ΔCt value between the patient and the control (ΔΔCt=ΔCt patient miR or cyclinG1- ΔCt control miR or cyclinG1), and ΔCt is the difference of Ct value between the target (miR-1 22or miR- 221or cyclinG1) and endogenous housekeeping references (RENUB6 and GAPDH) ***(Livak and Schmittgen, 2001)***.

2.7. Statistical analysis: The distributions of quantitative variables were tested for normality using Shapiro-Wilk's test ***(Shapiro and Wilk, 1965)***. For parametric data, comparison between different variables was done using Analysis of variance (ANOVA) test followed by Duncan's test ($p<0.05$ is considered significant). For non-parametric data, comparisons between different categories were done using Kruskal-Wallis test followed by Mann-Whitney U test. Comparison between the different groups regarding the categorical variables was analyzed using Chi-square test. Receiver operating characteristic (ROC) curves were constructed to detect the sensitivity, specificity, and the diagnostic efficacy of AFP, miR -122, miR-221 and cyclinG1 in HCV and HCC with its different stages. The data were statistically analyzed using Statistical Program for Social Science (SPSS) version 23 **(Chicago, Illinois, USA).**

3. Results and Discussion

3.1.Blood biochemical parameters for HCV, HCC and all HCC stages patients: There was a statistically significant increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in HCV patients compared to healthy subjects ($p < 0.001$) **(Figure 1A)**. In addition, HCC patients showed a statistically significant increase in serum ALT, AST, alkaline phosphatase (ALP) and total and direct bilirubin levels ($p < 0.001$), compared to healthy subjects **(Figure 1 A&B).** By contrast, there was a statistically significant decrease in serum albumin level in HCC patients ($p < 0.001$), compared to healthy subjects **(Figure 1C)**. Moreover, HCC stage I, II and III patients showed a statistically significant increase in serum ALT and AST levels ($p < 0.001$), compared to healthy subjects. On the other hand, only HCC stage II patients demonstrated a statistically significant increase in serum ALP activity ($p < 0.001$), compared to healthy subjects **(Figure 2A)**. HCC stage I,II and III patients showed a sharp statistically significant increase in serum total and direct bilirubin levels, compared to healthy subjects ($p < 0.001$) **(Figure 2B)**. By contrast, there was a statistically significant decrease in serum albumin level compared to healthy subjects ($p < 0.001$) **(Figure 2C)**. Non statistically significant differences in creatinine between HCV, HCC stages patients and normal controls were recorded **(Figure 2B)**.

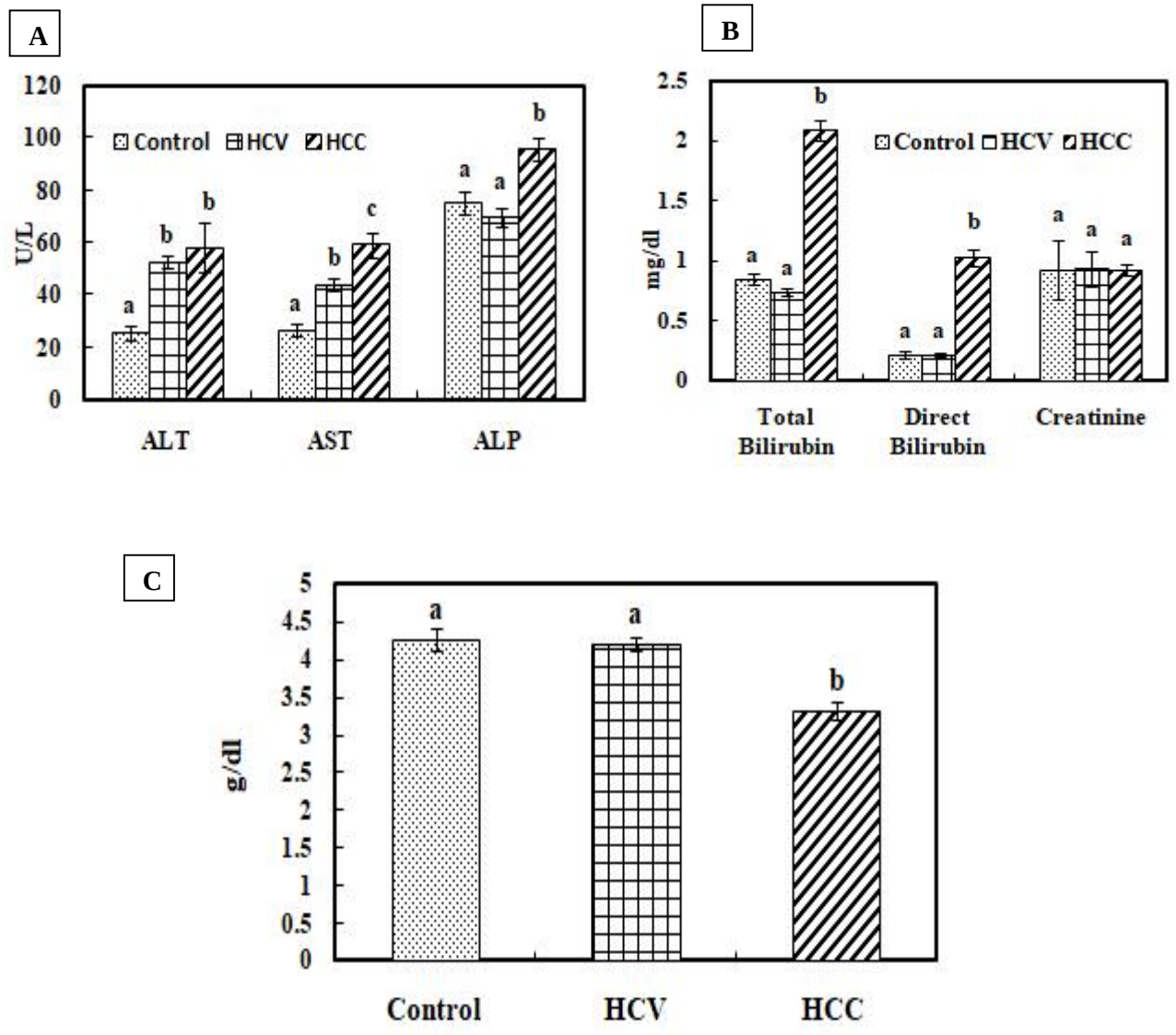

Figure 1: Serum ALT, AST & ALP activities (A), total & direct bilirubin and creatinine (B) and albumin (C) levels in control, HCV and all HCC patients. Values are expressed as mean ± standard error. Different letters denote significance at $p<0.05$(analysis of variance [ANOVA] test was applied followed by Duncan's test).

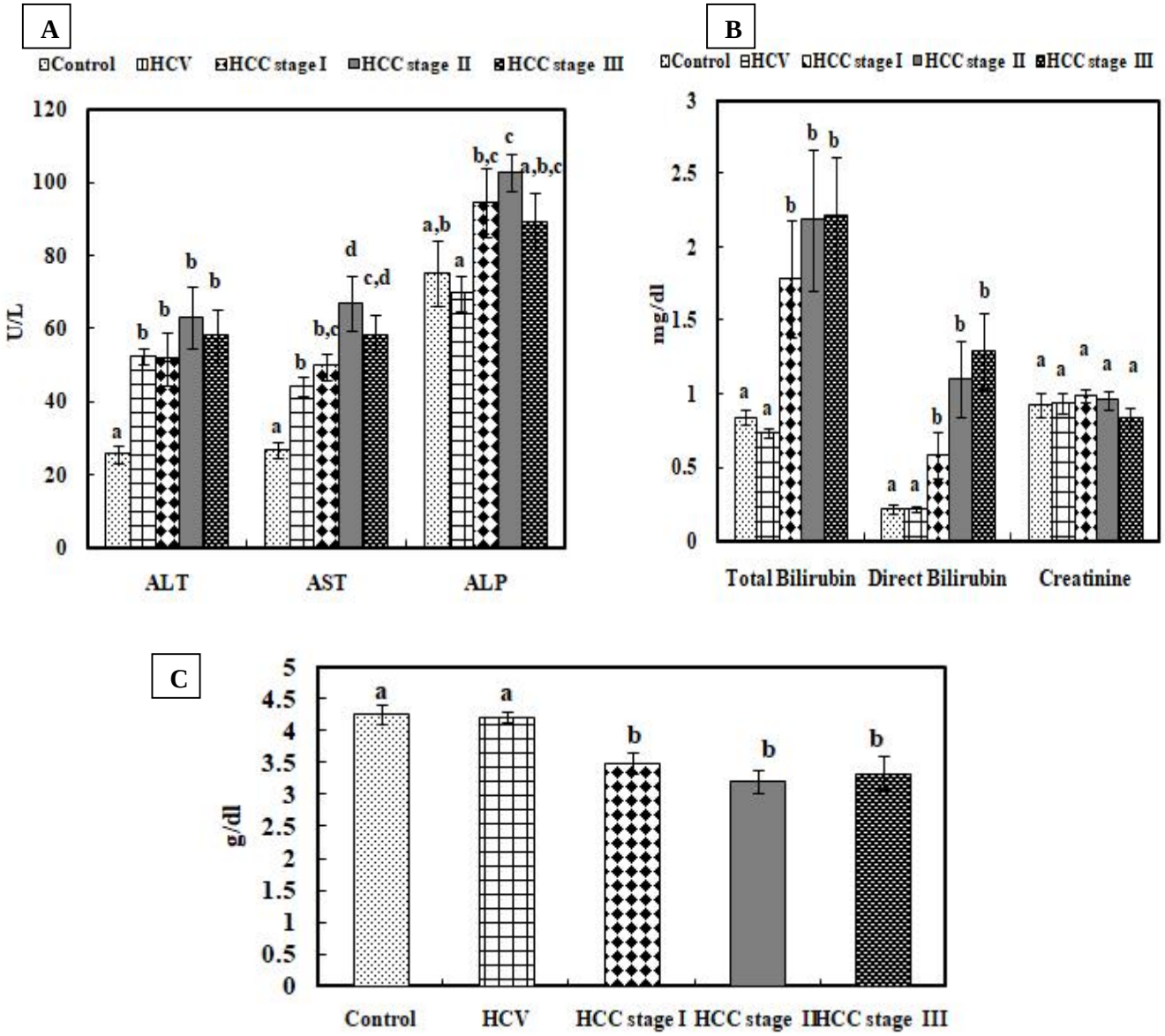

Figure 2: Serum ALT, AST & ALP activities (A), total & direct bilirubin and creatinine (B) and albumin (C) levels in control, HCV and different HCC stages patients. Values are expressed as mean ± standard error. Different letters denote significance at $p<0.05$(analysis of variance [ANOVA] test was applied followed by Duncan's test).

3.2. The relative expression level of AFP and its diagnostic performance in HCV and HCC patients: There was a statistically significant increase in serum AFP expression levels in HCV and HCC patients compared to the control group **(Figure 3A)**. There was a non-significant change in the serum level of AFP in stage I HCC patients, compared to HCV patients as well as healthy subjects. However, a sharp significant increase in AFP serum level was recorded in stages II and III HCC patients, compared to stage I HCC and HCV patients, as well as healthy subjects **(Figure 4A)**.According to the ROC curve analysis, AFP was found to be the gold standard as a single biomarker to discriminate between HCC stages (II& III) and HCV patients, with absolute sensitivity and specificity values. However, AFP was unable to differentiate between stage I HCC and HCV patients **(Figure 5)**. Accordingly, AFP is considered a poor diagnostic biomarker in differentiating stage I HCC patients from healthy subjects.

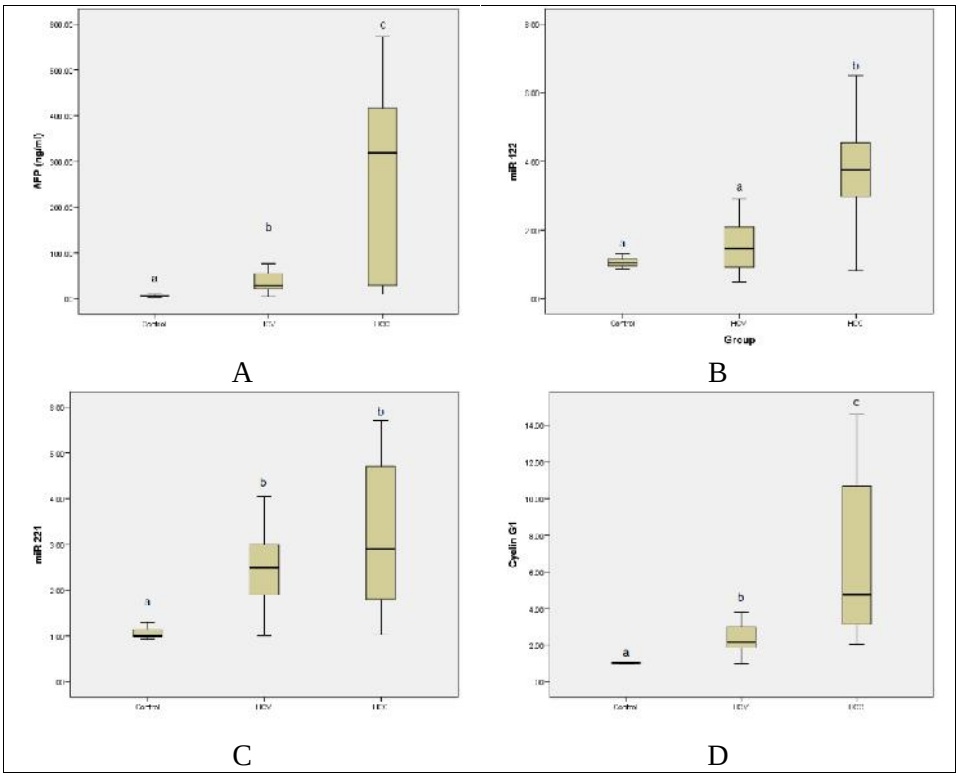

Figure (3): Box plots for AFP level (A), relative quantification of miR-122 (B), miR-221 (C) and cyclin G1 (D) in control subjects, HCV and all HCC patients. Box plots show the median and the interquartile range (Chi-square test). Different letters denote significance at $p<0.05$ (Kruskal-Wallis test followed by Mann-Whitney U test).

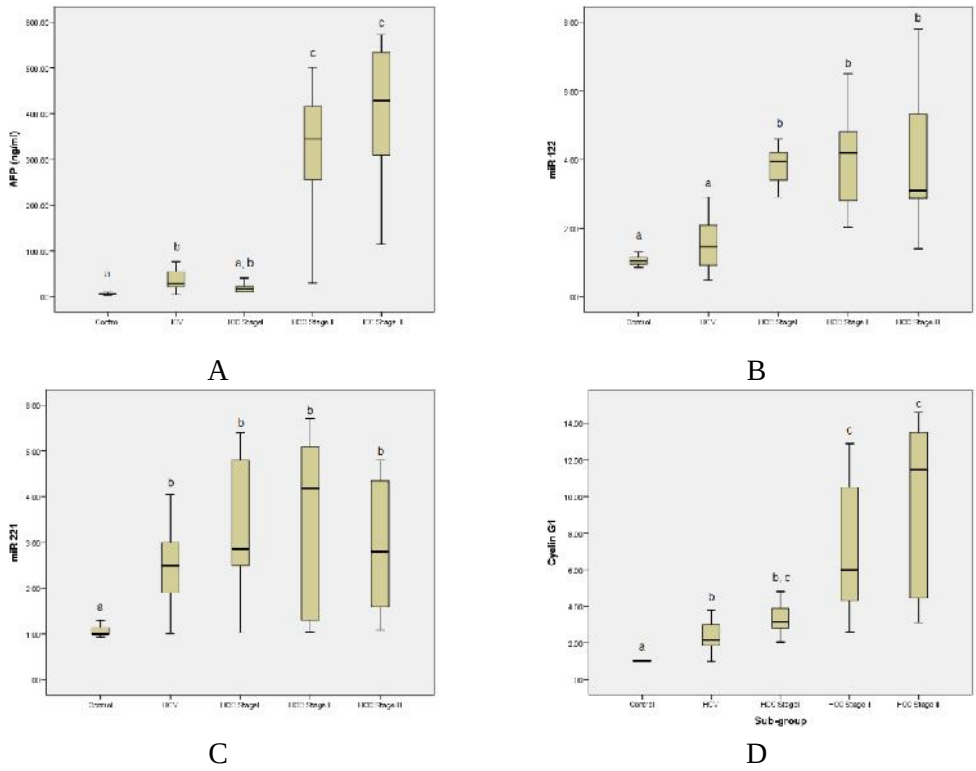

Figure 4: Box plots for AFP level (A), relative quantification of miR-122 (B), miR-221 (C) and cyclin G1 (D) in control subjects, HCV and different HCC stages.Box plots show the median and the interquartile range (Chi-square test). Different letters denote significance at $p<0.05$ (Kruskal-Wallis test followed by Mann-Whitney U test).

In agreement with the results of the current study**,** ***El-Saeid et al. (2012), Baghdady et al. (2014), Bahnassy et al. (2014), EL-Abd et al. (2015), Ghoneim et al. (2016), Amr et al. (2017), Demerdash et al. (2017)*** *and* ***El-Ahwany et al. (2019)*** found that serum AFP level was significantly increased in HCCand HCV patients compared to healthy subjects. The previous findings of ***Luo et al.*** **(2013**), suggested that the elevation of AFP serum level was attributed to the fact that most of studied patients were in late stage of HCC. By contrast, ***El-Garem et al. (2014)*** reported that AFP serum level was decreased in HCC patients, demonstrating that not all HCC tumors can secrete AFP and its serum level may be normal up to 40% of small tumor size HCC patients. It was also showed that AFP alone was not recommended for the diagnosis of HCC and its cut off value should be set at 200 ng/ml ***(Demerdash et al., 2017)***.

In agreement with the results of the current study, ***El-Garem et al. (2014)***reported that AFP was unable to differentiate between HCV and stage I HCC patients, suggesting a positive correlation between AFP serum level and tumor size. In addition, ***Demerdash et al. (2017)*** reported that the false negative results with AFP level may be as high as 40% for patients with early stage of HCC.

By contrast to our results, ***Choi et al. (2019)*** found that serum AFP level starts to increase 6 months before HCC diagnosis, while it remains unchanged in the controls. Consequently, AFP has a good performance in discriminating stage I HCC patients from controls. Beside, they found that AFP exhibits the best performance as a single biomarker in differentiating stage I HCC patients from controls, with an area under the curve of 0.77 at month 0, as the performance was maximized at month 0, then decreased at months 6 and 12, suggesting that because of the median size of HCC in their study was only 3.3 cm, AFP might provide a chance of early HCC detection before ultrasonography can pick up the lesion, particularly in cirrhotic liver with the appearance of nodularity on imaging ***(Wong et al., 2014)*.**

3.3. The relative expression levels of miR-221, cyclin G1 and their diagnostic performance in HCV and HCC patients: HCV and HCC patients demonstrated a significant increase in serum miR-221 and cyclin G1 expression levels, compared to healthy subjects **(Figure 3 C&D)** .In addition, stage I, II & III HCC patients demonstrated a significant increase in serum miR-221 and cyclin G1 expression levels, compared to healthy subjects **(Figure 4 C&D).** Moreover, by using ROC curve analysis, serum cyclinG1 was able to discriminate between HCV and HCC patients, while serum miR-221 was unable to differentiate between both groups **(Figure 5A)**.ROC curve analysis demonstrated that serum miR-221 and cyclin G1 were able to differentiate between HCV and stage I HCC patients, whereas only cyclin G1 was able to differentiate between HCV and stage II & III HCC patients, demonstrating the importance of miR-221 in the induction of proliferation of early-stage HCC cells **(Figure 5 B, C&D)**.

In agreement with the results of the current study, ***Luo et al. (2013), Amr et al. (2017)*** *and* ***Demerdash et al. (2017)***reported a significant increase in serum miR-221 level of HCV and HCC patients, compared to healthy subjects. However, ***Meng et al. (2007)*** demonstrated an increase in serum miR-221 level in HCC patients only, compared to healthy subjects. In addition, ***Ladeiro et al. (2008)*** and ***Huang et al. (2009)*** found that miR-221was significantly upregulated in HCC patients compared to healthy subjects. By contrast, ***Qi et al. (2011)*** showed that there is no statistical difference in serum miR-221 level between HCV or HCC patients and healthy subjects.

Likewise, ***Awwad et al. (2021)*** reported an increase in serum miR -221 levels in HCV and HCC patients compared to healthy subjects. Unlike our results, miR-221 was able to discriminate between both groups with 85% sensitivity and 55% specificity. They suggested that miR-221 was found to induce proliferation of hepatic cell by repression of the cell cycle player p27. Therefore, the miR-221 was able to inhibit the cyclin-dependent kinase inhibitors CDKN1B/p27 and CDKN1C/p57 expression and so override some checkpoints and support uncontrolled proliferation. These kinase inhibitors are considered significant regulators of cell cycle progression, and its inhibition is linked to HCC patients' poor prognosis. Moreover, miR-221 was shown to modulate the angiogenic properties of human umbilical vein endothelial cells through directly regulating downstream targets, such as c-kit, p27Kip1, p57Kip2 and cyclin G1 (ie: there was a negative correlation between miR-221 and cyclin G1 serum levels) ***(Kerr et al., 2011; Ali et al., 2017)***.

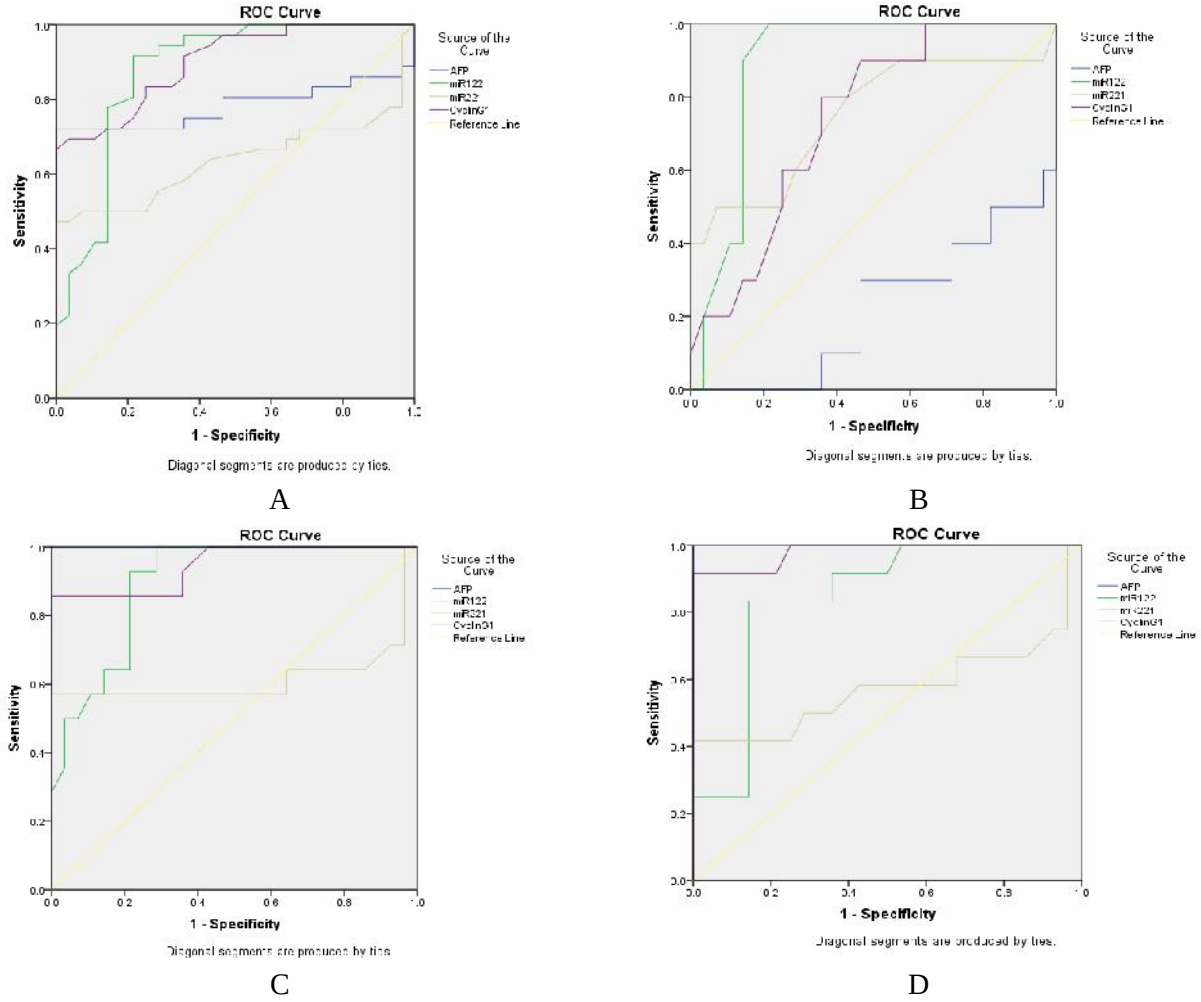

Figure 5: Receiver operating characteristic (ROC) curves analysis for AFP, miR-122, miR-221 and cyclin G1 to discriminate between HCV and HCC patients (A),HCV and stage I HCC patients (B), HCV and stage II HCC patients (C), HCV and stage III HCC patients(D).

El-Garem et al. (2014) demonstrated that serum miR-221 level was down-regulated in HCC patients in comparison to healthy subjects, and by contrast there was a significant increase in serum miR-221 level in HCV patients, compared to healthy subjects. Also, there was a significant fold decrease in serum miR-221 levels of HCC patients compared to HCV patients. The authors postulated thatduring the progression of liver disease from chronic hepatitis to HCV, the increase in the activity of hepatic stellate cells was associated with an increase in the miR-221 expression level. Such high level of hepatic tissue miR-221 stimulated tumorigenesis, resulted in the appearance and developing of HCC. Circulating miR-221 level is significantly upregulated in the serum of HCV infected patients. It has some value in the differentiation between HCV patients with HCC and those without HCC, recording 87% sensitivity and 40% specificity. It may be able to serve as a promising non-invasive diagnostic marker for HCC.

Even though miR-221 was unable to discriminate between HCV and HCC patients, ROC curve analysis revealed that it was able to discriminate between HCV and stage I HCC patients, while AFP, the gold standard biomarker for HCC was unable to discriminate between HCV and stage I HCC patients, which makes miR-221 an eligible surrogate non-invasive biomarker in the diagnosis of early-stage HCC.

3.4. The relative expression level of miR-122 and its diagnostic performance in HCV and HCC patients:

There was no statistical difference in miR-122 expression level between HCV patients and control group. On the other hand, HCC patients manifested a significant elevation in serum miR-122 expression, compared to HCV patients and healthy subjects **(Figure 3B)**. In addition, stage I, II & III HCC patients manifested a significant increase in serum miR-122 expression level, compared to healthy subjects **(Figure 4 B).** On the other hand, by using ROC curve analysis, miR-122 was able to discriminate between HCV and HCC patients with 91.7% and 78.6% sensitivity and specificity respectively as well as different stages of HCC **(Figure 5)**.

The findings of ***El-Garem et al. (2014), Ghoneim et al. (2016), Demerdash et al. (2017)*** and ***Bharali et al. (2019)*** were in a good agreement with the results of the current study by reporting a significant upregulation in the expression level of serum miR-122 in HCC patients in comparison to healthy subjects. By contrast to our results, these authors reported a significant upregulation in the expression level of serum miR-122 in HCV patients in comparison to healthy subjects. The authors suggested that the hepatocytes are primary source of miR-122, which exists copiously in hepatocytes with much lower levels in plasma of healthy individuals. During the liver injury, due to HCV infection, enhanced intracellular miR-122 release into the circulation takes place as a strategy of cell adaptation for adverse conditions and plasma levels therefore upsurge.

Moreover, the increase in the expression of serum miR-122 in patients with HCC might be due to its downregulation in HCC tissues and subsequent elevation in circulation of HCC patients. Patients with high miR-122 had better prognosis and longer survival than others with lower miR-122 ***(Demerdash et al., 2017).Ghoneim et al. (2016)*** reported a relation between the viral load and the expression level of miR-122, as the increase in the expression level of miR-122 in patients was associated with high viral load. This might be linked to the virus itself, which can also enhance the secretion of miRs from host cells to influence the cellular physiology for their production. These assumptions may be correlated with our observations, where the gradual increase in viremia was associated with increased expression of miR-122 and HCC stages.

In addition, ***El-Garem et al. (2014)***revealed that the circulating miR-122 was upregulated in HCC patients suggesting that miR-122 may down regulate target mRNA of obscure tumor suppressor genes and in this way prompts further tumor development. MiR-122 can modulate p53 activity through the control on cyclin G1 in cyclin G1/p53 pathway. Once cyclin G1 presented abnormal expression, the increase in the number of hepatic cells was arrested in G1 phase, exerting certain regulatory functions to p53-dependent cell division pathway by accelerating cell division and inhibition of its apoptotic pathway resulting in tumor development. MiR-122 may act as oncogene and there was a correlation between miR-122 and cyclin G1.

Moreover, ***Qi et al. (2011)*** found that the level of miR-122 was significantly reduced in HCC patients postoperatively when compared to the preoperative level, suggesting that the elevation of circulating miR-122 is likely originated from HCC.

By contrast to our results, ***Ali et al. (2017),Amr et al. (2017)***and ***El-Ahwany et al. (2017)***founda significant decrease in serum miR-122 level in HCC patients and increase in its level in HCV patients compared to healthy subjects. Besides, ***Huang et al. (2009), Luo et al. (2013)*** and ***EL-Abd et al. (2015)*** reported a decrease in serum miR -122 level in HCC patients compared to healthy subjects.

According to ***Amr et al. (2017),*** plasma miR-122 was reported to be significantly downregulated in HCC patients compared to healthy controls, supporting its function as a tumor suppressor gene. MiR-122 has a central role in the suppression of HCC. The role of miR-122 is the suppression of oncogenic genes involved in diverse HCC hallmarks. Among these genes, Bcl-2 which can inhibit tumor cells apoptosis, Wnt1 which is responsible for the proliferation of cells, ADAM17 which is responsible for the metastasis and Ccgn1 which is responsible for the progression of the cell cycle. Cellular mRNA and protein levels of Bcl-w were suppressed by miR-122, which directly targets 3ˋ -UTR (a recognized binding site located in Bcl-w one of the antiapoptotic Bcl-2 family), and subsequently activates caspase-3 with resultant decline in cell viability. Also, miR-122 identified Wnt1, as a target related to HCC apoptosis, which was negatively regulated by miR-122 through binding to 3ˋ -UTR of Wnt1 ***(Xu et al., 2012)***.

Furthermore, miR-122 expression was reported to be significantly higher in HCV patients when compared to healthy controls, which can be explained by the leakage of miR-122 from apoptotic or necrotic cells into the blood. MiR-122 may contribute to the pathogenesis of chronic HCV due to its function in HCV replication, translation, and inflammation ***(Amr et al., 2017)***.

According to ***El-Ahwany et al. (2017),*** the expression levels of miR-122 declined during the process of hepatocarcinogenesis; hence miR-122 can function as a tumor suppressor. Their findings were consistent with previous studies, which reported a decline in the levels of miR-122 in HCC patients compared to the control individuals. On the other hand, the level of miR-122 increased in HCV patients, suggesting that during the process of hepatocyte injury, miR-122 level increases drastically, and then declines back again significantly after the liver has entered carcinogenesis. Also, they suggested that serum miR-122 might serve as a novel potential non-invasive biomarker for HCV-induced HCC. Also, ***Ali et al. (2017)*** reported that the over expression of miR-122 induces apoptosis and inhibits proliferation in HCC cell lines.

In addition***, El-Garem et al. (2014), Demerdash et al. (2017)***and ***Bharali et al. (2019)*** revealed that the altered expression of miR-122 was able to differentiate between HCV and all stages of HCC especially that it was effective in the differentiation between HCV and early stage (stage I) HCC patients.

Demerdash et al. (2017) and ***Bharali et al. (2019)*** reported that the miR-122 level was correlated with tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis (LC) and AFP serum level. Therefore, the serum miR-122 level was relatively low in early stage (stage I) HCC patients because of small tumor size (>2cm), then its level increased gradually by the increasing in the tumor size in the late stages of HCC ***(EI-Garem et al., 2014)***. By contrast, ***Amr et al. (2017), El-Ahwany et al. (2017)*** and ***Wei et al. (2020)*** revealed a decrease in the expression of miR-122 of HCC patients, therefore miR-122 was unable to differentiate between HCV and all stages of HCC.

Michael et al. (2020) and ***Wei et al. (2020)*** reported that the miR-122 expression level increased in advanced stage HCV infected patients (F4) and then its level decreases in the same patients when they became HCC patients. They suggested that the increase in miR-122 expression level in HCV patients (F4) was a normal respond from the hepatocytes to the HCV infection and then the decrease in its level in the same patients after the liver has entered carcinogenesis signifies miR-122 as a tumor suppressor gene. Besides, with the eventual loss of hepatocytes and development of fibrosis with proliferation of myelofibroblasts and accumulation of extracellular matrix the circulating miR-122 levels drop again ***(El-Ahwany et al., 2017)***. Moreover, the decreasing of miR-122 level in the same patients after they changed to HCC patients indicated that the HCC patients were in the early stage (stage I) of HCC ***(Amr et al., 2017)***.

3.5. The relative expression levels of the combination between miR-122,miR-221 &cyclin G1 and their diagnostic performance in HCV and HCC patients: The diagnostic significance of the combined markers was performed by ROC curve analysis and the combination of AFP+miR-122, AFP+cyclin G1, miR-122+cyclin G1 and AFP+cyclin G1+miR-122gave rise to AUC values higher than those of each marker individually to discriminate between HCV and all HCC patients, suggesting that the combination of those markers might be more reliable to discriminate between HCV and all HCC patients with high sensitivity and specificity **(Figure 6)**.

In agreement with the results of the current study, Luo et al. (2013) and Amr et al. (2017) proved that the combination between AFP and miR-122 increased the AUC values to 0.932 and 0.943, respectively ($p<0.001$) with high specificity and sensitivity in the discrimination between HCV and all HCC patients. They suggested that miR-122 has high diagnostic value and the diagnostic power of AFP was significantly superior to that of miR-122. Therefore, the combination between both markers would increase the diagnostic performance better than the AFP alone. These findings establish that serum miR-122 has some value as a diagnostic marker, both individually and in combination with AFP.

In addition, ***Awwad et al. (2021)*** reported that serum miR-221 can be considered as a useful additional biomarker with AFP in discriminating HCC from HCV patients. Even though serum miR-221 expression level is a more sensitive biomarker in the diagnosis of HCC than the traditional marker AFP, it was not specific for HCC only, as high serum miR-221 levels could be detected in different types of cancer. Therefore, the combination of serum miR-221

and other HCC specific tumor markers such as AFP may have benefit to resolve this limitation.

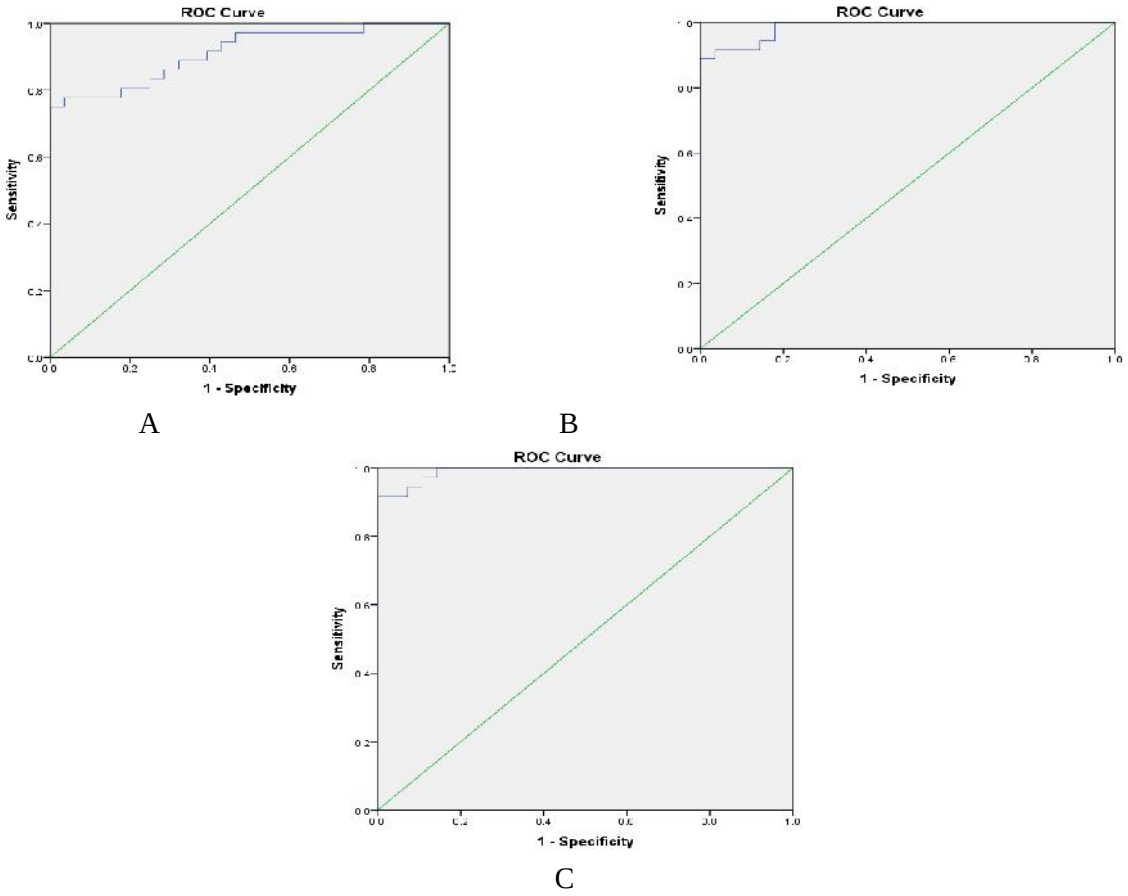

Figure 6: ROC curve of the combination between AFP+ cyclin G1 (A), miR-122 + cyclin G1 (B),AFP + miR-122 + cyclin G1 (C)to discriminate between HCV and all HCC patients.

3.6. The relative expression levels of the combination between miR-122,miR-221 &cyclin G1 and their diagnostic performance in HCV and all stages of HCC patients: The diagnostic significance of the combined markers was performed by ROC curve analysis which proved that the combination of miR-122+221, miR-122+cyclin G1, miR-221+cyclin G1 and miR-221+ cyclin G1+miR-122 gave rise to AUC values higher than those of each marker alone to discriminate between HCV and stage I HCC patients **(Figure 7)**. In addition, the combination of miR-122+cyclin G1 gave rise to AUC values higher than those of each marker individually to discriminate between HCV and stage II&III HCC patients **(Figure 8),** suggesting that the combination of those markers might be more reliable to discriminate between HCV and all stages of HCC patients with high sensitivity and specificity.

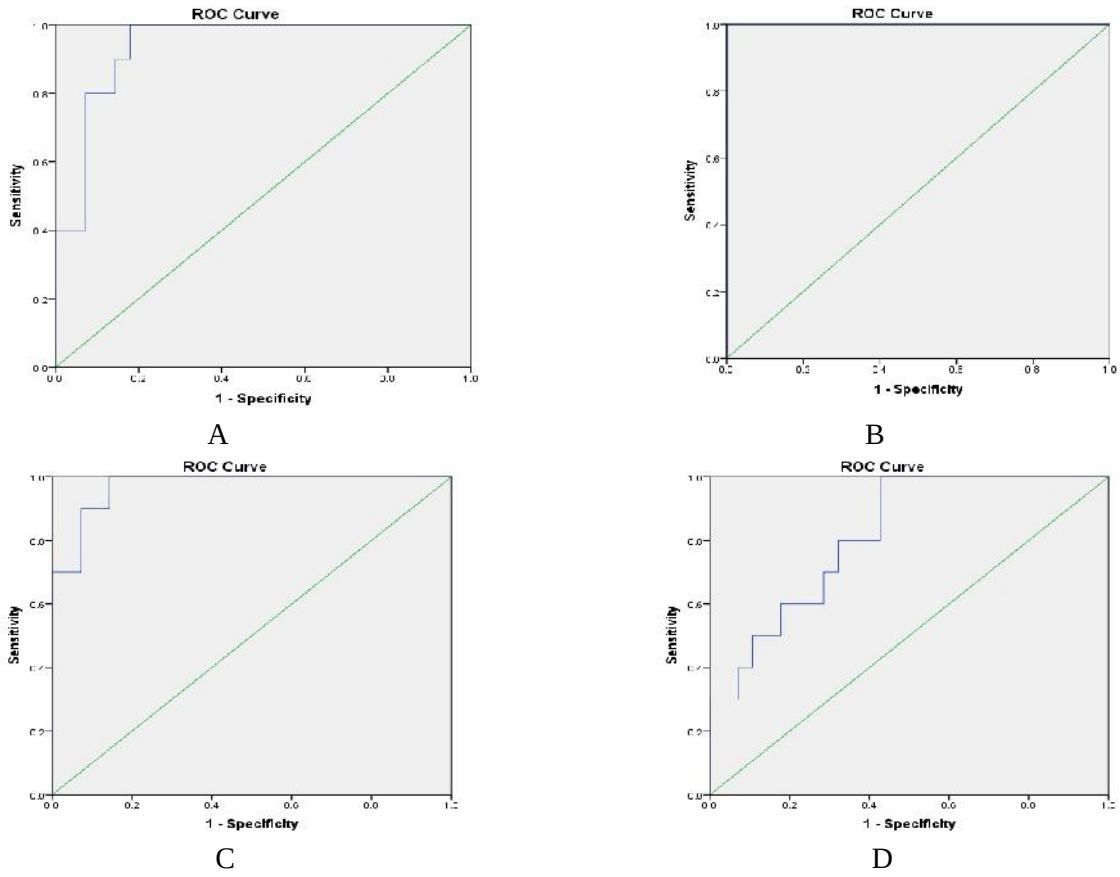

Figure 7:ROC curve of the combination betweenmiR-122 + miR-221 (A), miR-122 + miR-221 + cyclin G1 (B), miR-122 + cyclin G1 (C), miR-221 + cyclin G1 (D)to discriminate between HCV and stage I HCC patients.

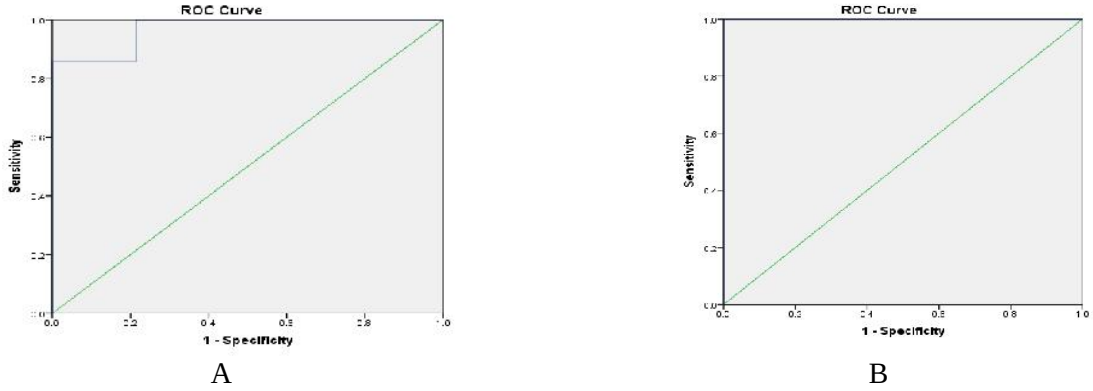

Figure 8: ROC curve of the combination betweenmiR-122+cyclin G1 to discriminate between HCV and stage II (A) or stage III (B) HCC patients.

4. Conclusion

The current study suggests that serum miR-122 and cyclin G1 may be used as non-invasive diagnostic biomarkers for HCC. In addition, serum miR-122, miR-221 and cyclin G1 either alone or combined can be useful biomarkers, rather than AFP, in discriminating between HCV and early stage I HCC patients.

Statement of conflict of interest

The authors declare that there is no conflict of interest.

References

Ali, H. E. A., Hameed, R. A., Effat, H., Ahmed, E. K., Atef, A. A., Sharawi, S. K., Ali, M., Abd Elmageed, Z.Y.& Abdel Wahab, A. H. A. (2017). Circulating microRNAs panel as a diagnostic tool for discrimination of HCV-associated hepatocellular carcinoma. Clinics and Research in Hepatology and Gastroenterology, 41(4), e51-e62.

Amr, K. S., Atia, H. A. E., Elbnhawy, R. A. E., & Ezzat, W. M. (2017). Early diagnostic evaluation of miR-122 and miR-224 as biomarkers for hepatocellular carcinoma. Genes and Diseases, 4(4), 215-221.

Awwad, A. M., El Wazzan, D. A., Salem, A. M.& Asser, S. L. (2021). Circulating microRNA-221 as a diagnostic biomarker for hepatitis C virus-related hepatocellular carcinoma. Microbes and Infectious Diseases, 2(1), 68-76.

Baghdady, I., Fouad, F., Sayed, M., Shoaib, A., Salah, Y., Elshayeb, E. & Hasan, A. E. (2014). Serum markers for the early detection of hepatocellular carcinoma in patients with chronic viral hepatitis C infection. Menoufia Medical Journal, 27(3), 544-550.

Bahnassy, A. A., Zekri, A. R. N., El-Bastawisy, A., Fawzy, A., Shetta, M., Hussein, N.,Omran, D. Ahmed, A. A. S.& El-Labbody, S. S. (2014). Circulating tumor and cancer stem cells in hepatitis C virus-associated liver disease. World Journal of Gastroenterology, 20(48), 18240.

Bharali, D., Banerjee, B. D., Bharadwaj, M., Husain, S. A. & Kar, P. (2019). Expression analysis of MicroRNA-21 and MicroRNA-122 in hepatocellular carcinoma. Journal of Clinical and Experimental Hepatology, 9(3), 294-301.

Choi, J., Kim, G. A., Han, S., Lee, W., Chun, S. & Lim, Y. S. (2019). Longitudinal assessment of three serum biomarkers to detect very early-stage hepatocellular carcinoma. Hepatology, 69(5), 1983-1994.

Demerdash, H. M., Hussien, H. M., Hassouna, E. & Arida, E. A. (2017). Detection of microRNA in hepatic cirrhosis and hepatocellular carcinoma in hepatitis C genotype-4 in Egyptian patients. BioMed Research International, 2017:1806069.

Dietrich, P., Freese, K., Mahli, A., Thasler, W. E., Hellerbrand, C. & Bosserhoff, A. K. (2018). Combined effects of PLK1 and RAS in hepatocellular carcinoma reveal rigosertib as promising novel therapeutic “dual-hit” option. Oncotarget, 9(3), 3605-3618.

El-Abd, N. E., Fawzy, N. A., El-Sheikh, S. M., & Soliman, M. E. (2015). Circulating miRNA-122, miRNA-199a, and miRNA-16 as biomarkers for early detection of hepatocellular carcinoma in Egyptian patients with chronic hepatitis C virus infection. Molecular Diagnosis andTherapy, 19(4), 213-220.

El-Ahwany, E. G., Mourad, L., Zoheiry, M. M., Abu-Taleb, H., Hassan, M., Atta, R., Hassanien, M.& Zada, S. (2019). MicroRNA-122a as a non-invasive biomarker for HCV genotype 4-related hepatocellular carcinoma in Egyptian patients. Archives of Medical Science, 15(6), 1454 - 1461.

El-Garem, H., Ammer, A., Shehab, H., Shaker, O., Anwer, M., El-Akel, W. & Omar, H. (2014). Circulating microRNA, miR-122 and miR-221 signature in Egyptian patients with chronic hepatitis C related hepatocellular carcinoma. World Journal of Hepatology, 6(11), 818 - 824.

El-Saeid, M. E., Mousa, M. &Hamdy, M. (2012). Evaluation of cystatin C, fibronectin and alpha-feto protein as biochemical markers in patients with liver diseases. Life Science Journal, 9(4).

Ghoneim, E. M., El-Aziz, A. M. A., Abd El-Mottaleb, T. M., El-Hendawy, G. R., El-Ezawy, H. M. & Khalil, F. O. (2016). Profiling of microRNA-122 in chronic hepatitis C. Menoufia Medical Journal, 29(4), 826 -834.

Huang, X. H., Wang, Q., Chen, J. S., Fu, X. H., Chen, X. L., Chen, L. Z., Li, W., Bi, J., Zhang, L. J., Fu, Q., Zeng, W. T., Cao, L. Q., Tan, H. X.& Su, Q. (2009). Bead-based microarray analysis of microRNA expression in hepatocellular carcinoma: miR-338 is downregulated. Hepatology Research, 39(8), 786-794.

Kandeel, A., Genedy, M., El-Refai, S., Funk, A. L., Fontanet, A. & Talaat M. (2017). The prevalence of hepatitis C virus infection in Egypt 2015: implications for future policy on prevention and treatment. Liver International, 37 (1):45-53.

Kashyap, D., Tuli, H. S., Garg, V. K., Goel, N. & Bishayee, A. (2018). Oncogenic and tumor-suppressive roles of MicroRNAs with special reference to apoptosis: molecular mechanisms and therapeutic potential. Molecular Diagnosis andTherapy, 22(2), 179-201.

Kerr, T. A., Korenblat, K. M. & Davidson, N. O. (2011). MicroRNAs and liver disease. Translational Research, 157(4), 241-252.

Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S. & Zucman-Rossi, J. (2008). MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology, 47(6), 1955-1963.

Le, Ngoc, C., Tran ThiThanh, T., Tran ThiLan, P., Nguyen Mai, T., Nguyen Hoa, T., Nghiem My, N., Le Van, T., Le Manh, H., Le Thanh, P., Nguyen Van Vinh, C., Thwaites, G., Cook, G., Heilek, G. M., Shikuma, C., Le, T., Baker, S., Rahman, M.& VIZIONS consortium. (2019). Differential prevalence and geographic distribution of hepatitis C virus genotypes in acute and chronic hepatitis C patients in Vietnam. PloSOne, 14(3), e0212734.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using realtime quantitative PCR and the 2ΔΔC(T) Method. Methods, 25(4): 402–408. doi:10.1006/meth.2001.1262.

Luo, J., Chen, M., Huang, H., Yuan, T., Zhang, M., Zhang, K. & Deng, S. (2013). Circulating microRNA-122a as a diagnostic marker for hepatocellular carcinoma. OncoTargets and Therapy, 6: 577-583.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S. T. & Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology, 133(2), 647-658.

Michael, T. G.,Helal, E. M. B.,Sayed, M. M., Agwa, S. H., Elwakeel, S. M.& Anwar, C. A., (2020). Serum microrna-122 levels in Egyptian patients with chronic hepatitis c virus genotype 4 infection before and after treatment with direct acting antiviral drugs. European Journal of Molecular and Clinical Medicine, 7(11), 1321-1333.

Ozakyol, A. (2017). Global epidemiology of hepatocellular carcinoma (HCC epidemiology). Journal of Gastrointestinal Cancer, 48(3), 238-240.

Qi, P., Cheng, S. Q., Wang, H., Li, N., Chen, Y. F. & Gao, C. F. (2011). Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. PloSOne, 6(12), e28486.

Shapiro, S. S. and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). Biometrika, 52: 591-611. http://dx.doi.org/10.1093/biomet/52.3-4.591. 22**).**

Uccello, M., Malaguarnera, G., Corriere, T., Biondi, A., Basile, F. & Malaguarnera, M. (2012). Risk of hepatocellular carcinoma in workers exposed to chemicals. Hepatitis Monthly, 12(10 HCC):e5943.

Uotila, M., Ruoslahti, E. & Engvall, E. (1981). Two-site sandwich enzyme immunoassay with monoclonal antibodies to human alpha-fetoprotein. Journal of Immunological Methods, 42(1), 11-15.

Wei, X. Y., Ding, J., Tian, W. G. & Yu, Y. C. (2020). MicroRNA-122 as a diagnostic biomarker for hepatocellular carcinoma related to hepatitis C virus: a meta-analysis and systematic review. Journal of International Medical Research, 48(8), 0300060520941634.

Whittaker, S., Marais, R. & Zhu, A. X. (2010). The role of signaling pathways in the development and treatment of hepatocellular carcinoma. Oncogene, 29(36), 4989-5005.

Wong, G. L. H., Chan, H. L. Y., Tse, Y. K., Chan, H. Y., Tse, C. H., Lo, A. O. S.& Wong, V. W. S. (2014). On-treatment alpha-fetoprotein is a specific tumor marker for hepatocellular carcinoma in patients with chronic hepatitis B receiving entecavir. Hepatology, 59(3), 986-995.

Xu, J., Zhu, X., Wu, L., Yang, R., Yang, Z., Wang, Q. & Wu, F. (2012). Micro RNA-122 suppresses cell proliferation and induces cell apoptosis in hepatocellular carcinoma by directly targeting Wnt/β-catenin pathway. Liver International, 32(5), 752-760.

Yasser, M. B., Abdellatif, M., Emad, E., Jafer, A., Ahmed, S., Nageb, L., Abdelshafy, H., Al-Anany, A. M.,Ezz Al-Arab, M. A.& Gibriel, A. A. (2021). Circulatory miR-221 & miR-542 expression profiles as potential molecular biomarkers in Hepatitis C Virus mediated liver cirrhosis and hepatocellular carcinoma. Virus Research,296, 198341.

Zhou, J., Yu, L., Gao, X., Hu, J., Wang, J., Dai, Z., Wang, J. F., Zhang, Z., Lu, S., Huang, X., Wang, Z.,Qiu, S., Wang, X., Yang, G., Sun, H., Tang, Z., Wu, Y., Zhu, H. & Fan, J. (2011). Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. Journal of Clinical Oncology, 29(36), 4781-4788.

Access this Article in Online	
	Website: www.ijarbs.com
Quick Response Code	Subject: Oncology
DOI:10.22192/ijarbs.2021.08.11.008	

How to cite this article:
Hanan H. A. Zedan, Amina M. Medhat, Laila A. Rashed, Mahmoud M. Said. (2021). Serum Micro RNAs 122,221 and Cyclin G1 and Their Combinations as Non-Invasive Diagnostic Biomarkers for Early-Stage Hepatocellular Carcinoma . Int. J. Adv. Res. Biol. Sci. 8(11): 64-79.
DOI: http://dx.doi.org/10.22192/ijarbs.2021.08.11.008