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ORIGINAL ARTICLE

Circulating MiRNA-21 and programed cell death (PDCD) 4 gene expression in hepatocellular carcinoma (HCC) in Egyptian patients

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KEYWORDS

miRNA-21;
PDCD4 gene;
Hepatocellular carcinoma;
CLD;
Apoptosis;
Real time-PCR

Abstract *Background:* Circulating microRNAs (miRNAs) are endogenous, small (17–25 nucleotides) non-coding RNAs that are overexpressed in many human cancers including hepatocellular carcinoma (HCC). Moreover, circulating miRNAs can reflect the level of tissue miRNAs, so could be potential tumor markers. miRNA-21 regulates post-transcriptional expression of tumor suppressor gene; programed cell death 4 (PDCD4) gene which implies that miRNA-21 might be a novel diagnostic and/or prognostic marker for cancer.

Objective: To evaluate the diagnostic and prognostic potential of circulating miRNA-21 and study the expression of PDCD4 gene as a target of miRNA-21 in HCC in Egyptian patients.

Subjects and methods: This study was conducted on 30 HCC patients, 20 chronic liver disease (CLD) patients due to HCV infection and 20 healthy subjects. Serum alpha fetoprotein (AFP) was measured for all participants. The relative plasma expression of each of miRNA-21 and PDCD4 gene was determined in whole blood samples using real-time polymerase chain reaction.

Results: The results revealed over expression of miRNA-21 and under expression of PDCD4 gene in HCC group ($p < 0.05$) compared to both CLD and healthy subjects, while no significant change was detected between CLD and healthy subjects. miRNA-21 expression was negatively correlated with PDCD4 gene expression. miRNA-21 expression increased significantly with presence of cirrhosis, increased number of focal lesions, larger size of tumor, advanced tumor stage and presence of vascular invasion. Receiver Operator of Characteristics (ROC) curve analysis of plasma miRNA-21 revealed that, at a cut-off value of 3.93 (fold expression), the sensitivity and specificity for differentiation of HCC cases were 93% and 90%, respectively.

Conclusion: Circulating miRNA-21 could be a novel early diagnostic and prognostic biomarker for detection of HCC. Approaches interfering with the miRNA-21/PDCD4-axis, or releasing PDCD4 expression, may have a strong basis for therapeutic uses in cancer in the future.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is associated with liver cirrhosis (LC) in 80% of cases [1]. In Egypt, the incidence rate of HCC has increased sharply in the last decade [2]. 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 [3].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 [4]. miRNAs regulate post-transcriptional gene expression, by binding to the 3′-untranslated region (3′-UTR) of specific target messenger RNAs (mRNAs), which in turn causes mRNA degradation or translational repression [5,6]. 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 [7]. miRNA-21 is one of the first oncogenic miRNAs with upregulation detected in many types of human cancer [8]. miRNA-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, such as phosphatase and tensin homolog (PTEN), tropomyosin 1 (TPM1), programed cell death 4 (PDCD4) and B-cell lymphoma 2 (Bcl-2) [9].

PDCD4 is a tumor suppressor gene that plays an important role in regulating apoptosis, invasion and metastasis [10]. Several reports described the regulation of PDCD4 by miRNA-21. In pancreatic ductal adenocarcinoma; inhibition of miRNA-21 reduces proliferation and increases cell death by increasing PDCD4 [11]. Asangani et al. [12] found a conserved potential site for miRNA-21 within the 3′UTR (3-untranslated region) of PDCD4 mRNA. Refs. [13,14] demonstrated the functionality of this site as well as the regulation of PDCD4 levels by miRNA-21 and induction of invasion, intravasation and metastasis by elevated miRNA-21. Thus, miRNAs modulate various cellular signaling pathways involved in cell growth, proliferation, motility and survival [15].

Better understanding of the molecular mechanisms involved in hepatocellular carcinogenesis contributes to the identification of novel prognostic and diagnostic biomarkers and therapeutic targets for HCC. So, this study aimed to evaluate the diagnostic potential of circulating miRNA-21 and study the expression of programed cell death 4 (PDCD4) gene as a target of miRNA-21 in Egyptian patients with HCC and correlating them to the clinical and path logical parameters of the patients.

2. Subjects 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. Enrollment of individuals in the study was conditioned by an obtained written informed consent. Seventy subjects were enrolled in the study and were divided into 3 groups. Fifty patients were randomly selected from the inpatient ward and outpatient clinic, National Liver Institute, Menoufia University from January 2014 to December 2014. Patients were subdivided to newly diagnosed naïve HCC patients and HCV positive chronic liver disease (CLD) cases. The HCC group comprised 30 patients, 26 males and 4 females, their ages ranged from 30 to 52 years. The diagnosis of HCC was based on clinical examination, laboratory tests, ultrasonography and spiral CT. The CLD group consisted of 20 cases, 15 males and 5 females, and their ages ranged from 35 to 51 years. CLD patients were diagnosed by ultrasonographical findings (shrunken liver, coarse echo pattern, attenuated hepatic vein and fine nodular surface) and biochemical evidence of parenchymal damage as well as liver biopsy. Patients with bacterial or other viral infection, chronic renal damage, Insulin-dependent diabetes mellitus (IDDM), other malignant diseases, or undergoing interferon administration or immune-suppressive or chemotherapy were excluded from this study. In addition 20 apparently healthy subjects, 18 males and 2 females, and their ages ranging from 32 to 53 years, age and gender matching, served as the control group.

All patients and control groups were subjected to full history taking, complete clinical examination, abdominal ultrasonography and/or CT.

2.2. Laboratory investigations

Ten ml venous blood samples were collected from patients and controls and divided into three parts. The first part was collected in plain tube and used for routine laboratory investigations, including liver function tests [using fully automated auto 111 analyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA)] and immunoassay; serum HBs-Ag and HCV-Ab [using (Abbott Laboratories, Abbott Park, IL, USA)]. Also, serum AFP concentration was measured using the Automated Chemiluminescence System (ACS: 180 provided by Siemens Medical Solutions Diagnostics Corporation, USA). The second part was collected in an ethylene diamine tetra acetic acid (EDTA) containing tube and used for CBC assessment using Sysmex K-21, (Sysmex Corporation, Kobe, Japan). The third part was collected in an EDTA containing tube and used immediately for RNA – miRNA extraction and molecular testing.

2.3. Molecular testing

Real time PCR technology (using 7500 fast real time PCR – TaqMan® microRNA and RNA Control Assay) was used for assessments of miRNA-21 and its control gene (RNU43) and the tumor suppressor gene PDCD4 and its control gene [Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)].

2.4. Extraction and cDNA synthesis

2.4.1. RNA extraction and cDNA synthesis

RNA was extracted from fresh EDTA treated blood sample using PureLink® RNA Mini Kit (Ambion, Life Technology)

and QIAzol (Lysis solution) according to the manufacturer's instructions. RNA quantity was tested by spectrophotometer at optical density; OD_{260} (RNA concentration = Diluted volume (μg/mL) × OD_{260} × dilution factor). Then single stranded cDNAs were synthesized using High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, CA, USA). Random Hexamer Primer (2 μL), Reaction Buffer (2 μL), RNase Inhibitor (1 μL), dNTP Mix (0.8 μL), MultiScribe™ Reverse Transcriptase (1 μL), and nuclease-free water (3.2 μL) were mixed. Then cDNA synthesis was done where RNA samples (10 μL) were added, mixed and briefly centrifuged with the previous prepared components. The programing of thermal cycler condition was as follows: heating at 25 °C for 10 min, 37 °C for 120 min, then, termination of the reaction by heating at 85 °C for 5 min.

2.4.2. miRNA extraction and cDNA synthesis

miRNAs were extracted from fresh EDTA treated blood sample using Qiagen miRNA Extraction kit and QIAzol (Lysis solution) according to the manufacturer's instructions. Single-stranded cDNAs were generated using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) via mixing RT Primer (3 μL), 10X RT Buffer (1.5 μL), RNase Inhibitor (0.19 μL), dNTP mix (0.15 μL), MultiScribe™ Reverse Transcriptase (1 μL), and nuclease-free water (4.16 μL). RNA samples (5 μL) were added, mixed and briefly centrifuged with the previous prepared components. The programing of thermal cycler condition was as the following: hold for 30 min at 16 °C, hold for 30 min at 42 °C, then, termination of the reaction by heating at 85 °C for 5 min.

2.5. Amplification

Determination of miRNA-21 levels was done by TaqMan miRNA Assay and PDCD4 expressed levels by TaqMan gene expression assay using Universal TaqMan master mix (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's protocol. Fluorescence measurements were made in every cycle (Figs. 3 and 4) and the cycling conditions used for amplification of genes of interest were: initial denaturation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 40 cycles of 60 °C for 60 s. The primers for miRNA-21, RNU43 (reference control gene), PDCD4 and GAPDH (reference control gene) were supplied by Qiagen Germany. miRNA-21 Forward Primer: 5-AGAAATGCCTG GGTTTTTTTGGTT-3 and miRNA-21 Reverse Primer: 5-T

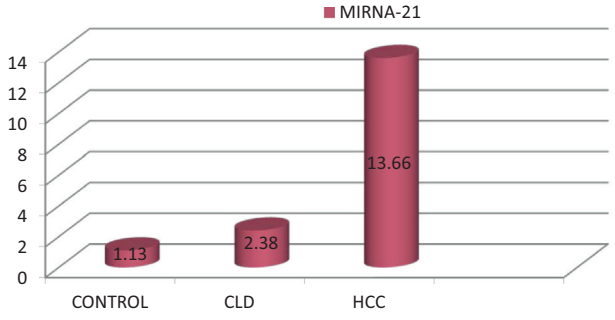

Figure 1 Mean RQ of circulating miRNA-21 in HCC, CLD and control groups.

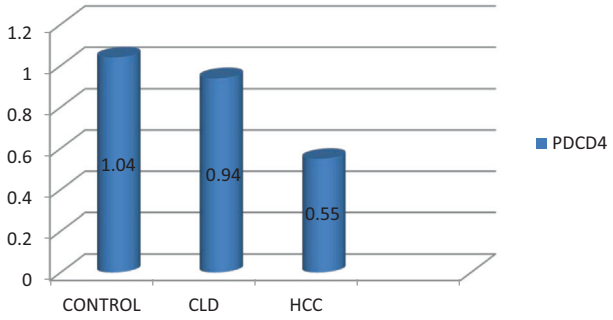

Figure 2 Mean RQ of PDCD4 gene expression differences between HCC, CLD and control groups.

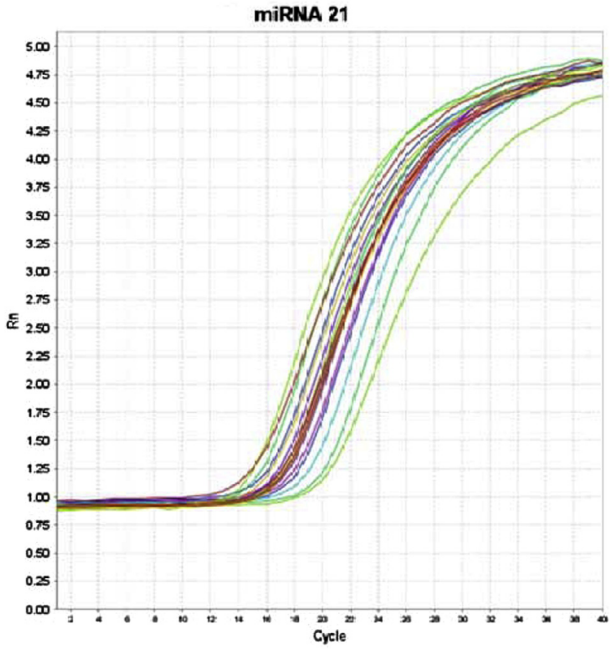

Figure 3 Amplification Plot (Rn vs. Cycle) of miRNA-21.

TGGGAATGCTTTTCAAAGAAGGT-3.RNU43 probe sequence: GAACTTATTGACGGGCGGACAGAAACTGTGT GCTGATTGTCACGTTCTGATT. PDCD4 Forward Primer: 5-CTTGAAGGTTAAGATGTGGGAGTGT-3 and PD CD4 Reverse Primer: 5-AGCTAAACTCCAGAACTTTT CCATT-3. GAPDH Forward Primer: 5-TGCCCAGTTGAA CCAGGCG-3 and GAPDH Reverse Primer: 5-CGCGGAG GGAGAGAACAGTGA-3.

2.6. Quantification

Quantification of gene expression of interest was accomplished by measuring the fractional cycle number at which the amount of expression reached a fixed threshold (Ct), which was directly related to the amount of product. The relative quantification given by the Ct values was determined and the control gene Ct subtracted to achieve ΔCt [ΔCt = Ct (gene of interest) – Ct (control gene)]. Then relative expression level was determined as $2^{(-\Delta\Delta Ct)}$, where ΔΔ Ct = ΔCt (target sample) – ΔCt (reference sample).

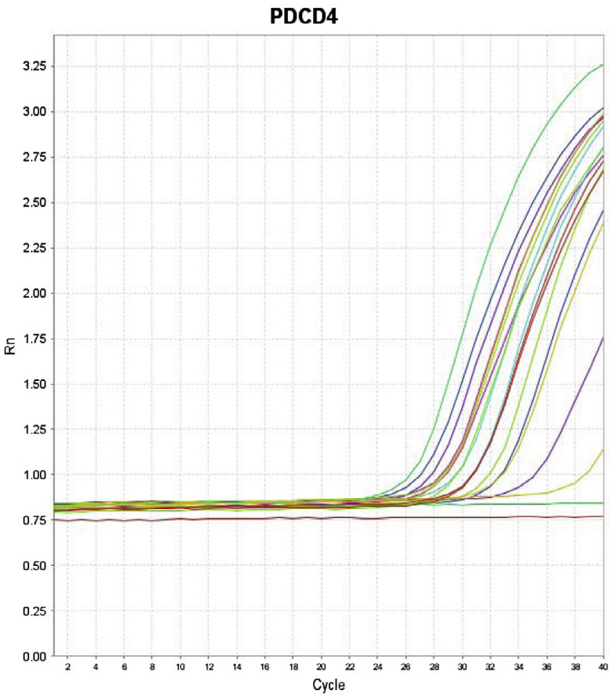

Figure 4 Amplification Plot (Rn vs. Cycle) of PDCD4 gene.

2.7. Statistical analysis

Data were collected, tabulated and statistically analyzed by IBM® SPSS® (Statistical Package for the social Science) version 18.0 statistical package (SPSS, Inc, Chicago, IL, USA). ANOVA, Student's t test and Pearson's correlation tests were performed at 5% level of significance. The diagnostic performance for miRNA-21 and AFP to discriminate HCC cases from those without HCC was evaluated using Receiver Operating Characteristic (ROC) curve analysis. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and overall accuracy for each marker were determined.

3. Results

A total of 70 subjects were enrolled in this study, 30 HCC patients (86% were males) with a mean age of 43.47 ± 4.37 years, 20 CLD patients (75% were males) with a mean age of 42.00 ± 6.88 years, and 20 healthy volunteers (90% were males) with a mean age of 42.65 ± 5.72 years. The studied groups were homogenous in terms of age and gender ($p > 0.05$).

Comparison between the studied groups regarding mean relative quantities (RQs) of miRNA-21 and PDCD4 gene expression revealed that there were significant increase in miRNA-21 and significant decrease in PDCD4 gene expressions in HCC group compared to each of CLD and control groups ($p < 0.05$). However, they showed no significant difference between CLD and control groups ($p > 0.05$) (Figs. 1 and 2).

Table 1 showed significant inverse correlation ($p < 0.01$) between miRNA-21 and PDCD4 gene expression RQs in HCC group.

Relationship between mean RQs of miRNA-21 and PDCD4 gene expression and pathological characteristics of HCC patients: The mean RQ of miRNA-21 showed significant increase with presence of cirrhosis, increased number of focal lesions, larger size of tumor, advanced tumor stages (stages 3 & 4 vs stages 1 & 2) and presence of vascular invasion. Meanwhile, the mean RQ of PDCD4 gene expression showed a significant decrease with increased number of focal lesions, larger sized tumors and advanced stages of HCC (Tables 2 and 3). However, no significant difference in serum AFP was detected regarding the aforementioned variables (data not shown).

The RQ of circulating miRNA-21 showed a highly significant elevation in HCC patients with early tumor development (single focal lesion, tumor size < 3 cm, and collectively TNM 1 and 2 stages) ($p < 0.01$) compared to each of the control and CLD patients groups (Table 4), while no statistical difference was detected regarding RQ of plasma PDCD4 gene expression (data not shown).

Regarding correlation between the mean RQ of each of miRNA-21 and PDCD4 gene expression and some biochemical parameters in the studied groups: There was a significant positive correlation ($p < 0.01$) between miRNA-21 and AFP and significant inverse correlation ($p < 0.05$) between PDCD4 and each of total and direct bilirubin in HCC group (Table 5). A significant positive correlation was detected between miRNA-21 and ALT as well as between PDCD4 gene expression and GGT in CLD group (data not shown). Meanwhile, No statistically significant correlation was identified between miRNA-21 and PDCD4 gene expression and any of these studied parameters in control group (data not shown).

Receiver Operator of Characteristics (ROC) curve analysis of AFP and miRNA-21: displayed that the best cut-off of serum AFP for differentiation of HCC cases from those without HCC was 91.7 ng/ml, at this cut-off; the sensitivity, specificity, PPV, NPV and overall accuracy were 75.2%, 92.3%, 90.2%, 69.2%, and 77.0%, respectively. For miRNA-21 the best cutoff was 3.93 RQ with sensitivity, specificity, PPV, NPV and overall accuracy of 93%, 90%, 94.4%, 87.5%, and 92.5%, respectively. Combined use of both parameters was superior to the use of AFP alone as the specificity, PPV, and overall accuracy were 97%, 96%, and 92%, respectively (Figs. 5 and 6 and Table 6).

4. Discussion

HCC as other malignancies is attributed to accumulated genetic alterations. As an oncomir, miRNA-21 is upregulated in a variety of human malignancies. Overexpression of

Table 1 Pearson's correlation between RQs of miRNA-21 and PDCD4 gene expressions in HCC Group.

Parameters	HCC group	
	MicroRNA-21 RQ (folds)	
	r	*P*-value
PDCD4 RQ (Folds)	−0.428	< 0.01

RQ: relative quantity. $P < 0.01$: highly significant. PDCD4 gene: programed cell death 4.

Table 2 Comparison between pathological criteria of HCC regarding RQ of miRNA-21 in HCC group.

Studied variables	MiRNA-21 expression (fold change) in HCC group (No = 30) (M ± SD)	Student – *t* test	*P*-value
Cirrhosis			
Positive (No = 26)	15.11 ± 12.7	4.39	< 0.01
Negative (No = 4)	3.8 ± 1.3		
Number of focal lesions			
Single (No = 12)	6.15 ± 3.24	3.65	< 0.01
Multiple (No = 18)	18.6 ± 13.9		
Size of tumor			
< 3 cm (No = 9)	5.6 ± 2.5	4.08	< 0.01
⩾3 cm (No = 21)	18.2 ± 13.6		
Vascular invasion			
Positive (No = 19)	17.4 ± 14	2.98	< 0.01
Negative (No = 11)	7.03 ± 4.4		
Enlarged liver			
Mild (No = 27)	13.44 ± 12.8	0.206	> 0.05
Moderate (No = 3)	15.04 ± 11.4		
Enlarged spleen			
Mild (No = 20)	14.71 ± 16.72	0.296	> 0.05
Moderate (No = 10)	13.0 ± 10.21		
Stages of tumor (TNM)			
1&2 (no = 15)	8.1 ± 4.6	9.5	< 0.01
3&4 (no = 15)	19.1 ± 15.4		
		Anova	
Ascites			
Absent (No = 14)	10.37 ± 6.72	0.938	> 0.05
Mild (No = 13)	16.96 ± 16.74		
Moderate (No = 3)	14.12 ± 11.72		

TNM: tumor-node metastasis system. $p < 0.01$: highly significant. $p > 0.05$: non-significant.

Table 3 Comparison between pathological criteria of HCC regarding mean RQ of PDCD4 gene expression in HCC group.

Studied variables	PDCD4 gene expression (fold change) in HCC group (No = 30) (M ± SD)	Student *t* test	*P*-value
Cirrhosis			
Positive (No = 26)	0.69 ± 0.44	0.931	> 0.05
Negative (No = 4)	0.90 ± 0.18		
Number of focal lesions			
Single (No = 12)	0.81 ± 0.33	2.39	< 0.05
Multiple (No = 18)	0.50 ± 0.34		
Size of tumor			
< 3 cm (No = 9)	0.73 ± 0.29	2.68	< 0.05
⩾3 cm (No = 21)	0.29 ± 0.45		
Vascular invasion			
Positive (No = 19)	0.45 ± 0.44	1.73	> 0.05
Negative (No = 11)	0.78 ± 0.60		
Enlarged liver			
Mild (No = 27)	0.59 ± 0.53	0.627	> 0.05
Moderate (No = 3)	0.39 ± 0.44		
Enlarged spleen			
Mild (No = 20)	0.60 ± 0.50	0.15	> 0.05
Moderate (No = 10)	0.57 ± 0.54		
Stage of tumor (TNM)			
1&2 (no = 15)	0.97 ± 0.46	13.6	< 0.01
3&4 (no = 15)	0.19 ± 0.18		
		Anova test	
Ascites			
Absent (No = 14)	0.61 ± 0.49	0.072	> 0.05
Mild (No = 13)	0.55 ± 0.56		
Moderate (No = 3)	0.50 ± 0.67		

PDCD4 gene: programed cell death 4. $P < 0.05$: significant. $p < 0.01$: highly significant. $p > 0.05$: non-significant.

miR-21 promoted proliferation and protected against apoptosis in various tumors (e.g., breast, lung, colon, and liver cancers) [16,17].

Consistent with the above studies, this work demonstrated that miRNA-21 is upregulated in HCC where mean circulating miRNA-21 RQs in patients with HCC was significantly higher ($p < 0.05$) compared to that of patients with CLD and healthy individuals groups, which was in agreement with other researches [18–20].

Circulating miRNAs originate from tumor tissues, and they are present in a certain form in the blood and are resistant to RNase activities [21]. However, it remains unclear how circulating miRNAs originate from tumor tissues. It was suggested that miRNAs could be derived from dying or lysed tumor cells, invasive lymphoma cells, cells from tissues affected by long-term disease, or the active secretion of tumor cells [19].

Table 4 Comparison of RQ of circulating miRNA-21 between patients with early developed HCC and each of control and CLD groups.

Early tumor characteristics	Mean ± SD	*T* test	*P*-value
Single nodule ($n = 12$)	6.15 ± 3.24	T1 = 5.36	P1 < 0.01
		T2 = 3.7	P2 < 0.001
Size < 3 cm ($n = 9$)	5.6 ± 2.5	T1 = 5.36	P1 < 0.001
		T2 = 3.26	P2 < 0.01
No vascular invasion ($n = 11$)	7.03 ± 4.4	T1 = 4.44	P1 < 0.01
		T2 = 3.24	P2 < 0.01
Stage 1 and 2 ($n = 15$)	8.1 ± 4.6	T1 = 5.84	P1 < 0.001
		T2 = 4.36	P2 < 0.001

T1 and P1: HCC vs. 20 control subjects whose mean miRNA-21 RQ level is 1.13 ± 0.23.
T2 and P2: HCC vs. 20 CLD patients whose mean miRNA-21 RQ level is 2.38 ± 2.44.
P-value < 0.01: highly significant.

Table 5 Pearson's Correlation between the RQs of each miRNA-21 and PDCD4 gene and some studied parameters in HCC group.

Parameters	HCC Group			
	MiRNA21		PDCD4	
	R	*p*-value	*r*	*p*-value
AST (IU/L)	0.141	> 0.05	−0.094	> 0.05
ALT(IU/L)	0.129	> 0.05	−0.065	> 0.05
TP (g/dl)	−0.094	> 0.05	−0.052	> 0.05
Alb (g/dl)	−0.271	> 0.05	0.201	> 0.05
ALP (IU/L)	0.072	> 0.05	−0.016	> 0.05
GGT (IU/L)	0.170	> 0.05	−0.257	> 0.05
T.B (mg/dl)	0.249	> 0.05	−0.374	< 0.05
D.B (mg/dl)	0.314	> 0.05	−0.437	< 0.05
INR	0.218	> 0.05	0.102	> 0.05
AFP (ng/ml)	0.028	< 0.01**	0.078	> 0.05

PDCD4 gene: programed cell death 4. $P < 0.05$: significant. $p > 0.05$: non-significant.
** $P < 0.01$: highly significant.

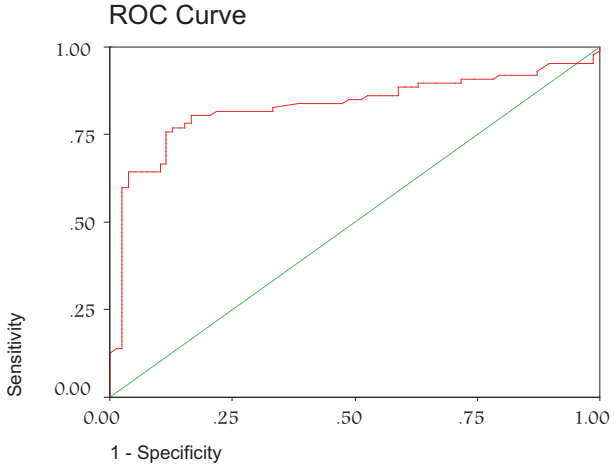

Figure 5 ROC curve analysis of AFP.

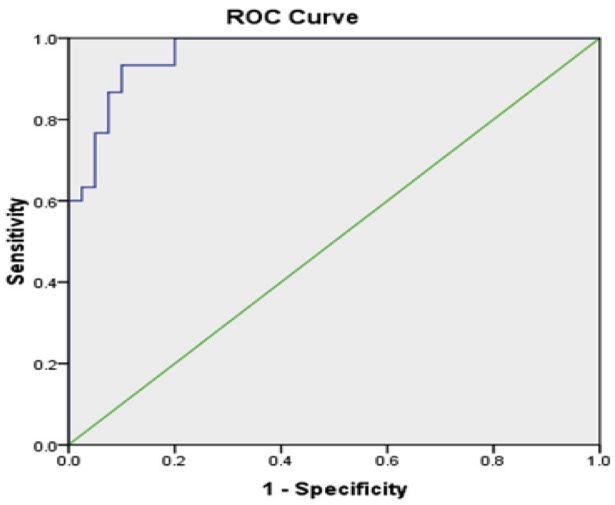

Figure 6 ROC curve analysis of miRNA-21.

On the contrary, other reports demonstrated that plasma miRNA-21 levels were higher in patients with hepatitis B than in those with HCC [22]. An additional study revealed that circulating miRNA-21 levels were lower in patients with hepatitis B than healthy individuals [23]. 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 and stage of HCC.

In the current study, a significant increase ($p < 0.01$) in the mean RQ of miRNA-21 was observed with cirrhosis and progression of HCC, as it showed a significant increase in multiple focal lesions, larger size of tumor, advanced tumor stage and presence of vascular invasion which indicate that circulating miRNA-21 could be a potential prognostic marker in HCC. Meanwhile, no significant change of the mean serum AFP levels was observed regarding the aforementioned parameters on comparing early and late HCC cases.

These results are consistent with the study done by Wang et al. [19] who revealed that circulating miRNA-21 levels were significantly higher in advanced HCC compared with early HCC groups, but serum AFP level was not changed until tumor appearance.

Also, Faltejskova et al., Hu et al., and Huang et al. [24–26] showed that overexpression of miRNA-21 was associated with advanced clinical stage and lymph node metastasis in breast cancer, colorectal cancer, laryngeal squamous cell carcinoma and HCC respectively. Furthermore, they indicated that patients with high miRNA-21 expression had poor prognosis and poor survival rates compared to patients with lower miRNA-21 expression.

Many studies have supported the hypothesis that miRNA-21 overexpression might promote HCC invasion and metastasis through direct or indirect mechanisms, and thus lead to poor prognosis [17,22,27].

Zhou et al. [28] demonstrated that miRNA-21 promoted side population cell migration and invasion by targeting PTEN (Phosphatase and tensin homolog), RECK (Reversion-inducing cysteine-rich protein with Kazal motifs), and PDCD4, and that it could also directly target MAP2K3 (mitogen-activated protein-2 kinase-3) and inhibit its expression during HCC carcinogenesis [29]. Therefore, patients with

Table 6 Sensitivity, specificity, PPV, NPV and accuracy of AFP and/or miRNA-21 in HCC group (No = 30) versus non HCC (CLD and Control) groups (No = 40).

Studied markers	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Serum AFP at a cut-off point of 91.7 (ng/mL)	75.2	92.3	90.2	69.2	77.0
Circulating miRNA-21 at cut-off point of 3.93 RQ	93	90	94.4	87.5	92.5
AFP and/or miRNA-21	88	97	96	90	92

PPV: positive predictive value.
NPV: negative predictive value.

high plasma miRNA-21 expression were shown to have a significantly shorter time-to-recurrence compared to those with low plasma expression [27]. Thus, identification of miRNA is associated with the prognosis of HCC patients, and may not only elucidate the underlying biological mechanisms involved in the development or progression of the disease, but also provide an opportunity to identify novel targets for HCC therapy [26].

The current study showed that there was a significant increase ($p < 0.01$) in the mean RQ of circulating miRNA-21 in patients with early development of HCC (single focal lesion, no vascular invasion, tumor size less than 3 cm or TNM stages 1 and 2) compared to each of CLD and control groups. Thus, plasma mean RQ of miRNA-21 could be used in the follow up of chronic liver disease patients for early detection of HCC development.

These findings were consistent with the results informed by Wang et al. [19] who stated that changes in plasma miRNA-21 are early and accurately reflect the process of the formation of tumors. Also, Tomimaru and Eguchi [27] reported that circulating miRNA-21 was an independent significant factor for recurrence and was reported to be more sensitive than AFP for the detection of HCC.

It is reported that, miRNA-21 functions as an anti-apoptotic factor and this role is at least partially due to the repression of a highly interconnected tumor suppressor network that includes multiple components of the TP53 (tumor protein), transforming growth factor beta (TGF β) and apoptotic pathways [30,31]. Also, several other tumor suppressors such as PTEN or PDCD4 are regulated by miRNA-21 in different cancer cell types [32].

Tumor suppressor PDCD4 gene is one of the miRNA-21 target genes [12]. PDCD4 gene was identified as a suppressor of transformation, tumorigenesis, tumor growth, progression and invasion [33]. It has been reported that loss or reduction of PDCD4 expression was found in some malignant tumors including renal-, lung- and glia-derived tumors [34,17].

In agreement with above studies, the present study showed that the expression of PDCD4 gene was downregulated in HCC patients ($p < 0.05$). Meanwhile, its level did not significantly differ between CLD and control groups.

This finding is consistent with researches showing reduced PDCD4 expression in lung cancer [35], HCC [36], breast cancer [37], glioma [38], pancreatic cancer [13] and esophageal cancer [39].

The current study also revealed a highly significant inverse correlation ($p < 0.01$) between mean RQs of miRNA-21 and PDCD4 mRNA in HCC patients, which could make it a potential regulator of PDCD4 as its over-expression could have led to decreased PDCD4-mRNA expression.

Zhu et al. and Jiao et al. [17,40] reported the same findings in tissue samples from HCC, colorectal cancer and malignant melanoma. PDCD4 has been shown to be regulated post-transcriptionally by miRNA-21 in breast [41], colon [12] and esophageal carcinoma [39].

miRNAs regulate mRNA expression by binding imperfectly to their target mRNA; interfering with translation initiation factor binding and relocating the mRNA to cytoplasmic processing bodies (P-bodies) [42]. Once the mRNA is in the P-body, it is not translated and may re-enter the cytoplasm, but can also be degraded over time with a long term expression of miRNAs [43], which would explain why miRNA-21 is inversely correlated with PDCD4 mRNA.

Also, this study revealed a significant decrease of PDCD4 expression in HCC patients with advanced tumor stages, increased number of focal lesions and increased tumor size.

PDCD4 protein expression was significantly downregulated in metastatic HCC tumor tissues and tumor tissues from stages III to IV patients, and was gradually reduced in HCC cell lines along with increasing invasive capacity. Inhibition of PDCD4 expression could promote migration and invasion of hepatocellular carcinoma cell line (HepG2) cells. Taken together, these data suggest that PDCD4 is a critical mediator through which miRNA-21 exerts its biological functions in HCC [17,44]. These results were in disagreement with those of Chen et al. [35] who showed that PDCD4 under expression in lung adenocarcinoma was not associated with tumor size, stage or nodal status indicating a potential tissue specific role of PDCD4 gene.

The fact that PDCD4 is down-regulated in a number of different cancers, including HCC, suggests that restoring its expression could be a promising mode of therapy [45]. Moreover, delivered PDCD4 facilitated apoptosis, inhibited pathways involved in cell proliferation and suppressed pathways that are known to be involved in tumor angiogenesis, also, regulated proteins involved in cell-cycle control and suppressed activator protein-1 (AP-1) activity [46]. Such studies show that it is possible to effectively deliver a functional PDCD4 gene to tumors which might help to treat un-resectable HCC tumors in order to decrease or prevent metastatic spread.

In the current study, the cut-off values and validity of serum AFP and circulating miRNA-21 for differentiation of HCC patients from those without HCC (CLD and healthy subjects) were determined by ROC curves.

ROC curve analysis indicated that, the best cut-off for serum AFP to differentiate between HCC cases and subjects without HCC was 91.7 ng/ml. At this cut-off, the sensitivity, specificity, PPV, NPP and overall accuracy were 75.2%, 92.3%, 90.2%, 69.2%, and 77.0%, respectively.

For circulating miRNA-21; ROC curve analysis indicated that the best cut-off to differentiate between HCC cases and non HCC subjects was 3.93 RQ (fold change). At this cut-off, the sensitivity, specificity, PPV, NPP and overall accuracy were 93%, 90%, 94.4%, 87.5%, and 92.5%, respectively with area under the curve (AUC) of 0.97. This was consistent with Tomimaru and Eguchi [27] who demonstrated that ROC curve analysis of circulating miRNA-21 revealed the sensitivity and specificity to be 87.3 and 92%, respectively, differentiating HCC patients from healthy adults and reported that, miRNA-21 could be a promising biomarker of HCC. This was in agreement with other studies [18–20].

Furthermore, combined use of serum AFP and circulating miRNA-21 for detection of HCC cases, had an advantage over the use of AFP alone as the sensitivity, specificity, PPV, NPP and overall accuracy were increased to 88%, 97%, 96%, 90%, and 92%, respectively. Tomimaru and Eguchi [27] suggested that the combination of circulating miRNA-21 and serum AFP enhanced the performance of AFP in discriminating HCC from healthy volunteers and patients with chronic hepatitis.

As a potential diagnostic biomarker for HCC, Liao et al. [20] suggested that circulating miRNA-21 possesses several unique advantages. First, circulating miRNA is non-invasive. Second, circulating miRNA expression levels are stable and reproducible [47]. Third, circulating miRNA-21 level cannot be influenced by both cirrhosis and viral status. Fourth, significant overexpression of circulating miRNA-21 was observed even in patients with early-stage HCC (which is the case in our study) [27]. 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 with small lesions (< 3 cm) 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 [48].

The results of this study augmented the aforementioned data as the circulating miRNA-21 RQ exhibited significant elevation in the early development of HCC (solitary focal lesion, absence of vascular invasion, tumor size < 3 cm, and TNM stages 1 and 2) compared to controls and CLD patients. Also, the sensitivity and NPV of miRNA-21 were 92% and 87.5% respectively and for AFP, they were 75.2% and 69.2%, respectively.

Also, the above results give an indication about the probability of using miRNA-21 as a potential therapeutic target and this was previously identified by Khare et al. [49] who proposed that miRNA-21 as a therapeutic target would be effective since single miRNA can control multiple deranged genes in HCC.

Moreover, miRNAs have also been shown to influence sensitivity of tumors to anticancer drugs. HCC cells transfected with pre-miRNA-21 were resistant to the cytotoxicity induced by IFN α/5-FU. Therefore, antagomirs targeting miR-21 might be useful in increasing drug efficacy [50].

However, the overexpression of circulating miRNA-21 is not limited to HCC as its expression was informed in other human tumors, such as gastric and lung cancers. This indicates that the increased expression of miRNA-21 is associated to cancer development regardless of its kind or origin and may not be specific to HCC. Therefore, miRNA-21 alone may not be a specific indicator for the diagnosis of HCC in routine clinical practice, but this marker will progress test performance when it is used in combination with other biomarkers as AFP [22].

5. Conclusion

The current study suggests that circulating miRNA-21 could be novel early diagnostic and prognostic biomarkers for the detection of HCC. Also, PDCD4 could be an important mediator through which miRNA-21 exerts its biological functions in HCC. Thus, approaches interfering with the miRNA-21/PDCD4-axis, or releasing PDCD4 expression, may have a strong basis for therapeutic uses in cancer in future. Large scale and multi-center studies are required to confirm these findings.

Declaration of conflict of interest

The authors declare that they have no conflict of interest.

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