Evaluation of serum Midkine as a novel biomarker for the diagnosis of hepatocellular carcinoma

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Introduction
Hepatocellular carcinoma (HCC) is the most common type of primary liver malignancy, accounting for about 90% of all primary hepatic malignancy. Most of them occur due to chronic liver disease, usually due to hepatitis B virus or hepatitis C virus.

Aim
The aim of the study was to assess the validity of serum midkine as a noninvasive diagnostic marker for HCC especially early stage of cirrhosis and compare the diagnostic performance of serum midkine, α-fetoprotein, and their combination in HCC.

Patients and methods
This study was conducted on 40 HCC patients, 24 liver cirrhosis patients and 16 age-matched and sex-matched healthy controls. Serum Midkine level was measured by the ELISA technique using Human Midkine ELISA Kit, catalog no: E-EL-H2297 96T, purchased from Elabscience (China).

Results
Midkine level was significantly higher in newly diagnosed HCC patients than in the nonmalignant control group (liver cirrhosis and healthy controls). Diagnostic performance of serum Midkine showed the best specificity (93.75%) in diagnosing HCC among healthy controls and the combination of Midkine and α-fetoprotein improved the diagnostic accuracy (92.86%) and enhanced the sensitivity (95.0%).

Conclusion
In conclusion, this study has shown that serum Midkine level in HCC patients is a good marker for the detection of HCC and in distinguishing HCC from cirrhotic patients. We also found that there was positive association between the level of Midkine and the advancement of the HCC, so this marker can be used as a prognostic marker in patients with HCC.

Keywords:
Alpha fetoprotein, biomarker, Hepatocellular carcinoma, midkine

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver malignancy, accounting for about 90% of all primary hepatic malignancy [1]. Most of them occur due to chronic liver disease, usually due to hepatitis B virus or hepatitis C virus [2].

HCC is a cancer that is difficult to be treated. When the cancer is detected at early stage, patients have a significantly high survival rate. Therefore, biomarkers for early diagnosis of HCC are important to decrease the mortality of HCC [2,3].

α-Fetoprotein (AFP) is the most commonly used biomarker in HCC and for the differentiation of HCC from cirrhosis without HCC. However, AFP has a decreasing performance as a serological test for surveillance for two reasons; first, fluctuating levels of AFP in patients with cirrhosis might indicate hepatitis B virus or hepatitis C virus infection aggravation and flaring of the underlying liver disease or HCC development. Also, only a small number of HCC at an early stage present with abnormal levels of AFP [4].

MDK is a basic heparin-binding growth factor of low molecular weight. It is encoded by the MDK gene on chromosome 11 in humans, also known as neurite growth-promoting factor 2 [5].

MDK activates several cell surface receptors to aid in modulating various biological activities. MDK has an important role in activities related to carcinogenesis such as proliferation, antiapoptosis, migration, angiogenesis, and transformation, in many types of tumors, including HCC [6].

MDK is increased in HCC and plays significant roles in cancer formation due to its carcinogenic properties [7].

In detecting early-stage HCCs [Barcelona Clinic Liver Cancer (BCLC) 0/A], the sensitivity of MDK was much higher than that of AFP (87.1 vs. 46.7%); also in very early-stage HCCs (BCLC 0), MDK showed an obviously higher sensitivity of 80% compared with 40% of AFP [8]. Serum MDK may act as a novel diagnostic tumor marker for the detection of early-stage HCC (BCLC 0/A) [6,9,10].

Patients and methods

This study was conducted on 40 HCC patients, 24 liver cirrhosis (LC) patients and 16 age-matched and sex-matched healthy controls. The patients were selected from the Tropical Medicine and Gastroenterology Department, Al-Rajhi Liver Hospital, Assiut University over a period of 1 year from March 2017 to March 2018. Formal consent was obtained from patients and controls. The study was approved by Ethical Committee of Faculty of Medicine Assiut University.

Classification of participants

(1) HCC group: 40 patients and they were classified according to the BCLC staging into:
   (a) Stage 0 (very early stage) HCC (10 patients)
   (b) Stage A (early stage) HCC (10 patients)
   (c) Stage B (intermediate stage) HCC (10 patients)
   (d) Stage C and D (late stage) HCC: (10 patients).

According to the Child–Pugh score, the HCC patients were divided into:
   (i) Class A (27 patients)
   (ii) Class B (eight patients)
   (iii) Class C (five patients).

(2) LC group (24 patients).

They were classified according to Child–Pugh score into:
   (a) Class A (eight patients)
   (b) Class B (eight patients)
   (c) Class C (eight patients).

(3) Control group: 16 apparently healthy persons, sex- and age-matched with both patients' groups.

Sample collection, storage, and handling

Random blood sample: 8 ml of venous blood was withdrawn under complete aseptic conditions and was divided into the following.

Two milliliter was collected into an EDTA containing tube for blood count. Two milliliter was collected into a sodium citrate containing tube for prothrombin time and concentration. Four ml was collected into a plain tube without an anticoagulant and was centrifuged at a speed of 2000–3000 rpm for 20 min and stored at −80°C for kidney functions, liver functions, AFP, and assay of Human Midkine (MDK) level.

Routine investigations

Serum urea, serum creatinine, and liver functions were done on COBAS Integra 400 plus, Roche (Germany). Prothrombin time and concentration: was done on Sysmex CA-1500 System (Siemens, Germany). Complete blood count: was done on ABX Pentra XL 80, HORIBA Medical (France).

Special investigations

Serum AFP: was done on a MAGLUMI fully an automatic chemiluminescence immunoassay (CLIA) analyzer (MAGLUMI 2000, China).

Serum Midkine level determination: was measured by the ELISA technique using human MDK (Midkine) ELISA Kit catalog no.: E-EL-H2297 96T, purchased from Elabscience (China).

Principle of the test

ELISA was based on sandwich immunoassay principle. The assay uses two highly specific monoclonal antibodies for the detection of the tested antigen; one antibody is immobilized into the microplate and the other one is labeled to form a sandwich complex (Ab-Ag-labeled Ab). Absorbance is measured spectrophotometrically at 450 ± 2 nm.

Results

Serum levels of MDK and AFP in patients and control groups

Midkine

The HCC group had significantly higher MDK than that with the control and LC (P = 0.000* and P = 0.003*, respectively), but there was no significant difference between the LC group and the control group ($P = 0.082^{ns}$) (Table 1).

AFP

The HCC group had significantly higher AFP than that with the control and LC (P = 0.000* and P = 0.000*, respectively), but there was no significant difference between the LC group and the control group ($P = 0.341^{ns}$).

Serum levels of MDK and AFP in HCC patients according to BCLC staging

MDK

Our results show that the serum level of MDK had a statistically significant increase with staging (P = 0.010*) (Table 2).

Serum levels of MDK and AFP in HCC patients according to the Child–Pugh score

MDK

The MDK level increased with increased Child score from A to C with significant difference between them (P = 0.020*) (Table 3).

Study of diagnostic performance of MDK and AFP in HCC

For the diagnosis of HCC and early-stage HCC

(1) HCC versus nonmalignant group (LC and control groups) (Table 4, Figs. 1 and 2):

At a cutoff of greater than 0.34 ng/ml MDK has better sensitivity than AFP. When using the currently recommended clinical cutoff for AFP (20 ng/ml), the sensitivity was 50.00% and the specificity was 97.0% with area under the curve (AUC) being 0.738.

In early-stage HCC patients (BCLC stage 0 and A) versus all controls (LC and healthy) a cutoff for MDK greater than 0.32 ng/ml MDK has better sensitivity than AFP. The combination of tumor markers shows better sensitivity than each marker alone.

(2) Diagnostic performance of MDK, AFP, and their combination for distinguishing HCC from high-risk patients (the LC group) (Table 5, Figs. 3 and 4).

MDK at a cutoff of greater than 0.47 ng/ml and AFP (>8 IU/ml) have same sensitivity. but has better specificity.

In early-stage HCC patients (BCLC stage 0 and A) MDK has better sensitivity than AFP. The combination of tumor markers shows better sensitivity than each marker alone.

MDK at a cutoff of greater than 0.47 ng/ml has better sensitivity than AFP (>20 IU/ml), AFP has better

Table 1 Serum levels of Midkine and α-fetoprotein in patients and control groups

	HCC (n=40)	LC (n=24)	Control (n=16)	P_1	P_2	P_3
AFP (IU/ml)						
Mean±SD	437.43±869.25	7.60±10.89	4.47±2.94	0.000*	0.000*	0.341 (NS)
Median (range)	36.6 (1.9–3750)	5.1 (0.5–55)	4.1 (0.8–11.5)			
MDK (ng/ml)						
Mean±SD	2.68±2.70	1.78±3.04	0.23±0.10	0.003*	0.000*	0.082 (NS)
Median (range)	1.51 (0.09–9.23)	0.32 (0.06–8.90)	0.20 (0.09–0.43)			

AFP, α-fetoprotein; HCC, hepatocellular carcinoma; LC, liver cirrhosis; MDK, Midkine; P_1, comparison between HCC and LC; P_2, comparison between HCC and control; P_3, comparison between LC and control. NS, no statistically significant difference (P>0.05).
*Statistically significant difference (P<0.05).

Table 2 Serum levels of Midkine and α-fetoprotein in hepatocellular carcinoma patients according to Barcelona Clinic Liver Cancer staging

	BCLC				P value
MDK (ng/ml)	0 (n=10)	A (n=10)	B (n=10)	C and D (n=10)	
Mean±SD	1.72±1.95	1.75±2.41	2.04±1.94	5.22±2.91	0.010*
Median (range)	0.84 (0.2–6.0)	0.6 (0.4–7.7)	1.3 (0.1–5.7)	4.6 (0.6–9.2)	
AFP (IU/ml)	0 (n=10)	A (n=10)	B (n=10)	C (n=10)	
Mean±SD	269.89±420.51	174.39±320.14	511.51±965.39	793.93±1344.45	0.517 (NS)
Median (range)	29.9 (2.2–1000.0)	20.7 (2.6–1000.0)	20.3 (1.9–3000.0)	123.0 (2.4–3750.0)	

AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; MDK, Midkine.

Table 3 Serum levels of Midkine and α-fetoprotein in hepatocellular carcinoma patients according to the Child–Pugh score

	Child–Pugh score			P value
	A (n=27)	B (n=8)	C (n=5)	
MDK				
Mean±SD	1.92±2.24	3.63±2.66	5.29±3.40	0.020*
Median (range)	0.65 (0.09–7.71)	3.27 (0.47–9.23)	4.3 (0.6–8.8)	
AFP				
Mean±SD	221.77±362.95	1005.33±1512.82	693.38±1210.28	0.267 ns
Median (range)	25.3 (1.9–1000.0)	123.0 (8.7–3750.0)	35.0 (2.4–2813.0)	

AFP, α-fetoprotein; MDK, Midkine.

Table 4 Hepatocellular carcinoma versus nonmalignant group (liver cirrhosis & control groups)

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC
HCC versus (LC+control groups)							
MDK (ng/ml)	>0.34	90.00	70.00	75.00	87.5	80.00	0.812
AFP (IU/ml)	>8	77.50	85.00	83.8	79.1	81.25	0.837
MDK and AFP	AFP>8 or MDK>0.34	95.00	65.00	73.1	92.9	80.0	0.800
Early stage versus (LC+control groups)							
MDK (ng/ml)	>0.32	95.00	65.00	57.6	96.3	75.0	0.803
AFP (IU/ml)	>8	75.00	85.00	71.4	87.2	81.7	0.829
MDK and AFP	AFP>8 or MDK>0.32	100.00	60.00	55.6	100.00	73.3	0.800

AFP, α-fetoprotein; AUC, area under the curve; HCC, hepatocellular carcinoma; LC, liver cirrhosis; MDK, Midkine; NPV, negative predictive value; PPV, positive predictive value.

Table 5 Diagnostic performance of Midkine, α-fetoprotein and their combination for distinguishing hepatocellular carcinoma from high-risk patients (liver cirrhosis group)

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC
HCC versus LC							
MDK (ng/ml)	>0.47	77.5	66.67	79.5	64	81.25	0.723
AFP (IU/ml)	>8	77.5	83.33	88.6	69.0	79.69	0.821
AFP (IU/ml)	>20	60.00	95.83	96.0	59.0	73.44	0.779
MDK and AFP	AFP>20 or MDK>0.47	85.00	66.67	81.0	72.7	80.0	0.758
MDK and AFP	AFP>8 or MDK>0.47	92.50	62.5	80.4	83.3	75.00	0.775
Early stage versus LC							
MDK (ng/ml)	>0.47	70.00	66.67	63.6	72.7	68.18	0.697
AFP (IU/ml)	>8	75.00	83.33	78.9	80.0	79.55	0.808
AFP (IU/ml)	>20	50.00	97.50	90.9	79.6	81.67	0.738
MDK and AFP	AFP>20 or MDK>0.47	80.00	80.00	66.7	88.9	80.0	0.800
MDK and AFP	AFP>8 or MDK>0.47	90.00	62.5	66.7	88.2	75.00	0.762

AFP, α-fetoprotein; AUC, area under the curve; HCC, hepatocellular carcinoma; LC, liver cirrhosis; MDK, Midkine; NPV, negative predictive value; PPV, positive predictive value.

specificity. In early-stage HCC patients (BCLC stage 0 and A) MDK has better sensitivity than AFP. The combination of tumor markers shows better sensitivity than each marker alone.

Discussion

In our study, there was a statistically significant difference between the mean value of MDK in patients with HCC compared with patients with LC (P = 0.003). Also, MDK levels were significantly higher and could distinguish HCC patients from controls (P = 0.000*). These results were in agreement with those of Zhu *et al.* [8] who found that the median values of the MDK levels in HCC patients were significantly higher than that of cirrhotic patients and healthy controls. Also Shaheen *et al.* [6] reported that MDK levels in the HCC group were much higher when compared with the LC group and with the healthy control group.

Makleda and colleagues [10–12] reported that MDK was significantly higher in HCC than in cirrhosis, chronic liver disease (CLD), and HC (healthy control). Also Ammo *et al.* [13] reported that there was significant increase in MDK in the HCC group when compared with the healthy control group.

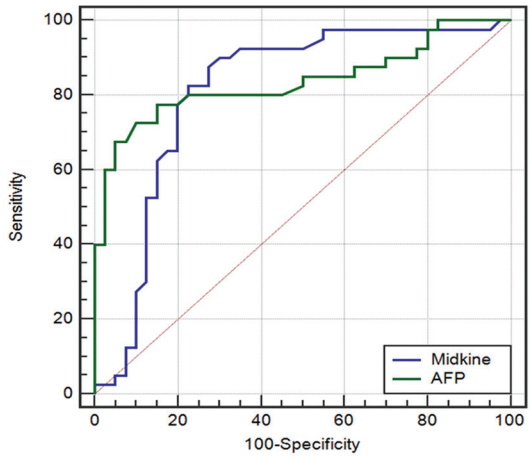
MDK is a mitogenic factor during carcinogenesis, it was demonstrated that MDK acts as an antiapoptotic factor in HepG2 cells; also, MDK inhibits the activity of caspase-3, which plays an important role in the apoptotic pathway. A higher serum MDK level could be used for detecting early HCC and metastasis and poor prognosis [6,8].

Higher blood MDK level might be essential for resistance of HCC circulating tumor cells to anoikis, and is responsible for promoting metastasis [14].

Anoikis is an important mechanism for functions such as creating a physiological barrier to cancer metastasis and preventing ectopic cell growth or attachment to an inappropriate extracellular matrix. Resistance to anoikis increases tumor cell survival during the processes of systemic circulation and distant colonization [15].

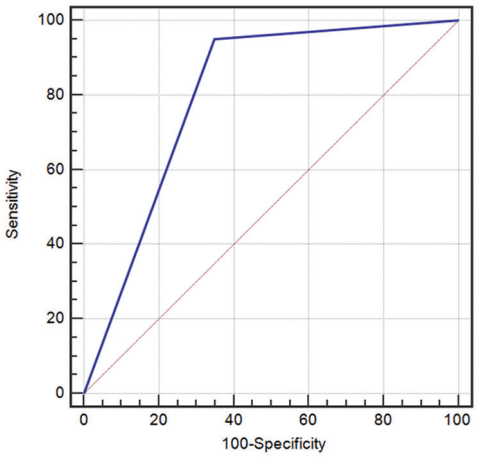
The present study has shown that there was significantly stepwise increase of serum MDK levels in patients with BCLC from stage 0, A, B, C up to stage D with statistically significant difference (P = 0.010*). Our results are in agreement with Vongsuvanh *et al.* [9] who reported that the serum level of MDK increased from BCLC A to BCLC B, with statistically significant difference. In contrast, Shaheen *et al.* [6] and Zhu

Figure 1



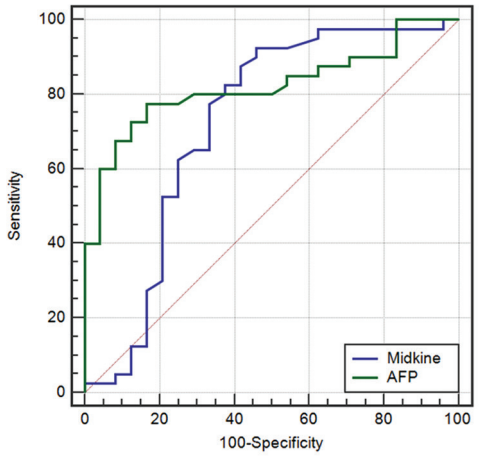
Receiver operating characteristic curves for Midkine, α-fetoprotein in the diagnosis of hepatocellular carcinoma versus (liver cirrhosis and control groups).

Figure 2



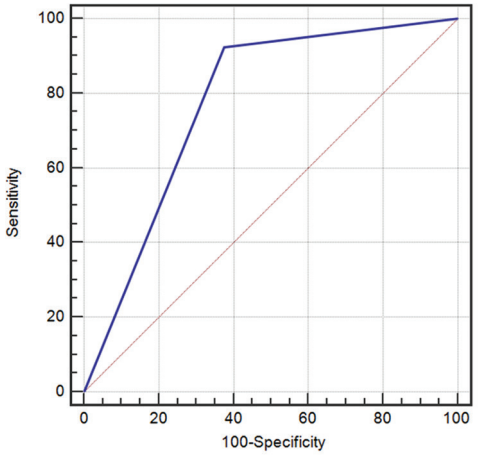
Receiver operating characteristic curves for the combination of Midkine and α-fetoprotein in the diagnosis of hepatocellular carcinoma versus (liver cirrhosis and control groups).

Figure 3



Receiver operating characteristic curves for Midkine, α-fetoprotein in the diagnosis of hepatocellular carcinoma versus liver cirrhosis.

Figure 4



Receiver operating characteristic curves for the combination of Midkine and α-fetoprotein in the diagnosis of hepatocellular carcinoma versus liver cirrhosis.

et al. [8] reported that no significant association was found between serum MDK and BCLC stage. Also Makleda *et al*. [10] reported that the serum level of MDK increased from BCLC A to BCLC B, but without statistically significant difference.

In our study, according to the Child–Pugh score, serum MDK level showed a significant increase in HCC patients with Child class B, C than HCC patients with Child class A. These results are in agreement with Vongsuvanh *et al*. [9]. We evaluated the diagnostic performance of MDK as a serological biomarker for HCC in distinguishing HCC patients from the nonmalignant group (LC patients and healthy controls) at an optimum cutoff value of greater than 0.34 ng/ml, which showed that MDK if better than AFP in sensitivity and negative predictive value (NPV).

These results agreed with those of Shaheen and colleagues [6,10,11] who reported that MDK had better sensitivity than AFP in the diagnosis of HCC. On the other hand, Vongsuvanh *et al*. [9] reported that AFP continued to have superior diagnostic performance than MDK.

When using the currently recommended clinical cutoff for AFP (20 IU/ml). our results showed that the sensitivity of AFP for diagnosing HCC decreased (50%) and specificity increased (97%). In agreement with our results, Takenaka *et al*. [16] reported that the sensitivity was 58.40% and the specificity was 95.70% in using the currently recommended clinical cutoff for AFP (20 ng/ml).

Shaheen *et al*. [6] reported that the AFP levels at a value of 20 ng/ml showed low specificity (53.3%) and increased sensitivity (62.5%).

In the current study, the results of the combination of the two markers improved the sensitivity (95.00%) and NPV (92.9%) while the specificity and positive predictive value (PPV) were decreased (65.00, 73.1%, respectively) than using either test alone.

Also, Shaheen and colleagues [6,8] reported that the combination of MDK and APF improved the detection rate of for early diagnosis of HCC than MDK alone. Also Hodeib *et al*. [11] reported that combined analysis of both MDK and AFP in HCC yielded a diagnostic value of (98%). Also Ammo *et al*. [13] reported that the sensitivity of HCC detection increased after a combination of AFP and MDK. In contrast, Vongsuvanh *et al*. [9] reported that combining biomarkers did not significantly improve the diagnosis of HCC compared with either test alone.

Since early detection is one of the key approaches to improve the survival of cancer patients, we further evaluated the diagnostic performance of these two markers in early-stage HCC patients (BCLC stage 0 and A) versus the nonmalignant group (LC group and control group). At an optimum cutoff for MDK greater than 0.32 ng/ml, it showed a sensitivity of 95.00%, specificity of 65.00%, PPV of 57.6%, NPV of 96.3%, and an AUC of 0.803, while at an optimum cutoff for AFP greater than 8 IU/ml, it showed.

Sensitivity (75.00%), specificity (85.00%), PPV (71.4%), NPV (87.2%), AUC (0.829), and MDK showed higher sensitivity and NPV than AFP. These results agreed with those of Zhu *et al*. [8,10,11] who reported that serum MDK showed an obviously higher sensitivity compared with AFP.

The present study showed that the results of the combination of the two markers improved the sensitivity (100.00%) and NPV (100%) than using each marker alone while the specificity (60.00%) and PPV (55.6%) decreased. Our results agreed with Zhu *et al*. [8] who reported that the combination of MDK and AFP further significantly improved the detection rate of very early HCC to 96.6%.

In the light of these results, our study showed that the combination of the two markers were important as they yielded a sensitivity of 100%.

We evaluated the diagnostic performance of these two markers in HCC patients versus the LC group.

Our results showed that at the optimum cutoff for MDK (>0.47 ng/ml), it showed a sensitivity of 77.5%), specificity of 66.67%), AUC of 0.723, PPV of 79.5%), NPV of 64.00%, and a diagnostic accuracy of 81.25%. At the optimum cutoff for AFP greater than 8 IU/ml, it showed an AUC of 0.821, sensitivity of 77.5%, specificity of 83.33%, PPV of 88.6%, NPV of 69.00%, and a diagnostic accuracy of 79.69%. AFP at a cutoff of 20 IU/ml which was the clinically recommended cutoff use, our results showed an AUC of 0.779, AFP showed a decreased sensitivity of 60.00%, NPV of 59.00%, and a diagnostic accuracy of 73.44% than MDK, while specificity (95.83%) and PPV (96.00%) were increased.

MDK had higher diagnostic accuracy (81.25%), same sensitivity, less specificity, PPV, NPV, and AUC than AFP.

In contrast, Zhu *et al*. [8] reported that when MDK was at a cutoff value of 0.387 ng/ml, AFP at a cutoff of 20 ng/ml, sensitivity of MDK was 86.9% which was much higher than that of AFP (51.9%). Also Shaheen *et al*. [6] reported that the sensitivity of MDK at a cutoff value 0.387 ng/ml was found to be much significantly higher when compared with that of AFP at a cutoff value of 20 ng/ml (92.5 vs. 62.5%). Also Makleda *et al*. [10] reported that MDK with a sensitivity of 100% was higher than that of serum AFP with a sensitivity of 72.5%. In contrast Vongsuvanh *et al*. [9] reported that when MDK was at a cutoff value of 0.44 ng/ml, AFP at a cutoff value of 20 IU/ml, AFP had a greater sensitivity (70.9%) than MDK (62.2%), suggesting that AFP is superior to MDK for HCC diagnosis.

Our results showed that a combination of MDK and AFP at an AFP cutoff value of greater than 8 IU/ml, MDK (>0.47 ng/ml) improved sensitivity (92.5%), NPV (83.3%) while the specificity and PPV have been decreased (62.5 and 80.4%, respectively).

These results agreed with those of Shaheen and colleagues [6,8,11,12] who showed that a combination of MDK and AFP improved sensitivity.

On the other hand, Vongsuvanh *et al*. [9] reported that combining biomarkers did not significantly improve the diagnosis of HCC compared with either test alone.

Also, we assessed the diagnostic performance of the two markers for distinguishing early-stage HCC versus LC patients. At an optimum cutoff value of MDK greater than 0.47 ng/ml, MDK showed a sensitivity of 70%) and specificity of 66.67%), respectively, with an AUC of 0.697, PPV of 63.6%, NPV of 72.7%, and diagnostic accuracy of 68.18%, while AFP at an optimum cutoff value is greater than 8 IU/ml which showed a sensitivity of 75% and specificity of 83.33%, AUC of 0.808, PPV of 78.9%, NPV of 80.00%, and a diagnostic accuracy

of 79.55%. AFP had higher sensitivity and specificity, PPV, NPV, AUC than MDK. However, AFP at a cutoff of 20 IU/ml, our results showed that AFP had higher specificity (97.5%) versus MDK (66.67%).

Similar results were obtained by Zhu *et al*. [8] who reported that the sensitivity of MDK at a cutoff value of 0.654 ng/ml was much higher than that of AFP at a cutoff value of 20 ng/ml. Also Shaheen *et al*. [6] reported that when MDK at a cutoff value of 0.387 ng/ml, AFP at a cutoff value of 20 IU/ml, the sensitivity of MDK versus AFP was 90 vs. 40%, respectively. Makleda *et al*. [10] also reported that serum MDK may serve as a novel diagnostic tumor marker for the detection of early-stage HCC (BCLC 0/A), as MDK had a greater sensitivity than AFP (88.9 vs. 66.7%, respectively). On the other hand, Vongsuvanh *et al*. [9] reported that when MDK at a cutoff value of 0.44 ng/ml, AFP at a cutoff value of 20 IU/ml, AFP had a greater sensitivity than MDK, suggesting that AFP is superior to MDK for HCC diagnosis.

Also, our results showed that the combination of MDK and AFP at an AFP cutoff of (>8 IU/ml) showed increased sensitivity (90%) and NPV (88.2%) than AFP or MDK each but lower specificity (62.5%.), AUC (0.762), and PPV (66.7%). These results were in agreement with Zhu *et al*. [8] who reported that the combination of MDK and AFP further significantly improved the detection rate of very early HCC from 80% to more than 96.6%, which was much higher than the AFP or MDK alone.

In conclusion, this study has shown that serum MDK level in HCC patients is a good marker for the detection of HCC and in distinguishing HCC from cirrhotic patients. We also found that there was a positive association between the level of MDK and the advancement of the HCC, so this marker can be used as a prognostic marker in patients with HCC. Additionally, results indicated that the combination of AFP and MDK may be used as a panel for early diagnosis of HCC patients as they yield 100% sensitivity and NPV.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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