MOLECULAR EVALUATION OF MIDIKINE (MDK) AS A POTENTIAL BIOMARKER TODETECT HEPATOCELLULAR CARCINOMA

Amira AbdelAziz[1]*, Moustafa A. Sakr[1], Ahmed Sharawy[2], Hisham A. Ismail[1]

[1]Molecular diagnostics and therapeutics department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt
[2]Clinical Pathology Department, National Liver Institute, Menoufia University, Egypt

**Corresponding author: Amira Abdelaziz Email: alnadiamira@yahoo.com*

ABSTRACT

The aim of this study was to assess the efficacy of non-invasive Midkineexpression level as diagnostic biomarkers of Hepatocellular Carcinoma and its relationship with diagnostic parameters. Thirty six subjects were enrolled in the present study, they were divided into three groups: Group (A) included 10 apparently healthy subjects as acontrol group; Group (B) included 14 HCC patients ; Group (C) included 12 HCV patients ; The studied groups under study were age and sex matched. Routine and specific (MDK) laboratory investigations were performed in all included subjects. The median age of the patients was 51 years (rang 44-58 years); where males represented the majority of the groups (69.4%). MDK expression level was detected by quantitative polymerase chain reaction (qRT-PCR). None of the patients were pretreated with local therapies. The MDK level was significantly higher in patient HCC than in the HCV and control groups (p=0.028), whereas MDK level was also higher in HCV patient than healthy patient. Further the correlation between the level of MDK and all laboratory parameters was tested. In this study there is a good positive correlation between MDK levels and SGPT (AST), and SGOT (ALT). The sensitivity and specificity of AFP were 75.7% and 86.4% respectively, while those of MDK were 64.3% and 77.3%, respectively, in early-stage HCC patients but when combinations between two markers lead to higher sensitivity 100% to predict HCC patient.

***Key words**: MDK, AFP, HCC, Egyptian patients*

INTRODUCTION

Hepatitis C virus (HCV) was first characterized by **(Choo et al., 1989)**. It was soon identified as the main causative agent of the disease previously known as post transfusion non-A, non-B hepatitis virus infection**.** HCV is an enveloped RNA virus and belongs to the genus Hepacivirus of the family Flaviviridae. The HCV genome consists of 9.6-kb single-stranded RNA of positive polarity. Its genome codes for a long polyprotein of approximately 3000 amino acids **(Lindenbach and Rice,2001).** RNA polymerase lacks proof reading activity and this may alter the detection, sensitivity to interferon

anti-viral activity and pathogenicity of the virus **(Yamane et al.,2013)**. Seven major HCV genotypes and several subtypes have been identified. Genotypes 1 and 2 are endemic in West Africa while genotype 3 is endemic to the Indian subcontinent. Genotypes 4 and 5 are primarily found in Africa, and genotype 4 is particularly endemic in Egypt and Central Africa. Genotype 6 is endemic of Asia **(Agha et al., 2004; Lavanchy, 2011; Simmonds, 2013)**. In Egypt the situation is very critical; Hepatitis C virus constitutes an epidemic in Egypt which is having the highest prevalence in the world**.**

Hepatocellular carcinoma (HCC) has an annual incidence of 7.9% in men and 6.5% in women (fifth and seventh worldwide respectively) **(Jemal et al.,2011)**. In regions with a high prevalence of hepatitis C virus (HCV) and hepatitis B virus (HBV) infections, the incidence and prevalence of HCC has progressively increased **(El-Serag, 2012)**. In Egypt, due to the high prevalence of HCV and HBV infections, the incidence rate of HCC has doubled in the past ten years **(El-Garemet al., 2013).** Important environmental risk factors for HCC include chronic hepatitis virus infections, alcohol abuse, and non-alcoholic steatohepatitis (NASH). These risk factors are also relevant aetiologic factors for cirrhosis **(Bosch et al.,2004)**. HCV increases HCC risk by promoting cirrhosis and causing specific genetic lesions to the infected liver cells**(Yu et al.,2006).** Daily alcohol consumption and Aflatoxin food contamination have been associated with increased risks of HCC **(Schwartz et al.,2013; Magnussen and Parsi .,2013)**.For diagnosis of HCC it was recommend using a combination of liver ultrasound and serum alpha fetoprotein (AFP). Liver ultrasound is recommended as the primary surveillance modality for HCC; it has a modest sensitivity of approximately 60% and a higher specificity of approximately 85–90% **(Singal et al., 2009)**. Computed axial tomography (CT) and magnetic resonance imaging (MRI) scans have not been adequately tested for HCC surveillance, but are used in clinical practice **(Mohamoud et al., 2013).** CT and MRI are associated with increased detection of more HCC than ultrasound but they are also associated with a higher false-positive rate **(Kobayashi et al., 1985)**. AFP measurement is also commonly used for HCC surveillance because it is relatively inexpensive, simple to perform, and is widely available.AFP alone is not recommended as a HCC surveillance test due to its low sensitivity and specificity for detecting HCC **(Paul et al., 2007)**.AFP levels may not discriminate between benign liver and HCC. There is an urgent need to identify additional tumor markers or diagnosis of HCC and must find other markers for early diagnosis of HCC that have a role in the early treatment. MDK is also up-regulated in the majority of human malignant tumours and contributes to tumour development and progression by enhancing the growth, survival, migration, epithelial-mesenchymal transition and angiogenic activity of these cells **(Kadomatsu and Muramatsu, 2004; Muramatsu, 2010, 2011;Erguven et al., 2012)**.

The aim of this work is to evaluate the value of non-invasive MDK expression to use as a potential tumor biomarker for HCC in Egyptian patient and their accuracy in early detection in expression with conventional tumor marker (AFP).

PATIENTS AND METHODS

Patient

This case-control study was performed on 36 patients with chronic liver

diseases infection being referred at the National Liver Institute Hospital, Menoufia University, Egypt. This study was approved during period from December 2014 to October 2015. Patients in this study 25 of them male and 11female are containing 10 healthy volunteer as control group.

All of the patients were have positive of serum hepatitis C virus identified by serology and confirmed by qualitative PCR to detect HCV-RNA. HCC patients group had focal lesion that were detected by ultrasonography and computed tomography (CT) scan. All patients were negative serum hepatitis B surface antigen (ELISA). Blood samples were obtained only from patients who gave informed consent. A full history was taken from all patients and control. Exclusion criteria included: patients with chronic HBV infection or any other known cause for chronic hepatitis other than HCV, previous treatment for HCC or antiviral therapy and any associated malignancies other than HCC.

The enrolled patients were subjected to full medical history taking, thorough clinical examination, and laboratory investigations including complete blood picture, complete liver and kidney profile, viral or autoimmune liver markers, serum alpha-fetoprotein,anti-HCV titer, HBsAg, and HBc-Ab using commercial assays.

qRT-PCR for MDK

All blood samples of the HCC, HCV and control groups were gained by venipuncture with EDTA as anticoagulant. The whole blood collected frozen immediately at -80ºC until analyzed. The RT-PCR (real time polymerase chain reaction) is a technique in molecular genetics that create multiple copies of a rare piece of RNA even in samples containing only minute quantities of RNA. RNA isolation from whole blood was done by Pure Link RNA mini kit (Thermo scientific, USA) according to the manufacture instructions.The cDNA Synthesis byHiSenScriptRH$^{(-)}$cDNA synthesis kit (iNtRon, Korea)is designed for the sensitive and high yields synthesis and analysis of full-length cDNA copies from either a total or poly (A) + RNA sample. It optimized for a Reverse Transcription (RT ase) reaction that can utilize any amount of total RNA from 1 µg per reaction and is application and is applicable for the synthesis of full-length first strand cDNA. Real-time PCR reaction with intercalating dye (SYBR green dye).is a 2X concentration premix type reagents, which contains all reagents for real-time PCR reaction except for primers. This makes preparation of reaction mixture easer. The reaction was done by RealMOD Green Real-time PCR Master Mix Kit (iNtRon, Korea). Intercalating dye in the master mix enables the analysis of many different targets without having to synthesize target- specific labeled probes. The results of MDK were expressed as fold change.

Statistical analysis

Statistical analyses were conducted using (SPSS version 20.0) **(**Statistical Package for the Social Sciences) software for analysis. According to the type of data qualitative represent as number and percentage. Chi square test was used to examine the relationship between

categorical variables. Differences between parametric quantitative independent multiple groups was detected by ANOVA. Kruskal Wallis test is applied for statistical comparison between more than two sets of data if one or both of them have a skewed distribution. The ROC was constructed to obtain the most sensitive and specific cutoff value for MDK. Non parametric correlation was done by Spearman's correlation. P value was set at <0.05 for significant results &<0.001 for high significant result.

RESULTS AND DISCUSSION

In this study, a total of 36 persons were recruited and enrolled including 14 newly diagnosed HCC patients (10 were males (71%) and 4 were females (29%), 12 patients with liver cirrhosis (10 males (83%) and 2 females (17%), and 10 healthy individuals with median age of 45 years old (5 males (50%) and 5 females (50%)) selected over the period from December 2014 to October 2015, to investigate the diagnostic utility of MDK in patients with newly diagnosed hepatocellular carcinoma (HCC).

The values of the hematological, and biochemical variables of the groups under study are summarized in **Table (1)**. Gender and age were not significantly different between the studied groups. Serum levels of ALT (SGPT), AST (SGOT), total bilirubin, and INRwere significantly higher in HCC than other groups; whereas albumin, hemoglobin levels, and platelets count were significantly lower in HCC patients versus the other groups. Total WBCs count and Creatinine were not significantly different between studied groups.

MDK and AFP levels and their correlation with the laboratory findings in HCC

Table 2 and Figure 1 show the level of the MDK detected from the whole blood by noninvasive technique and tested by qRT-PCR in HCC cases and other groups normalized GAPDH as internal control. We found that the expression level of MDK was significantly higher in HCC and HCV group in comparing of healthy control group (p=0.021). In the other hand, the median level of serum AFP showed a significant increase in HCC group versus HCV, and healthy control groups (P =0.02). Further we analyzed the correlation between MDK, with the laboratory findings of HCC group, We found that, there were a statistically significant positive correlation between MDK with serum ALT, SGOT, and AFP level. Moreover, no statistical significant correlation was found among the studied MDK and the other parameters

Table 1.Laboratory data of the studied subjects (n=36)

Variable	Mean ± SD			
	HCC(n=14)	HCV(n=12)	Control(n=10)	P value
ALT (up to40 U/L)	(48.57±23.7) [2]	(43±11.8)2	(20.3±7.3)1	0.001**
SGOT (up to 50 U/L)	(50.85±26.6)[2]	(37.08±8.3) 1	(19.7±5.3) 1	0.001**
Total Bil. (~1 mg/dL)	(1.00±.34)	(.6833±.071) 1	(.63±.094)1	0.00**
Albumin(3.5:5.5 gm/dL)	(3.23±.617) [2]	(3.75±.137) 1	(3.9±.161) 1	0.00**
Glucose	(104.85±37.10)[2]	(80.58±6.55)1	(81.3±14.74)1	0.028*
Creatinine	(.9121±.39)[2]	(.75±.10)1	(.6930±.14)1	0.118
Plat.(150:450x103/mm3)	(125.5±43.27)[3]	(237±31.5)1	(322.6±66.42)1	0.00**
Prothrombin time	(13.41± 1.2)	(12.28± 0.1)1	(11.81±0.64)1	0.003*
Prothrombin conc.	(77.49±12.4)	(88.83±10.3)1	(91.0±8.7)1	0.008*
INR	(1.186±0.109)2	(1.133±0.12)1	(1.06±0.07) 1	0.033*
RBCs	(5.00±2.38)2	(5.00±2.38)2	(4.6700±0.3)1	0.674
WBCs(3.9:11x103/mm3)	(3.9143±0.71)2	(4.4667±0.27)1	(4.40±0.3) 1	0.015*
Hgb (11.5:16.5gm/dL)	(11.92±1.56) 2	(13.58±0.59)1	(12.73±0.87) 1	0.003*

N.B; Groups bearing different numbers are significantly different from each other at P<0.05 * show statistical significant difference

The diagnostic accuracy of MDK to detect HCC cases

We used ROC curve analysis to compare the accuracy of noninvasive level of MDK and AFP to predict patients with HCC. It was found that MDK could predict HCC at level of 2.39 fold with sensitivity 64.3% and specificity 77.3% while with AFP could predict HCC at level of 90ng/dl with sensitivity 75.7% and specificity 86.4% **(Table 3, Fig 2,3,4**). Whereas with combination of the expression level of MDK and the AFP serum level, the sensitivity to predict the HCC cases was increased to 100%with same specificity level 77.3%.Collectively from these result it was found that the combination of MDK expression level with conventional tumor marker AFP is more sensitive but bad specific in prediction of HCC. Overall, these results suggest that MDK could serve as a useful tumor biomarker for prediction of HCC only when combined with AFPand in turn it will have better implications on the HCC treatment outcome.

Table2.Comparison of the mean values of fold change of the studied MDK and AFP median values as markers for differentiatingHCC Group from the other Groups

Variable	Mean ±SD			
	HCC (n=14)	**HCV (n=12)**	**Control (n=10)**	**P value**
MDK fold change to GAPDH	4.5414±4.41540	2.5484±1.33144	1.1021±0.36309	0.021*
AFP (up to 10ng/ml)	290.21429±202.7600	3.3833±1.35501	1.1200±0.44171	0.020*

Groups bearing different numbers are significantly different from each other at P<0.05

* show statistical significant difference

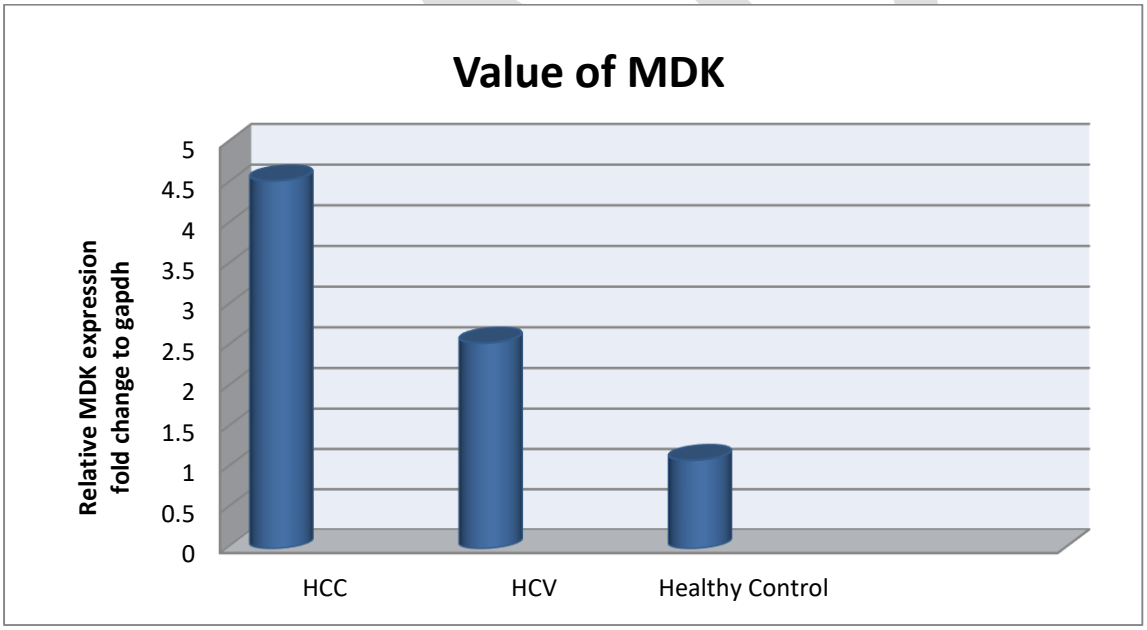

Fig.1. MDK level between the studied groups (p<0.001).

Table 3. Association, agreement and validity of AFP and MDK regard HCC

Variable	Sensitivity	Specificity	Cut off	AUC	P value
AFP	75.7%	86.4%	>90	.968	0.00**
MDK	64.3%	77.3%	>2.39	.726	.024
MDK &AFP	100%	77.3%	–	0.98	0.00**

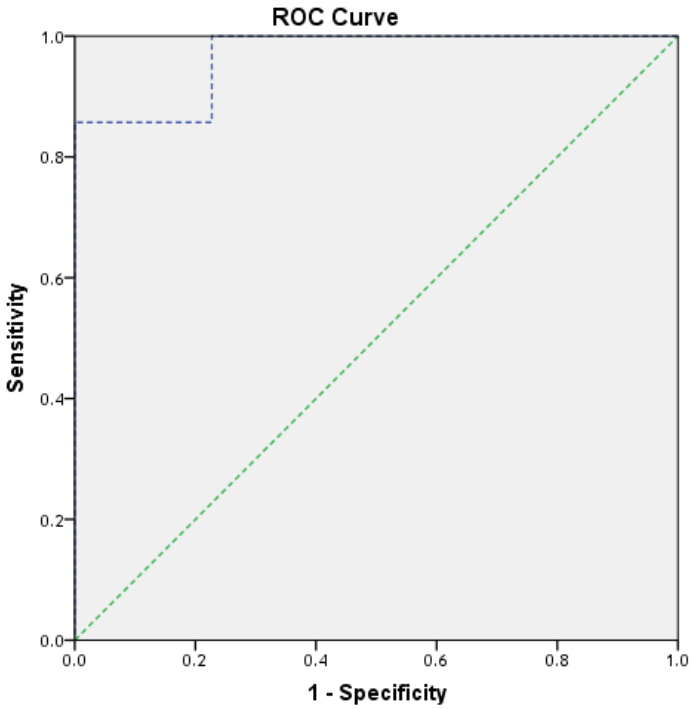

Fig. 2. ROC curve of AFP for HCC cutoff value

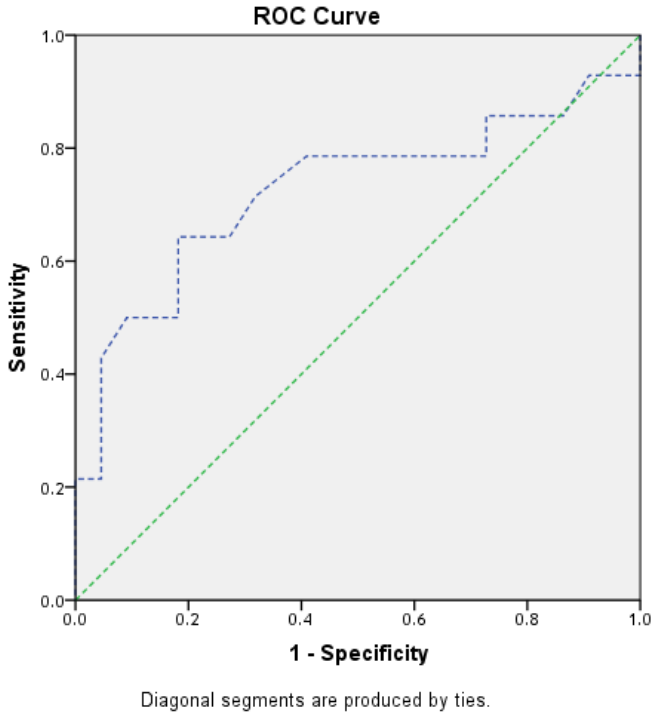

Fig. 3. ROC curve of MDK for HCC cutoff value

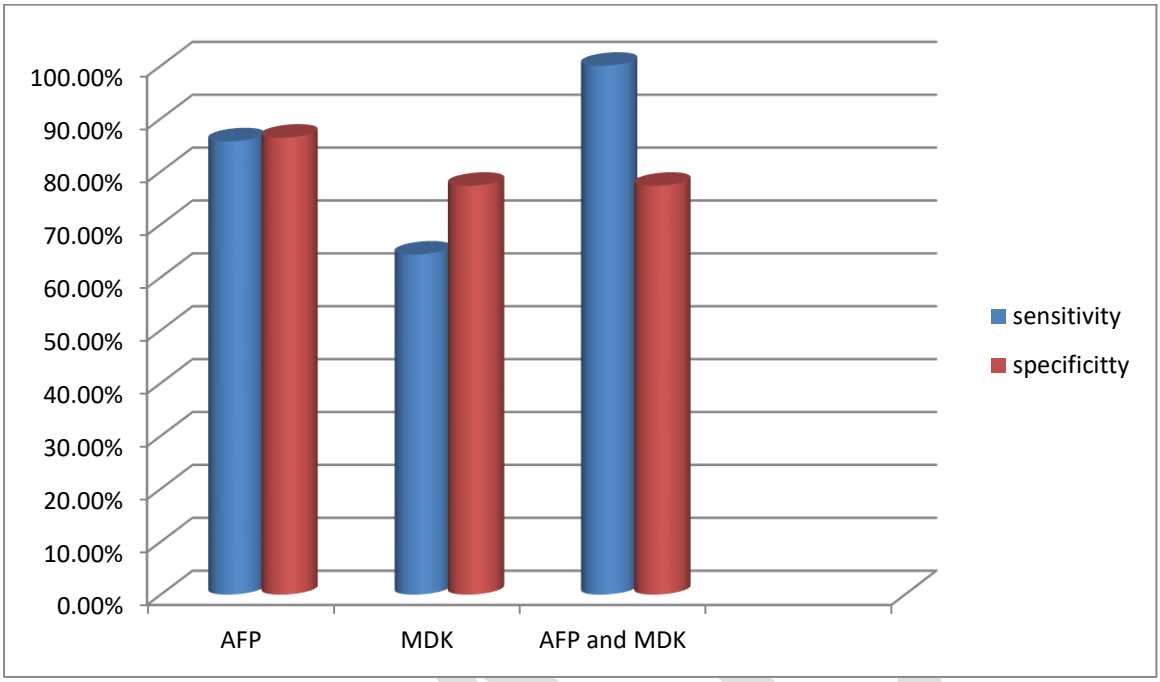

Fig 4. Comparison of MDK, AFP and (AFP with MDK) sensitivity and specificity for HCC predication

To date, about 70%to 90% of HCC patients have an established backgroud of chronic liver disease or cirrhosis, which was caused by +HBV or HCV infection **(El-Serag and Rudolph, 2007)**. A large epidemiological study performed on HCC patients in Egypt, revealed that HCV is the predominant cause of the underlying liver cirrhosis constituting of about 91.32% of HCC cases, while chronic HVB infection was reported in 2.51% **(Shaker et al., 2013).**The only biochemical marker, AFP, is not sufficient enough to identify a substantial proportion of HCC cases. Systematic reviews provided evidence supporting that the use of AFP as a diagnostic and screening test for HCC is limited (**Colli et al .,2006**).Alpha-fetoprotein (AFP) is the commonly used serological marker to detect HCC; however, it is not satisfactory due to low sensitivity and specificity **(Mao et al., 2010)**. Therefore, AFP is not sufficient as a seldom tool for screening and diagnosing of HCC. New sensitive markers are needed for early identification to improve clinical outcomes of HCC patients.MDK plays a significant role in carcinogenesis related activities, such as proliferation, migration, antiapoptosis, mitogenesis, transformation, and angiogenesis, in many types of solid tumors, including hepatocellular carcinomas **(Kato et al., 2002;Muramatsu, 2002).** MK is involved in various physiological processes, such as development, reproduction and repair **(Erguven *et al*., 2012)**. Furthermore, MK plays important roles in the aetiology of inflammatory and malignant diseases **(Erguven*et al*., 2012).**In our study the detection rate MDK was significantly higher in patients with HCC than in patients with HCV and healthy patient (P<0.013*).Results of the present work showed that serum levels of AFP and

MDK were significantly elevated in HCC patients by comparing these values with that of the other studied groups. These findings were in agreement with Shaheen *et al*. **(Shaheen*et al*.,2015)**who found that the median values of the MDK levels in the HCC patients were significantly higher than that of cirrhotic patients and the healthy control subjects. Though the median values of MDK levels in the cirrhotic patients were higher than that in the control group, values did not reach significance**.** In this study, we hypothesized that MDK could be a promising marker for HCC, particularly when combined with AFP. Indeed, this combination showed a diagnostic advantage for the early diagnosis of HCC compared with AFP alone.Alpha-fetoprotein showed the greatest sensitivity and specificity than MDKfor comparing HCC patients with HCV patients and controls. Thus, despite itsdiagnostic limitations, AFP is still the best single marker for the diagnosisof HCC and will continue to play a role in in the diagnostic algorithm of HCC. On the other hand, MDK alone was less sensitive and specific than AFP alone for comparing HCC patients with HCV patients as well as with controls. Therefore, MDK alone is not a valid substitute for AFP as a screening test in HCC surveillance. However, the MDK elevation observed in the HCC group indicates that the combination of MDK and AFP could improve the diagnostic accuracy of the screening**. In conclusion,** the combination of MDK and AFP showed a better diagnostic yield than AFP alone, and this combination may therefore have clinical relevance. AFP is superior to MDK when used individually. MDK is complementary marker to AFP in clinical use for the diagnosis of HCC.Additional studies with high sample size were required to determine molecular evaluation of these markers in HCC.

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