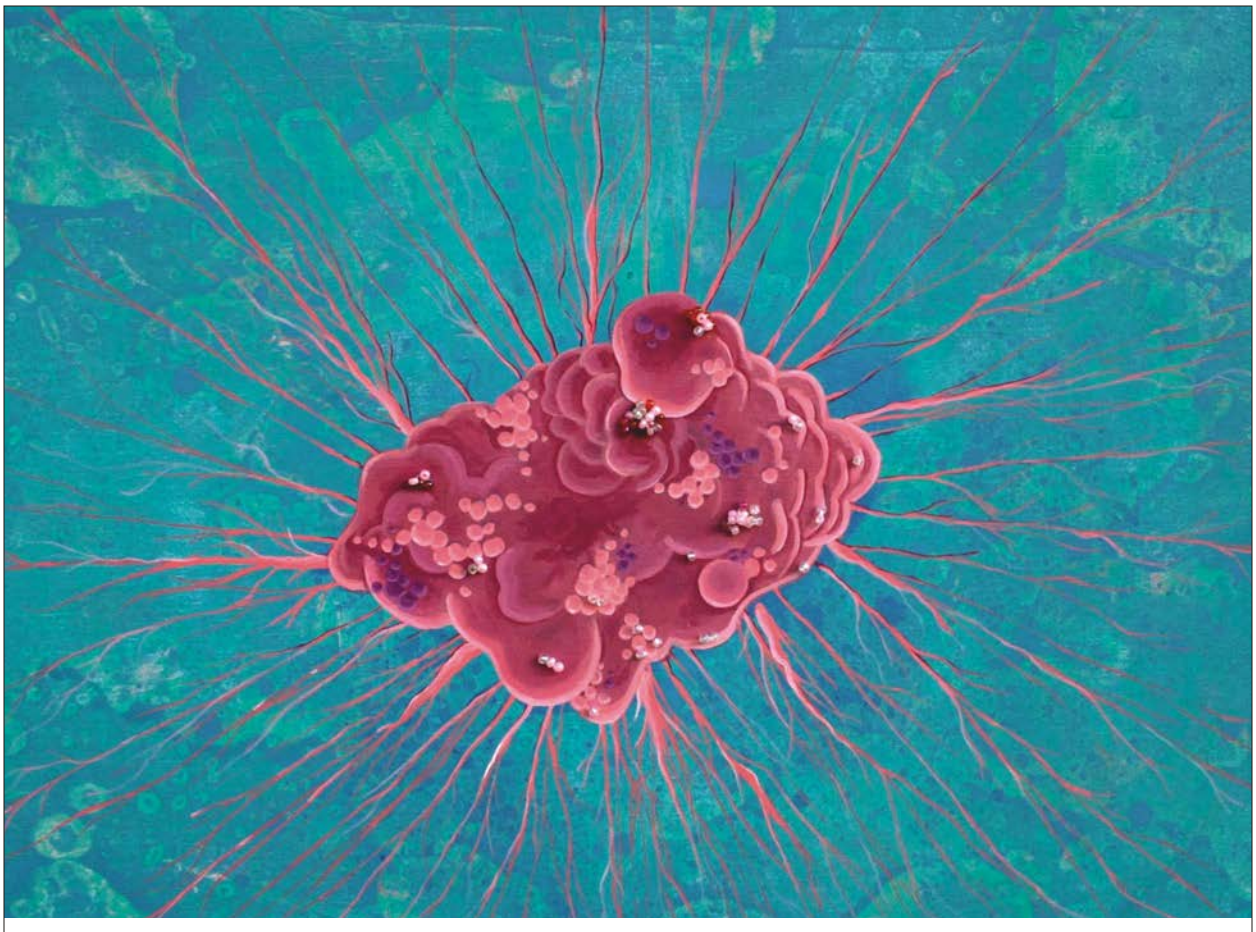

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Research Article
Serum MicroRNA-1246 as a Potential Biomarker for HCV-related Early-stage Hepatocellular Carcinoma

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Abstract

Background and Objective: MicroRNAs had been implicated in several malignancies. Abnormal circulating microRNA-1246 expression had been detected in HCC patients in an expanding number of studies. However, the information in literature describing the pertinent ramifications of miR-1246 in early-stage HCCs are rare and heterogeneous. This study was designed to assess the diagnostic accuracy of serum miR-1246 level in early-stage HCV-related HCC patients contrasted with chronic hepatitis C (CHC), liver cirrhosis (LC) and healthy control (HC). **Materials and Methods:** Two hundred HCV outpatients were doled out into 3 groups, HCC group (n = 100), CHC group (n = 30) and LC group (n = 70). Another hundred (100) age- and sex-matched healthy controls (HC) were likewise enlisted. The serum expression level of miR-1246 (by quantitative Real-Time PCR), AFP and prothrombin induced by vitamin K absence-II (PIVKA-II) were tested. **Results:** In HCC patients, in contrast to AFP, the serum expression levels of PIVKA-II and miR-1246 were statistically significantly increased discriminating HCC patients and early-stage HCCs not only from non-HCC patients (CHC, LC) yet additionally from HC. PIVKA-II and miR-1246, either individually or combined, had excellent diagnostic accuracies and performances as demonstrated by their ROCs and high AUCs >0.7. This serum over-expression positively correlated with the clinicopathological characteristics of both HCC and non-HCC patients. **Conclusion:** Serum miR-1246 level was significantly higher in HCC patients compared with non-HCC and HC and reliably discriminate early-stage HCV-related HCCs particularly when combined with PIVKA-II.

Key words: Early-stage HCC, microRNAs, miRNA-1246, HCV

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the commonest and deadliest primary liver malignant growth, the 5th commonest malignancy in male, the seventh most common malignant tumor in females worldwide and the 3rd reason for cancer-related passings with high incidence in Sub-Saharan Africa and Eastern Asia[1,2]. Despite the marked advances in diagnostic interventions and the significant clinical usage of abundant remedial modalities, HCC had an extremely terrible prognosis with poor overall survival and high incidence of early tumor recurrence (ETR)[3]. This high death rate is attributable to the absence of solid and approved techniques for the early determination of HCCs[4]. Over 90% of HCCs develop on top of liver cirrhosis due to CHC, CHB, steatohepatitis or obesity[5].

Unfortunately, the ongoing therapeutic approaches of HCC including transarterial chemoembolization (TACE), radiofrequency ablation (RFA), ethanol injection, surgical resection and orthotopic liver transplantation (OLT) are useful just at early stages of the disease. Sorafenib, the only FDA-approved medicate for advanced HCC, has restricted survival benefits[6]. Besides, the utilization of alpha-fetoprotein (AFP) is never again recommended by international surveillance guidelines due to its inferiority in sensitivity, specificity and diagnostic performance particularly in early-stage HCC[7]. Although the recent radiological techniques could detect small nodules down to less than 1 cm, it represents a financial burden and a debate in distinguishing cirrhotic from malignant small nodules[8].

In this manner, the identification of a solid modality or a novel biomarker for efficient diagnosis of early-stage HCC and tracing of ETR has been a subject of utmost enthusiasm for all scientists. However, this is much troublesome because of the concurrence of inflammation as well as liver cirrhosis in HCC. The ideal biomarker ought to non-invasive, affordable, quantifiable in serum or urine, ethnically-explicit and practically applicable in both the developed and developing countries.

Recently, a wide variety of novel biomarkers which are protein-, RNA-, DNA- or antibody-based have been proposed to fortify the present surveillance techniques and predict early-stage HCCs in patient's at-risk[9,10]. MicroRNAs (miRNAs), discovered in 1993 are small non-coding RNAs of 20-24 nucleotides long that are produced by drosha RNase III protein in the nucleus and dicer protein in the cytoplasm. They function as guide molecules in RNA silencing and regulate protein expression by fine-tuning gene expression. MicroRNAs are implicated in several processes, for example cell development, apoptosis, proliferation and differentiation, genomic instability, immune response and tumor metastasis and play a pivotal role in tumorigenesis[11].

The dysregulation and altered expression of microRNAs are linked to various human ailments and malignancies, including HCCs[12]. Some dysregulated miRNAs may function as oncogenes and others as tumor-suppressors[13]. MicroRNAs are present in almost all tissues and secreted from the cells to various body fluids [such as plasma, serum, urine, saliva, sputum, etc. When emitted to peripheral blood, miRNAs circulate either encapsulated in exosomes or tied to protein or lipids. MicroRNAs particularly the tumor-derived miRNAs are impervious to endogenous ribonuclease activity in plasma or serum and resist degradation by pH extremes, boiling or storage[14].

The determination of disease-specific circulating miRNAs has turned into a noteworthy focus of cancer researches as they are non-invasive, sensitive, stable, exceptionally reproducible, circling cell-free in blood, have an amazing tissue specificity and could be detected in blood by the highly-sensitive droplet digital PCR (ddPCR) and quantitative real-time (qRT) PCR assay[15].

Few studies assessed the circulating expression level of MicroRNAs either individually or in panels in Egyptian patients with HCV-related early-stage HCCs[16, 17]. MicroRNA-1246 is one of the recently contemplated microRNAs and has been depicted to be embroiled in several malignancies including HCCs. MicroRNA-1246 gene is located on chromosome II (2q31.1) of the human genome serving as a transcriptional target of p53 and is implicated in the regulation of the cell cycle, apoptosis and senescence[18]. MicroRNA-1246 may be involved in the regulation of cancer cell proliferation, cell motility, invasion and metastasis[19,20].

Scientists have exhibited that miR-1246 could serve as a proto-oncogene in numerous malignant growths, for example, breast, colon, lung and gastric cancers[21]. The anomalous microRNA-1246 expression had been identified non-invasively from plasma or serum samples of cancer patients in a large number of studies, however, no comprehensive conclusion has been elucidated.

As most HCCs occur on top of liver cirrhosis, it might be difficult to distinguish regeneration nodules from early-stage HCC. Lamentably, the data in literature describing the relevant diagnostic implication of miR-1246 in early-stage HCCs particularly the HCV-related HCC are infrequent and inconsistent. Moreover, it isn't clear if the serum expression levels of miR-1246 may discriminate between cirrhotic nodules and early-stage HCC or not.

This study aimed to study the diagnostic value of serum expression level of miR-1246 in HCV-related HCC Egyptian patients compared to liver cirrhotic patients (LC), chronic hepatitis C patients (CHC) and healthy controls (HC). Additionally, to evaluate the reliability of serum level of miR-1246 either alone or in blend with PIVKA-II in discriminating early-stage HCCs.

MATERIALS AND METHODS

Material: About 200 adult consecutive Egyptian outpatients attending department of internal medicine at Specialized Medical and Mansoura University Hospitals with a confirmed diagnosis of hepatitis C virus infection and receiving long-term follow-up were initially enrolled in this study. They were relegated into 3 groups, HCC group (n = 100), CHC group (n = 30) and LC group (n = 70). Another hundred (100) age and sex-matched healthy volunteers as a control group (HC) were likewise selected. The study was initiated in June, 2016, proceeded through 2019. The study was approved by the Ethical Committee and Institutional review board of Mansoura Faculty of Medicine in Egypt (MFM-IRB, Code No: R.19.07.561). A written informed conscious consent was acquired from all participants before their participation.

Inclusion criteria: The diagnosis of chronic hepatitis C. All patients were HCV positive.

Exclusion criteria: Age below 18 years and over 70 years, a history of malignancy of any type other than HCC within the last 5 years, a history of solid organ transplantation or previous bone marrow transplantation, antiviral treatment and local or systemic tumor-specific treatment within the last month. Additionally, we excluded patients with chronic renal failure, bone disease, thyroid disorders, inflammatory bowel diseases, cardiac failure, systemic infection (e.g., HIV, bacterial and fungal), other causes of liver disease (e.g., alcohol consumption, chronic hepatitis B or D, primary biliary cirrhosis (PBC), metabolic liver disease, non-alcoholic steatohepatitis and autoimmune hepatitis). The control group had no clinical, radiological or biochemical evidence of liver disease or any medical illness at enrolment.

Methods: Initially, all participants were submitted to a thorough history taking with detailed physical examinations and relevant medical history. At the day of study incorporation, 5 mL of venous blood was obtained from all participants and the serum samples were centrifuged at 3400 rpm for 7min at room temperature then centrifuged at 12000 rpm for 4 min at 4°C to remove any remaining cells then aliquoted and stored at -80°C until assayed. Laboratory liver biochemical profile was done including total and direct bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), serum albumin (ALB), gamma-glutamyl transpeptidase (GGT), prothrombin time (PT), international normalized ratio (INR). Complete blood count (CBC), HBsAg, HCV-Ab, HCV PCR quantitative, serum creatinine, C reactive protein (CRP), erythrocyte sedimentation rate (ESR), serum creatinine and fasting blood sugar (FBS) were additionally done. Tumor markers including alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3) and des-gamma carboxy-prothrombin (DCP) (also known as PIVKA II) were done using commercially available ELISA assay kits according to the manufacturer protocols (Sanko Junyaku, Tokyo, Japan).

Chronic hepatitis C: Diagnosed by HCV antibody-positive test (third-generation enzyme-linked immunosorbent assay, ELISA) and confirmed by detecting the viral load (HCV RNA) using quantitative Real-Time PCR (qRT-PCR) with COBAS TaqMan HCV Test (TaqMan HCV, Roche Molecular Systems Inc., Branchburg, NJ, USA, lower limit of detection: 15 IU mL^{-1}).

Liver cirrhosis: Diagnosed clinically (for example ascites, esophageal varices, fundic varices, splenomegaly, jaundice, hepatic encephalopathy), imaging (ultrasound), lab liver profile or by liver biopsies (if accessible as indicated by modified-knodell histological activity index). The severity and stage of liver cirrhosis were evaluated by the model of end-stage liver disease (MELD score) and Child-Pugh scores were assessed at the time of the study inclusion.

HCC: Diagnosed by contrast-enhanced ultrasound technique (CE-US) utilizing Sequoia 512 equipment (Acuson, Mountain View, CA), 4-phase multi-detector computed tomography scan (CT), dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)[22]. Stage of HCC was determined according to Barcelona Clinic Liver Cancer staging system (BCLC)[23]. Diagnosis of HCC was affirmed if there is one of the following three items based on the American Association for the Study of Liver Diseases (AASLD) practice guidelines[24]:

- One or more of liver nodules > 1 cm in diameter in CT or MRI
- Early arterial enhancement with α-fetoprotein (AFP) $\geq$400 ng mL^{-1}
- Typical features of dynamic imaging (early arterial phase enhancement and delayed venous phase washout) regardless of AFP

Early-stage HCC: Affirmed if the tumor size ≤3cm, BCLC stage (0+A), Child-Pugh score A, negative AFP and/or PIVKA-II and absence of local or distant metastasis.

RNA extraction and quantification: Total RNA including miRNA fractions was extracted from 200 µL serum of previously thawed samples using miRNeasy Mini kit according to manufacturer's instructions (Qiagen, Carlsbad, California, USA). To remove any contaminating DNA in the total RNA, 1 µL 2 U μL^{-1} DNase (Qiagen) was utilized and the final elution volume was 20 µL. Prior to the RT reaction, the serum RNA preparations were both qualified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) at 260 and 280 nm ($A_{260/280}$). Only RNA samples exhibiting $A_{260/280}$ ratios of 1.9-2.1 were selected. The serum RNA preparations were then quantified on a Nanodrop 2000 spectrofluorometer (NanoDrop Technologies, Wilmington, Delaware, USA). The cDNA was synthesized from the secluded RNA utilizing the miScript II RT kit (Qiagen) as indicated by the producer directions. The relative expression of miRNAs was measured by miScript SYBR Green PCR kit (Qiagen), which incorporates miScript universal primer and quantiTect SYBR green PCR master mix. The reactions were performed in 384 well plates with 10 mL total volume/well and contained 1X SYBR green master mix, 200 nmol L^{-1} forward primers (miRNA-specific primer) and 200 nmol L^{-1} universal primers, using 3ng cDNA/well. The conditions included initial denaturation at 95°C for 15 min, trailed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. All the samples were performed in duplicates on ViiA7 real-time PCR system (Applied Biosystem, Foster City, CA, USA). The miR-16 was utilized as an endogenous control (standardization factor)[25]. The cycle threshold (Ct) values were calculated utilizing SDS 2.0.1 software (Applied Biosystems). The relative gene expression analysis of the target miRNAs (miR-1246) was performed using the 2-delta-delta-cycle threshold method (2-ΔΔ-Ct) as previously described and was displayed in $\log_{10}$ base[26].

Statistical analysis: Statistical analysis was executed using SPSS software version 20.0 (SPSS, Inc., Chicago, IL, USA). Quantitative (continuous) data were expressed as mean±standard deviation (SD) or median and inter-quartile range (IQR) while qualitative data were expressed as number and percentage. Categorical variables were compared using the chi-square (χ^2) test or Fisher's exact test. Subgroups were compared using the Mc-Nemar test. One-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's honest significant difference (HSD) test was applied for multiple comparisons. Spearman's rank correlation coefficient was used to test correlations between two variables. The nonparametric Mann-Whitney U test was utilized to study the different associations between the relative expression levels of circulating miRNA and the clinicopathological characteristics. The diagnostic accuracy of miRNA was assessed by receiver operating characteristic (ROC) analysis and calculation of the area under the curve (AUC) and optimal cut-off value. Sensitivity, specificity, predictive values and likelihood ratios were calculated. AUC was used as an accuracy index for evaluating the diagnostic performance of the selected miRNA. The p-values were two-sided and displayed as Log10 for AFP, PIVKA-II and miR-1246. The $p<0.05$ was considered significant.

RESULTS

The demographic data, clinical and lab attributes of all participants were exhibited in Table 1. The age range was 18-70 years. Male to female ratio was 1.29, 169:131. Early-stage HCC was diagnosed in 24 out of 100 patients. There were no statistical significant differences between the examined groups in regards to age, gender and serum creatinine ($p>0.05$). With respect to liver functions and platelets, there were statistically significant contrasts between the groups, being significantly deteriorated in HCC than non-HCC patients ($p<0.05$). Notwithstanding, no statistically significant differences regarding liver functions and platelets were seen between CHC and LC ($p>0.05$).

The serum expression levels of AFP, PIVKA-II and miR-1246 in the considered groups were shown in Table 2. There were statistical and significant differences between the studied groups ($p<0.001$). Likewise, there were statistically and significantly increments in their serum expression levels in total HCC patients contrasted with non-HCC patients or HC ($p1<0.0001$). Looking at early-stage HCC and advanced HCC, AFP and PIVKA-II had statistical significant difference being increased only in advanced HCC ($p2 = 0.0001$), while serum miR-1246 had no statistical significant difference being significantly increased in both early-stage and advanced HCC ($p2 = 0.094$). Only the serum expression levels of miR-1246 had a statistically significant increase in early-stage HCCs versus non-HCCs patients (p3 0.0001), while AFP and PIVKA-II had no statistically significant difference in early-stage HCC and non-HCCs ($p3>0.05$). Along these lines, just the serum miR-1246 level could segregate early-stage HCC from CHC and LC. There were no significant contrasts between CHC and LC groups in regards to AFP, PIVKA-II and miR-1246 ($p4>0.05$).

Table 1: Demographic, clinical and lab characteristics of patients in the studied groups

		Non-HCC (100)			
	Control (100)	CHC (30)	LC (70)	HCC (100)	
Parameters	Mean±SD				ANOVA
Age (years)	57.30±1.3	56.15±1.1	55.39±1.4	61.0±5.4	0.131
Male/female (169/131)	54/46	18/12	36/34	59/41	0.347
S. creatinine (mg dL^{-1})	0.90±0.4	1.1±0.6	1.3±0.5	1.4±0.4	0.171
AST (U L^{-1})	39.60±1.9	91.8±3.12	103.8±3.1	113.1±2.08	0.035
ALT (U L^{-1})	41.45±1.6	99.0±1.9	76.0±1.7	117.65±3.8	0.026
S. bilirubin (mg dL^{-1})	1.10±0.16	1.5±0.14	1.4±0.2	2.8±0.8	0.032
S. Albumin (g dL^{-1})	4.60±1.2	3.2±1.4	2.9±1.3	2.1±1.7	0.024
INR	1.10±0.1	1.2±0.2	1.3±0.2	2.8±0.4	0.021
Platelets (10^4 μL^{-1})	20.60±0.23	14.3±0.5	13.5±0.5	11.6±0.6	0.031
Child-Pugh score (n: A/B/C)	-	27/2/1	33/27/10	1/16/83	-
AFP (-ve/+ve)	100/0	23/7	58/12	24/76	-
PIVKA-II (-ve/+ve)	0/100	25/5	62/8	19/81	-
p>0.05					

Data were expressed as M±SD: Mean±standard deviation, INR: International normalized ratio, LC: Liver cirrhosis, HCC: Hepatocellular carcinoma, AST: Aspartate transaminase, ALT: Alanine transaminase, AFP: Serum alpha-fetoprotein level (ng mL^{-1}), PIVKA-II: Prothrombin induced by vitamin K absence II, AU: Armstrong unit, P: Probability, ANOVA: One way analysis of variance, p<0.05 was considered to be statistically significant, PIVKA-II was negative if <65 mAU mL^{-1}, AFP was negative if <20 ng mL^{-1}

Table 2: Serum biomarkers (AFP, PIVKA-II and miR-1246) in all participants of the studied groups

			Non-HCCs			HCC				
		Control								
Biomarkers		(HC)	CHC	LC	Total	Early	Advanced	Total	ANOVA	p-values
	N		30.00	70.00	100.00	24.00	76.00	100.0		
Log_{10} AFP	Mean	0.82	1.01	1.24	1.13	1.182	2.858	2.3	p<0.001	p1= 0.0001, p2 = 0.0001, p3 = 0.539
	SD	0.357	0.27	0.44	0.44	0.11	0.289	0.83		
	SE	0.042	0.047	0.018	0.037	0.02	0.042	0.098		
	Median	0.95	1.12	1.17	1.04	1.19	2.939	2.73		
	Range	1.22	1.04	0.61	1.79	0.41	1.12	2.16		
Log_{10} PIVKA-II	Mean	1.35	1.34	1.51	1.48	1.60	2.83	2.52	p<0.001	p1= 0.0001, p2 = 0.0001, p3 = 0.195
	SD	0.19	0.25	0.28	0.26	0.38	0.323	0.56		
	SE	0.02	0.08	0.054	0.02	0.08	0.047	0.066		
	Median	1.34	1.98	1.35	1.43	1.82	2.83	2.74		
	Range	0.76	1.13	0.91	0.89	1.31	1.77	1.77		
Log_{10} miR-1246	Mean	0.67	0.94	1.14	1.03	1.79	1.86	1.84	p<0.001	p1= 0.0001, p2 = 0.094, p3 = 0.0001
	SD	0.38	0.34	0.51	0.49	0.12	0.193	0.175		
	SE	0.045	0.51	0.14	0.04	0.02	0.028	0.021		
	Median	0.48	1.23	1.84	1.15	1.77	1.826	1.82		
	Range	1.04	0.87	0.27	1.66	0.51	0.98	0.98		
			p4>0.05		p3					
			p4			p2				
			p1							

Data were expressed as Mean and standard deviation (SD), SE: Standard error of mean, HCC: Hepatocellular carcinoma, AFP: Alpha-fetoprotein, PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, ANOVA: One way analysis of variance, p1: Compared total HCCs with either non-HCCs or HC, p2: Compared early HCC with advanced HCCs, p3: Compared early HCCs with total non-HCCs, p4: Compared CHC and LC, p-value was calculated with Log_{10} and was significant at 0.05

Table 3 demonstrated the correlations between the serum expression levels of AFP, PIVKA-II, miR-1246 and the clinicopathological characteristics in HCC patients. Serum AFP, PIVKA-II and miR-1246 were significantly positively correlated with the clinical and BCLC stages of HCCs (p<0.05). The serum expression level of miR-1246 was significantly positively correlated with AFP (p = 0.303, r = 0.010) and PIVKA-II (p = 0.302, r = 0.010).

The sensitivity (Sn.), specificity (Sp.) and diagnostic accuracies (Acc.) of serum expression levels of serum miR-1246 and PIVKA-II in early-stage HCC patients were exhibited in Table 4. In early-stage HCC, the diagnostic performances of miR-1246 at a cut-off 9.5fold change was significantly higher than that of PIVKA-II at a cut-off 65 mAU mL^{-1} (Acc. 92.45%, 74.49%, respectively). Strangely, the diagnostic accuracy of miR-1246

Table 3: Spearman's rho correlations between serum biomarkers, clinicopathological characteristics in HCC patients

Spearman's rho	PIVKA-II	miR-1246	BCLC-stage	Clinical stage	PLT	ALT
AFP						
r	0.478*	0.303**	0.817**	0.636**	-0.014-	0.048
p	0.011	0.010	0.000	0.000	0.835	0.481
PIVKA-II						
r	1.000	0.302**	0.679**	0.733**	-0.068-	0.128
p	0.0	0.010	0.000	0.000	0.318	0.581
miR-1246						
r	0.302**	1.000	0.262**	0.740**	-0.147-*	0.331
p	0.010	0.0	0.006	0.000	0.019	0.052

**Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed), BCLC: Barcelona clinic liver cancer staging system, AFP: Alpha-fetoprotein, PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, ALT: Alanine transaminase, PLT: Platelets, P: Probability, r: Correlation coefficient

Table 4: Diagnostic performances of serum biomarkers expression levels in the diagnosis of early-stage HCC patients

Parameters	AFP	PIVKA-II	miR-1246	Combined miR-1246 and PIVKA-II
Sensitivity (Sn.) (%)	69.86%	71.92%	96.25%	98.65%
Specificity (Sp.) (%)	52.63	69.7%	82.14%	95.83%
PLR	1.47	2.54	5.38	23.68
NLR	0.57	0.34	0.05	0.01
Diagnostic accuracy (%)	63.96%	74.49%	92.45%	98.00%
P-value	0.266	0.051	0.000	0.000
Cut-off value	20.0 ng mL^{-1}	65.0 mAU mL^{-1}	9.5 (2-ΔΔCt)	
AUC	0.600	0.698	0.866	0.936
95% CI	0.431-0.769	0.593-0.913	0.779-0.973	0.859-1.000
Standard error	0.086	0.081	0.050	0.039

AFP: Alpha-fetoprotein, PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, AUC: Area under the curve, PLR: Positive likelihood ratio, NLR: Negative likelihood ratio, 95% CI: 95% confidence interval, P: Probability, HCC: Hepatocellular carcinoma

Table 5: Diagnostic performances of serum biomarkers expression levels in the diagnosis of all HCC patients

Parameters	AFP	PIVKA-II	miR-1246	Combined miR-1246 and PIVKA-II
Sensitivity (Sn.) (%)	79.94%	86.57%	98.44%	98.88%
Specificity (Sp.) (%)	72.0%	73.33%	87.50%	100.0%
PLR	2.67	3.25	25.88	-
NLR	0.31	0.18	0.01	0.01
Diagnostic accuracy (%)	77.75%	84.15%	97.22%	99.06%
p-value	0.006	0.002	<0.0001	<0.0001
Cut-off value		65.00 mAU mL^{-1}	9.5 (2-ΔΔCt)	
AUC	0.715	0.795	0.901	0.975
95% CI	0.602-0.958	0.655-0.968	0.831-0.980	0.918-1.000
Standard error	0.091	0.080	0.038	0.02

AFP: Alpha-fetoprotein, PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, AUC: Area under the curve, PLR: Positive likelihood ratio, NLR: Negative likelihood ratio, 95% CI: 95% confidence interval, P: Probability, HCC: Hepatocellular carcinoma

was significantly expanded when combined with PIVKA-II (Acc. 98%, Sn. 98.65%, Sp.95.83%).

The sensitivity (Sn.), specificity (Sp.) and diagnostic accuracies (Acc.) of serum expression levels of serum miR-1246 and PIVKA-II in total HCC patients were displayed in Table 5. In all HCC patients, the diagnostic performances of miR-1246 at a cut-off 9.5 fold change was significantly higher than that of PIVKA-II at a cut-off 65 mAU mL^{-1} (Acc. 97.22, 84.15%, respectively). Interestingly, the diagnostic accuracy of miR-1246 was significantly increased when joined with PIVKA-II (Acc. 99.06, Sn. 98.8%, Sp.100%).

Figure 1 showed the differential expression level of serum miR-1246 in various studied groups. The serum miR-1246 expression level is higher in advanced HCCs than early-stage HCCs yet not significant. No noteworthy contrasts were noticed between CHC and LC. Nevertheless, the differences between HCC (early or advanced) and non-HCC (CHC or LC) were statistically significant. This could discriminate patients with early-stage HCC from non-HCC patients (CHC or LC).

The receiver operating characteristic (ROC) analysis of serum biomarkers for the diagnosis of early-stage HCCs were shown in Fig. 2. The AUC were significantly high for serum miR-1246 was 0.866 (0.779-0.973). The AUC for the combined (miR-1246 and PIVKA-II) was 0.936 (0.859-1.000) indicating incredible diagnostic performance in diagnosing early-stage HCCs. However, the AUC for AFP and PIVKA-II were below 0.7 indicating poor diagnostic performances for detection of early-stage HCCs.

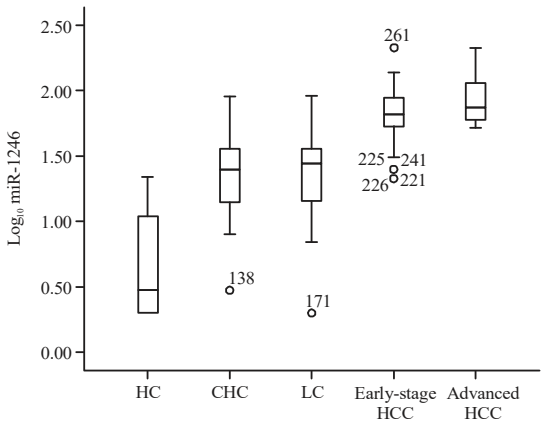

Fig. 1: Differential expression level of serum miR-1246 biomarkers in the different studied groups

HC: Healthy controls, CHC: Chronic hepatitis C, LC: Liver cirrhosis, HCC: Hepatocellular carcinoma, Log_{10} was used for the serum expression levels

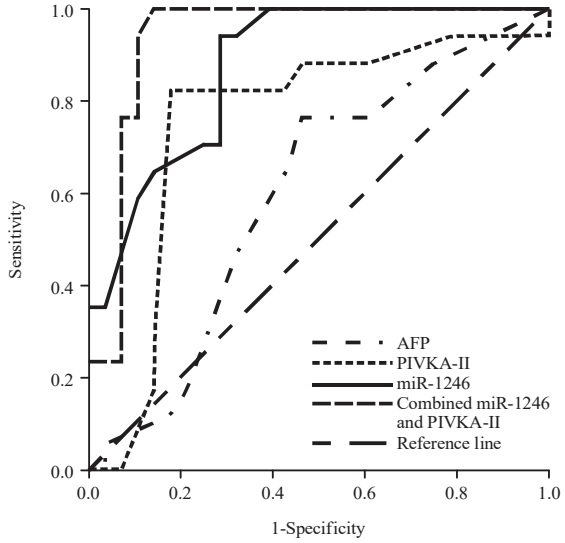

Fig. 2: Receiver operating characteristic (ROC) curve of the individual and combined serum biomarkers for the diagnosis of early-stage HCCs

AFP: Alpha-fetoprotein, PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, HCC: Hepatocellular carcinoma

In Fig. 3, the AUC (95% CI) of miR-1246 was 0.901 (0.831-0.980) indicating magnificent diagnostic performance in diagnosing total HCCs. While the AUC for AFP and PIVKA-II were 0.715 and 0.795, respectively. In Fig. 4 the AUC (95% CI) of the combined miR-1246 and PIVKA-II miR-1246 was 0.975 (0.918-1.000) indicating excellent diagnostic performance in diagnosing total HCCs. In rundown, the increased serum expression level of miR-1246 had an AUC scope of 0.8-0.9 in either

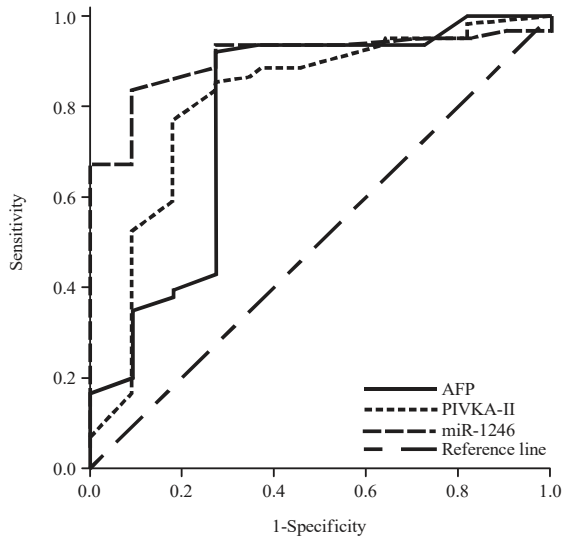

Fig. 3: Receiver operating characteristic curve (ROC) of the individual serum biomarkers for the diagnosis of all HCCs patients

AFP: Alpha-fetoprotein, PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, HCC: Hepatocellular carcinoma

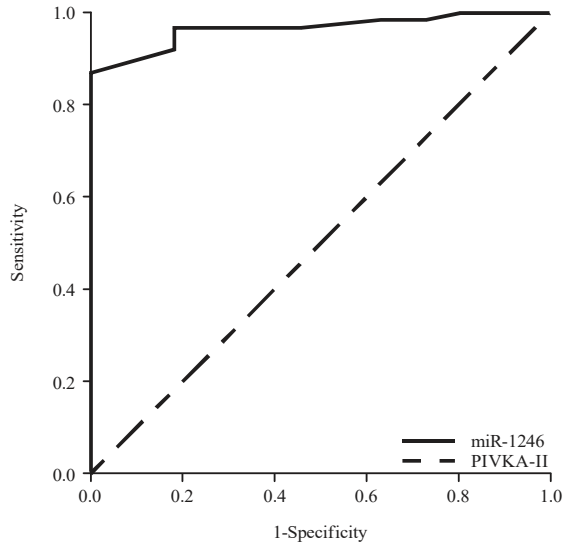

Fig. 4: Receiver operating characteristic curve (ROC) of the combined serum (miR-1246 and PIVKA-II) for the diagnosis of all HCCs patients

PIVKA-II: Prothrombin induced by vitamin K absence-II, miR-1246: microRNA-1246, HCC: Hepatocellular carcinoma

early-stage or total HCCs and when joined with PIVKA-II, the AUC expanded significantly above 0.9 indicating high diagnostic accuracy for detection of early-stage or advanced HCCs.

DISCUSSION

In this study, the dependability of the recently described miRNA-1246 either alone or combined with PIVKA-II in the diagnosis of HCV-related HCCs was assessed. Additionally, its appropriateness in separating early-stage HCCs from chronic hepatitis C (CHC), liver cirrhosis (LC) or healthy controls (HC) was inspected. The serum expression level of miRNA-1246 was assessed alone or in combination with PIVKA-II. This study demonstrated that patients with HCCs had statistically significantly higher serum expression levels of miRNA-1246 and PIVKA-II compared to non-HCC patients (CHC, LC) and healthy controls. However, just serum miR-1246 expression level had significant increment in early-stage HCC. This fascinating finding separates HCC patients from healthy controls as well as discriminates early-stage HCCs patients from patients with chronic hepatitis or liver cirrhosis.

Besides, the differential expression of this miRNA-1246 in HCC patients fundamentally and significantly correlated with clinicopathological characteristics and tumor phase of HCC. Despite the increased serum miRNA-1246 in both CHC and LC patients, no significant contrasts in the expression level of serum miR-1246 were seen among CHC and LC patients.

A wide variety of studies evaluated the capability of various miRNAs to differentiate HCC patients from healthy controls. Zekri *et al.*[27] recognized three serum microRNA panels that have considerable and impressive diagnostic accuracies in the early identification of HCV-related HCC in both normal controls and patients at-risk. The indicative exactness of these panels improved significantly when joined with AFP. Khairy *et al.*[16] identified five serum miRNAs, (miR-126, miR-129, miR-155 and in particular miR-203) which could recognize HCV-associated HCC from HCV-associated chronic liver disease and healthy controls proposing their potential convenience as HCC biomarkers. Another study by Motawi *et al.*[17] showed a panel of four microRNAs (miR-19a, miR-195, miR-192 and miR-146a) with a high indicative precision for HCC. This miRNA panel could distinguish HCC patients from chronic hepatitis or advanced fibrosis (F3-F4) patients. Qi *et al.*[28] demonstrated significant increase in the serum miR-122 in HBV-related HCC patients contrasted to healthy controls. Additionally, this serum up regulated miR-122 was significantly reduced postoperatively serving as a potential non-invasive biomarker for HCC diagnosis in healthy subjects. Notwithstanding, it couldn't segregate HCC patients from chronic HBV infection.

Unfortunately, nothing from what was just mentioned referenced investigations assessed the serum expression level of miR-1246 neither its combination with PIVKA-II in the detection of early-stage HCC patients. Besides, the outcomes in many studies were heterogeneous and conflicting meddling with their appropriateness and applicability in clinical settings.

In an exceptionally recent investigation, Ahmed *et al.*[29] revealed differential expression in the level of serum miR-210 and miR-1246 of HCC patients. Both miRNAs were significantly and fundamentally higher in metastatic liver tumors contrasted with patients with primary HCCs. The sensitivity and specificity of miR-1246, for differentiating HCC from metastatic malignancies in the liver were found to be 72.2% and 67.8%, respectively. Nevertheless, the authors didn't evaluate the above mentioned miRNAs neither in chronic liver disease nor in early-stage HCC. They just mentioned a differential expression of miR-1246 in primary HCC with or without liver cirrhosis. In the current study, other than affirming that serum miR-1246 was fundamentally increased in patients with HCC, it was exhibited that serum miR-1246 has a significantly high indicative value in early-stage HCCs, particularly when joined with PIVKA-II.

Chuma *et al.*[30] demonstrated that miRNA 1246 was significantly upregulated in HCC patients with early tumor recurrence (ETR) after curative resection, contrasted with those without ETR (AUC = 0.762) and accordingly could discriminate between HCC cases with and without ETR. Additionally, the increased expression of miR 1246 was associated with violent tumor attributes and was distinguished as an autonomous hazard factor for overall survival. NG *et al.*[31] showed that early-phase up-regulation of circling plasma miR-1246 in HCC recipients after liver transplantation (LT) was not just a potential indicator of intense hepatic damage yet in addition a prognostic biomarker for HCC recurrence and poor survival. As already mentioned these two studies exhibited the diagnostic and prognostic value of miR-1246 in just HCC patients with or without tumor recurrence after either curative resection or LT. In contrast, this study postulated that serum miR-1246 was significantly upregulated in early-stage HCC and could discriminate early-stage HCCs from liver cirrhosis and chronic hepatitis particularly when combined by PIVKA-II. Likewise, miR-1246 was significantly increased in CHC and LC contrasted with HC.

Recently, Chai *et al.*[32] revealed that endogenous and circling plasma exosomal miR-1246 were particularly raised in HCC, corresponded with a more terrible prognosis and could advance tumor progression through the Oct4/miR-1246 axis in HCC cancer stem cells (CSCs). Additionally, they showed higher miR-1246 expressions in HCC tissue samples than

nearby non-tumor tissues utilizing miRNA in situ hybridization. Wang *et al*.[33] showed that serum exosomal expression level of miR-122, miR-148a and miR-1246 was significantly higher in HCC group (particularly HBV-related HCC) than LC and normal control (NC) groups. As opposed to the present study, at the final validation step of Wang's study, nothing from what was just mentioned three exosomal microRNAs was contrastingly expressed among HCC and CH groups. Furthermore, the ROC analysis for miR-1246 was AUC (0.785) which is lower than AUC obtained in the current study. Additionally, when joined with AFP, just miR-122, miR-148a showed higher diagnostic values.

In a recent study by Moshiri *et al*.[34], the circling plasma levels of miR-106b-3p, miR-101-3p and miR-1246 were exhibited to be essentially and significantly over-expressed in HCC patients contrasted with liver cirrhotic patients and healthy controls. These miRNAs individually or in blends displayed incredible diagnostic and analytic exactnesses as indicated by their ROCs and AUCs. Nevertheless, the authors found that serum levels of miR-106b-3p and to a lesser extent serum miR-1246 held their up-regulation in HCC patients while serum miR-101-3p level was down-regulated.

Albeit increased circulating miR-1246 expression level was exhibited from plasma or serum samples to be associated with an increasing number of malignancies particularly HCCs, the information in literature portraying its diagnostic ramifications in early-stage HCCs are scarce and inconsistent. In addition, no thorough supposition or pertinent conclusion has been hypothesized until now. The reasons of this heterogeneity might be the source of samples (tissue, exosomal, serum or plasma), the various sorts of control (healthy controls, chronic hepatitis or liver cirrhosis), distinctive etiology (HCV- or HBV-related HCC) and the disease stage. Other sources of heterogeneity may be the sample size, tissue collection steps, pre-analytic and analytic procedures.

This study selected different control groups including patients with CHC, liver cirrhosis as well as healthy individuals with a larger sample size compared with previous publications. Additionally, the sampling source was the serum instead of plasma or exosomes to abolish the impact of platelet-derived miRNAs which could influence the expression level of plasma miR-1246. The pre-analytic serum samples' collection and diagnostic techniques were finished by the standard producer protocols[26].

The over-expression of circulating miR-1246 levels in patients with HCC on top of cirrhotic liver may be clarified by several factors, apoptosis, cell lysis and dynamic secretion from malignant cells. Other than the tumor cells, the tumor encompassing tissue, the penetrating cells and the field-effect may take part in increasing the circulating levels of miRNAs. This hypothesis was bolstered by the finding on miR-494 in tumor-extended myeloid suppressor cells[35]. Another supporting published study showed a hormone-like activity for circulating miR-21 and miR-29 which initiate/promote the pro-inflammatory reaction interceded by toll-like receptor[36].

CONCLUSION

The differential serum expression level of miR-1246 was demonstrated in the present study to discriminate HCV-related HCC patients not only from chronic hepatitis C and liver cirrhosis but also from healthy controls. Strikingly, high serum miR-1246 was demonstrated to be an astounding pointer of early-stage HCCs. The indicative exactness of miR-1246 in early-stage HCCs was tremendously increased whenever joined with PIVKA-II. In addition, the up-regulation of the serum expression level of miR-1246 was positively correlated with the clinicopathological features of HCC patients. Along these lines, serum microRNA-1246 could be considered as a dependable biomarker for detection of HCV-related early-stage HCC.

SIGNIFICANCE STATEMENT

This study elucidated the significant increment in the serum expression level of miR-1246 in patients with HCV-related early-stage HCC. This up-regulated serum expression level of miR-1246 especially when combined with serum PIVKA-II could distinguish early-stage HCC from liver cirrhosis.

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