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Circulating microRNA-21 as biomarker for diagnosis in Egyptian patients with hepatocellular carcinoma

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Abstract
Background: Circulating microRNAs are endogenous, approximately 22 nucleotide non-coding RNAs that are overexpressed in many human cancers including hepatocellular carcinoma (HCC). Moreover, circulating miRNAs can reflect the level of serum miRNAs, so could be potential tumor markers. miRNA-21 regulates post-transcriptional expression gene which implies that miRNA-21 might be a novel diagnostic marker for cancer**.**
Objective: To evaluate the diagnostic potential of circulating miRNA-21 as a target of miRNA-21 in HCC of Egyptian patients.
Patients and methods: This study was conducted on 35 patients had primary HCC without HCV, 35 patients infected with HCV and had primary HCC and 30 healthy subjects as a control. Serum alpha fetoprotein (AFP) was measured for all participants. The miRNA-21 was determined in whole blood samples using real-time PCR. The study was approved by ethics committee of Faculty of Medicine and National Liver Institute, Menoufia University, Egypt.
Results: The results showed that the level serum AFP was a significantly elevated in both HCC (152.48 ± 126.90) and combined HCC + HCV (192.98 ± 132.55) groups when compared with its corresponding level in control group (3.37 ± 2.10) ($p< 0.05$. The data revealed up-regulation of micro-RNA 21 in both HCC (2.31 ± 0.69) and combined HCC+ HCV (4.99 ± 1.46) groups when compared with its corresponding level in control group (0.64 ± 0.94) ($p< 0.05$). Also, micro-RNA 21 expression was significantly increased in combined HCC + HCV group when compared with its level in HCC group ($p< 0.05$). Receiver Operator of Characteristics (ROC) curve analysis of plasma miRNA-21 revealed that, at a cut-off value in groups (2,3) > 2.0 and 2.3 while the sensitivity and specificity for differentiation of HCC cases were 91.43% and 94.29% and 86.67% and 90 %, respectively. while (ROC) curve analysis of AFP revealed that, at a cut-off value in groups (2,3) > 5.8and 6.4 while the sensitivity and specificity for differentiation of HCC cases were 86.57% and 74.29% and 80% and 93.33 %, respectively.
Conclusion: Circulating microRNA-21 level could be used as a biological marker for HCC and it might be beneficial in early diagnosis. MicroRNA**-**21 and AFP could be used as co-biomarkers for HCC diagnosis.

Keywords: hepatocellular carcinoma, hematological parameters, AFP, miRNA-21, Real time-PCR

1. Introduction

The liver is the largest and important organ in the body. It plays many vital functions in maintenance, performance and regulating homeostasis of the body such as bile production, stores vitamins, minerals, proteins, fats and glucose storage from diet, removes damaged RB in the co-ordination with spleen (Solomon *et al.*, 2017) [1].

Liver tumors are classified into two major categories, primary liver tumors and metastatic liver tumors. The primary tumor originates in the liver, while the metastatic tumor spreads to the liver from other organs of the body, accessing the liver through the portal vein or the hepatic artery (McNally, 2010) [2].

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and normally develops as a consequence of underling liver disease and often associated with cirrhosis (Li *et al.*, 2013) [3]. Variety of risk factors contribute to the initiation of HCC (Aravalli *et al.*, 2013) [4].

In Egypt, the incidence rate of HCC has increased sharply in the last (El-Zayadi *et al.*, 2010) [5]. The development and progression of HCC is a complex process, which involves the dysregulation of oncogenes and tumor suppressor genes. It has previously been reported that microRNAs (miRNAs) are essential in oncogenesis by the regulation of oncogenes and tumor suppressor genes (Mao *et al.*, 2015) [6].

miRNAs are approximately 22 nucleotide, noncoding, endogenous RNA molecules with an important role in various cellular biological processes**,** including embryonic development, cell differentiation, and tumorigenesis (Hu *et al.*, 2013) [7]. miRNAs regulate post-transcriptional gene expression, by binding to the 3′- un-translated region (*3′*-UTR) of specific target messenger RNAs (mRNAs), which in turn causes mRNA degradation or translational repression (Valencia-Sanchez *et al.*, 2006; Ebert and Sharp, 2012) [8, 9].

In humans, more than 50% of miRNA genes are located at fragile sites or in cancer-associated genomic regions that are frequently involved in chromosomal abnormalities, such as loss of heterozygosity, amplification and breakpoints (Aravalli *et al.*, 2013) [4]. miRNA-21 is one of the first oncogenic miRNAs with upreg**-** ulation detected in many types of human cancer (Schetter *et al.*, 2008; El Gedawy *et al.*, 2017) [10, 11]. Mi RNA-21 has also been implicated in multiple malignancy**-** related processes, including cell proliferation, apoptosis, invasion and metastasis, by down

regulating the expression of specific target genes (Zhu *et al.*,2007) [12]. This study aimed to evaluate the diagnostic potential of circulating miRNA -21 in Egyptian patients with HCC.

2. Patients and Methods

2.1 Study Population

The work has been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. The study was approved by ethics committee of Faculty of Medicine and National Liver Institute, Menoufia University.

This study was conducted on 100 individuals admitted to the outpatient clinics of National Liver Institute, Menoufiya University, Egypt from the period January 2017 to October 2017. They were divided into three groups.

1. **Frist group:** Control group**,** consisted of 30 healthy subjects (24 males and 6 females, with a mean age of 45.67 ± 5.99 years).
2. **Second group:** (HCC without HCV) group consisted of 35 patients had primary HCC (29 males and 6 females, with a mean age of 48.03 ± 4.71 years). The diagnosis of HCC was based on clinical examination, laboratory tests, ultrasonography and spiral CT.
3. **Third group:** (HCC+HCV) group consisted of 35 patients infected with HCV and had primary HCC (29 males and 6 females, with a mean age of 46.71 ± 5.17 years). The diagnosis of HCC was based on clinical examination, laboratory tests, ultrasonography and spiral CT.

The criteria for exclusion in this study

None of the patients had bacterial or other viral infection, chronic renal damage, insulin-dependent diabetes mellitus (IDDM) and other malignant diseases. In addition, the patients did not receive treatment using any drug, hepatic artery mobilization, or percutaneous ethanol injection prior to surgical resection to exclude patients with other cancers or with accompanying serious infections.

All patients and control groups were subjected to the following

1. Complete history taking,
2. Complete clinical examination,
3. Abdominal ultrasonography and/or CT**.**

2.2 Laboratory investigation

Ten ml venous blood samples were collected from patients and controls and divided into three parts**.**

1. **Frist part:** 3 ml of blood were collected in a plain tube, left to clot, centrifuged and serum was separated to determine routine liver function tests and Alfa feto protein by using R and OX RX Imola fully automated system and (AFP) was done by Cobas - Roche fully automated system by electro-chemiluminescence method (ECL).
2. **Second part:** 1.8 ml of blood was collected on 0.2 ml sodium citrate (3.8%) for prothrombin time (PT) in which, the assay done using Thrombrel-S (human thromboplastin containing calcium).
3. **Third part:** 5 ml of blood were collected on two tubes containing an ethylene di amine tetra acetic acid (EDTA)
4. and were used, 1 ml for CBC by using by Sysmex kx-21N automated hematology analyzer and 4 ml used immediately for RNA – miRNA extraction and molecular testing.

2.3 Extraction of miRNA

Magnetic particle with glass fibre affinity method

RNA was isolated with reagents provided in the MagNA Pure Compact Nucleic Acid Isolation Kit I (Cat. no. o3730964001) supplied by (Roche, Germany).

Briefly, 200 µl blood samples, together with an internal control system (Cook *et al.,* 2004) [13]. (Lyophilized 5,000 copies of internal control, was suspended in 10 µl of pre-warmed lysis buffer before addition to the specimen).

2.3.1 Steps of extraction

1. PBS was added to the tube containing the sample to reach a final volume of (200µl).
2. Lysis buffer (200 µl) and 40 µl Proteinase K were added to the tube and mixed followed by incubation at 72°C for 10 minutes in the thermal block of the extractor.
3. The sample was then mixed with 150 µl isopropanol with MGP.
4. The sample was then transferred to the upper compartment of the Tip hold on magnet rack to collect the magnetic particle with nucleic acid on wall of tip followed by vigorous pipetting and suction to get rid of the contaminants (proteins, lipid, carbohydrate and salts) in the lower compartment, while the magnetic glass fibber fleece traps the RNA.
5. Washing was done twice using 800µl of the two washing buffer I well 4, 5. The wash buffer was transferred to the upper compartment of the Tip hold on magnet rack to collect the magnetic particle with nucleic acid on wall of tip followed by vigorous pipetting and suction to wash the RNA. This was done twice wash buffer II, III well 6, 7.
6. Elution of the trapped RNA was done using 40 µl of elution buffer well.

2.3.2 Measurement of RNA concentration

Measurements of RNA concentration were done by using a spectrophotometer system (Beckman, DU series 650, Beckman Instruments, Inc., California, USA).

According to (Sambrook *et al.,* 2001) [14]. The amount of ultraviolet radiation absorbed by a solution of RNA is directly proportional to the amount of RNA in the sample. The concentration of RNA in a sample can be determined given that a 40µg/ml solution of single- strand RNA has an absorbance of 1.0 at a wavelength of 260nm.

The purity of a RNA preparation can be judged by examining the ratio of absorbance at 260nm: 280nm. Pure RNA preparation has absorbance ratio of 1.8 to 2.0. Protein or phenol contamination will lower the absorbance ratio and result in reduced accuracy of RNA quantitation.

$$1\ O.D._{260} \equiv 40\ \mu g \text{ of ss RNA /ml}$$
$$\equiv 40\ \mu g /1000\ \mu l$$
$$\equiv 0.04\ \mu g / \mu l$$

Thus concentration of RNA expressed in µg / µl

$$= O.D._{260} \times 0.04 \times \text{dilution factor}$$

2.4 cDNA synthesis

2.4.1 Protocol

The EPIK™ miRNA Select protocol is a two-step protocol consisting of

Step 1: Reverse transcription with miRNA-specific RT-oligonucleotide and EPIK™ cDNA synthesis kit.

Step 2: Real-Time PCR using Sensi SMART™ SYBR Master Mix and amplification primers It is critical for the success of the experiment to follow the protocol carefully, from first-strand cDNA synthesis to real-time PCR amplification (approximately 2 hours). However, the procedure can be paused after the first-strand cDNA synthesis and the undiluted cDNA may be stored at -20 °C for up to three days.

2.4.2 Workflow

Workflow for miRNA-21 and internal control (cel-miR-39)

When working with the EPIK™ miRNA Select Assays, it can be difficult for a single user with a single qPCR machine to run all the plates in one day. We suggest that in order for all the samples to be treated the same, storage should occur just after cDNA synthesis. All the cDNA reactions must be treated identically, so if it is not possible to run all the qPCR within one day, all the cDNA reactions must be frozen at -20 °C once completed. This will ensure that all cDNA reactions are subjected to the same number of freeze thaw cycles prior qPCR. The user should allow sufficient time so that all the real-time data can be collected in as short a time as possible, that the cDNA stored at -20 °C for no more than three days.

i) Assemble reagents

Assemble the reaction as indicated in Table (1)

The most consistent results can be obtained by preparing a mastermix with template RNA, EPIK 5x RT buffer, water and EPIK RT enzyme in the proportions shown. The EPIK RT Enzyme should be added to the master mix last, right before dispensing of the master mix into the PCR tubes.

Table 1: Singleplex setup

Reagent	Volume(µl)/Reaction kit
Template RNA (up to 100 ng)	X µl (up to 6 µl)
EPIK 5x RT Buffer	4 µl
RT primer	0.4 µl
EPIK RT Enzyme	1 µl
Nuclease-free water	up to 20 µl

ii) Store cDNA

If desired, undiluted cDNA reactions can be stored at -20 °C for up to three days. It is recommended to store cDNA in "low-nucleic acid binding" (presiliconized) tubes.

2.5 Real-time PCR amplification and detection

In this step, the cDNAs (miRNA-21 and internal control) are amplified by real-time PCR (Real-time 7500 Fast PCR System; applied biosystems) using SensiSMART SYBR Master Mix (Chan, 1999) [15].

Important: Keep an reagents on ice (or at 4 °C) at all times during set up.

2.5.1 Prepare reagents

A. Thaw 2x SensiSMART PCR Master Mix and cDNA reactions on ice.
B. Mix by quickly vortexing and spin down.
C. Dilute the cDNA reaction 10 fold with DEPC-water (provided).

2.5.2 Assemble the real-time PCR reagents

Prepare each qPCR reaction in the proportion indicated in

Table 2: Mix by vortexing and spin down.

Reagent	Volume
2x SensiSMART SYBR Master Mix	10 µl
Diluted cDNA reaction	5 µl
PCR primers	2 µl
DEPC-water	3 µl
Total volume	20µl

2.5.3 Real-Time PCR amplification

Perform real-time PCR amplification according to the following cycling parameters Tab: (3) and Fig: (1).

Table 3

cycles	Temp	Time	Notes
1	95˚C	10 min	Polymerase activation
	40˚C	5 min	
40	95˚C	10 s	Denaturation
	60˚C	30 s	Annealin/Extension (Acquire at end of step

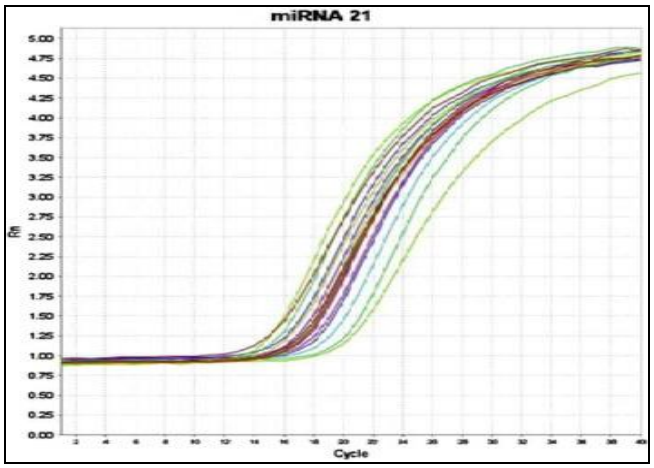

Fig 1: Amplification Plot (Rn vs. Cycle) of miRNA-21.

The primer of miRNA-21were supplied by Qiagen, Germany as follows:

Forward Primer*:*

5-AGAAATGCCTGGGTTTTTTTGGTT-3

Reverse Primer*:*

5-TTGGGAATGCTTTTCAAAGAAGGT-3

The house-keeping gene for MiRNA-21 is (cel-miR-39) as a reference control gene.

2.6 Quantification

Manual Calculation of RQ (Gene Expression)

Δ CT= Ct (Target) — Ct (Reference)

Mean Control = (Ctrll + Ctrl2+ Ctr13+….) /*N*

$\Delta\ \Delta$ CT = Δ CT (Target) — Ct (Mean control)

Normalized Target Gene Expression Level (RQ)= $2^{(-\Delta\ \Delta\ CT)}$

Δ = Delta

CT= Threshold Cycle

RQ = Relative Quantification

2.7 Statistical analysis

Results are expressed as mean ± standard deviation or number (%). Comparison between categorical data [number (%)] was performed using Chi square test or Fisher exact test if cell count was less than 5. Test of normality, Kolmogorov-Smirnov test, was used to measure the distribution Sof data. Accordingly, comparison between normally distributed variables in the three groups was performed using one way analysis of variance (ANOVA) followed by Tukey test if significant results was recorded. In not normally distributed data, comparison between variables in the three groups was performed using Kruskal-Wallis test followed by Mann Whitney test if significant results was recorded. Receiver operating curve (ROC) test was used discriminate between diseased (HCC) and un-diseased (control) groups and to calculate the diagnostic indices (sensitivity, specificity, positive and negative predictive values and accuracy) of both AFP and micro-RNA 21. Statistical Package for Social Sciences (SPSS) computer program (version 20 windows) was used for data analysis. P value ≤ 0.05 was considered significant.

3. Results

3.1 Hematological parameters

Table 4: showed that the mean values of RBCs, WBCs, platelet counts and hemoglobin concentration were significantly decreased in both HCC and combined HCC+HCV groups when compared with their corresponding levels in control group (p< 0.05)

	Control (n= 30)	HCC (n= 35)	HCC+HCV (n= 35)	P value			Overall p value [#]
				Control vs HCC	Control vs HCC+HCV	HCC vs HCC+HCV	
RBCs	5.03 ± 0.35	3.86 ± 0.34	3.80 ± 0.46	0.001*	0.001*	0.828	0.001*
Hb	14.38 ± 1.05	11.11 ± 1.14	10.91 ± 1.47	0.001*	0.001*	0.769	0.001*
Platelets	277.50 ± 55.37	102.06 ± 21.72	103.89 ± 1.68	0.001*	0.001*	0.978	0.001*
WBCs	7.79 ± 1.68	3.36 ± 0.52	3.24 ± 0.78	0.001*	0.001*	0.887	0.001*

Data are expressed as mean ± SD.

[#]= One way ANOVA test.

p> 0.05= not significant.

*p< 0.05= significant.

3.2 Alpha-feto protein (AFP)

Table 5: Showed that the value AFP was significantly elevated in both HCC (152.48 ± 126.90) and combined HCC + HCV (192.98 ± 132.55) groups when compared with its corresponding level in control group (3.37 ± 2.10) (p< 0.05).

Control (n= 30)	HCC (n= 35)	HCC + HCV (n= 35)	P value			Overall p value [$]
			Control vs HCC [$$]	Control vs HCC+HCV [$$]	HCC vs HCC+HCV [$$]	
3.37 ± 2.10	152.48 ± 126.90	192.98 ± 132.55	0.001*	0.001*	0.056	0.001*

3.3 Micro-RNA 21

Table: (6) showed that the mean relative quantities (RQs) of micro-RNA 21 expression was significantly elevated in both HCC (2.31 ± 0.69) and combined HCC+ HCV (4.99 ± 1.46) groups when compared with its corresponding level in control group (0.64 ± 0.94) (p< 0.05). Also, micro-RNA 21 expression was significantly increased in combined HCC + HCV group when compared with its level in HCC group (p< 0.05).

Table 6

Control (n= 30)	HCC (n= 35)	HCC+HCV (n= 35)	P value			Overall p value [$]
			Control vs HCC [$$]	Control vs HCC+HCV [$$]	HCC vs HCC+HCV [$$]	
0.64 ± 0.94	2.31 ± 0.69	4.99 ± 1.46	0.001*	0.001*	0.001*	0.001*

3.4 Receiver operating curve (ROC)

3.4.1 AFP

In HCC group, the area under ROC curve was 0.904 and cutoff value was > 5.8 (ng/ml) leading to 68.57% sensitivity, 80.0% specificity, 80% positive predictive value, 68.6% negative predictive value and 73.85% accuracy.

While in combined HCC + HCV group, the area under ROC curve was 0.967 and cutoff value was > 6.4 (ng/ml) leading to 74.29% sensitivity, 93.33% specificity, 92.9% positive predictive value, 75.7% negative predictive value and 83.08% accuracy (Table. (7); Figs. (2,3)).

Table 7: Diagnostic indices of AFP in both HCC and HCC + HCV groups.

	HCC Without HCV	HCC + HCV
Cutoff	> 5.8	> 6.4
Area under ROC	0.904	0.967
Sensitivity	68.57%	74.29%
Specificity	80.0%	93.33%
Positive predictive value (PPV)	80.0%	92.9%
Negative predictive value (NPV)	68.6%	75.7%
Accuracy	73.85%	83.08%

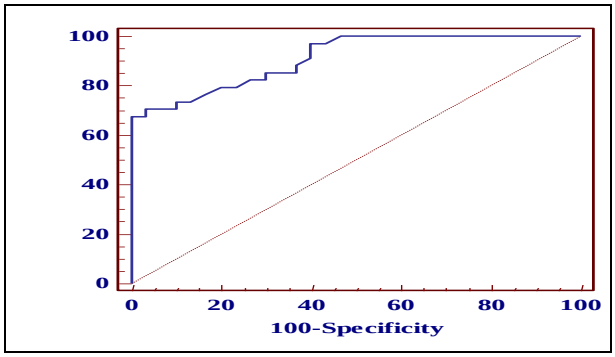

Fig 2: ROC curve of AFP in HCC Without HCV group

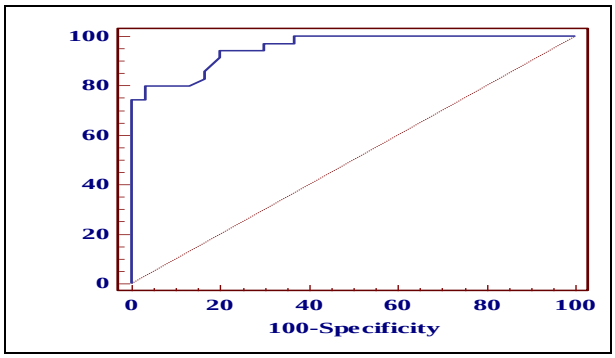

Fig 3: ROC of AFP in HCC+HCV group.

3.4.2. Micro-RNA 21

In HCC group, the area under ROC curve was 0.902 and cutoff value was > 2.0 leading to 91.43% sensitivity, 86.67% specificity, 88.9% positive predictive value, 89.2% negative predictive value and 89.2 % accuracy.

While in combined HCC + HCV group, the area under ROC curve was 0.991 and cutoff value was > 2.30 leading to 94.29% sensitivity, 90.0% specificity, 91.67% positive predictive value, 93.1% negative predictive value and 92.31% accuracy (Table (8); Figs.(4,5)).

Table 8: Diagnostic indices of micro-RNA 21 in both HCC Without HCV and HCC + HCV groups.

	HCC Without HCV	HCC + HCV
Cutoff	> 2.0	> 2.30
Area under ROC	0.902	0.991
Sensitivity	91.43%	94.29%
Specificity	86.67%	90.0%
Positive predictive value (PPV)	88.9%	91.67%
Negative predictive value (NPV)	89.7%	93.1%
Accuracy	89.2%	92.31%

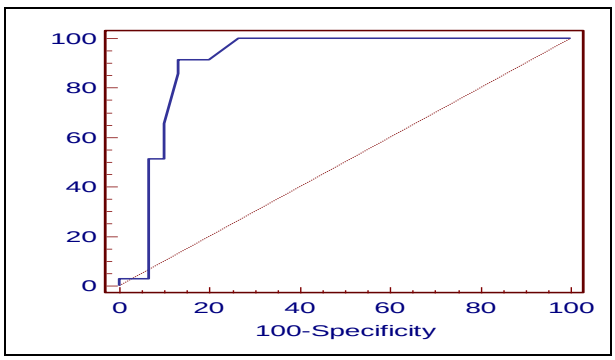

Fig 4: ROC curve of micro-RNA 21in HCC Without HCV group.

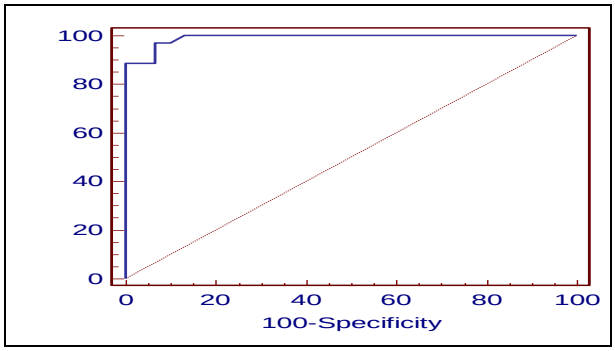

Fig 5: ROC curve of micro-RNA 21in HCC + HCV group.

4. Discussion

According to the World Health Organization, HCC is the fifth most common tumor worldwide and the second most common cause of cancer-related death. Male-to-female predominance is greater than 2:1 with liver cancer, and approximately 83% of the estimated 782,000 new HCC cases in 2012 occurred in less developed regions of the world (Song *et al.*, 2017; Heimbach *et al.*, 2018) [16, 17].

In the present study, the data revealed that the mean values of RBCs, WBCs, platelet counts and hemoglobin concentration were significantly decreased in both HCC and combined HCC+ HCV groups when compared with their corresponding levels in control group ($p< 0.05$).

These results agreed with (Selvamani and Thomas, 2017) [18] who studied the Evaluation of Haematological Abnormalities Decompensated Chronic Liver Disease Patients and stated that decreased RBCs and most common anemia in primary HCC patients are normochromic normocytic anemia as inferred from the study, from the other hand leucopenia and thrombocytopenia are present in most patients and is commonly present in the patients with splenomegaly and with the history of bleeding tendencies, also these results agreed with (Solomon *et al.*, 2017) [1] who showed the Study on Hematological Abnormalities in Chronic Liver Diseases and this might be due to the direct damage of the bone marrow and the blood elements are sensitive to the oxidative stress and their plasma membranes contain a highly percentage of polyunsaturated fatty acids (Carr, 2016) [19] and this leads to increasing lipid peroxidation products.

MiRNA-21(miR-21) is one of the first oncogenic miRNAs with upregulation detected in many types of human cancer (Schetter *et al.*, 2008) [10].

In recent years, the diagnostic value of circulating miR-21 in various human cancers has been studied intensely (Shan *et al.*, 2015) [20]. Several studies reported the significance of circulating miR-21 in HCC with inconsistent results (Qin *et al.*, 2013) [21].

Wang *et al.*, (2014) [22] reported that HCC patients had higher serum levels of miR-21 than healthy controls.The high levels of miR-21 expression in cancers have been reported to be correlated with tumor proliferation, invasion and metastasis so it's a its prognostic value for HCC (Luo *et al.*, 2017) [23].

The present study conducted on (35) newly diagnosed untreated HCC + HCV patients and (35) newly diagnosed untreated HCC without HCV selected from national liver institute in addition to (30) healthy person subjects matching age and gender of the patients were used as a control group.

In the present work, the circulating micro-RNA-21 RQ

revealed that significantly overexpressed in the serum of HCC group (2.31 ± 0.0.69) and combined group (HCC + HCV)(4.99 ± 1.46)when compared with its corresponding control group (0.64 ± 0.94) ($P < 0.05$). In addition micro-RNA 21 RQ expression was significantly increased in combined (HCC + HCV) group when compared with its level in HCC group ($P = 0.05$).

These results agreed with (Karakatsanis *et al.,* 2013; El Gedawy *et al*., 2017) [24, 11] who stated that micro-RNA-21 expression is increased in primary hepatocellular carcinoma patients and in malignant hepatocytes, when compared with non-tumor tissues. In addition the results of (*Hu et al.,* 2015) [25] suggested that miR-21 participated in HCC development through promoting cell proliferation. Also the present data agreed with (Liao *et al.,* 2015; Yoon *et al.,* 2018) [26, 27] who reported that Elevated miR-21 expression might represent a biomarker for HCC prognosis and their data showed that a significantly up-regulated in tumor tissues as compared with that observed in paired adjacent non-tumor tissues.

Zhu *et al.,* (2012) [28] reported that miR-21 promoted proliferation and protected against apoptosis in various tumors (e.g., breast, lung, colon, and liver cancers).

On the contrary, other reports demonstrated that plasma miRNA-21 levels were higher in patients with hepatitis B than in those with HCC (Xu *et al.,* 2011) [29]. An additional study revealed that circulating miRNA-21 levels were lower in patients with hepatitis B than healthy individuals (Qi *et al.,* 2011) [30]. The differences in these studies might be due to different sample sizes or sample selection, differences in the RNA extraction procedures or internal controls used or different ethnic groups (El Gedawy *et al*., 2017) [11].

Wang *et al.,* (2015) [31] who stated that changes in plasma miRNA-21 are early and accurately reflect the process of the formation of tumors. Also, (Tomimaru *et al.,* 2012) [32] reported that circulating miRNA-21 was an independent significant factor for recurrence and was reported to be more sensitive than AFP or the detection of HCC.

In the current study, the cut-off values and validity of serum AFP and circulating miRNA-21 for differentiation of HCC patients from healthy subjects were determined by ROC curves.

ROC curve analysis indicated that, the best cut-off for serum AFP to differentiate between HCC cases alone in group 2 and (HCC + HCV) cases in group 3 was $>$ 5.8 ng/ml and $>$ 6.4 ng/ml respectively. At this cut-off, the sensitivity**,** specificity, PPV, NPP and overall accuracy were 68.57%**,**74.29% 80%, 93.33%, 80%, 92.9, 68.6%, 75.7, 73.85% and 83.08%, respectively.

For circulating miRNA-21 ROC curve analysis indicated, the best cut-off to differentiate between HCC cases alone in group 2 and (HCC + HCV) cases in group 3 was $>$ 2 RQ in group2 and was >2.3 RQ in group3. At this cut-off, the sensitivity**,** specificity, PPV, NPP and overall accuracy were 91.43%**,**86.67% 88.9%, 89.7%, 89.2%, 94.29%, 90.0%, 91.67%, 93.1% and 92.31%, respectively.

As a potential diagnostic biomarker for HCCsugg (*Hu et al.,* 2015) [25] suggested that circulating miRNA-21 possesses several unique advantages. First, circulating miRNA is non-invasive**.** Second, circulating miRNA expression levels are stable and reproducible (Chen *et al.,* 2008) [33]. Third, circulating miRNA-21 level cannot be influenced by both cirrhosis and viral status. Fourth, significant overexpression of circulating miRNA-21 was observed in patients with early-stage HCC (which is the case in our study) (Tomimaru *et al.,* 2012) [32]. Meanwhile, AFP level of 400 ng/ml is considered as an indicator of HCC in general, and such high level might not be reached at an early HCC stage. As a result, about one-third of all HCC cases are not diagnosed in the early tumor stage. Therefore, circulating miRNA-21 may serve as a novel co-biomarker to AFP to improve the diagnostic accuracy of early-stage HCC (Corvalan, 2012) [34].

5. Conclusion

From the last results of the current study, the following could be concluded**:**

- Circulating microRNA-21 level could be used as a biological marker for HCC and it might be beneficial in early diagnosis.
- MicroRNA-21 and AFP could be used as co-biomarkers for early diagnosis of HCC**.**

6. Recommendations

- Large scale study is needed to confirm the results of the current study.
- The study has proved that miRNA-21 could be used as diagnostic markers for liver cancer.
- It could be of interest to examine the serum level of miRNA-21 at early stages of HCC development.
- After confirmation of the results, miRNA-21 could be used as prognostic marker for liver cancer and follow up.
- MiRNA-21 profiling analysis can predict the recurrence of HCC after surgical resection.
- It is conceivable that the ability of using miRNA-21as a potential therapeutic targeting for some cancers.

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