Evaluation of Midkine as a Diagnostic Serum Biomarker in Hepatocellular Carcinoma

Running title: Serum MDK in Diagnosis of HCC

Wen-Wei Zhu¹,²#, Jia-Jian Guo¹,²#, Lei Guo¹,²#, Hu-Liang Jia¹,²#, Ming Zhu³, Ju-Bo Zhang¹,², Christopher A. Loffredo⁴, Marshonna Forgues⁵, Hua Huang⁶, Xu-Jian Xing¹,², Ning Ren¹,², Qiong-Zhu Dong¹,², Hai-Jun Zhou¹,², Zheng-Gang Ren¹,², Nai-Qing Zhao³, Xin Wei Wang⁵, Zhao-You Tang¹,², Lun-Xiu Qin¹,²* and Qing-Hai Ye¹,²*

¹ Liver Cancer Institute and Zhongshan Hospital, Institutes of Biomedical Science, Fudan University, Shanghai, China.
² Key Laboratory of Carcinogenesis and Cancer Invasion (Fudan University), Ministry of Education, China.
³ Department of Biostatistics, School of Public Health, Fudan University, Shanghai, China.
⁴ Lombardi Cancer Center, Georgetown University, Washington DC USA
⁵ Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
⁶ Department of Abdominal Surgery, Fudan University Shanghai Cancer Center, Shanghai, China

# Authors contributed equally to this work.

*Corresponding to: Qing-Hai Ye, MD, PhD, Liver Cancer Institute and Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China, e-mail: ye.qinghai@zs-hospital.sh.cn; or Lun-Xiu Qin, MD, PhD, Liver Cancer Institute and Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China, Fax: +86 -21-5423 7960, e-mail: qin.lunxiu@zs-hospital.sh.cn

Contributorship statement: Conception and design: Wenwei Zhu, Lunxiu Qin, Qinghai Ye, Haijun Zhou, Ning Ren, Zhaoyou Tang
Administrative support: Zhaoyou Tang, Xin Wei Wang, Zhenggang Ren
Provision of study materials or patients: Jiajian Guo, Lei Guo, Ju-Bo Zhang, Qinhai Ye, Lunxiu Qing, Xin Wei Wang
Collection and assembly of data: Jiajian Guo, Lei Guo, Huliang Jia, Christopher A. Loffredo, Marshonna Forgues, Hua Huang, Xujiang Xing, Qiongzu Dong
Data analysis and interpretation: Wenwei Zhu, Ming Zhu, Naiqing Zhao
Manuscript writing: Wenwei Zhu, Lunxiu Qin, Qinghai Ye.
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Translational relevance: Early diagnosis still represents the best chance for successful treatments and improved outcomes of patients with HCC. It is necessary to identify new serologic biomarkers with both sufficient sensitivity and specificity to detect HCCs at an early stage. In our previous study, MDK was identified as one of the candidate biomarkers for HCC. In this study, we used a total of 933 participants including HCCs and hospital controls from different medical centers to further investigate the diagnostic value of MDK in clinical practice for HCC. This is, by far, the largest study on the diagnostic role of serum MDK in HCC and our findings suggest that serum MDK may serve as a novel diagnostic marker in early detection of HCCs especially for those with negative AFP and/or at an early stage. Moreover, monitoring of serum MDK after surgery is useful in evaluation of treatment response and early recurrence of HCC.

ABSTRACT

Purpose: To evaluate the value of serum midkine (MDK) as a diagnostic biomarker in hepatocellular carcinoma (HCC), particularly for those with negative alpha-fetoprotein (AFP) and at an early-stage.

Experimental Design: MDK expression in tumors was assessed by immunohistochemistry from 105 patients with HCC or liver cirrhosis. Serum MDK levels were detected by enzyme-linked immunosorbent assay in 933 participants including HCCs and hospital controls from different medical
centers. Sensitivities and specificities of serum MDK in diagnosing HCC according to AFP level and Barcelona Clinic Liver Cancer (BCLC) stage were analyzed.

**Results:** MDK levels were significantly elevated in HCC tissues as well as serum samples. The sensitivity of serum MDK for HCC diagnosis was much higher than that of AFP (86.9% vs 51.9%) with similar specificities (83.9% vs 86.3%). Notably, serum MDK had an outstanding performance in distinguishing AFP-negative HCCs from different controls: in those AFP-negative HCCs, the sensitivity could reach as high as 89.2%. Moreover, ROC curves analysis also showed that serum MDK had a better performance compared with AFP in distinguishing early-stage HCCs as well as small HCCs. Even in very early-stage HCCs, MDK showed an obviously higher sensitivity comparing with AFP (80% vs 40%). Furthermore, serum MDK level was significantly decreased in HCC patients after curative resection and re-elevated when tumor relapse occurred.

**Conclusions:** Serum MDK is significantly elevated in most HCCs including those with negative AFP and at an early stage which may serve as a novel diagnostic marker in early diagnosis and postoperative monitoring of HCCs.

**Keywords:**
Hepatocellular carcinoma; Serum midkine; Alpha-fetoprotein; Early diagnosis

**INTRODUCTION**

Liver cancer is the fifth most common cancer but the second leading cause of cancer death in men worldwide (half of these cases and deaths are estimated to occur in China), and hepatocellular carcinoma (HCC) represents the major histological subtype which accounts for 70% to 85% of the total liver cancer burden worldwide.\(^1\) Owing to the diagnostic and therapeutic progress during the past decades, the HCC outcome has been improved in a proportion of patients who were diagnosed at an early stage and received curative treatments.\(^2\)\(^,\)\(^3\) However, only about 10%-20% of patients are currently eligible for potentially curative therapies at the time of diagnosis,\(^4\)\(^,\)\(^5\) most of HCC patients are diagnosed at an advanced stage and their prognosis remain very dismal.\(^6\) Thus, early detection and diagnosis of HCC still present the best chance for successful treatments and improved outcomes.\(^7\) Alpha-fetoprotein (AFP) has been widely used as a serological diagnostic tumor marker for HCC.
However, serum AFP is elevated in only about 33%-65% of small HCCs and nonspecific elevation of serum AFP has been found in 15%-58% of patients with chronic hepatitis and 11%-47% of liver cirrhosis, thus there is a debate regarding the roles of AFP in early diagnosis and, particularly, surveillance of HCC. Many alternative novel biomarkers, such as AFP-L3, DCP, GP73 and GPC3, have been investigated; however, their diagnostic values regarding early HCCs remain controversial. Thus, it is necessary to identify new serologic biomarkers with both sufficient sensitivity and specificity to detect HCCs at an early stage and/or with negative AFP.

MDK, a 13-kDa small heparin-binding growth factor, was originally discovered in embryonal carcinoma cells and involved in the early stage of retinoic acid–induced differentiation. In our previous gene expression profiling study, MDK was identified as one of the five important potential novel biomarkers for early detection of HCCs. Additionally, mounting evidence has indicated that MDK plays a significant role in carcinogenesis-related activities, such as proliferation, migration, anti-apoptosis, mitogenesis, transformation and angiogenesis in many types of solid tumors, including HCCs. However, the diagnostic value of serum MDK for HCCs, particularly for those with negative AFP and/or at the early stage, has not yet been investigated comprehensively, which is the aim of this study.

PATIENTS AND METHODS

Cell lines
L-O2 and Chang liver (both normal liver cell lines), Bel-7402 and Huh-7 were from Cell Bank of the Chinese Academy of Sciences; HCCLM3 and MHCC97H were established from the same parental cell line at our institute; PLC was from Japanese Cancer Research Bank; HepG2 and Hep3B were from American Type Culture Collection. They were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO2.

Tissue specimens
HCC tissues, including the tumor and corresponding peri-tumor liver tissues, were obtained from 88 HCC patients among whom 61 matched serum samples were also obtained to investigate the relationship between serum MDK and tissue MDK immunoreactivity, while cancer-free cirrhotic liver tissues were collected from 17 HBV-related liver cirrhosis patients with portal hypertension without any evidence of HCC. All the patients have undergone surgery at Zhongshan Hospital, Fudan University (Shanghai, China) from 2002 to 2007. The clinicopathological characteristics of these HCC
samples are summarized in Supplementary Table S1.

**Serum samples and study design**

Three independent cohorts with a total of 933 participants including 388 HCCs and 545 different controls were enrolled in this study from different medical centers in two countries.(Fig. 1) Learning set one (Cohort A) consisted of 707 serum samples: 252 HCCs and 455 different hospital controls (129 liver cirrhosis, 47 benign liver tumor, 69 gastrointestinal malignant tumor patients, and 210 healthy individuals) who have received treatment or physical examination at Zhongshan Hospital, Fudan University or Shanghai Public Health Center (Shanghai China). The liver cirrhosis cases enrolled in this present study were routinely followed-up at least one year, only those patients without liver tumor were included. The clinical and biochemical information of non-HCC liver cirrhosis form Cohort A were shown in Supplementary Table S2. Most of the HCC patients from this cohort have a history of hepatitis B virus (HBV) infection or HBV-related liver cirrhosis; Learning set two (Cohort B): another 100 serum samples with HCV-related diseases (50 with HCC and 50 with cancer-free liver disease) were recruited from the National Cancer Institute of Cairo University and Kasr El Aini Medical Center, respectively, in Cairo, Egypt. Most have a history of chronic HCV infection, as previously described20; Independent validation set (Cohort C): consisting of 86 HCCs at early stage (BCLC-0/A) and 40 HBV-related liver cirrhosis from Shanghai, China. The clinicopathological characteristics of the participants from above three Cohorts were presented in Supplementary Table S3 and Supplementary Table S4. All of the pathological diagnoses were confirmed by two experienced pathologists after surgery or liver biopsy. Among 252 HCC patients in Cohort A, 240 patients with complete follow-up data were followed up until March 2010 with a median observation time of 33.3 months as described previously.21

The characterization and validation of serum MDK in diagnosing HCCs was performed in multiplex assay and were divided into two study phases (Fig. 1). In phase one (characterization) study, serum MDK was detected and the diagnostic performance was evaluated preliminary in two separate Cohorts (Cohort A and B) with different make up from two countries. Meanwhile, 36 randomly selected HCC patients from the Cohort A whose serum samples were collected again at the 4th week after surgery, and 20 patients in the same Cohort who received re-resection for postoperative recurrent HCC were evaluated for the monitoring role of serum MDK in response to curative resection and early recurrence. In phase two (validation) study, 126 serum samples were evaluated in a blinded manner (the statistician had no prior information related to the samples). Those samples from this independent
validation cohort had never been used in the learning process to avoid optimism in reporting performance.

All serum samples were obtained by venipuncture and immediately (less than half an hour) centrifuged at 3000g for 10 min. The serum was stored frozen at −80°C until use. All patient-derived samples were collected and archived under protocols approved by the institutional review boards of the parent institutions with written informed consent for collection of blood, tissue, and clinical follow-up information.

**Tissue Microarrays and Immunohistochemistry**

Tissue microarrays (TMAs) and immunohistochemistry (IHC) were constructed and performed as described previously \(^{21}\) (see details in the Appendix). IHC evaluation was determined independently by three pathologists without prior knowledge of the patients’ information. The mean percentage value of the two cores was considered representative of one tumor. MDK was considered positive if more than 10% of cells showed moderate or intense staining within each cylinder according to a previous study. \(^{22}\)

**Enzyme-linked Immunosorbent Assays (ELISAs)**

Serum MDK concentrations were determined by ELISA using a commercial kit (BioVendor, LLC, Candler, NC, USA). The assay was performed according to the manufacturer’s instructions (see details in Appendix) and values were reported as ng/ml. All specimens were tested blindly and in triplicate.

**Statistical Analyses**

Statistical analyses were performed using SPSS 17.0 and MedCalc software. The significance level is 0.05. Chi-square test or Fisher’s exact test was used to analyze the categorical data. The quantitative variables were analyzed by Student \(t\)-test or the Mann-Whitney U test. Pearson correlation test was used to investigate the correlation between two quantitative variables. Kaplan-Meier curve was used to describe the survival characteristics of patients. The log-rank test was used to compare patients’ survival between the subgroups. Cutoff values of serum MDK in diagnosing HCCs were obtained through the K-fold cross-validation method (see details in Appendix). Pairwise comparison of ROC curves were produced for the two variables (AFP and MDK) to investigate their capability to distinguish between HCC and non-HCC. \(^{23}\) Logistic regression model including both AFP and MDK as covariate was also fitted to combine diagnose information of two biomarkers (detailed in Appendix).

**RESULTS**
Over-expression of MDK in HCC Tissues
To evaluate the role of MDK in HCC development, we first investigated the expression of MDK in HCC cell lines, tumor tissues and paired serum samples. Elevated expression of MDK was observed in HCC cell lines as well as in culture medium compared with that in normal liver cell lines (Fig. 2A; Supplementary Fig. S1). TMA based IHC analysis showed that MDK was expressed in 72% (63/88) of the HCCs, which was significantly higher than that of peri-tumor liver tissues (12/88; 14%; P<.001) and cancer-free cirrhotic liver tissues (2/17; 12%; P<.001) (Supplementary Table S5), and the positive staining of MDK in HCC tissues were mostly confined to the cytoplasm of HCC cells with a diffuse pattern (Fig. 2B). Western blot assay of tissue samples from 6 representative patients of the same origin further confirmed that MDK were significantly increased in HCCs compared with their noncancerous tissues (Fig. 2C). Moreover, the correlation between MDK protein expression levels in 61 paired HCC tissues and serum samples was also analyzed. We noticed that MDK expression in tumor tissues positively correlated with the serum samples (r=0.670, P<.0001), and the median serum MDK level in these patients with MDK IHC-positive tumors (n=48) was significantly higher than that with IHC-negative(n=12) tumors (P<.001, Fig. 2D).

Serum MDK Level is Elevated in HCCs
To investigate the role of MDK as a tumor marker for HCC, serum MDK levels were first analyzed by ELISA in Cohort A: 252 HBV-related HCCs and 455 hospital controls from Shanghai, China. As shown in Fig. 2E, the median serum MDK level in HCCs (1.204 ng/ml; range, 0.850–1.710) was significantly elevated compared with that in healthy individuals (0.195 ng/ml; range, 0.150–0.417, P<.0001) and patients with different types of liver diseases [0.739 ng/ml (range, 0.483–1.231, P<.05) in patients with benign liver tumors; 0.265 ng/ml (range, 0.093–0.540, P<.0001) in liver cirrhosis patients]. More importantly, the median serum MDK level in HCCs was also significantly higher than that in patients with gastrointestinal malignant tumors (0.470 ng/ml; range, 0.296–0.633, P=.0001). Although, serum AFP levels were found to be significantly associated with aggressive clinicopathological features such as poorly tumor differentiation (P=.019), micro-vascular invasion (P<.001), larger tumor size (P=.002) and advanced tumor stage (P=.002), no significant correlation was found between serum MDK levels and the clinicopathological parameters mentioned above (Supplementary Table S6). In addition, elevated AFP levels were correlated with poor overall survival (OS: P=.002) and early time to tumor recurrence (TTR: P=.008) in HCC patients which may serve as an independent predictor for the outcome; however, no obvious association was found between serum
MDK levels and patients' survival or tumor recurrence (Supplementary Fig. S2 and Supplementary Tables S7). We also tested serum MDK levels in another Cohort (Cohort B; HCV-related HCCs and non-cancer liver disease from Egypt) and it was also found to be significantly elevated in HCCs compared with the controls ($P=.001$)(supplementary Fig. S3).

**Better Diagnostic Performance of Serum MDK Compared with AFP in HCCs**

We next analyzed the ROC curves to evaluate the sensitivity and specificity of serum MDK for HCC diagnosis in Cohort A (Fig. 3A). The area under the ROC (AUROC) curve (95% confidence interval, CI) of MDK (0.915; 95% CI: 0.894–0.936) was found to be much larger than that of serum AFP (0.754; 95% CI: 0.715–0.794; $P<.001$). The sensitivities and specificities at various cut-off values of MDK and AFP according to their ROC curves were calculated and shown in Supplementary Table S8. Although MDK and AFP were found to have similar specificities for HCC diagnosis at different cut-off values, the sensitivities of MDK were significantly higher than that of AFP. Moreover, multivariate logistic regression model indicated that the combination of MDK and AFP could improve the diagnostic performance significantly (Supplementary Fig. S4).

The diagnostic cut-off values of serum MDK were obtained through the analysis of K cross-validation. To reduce variability, multiple rounds of cross-validation were performed, and the final results were averaged over the rounds randomly (Supplementary Table S9). The optimal cut-off value of MDK according to the 5-fold cross-validation analysis was 0.654ng/ml, which was used in the following study; While 20ng/ml, the currently recommended clinical cut-off value was used for AFP. At the cut-off value of 0.654ng/ml, the sensitivity of MDK for HCC diagnosis was 86.9%, which was much higher than that of AFP (51.9%). Meanwhile, the distribution pattern of AFP and MDK in HCCs, healthy controls as well as liver cirrhosis is demonstrated in Fig. 3B. Nonspecific elevation of serum AFP was found in 36.4% (47/129) of patients with liver cirrhosis, which was strikingly higher than 13.2% (17/129) of MDK using the cut-off value mentioned above. Positive predictive value (PPV) and negative predictive value (NPV) for identifying HCCs through this cut-off value according to different prevalence are presented in Supplementary Fig. S5.

In addition, patients with advanced stage HCCs (BCLC B/C) had significantly higher AFP positive rate than that of early-stage tumors (BCLC 0/A; $P=.021$); however, no significant association was found between serum MDK levels and BCLC stages (Fig. 3C). More importantly, the sensitivity of MDK was independent of serum AFP levels ($r=.0443, P=.483$; Supplementary Fig. S6); even in those
with negative AFP (<20ng/ml; n=121), the mean serum MDK level was 1.759 ng/ml and the sensitivity could reach as high as 89.2% (Fig. 3D and Supplementary Fig. S7).

**Performance of Serum MDK for the Diagnosis of HCCs with Negative AFP and at Early-stage**

To further evaluate the diagnostic performance of MDK in early detection and diagnosis of HCC, we next focused on a subset of patients with negative AFP and early-stage HCC in Cohort A. BCLC stage system, the currently widely accepted prognostic classification system for HCC\(^{24, 25}\), was adopted in our study. ROC curves analysis suggested that serum MDK had a better performance compared with AFP for distinguishing early-stage HCCs as well as small HCCs(tumor size<5cm) from non-HCC controls including liver cirrhosis (Fig. 4A and B). In detecting early-stage HCCs (BCLC 0/A), the sensitivity of MDK was much higher than that of AFP (87.1% vs 46.7%); even in very early-stage HCCs (BCLC 0; n=30), MDK showed an obviously higher sensitivity of 80% compared with 40% of AFP (Table 1). Similar results were reached in considering small HCCs (Table 1) and TNM early-stage HCCs (see details in appendix). More importantly, the diagnostic performance of serum MDK was also carefully investigated in AFP negative (<20ng/ml) HCCs. We noticed that serum MDK had an outstanding performance for distinguishing AFP negative HCCs from non-HCC controls (AUROC:0.926; 95% CI:0.903-0.949) including liver cirrhosis (AUROC:0.931; 95% CI:0.898-0.964)(Fig. 4C).

These results suggested that serum MDK level is a much more sensitive tumor marker superior to AFP for the early detection of HCCs.

**Validation of the Early Diagnostic Values of MDK for HCCs in Another Independent Cohort**

To further assess the robustness of the serum MDK level as a novel early diagnostic marker in HCCs, we validated externally in another independent Cohort of 86 HCCs with early stage (BCLC-0/A) and 40 patients with liver cirrhosis blindly (the validation Cohort; Fig. 1). The serum MDK level (1.093ng/ml; range, 0.813–1.780) of HCCs was also significantly increased compared with that of live cirrhosis (\(P<.001\)), which was quite similar to that of the early-stage HCCs derived from the learning Cohort (\(P=.295\); Supplementary Fig. S7). No significant correlation between serum MDK levels and clinicopathological parameters as well as disease recurrence were found (Supplementary Tables S10). Using the same cut-off value of 0.654ng/ml, the sensitivity of MDK for HCC diagnosis in this
validation set was 86.04%, much higher than that of AFP (51.5%), with a higher specificity (90% for MDK; 35% for AFP) as regarding of cirrhotic liver disease.

**The Roles of Serum MDK Levels in Monitoring Treatment Response of HCCs**

The postoperative dynamic changes of serum MDK levels were monitored in 36 HCCs randomly selected from the Cohort A whose serum MDK levels were positive before surgery. At the fourth week after curative resection, serum MDK levels were significantly decreased from 1.362±0.362 ng/ml to 0.482±0.281 ng/ml (P<.001), a lower level similar to that of liver cirrhosis (Fig. 5A). Moreover, in 20 cases with tumor relapse, the decreased serum MDK levels after operation were elevated again at the time of tumor recurrence. A significant correlation (r=.984; P<.001) was found between the baseline MDK levels before the first operation and that at time of tumor recurrence (Fig. 5B and C).

Notably, in two patients out of the above 36 randomly selected cases with documented recurrence according to our follow up, the serum MDK level was elevated greater than the postsurgery nadir (4 weeks) at the time of recurrence. These data suggested that MDK could be a sensitive tumor marker to monitor the treatment response and post-operation tumor recurrence in HCC patients.

**DISCUSSION**

It has been estimated that 70%-90% of HCC patients have an established background of chronic liver disease or cirrhosis, the major causes of which are HBV or HCV infection. Over 50% (340,000 cases) of all HCCs worldwide are associated with HBV infection and near 30% (195,000 cases) are HCV infection-related. Once cirrhosis is established, the annual risk of developing HCC is estimated to be as high as 3%-4%. Screening for HCC using serum AFP or combined with ultrasonography in these high-risk populations is still the essential way for detection and diagnosis of HCC at an earlier stage, when curative therapies are likely to be more successful.

Based on the specific screening requirements for HCC in the context of chronic liver disease and cirrhosis, and the general criteria for an adequate screening test to detect disease, an ideal tumor marker for screening, early detection and diagnosis of HCCs should have the following characteristics: (1) is a secreted protein that can be detected in serum or urine which will facilitate patients examination with minimal invasiveness to them; (2) has a high sensitivity which is elevated in most of HCCs including those at an early stage; (3) has a high specificity which can differentiate HCCs from other chronic liver diseases/cirrhosis and benign liver tumors; (4) can reflect the tumor
treatment response, such as postoperative therapeutic efficacy and relapse monitoring; (5) has a good repeatability and cost-effectiveness. Currently, AFP is still the only widely used serologic tumor marker in screening and diagnosing HCCs. However, serum AFP has only a sensitivity of 39%-65% and a specificity of 76%-94% for the presence of HCCs, and it shows a tumor burden-dependent manner which hinders its clinical use. Although tremendous efforts have been applied to identify improved HCC biomarkers such as AFP-L3, DCP, GPC3, and GP73, to date, none has been demonstrated to be superior to AFP in clinical performance. Therefore, an additional biomarker favoring early detection and diagnosis of HCC is still urgently needed.

In this study, the circulation levels of MDK, a small secreted protein which was identified as one of the five novel candidate diagnostic biomarkers for HCCs in our previous gene expression profiling study, was analyzed in 388 HCC patients and 545 different controls including patients with differential diagnostic-based diseases such as HBV/HCV-related liver cirrhosis, benign liver tumors, and healthy individuals from different countries. We found serum MDK was significantly elevated in HCC patients compared with the controls ($P<.001$). At the “optimal” cut-off value of 0.654ng/ml which was generated from a 5-fold cross-validation analysis, only 13.2% (17/129) of liver cirrhosis patients exceeded the threshold; by contrast, 36.4% (47/129) of these patients were above the cut-off value of AFP (20ng/ml). Additionally, ROC curves demonstrated a higher classification power of MDK with respect to AFP among healthy controls, liver cirrhosis and HCCs. These indicate that MDK is a novel marker with a lower false-positive rate in diagnosing and differentiating HCC from liver cirrhosis.

More importantly, the elevation of serum MDK was independent of AFP level because a similar positive rate was observed when stratified by different serum AFP status and no correlation between these two markers was found. Even in those AFP-negative HCCs, the serum MDK level was also increased dramatically and the diagnostic sensitivity could reach as high as 89.2%, which is much higher than that of the other reported biomarker such as serum GP73 (57%). These indicate that MDK may become a novel diagnostic tumor marker superior to AFP for HCC.

Despite the variation of practice pattern worldwide, surgical resection, liver transplantation and ablative therapies are the currently therapeutic options with curative intent for HCC patients. The 5-year survival rate after curative treatment for patients with early-stage HCC is more than 50%, whereas the 5-year survival rate for patients with advanced-stage disease remains very dismal (less than 5%). Therefore, early detection and diagnosis of HCC are extremely important in improving the survival of the patients. In a recent large case-control multicenter phase II biomarker study to
investigate AFP, DCP and AFP-L3 in patients with HCC and those with cirrhosis, AFP was found to be the most accurate diagnostic marker for HCCs with early and very early stage, the diagnostic sensitivity and specificity of which being 66% and 81% (cutoff value 10.9 ng/ml) for early stage HCC. However, its sensitivity decreased dramatically with the elevated cutoff value. In our present study, MDK showed a superior diagnostic performance than AFP in those HCCs with early stage (BCLC 0/A) (sensitivity being 87.1% vs 46.7%), which was also obviously higher than other reported markers such as DCP (61%) and GPC3 (56.3%). Even in those patients with very early stage (BCLC 0), MDK showed a much higher sensitivity of 80.0% comparing with 40.0% of AFP. Additionally, the combination of MDK and AFP further significantly improved the detection rate of very early HCC from 80% to over 96.6%, which was much higher than the simultaneous use of GPC3 and AFP (from 56.3% to 75%). Thus, combination of MDK and AFP may be a promising strategy for early diagnosis of HCC in the future. It is undeniable that there are many differences in patient populations studied for each marker and combination of markers as well as molecular/genetic heterogeneity of HCCs that different types of HCC may have different markers. However these results strongly indicate that MDK may serve as a more valuable tumor marker than AFP in early detection of hepatitis B virus-related HCCs.

Monitoring response to therapies and tumor recurrence is another important role of tumor marker. In our present study, radical resection of HCCs resulted in a significant reduction in serum MDK to a lower level that similar to liver cirrhosis, and the decreased serum MDK was increased again at the time of tumor recurrence. This provides preliminary evidence of a relationship between serum MDK levels and HCC recurrence that warrants additional investigation.

Although we tested preliminarily the serum MDK in another independent cohort of HCV-related liver diseases including HCCs from Egypt and found it was significantly elevated in HCCs compared with the controls (P=0.001), most of these HCCs were at late stage and they were not treated by surgery or other curative therapies without detailed clinical data, therefore the association between MDK expression and clinic-pathological parameters as well as the value of MDK in detection of HCV-related HCCs were not analyzed in this study, which need to be further investigated later.

In the present study, we demonstrates that serum MDK may serve as a novel diagnostic tumor marker for the detection of HCCs, particularly for those with negative AFP and/or at an early stage. However, most of the HCCs in this study are HBV-related, according to the guidelines on phases of evaluating an early detection biomarker for cancer developed by the National Cancer Institute’s Early Detection
Further validation using larger cohort of serum HCC samples with hepatitis B and hepatitis C infectious liver disease, non-alcoholic fatty liver disease (NAFLD), and alcohol-induced liver disease (ALD) from multiple centers in a prospective, randomized controlled trial is needed.

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References:


**Figure legend**

**Fig. 1** Distribution of patients eligible for diagnosis and early detection of HCC (flowchart). Different study subjects present in the following study: patients with liver cirrhosis (LC), patients with benign liver tumor (BLT), patients with gastrointestinal (GI) malignant tumors, and healthy individuals (NC).

**Fig. 2** Up-regulation of MDK in tumor tissues and corresponding serum samples in HCCs: (A) Expression of MDK in seven established hepatoma cell lines and two human normal liver cell lines. (B) MDK expression detected by immunohistochemical staining in tissue microarrays of liver cirrhosis and HCCs. (a) Overview of the tissue microarrays (up) and two representative cylinders (down). (b) HBV-induced liver cirrhosis, positive staining for MDK was shown in bile duct (arrow). (c) Strong positive and (d) negative expression of MDK in HCC tissues. (Bar, 50 µm) (C) Representative western blot showing the expression of MDK protein in tumor tissue (T) and paired peritumor tissue (N) from six HCC patients. (D) Relationship between serum MDK and MDK immunoreactivity in 61 patients with hepatocellular carcinoma. (E) Comparison of serum MDK levels between the learning set of patients with HCC and different controls. Serum MDK levels of HCC patients are significantly higher than that of different controls (black dots represent outliers).

**Fig. 3** Comparison of the diagnostic performance of serum MDK and AFP for HCCs: (A) Sensitivities and specificities of MDK and AFP for HCC diagnosis was compared through the analyses of ROC curves in the learning Cohort A (n=707): The area under the ROC (AUROC) curve of serum MDK was much larger than that of AFP (P<0.000). (B) The distributing patterns of serum MDK (up) and AFP (down) in HCC, liver cirrhosis patients (LC) and healthy controls (NC) tested. Each dot represents individual cases. The red dotted lines represent the cutoff value for MDK and AFP. (C) The different sensitivities of MDK and AFP in detecting early and advanced-stage HCC patients. (D) Similar positive rate (present above the bar) of serum MDK (using the cut off value 0.654ng/ml) was observed in HCC patients with different AFP levels.

**Fig. 4** The performance of MDK and AFP for detecting early stage HCC, small HCC and AFP negative HCC in the learning Cohort A. (A) ROC curves for cases and controls assigned to early stage HCCs (BCLC-0/A) versus non-HCC controls (left) and liver cirrhosis (LC, middle); the relative expression pattern of serum MDK in early stage HCCs and liver cirrhosis with different
etiology (right). (B) Comparison of the ROC curves of AFP and MDK for distinguishing small HCCs (tumor size $<$ 5 cm) from liver cirrhosis (middle) and other non-HCC controls (left); the relative expression pattern of serum MDK in small HCCs and liver cirrhosis with different etiology was shown (right) (C) ROC curve evaluating those with AFP negative HCCs and cirrhosis (middle) and non-HCC controls (left); the relative expression pattern of serum MDK in AFP negative HCCs as well LC patients (right).

**Fig. 5.** The role of MDK in evaluating therapy response and surveillance of HCC after curative resection. (A) At the 4th week after curative resection, the serum MDK levels of most HCC patients were decreased to less than 0.654 ng/ml. Blue dots represent each patient (before and after surgery). (B) In 20 patients with documented recurrence in Cohort A, the serum MDK levels were increased again to the preoperative levels. (C) A significant correlation ($r=0.984$; $P<0.001$) was found between MDK levels before the first operation and after recurrence. Gray lines represent 0.654 ng/ml.
Figure 1
Figure 3

(A) ROC curve for Serum MDK and Serum AFP.

(B) Scatter plots for Serum MDK and Serum AFP in different stages: NC, LC, HCC.

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<td>AFP</td>
<td>0.754</td>
<td>0.020</td>
<td>&lt;0.000</td>
<td>0.715</td>
</tr>
</tbody>
</table>

(C) Comparison of positive rates between early and advanced stages for AFP and MDK.

(D) Analysis of AFP levels in different categories: ≤20ng/ml, 20-200ng/ml, >200ng/ml.

MDK (-): 89.2%, MDK (+): 84.9%, P=0.652
Figure 4
Figure 5

A

Serum MDK (ng/ml)

Presurgery  Postsurgery

4 weeks (n=36)

B

P=0.358

Serum MDK (ng/ml)

Presurgery  Recurrence

C

MDK presurgery (ng/ml)

MDK at recurrence (ng/ml)

rs=0.948

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<table>
<thead>
<tr>
<th>Variable</th>
<th>Cut off value</th>
<th>Tumor size</th>
<th>BCLC stage&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ng/ml)</td>
<td>≤2 cm N(%)</td>
<td>2-5 cm N(%)</td>
</tr>
<tr>
<td>MDK</td>
<td>0.654</td>
<td>24 (80.0%)</td>
<td>88 (88.0%)</td>
</tr>
<tr>
<td>AFP</td>
<td>20</td>
<td>12 (40.0%)</td>
<td>45 (45.0%)</td>
</tr>
<tr>
<td>AFP-2</td>
<td>200</td>
<td>6 (20.0%)</td>
<td>29 (29.0%)</td>
</tr>
<tr>
<td>Combined MDK and AFP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.654;20</td>
<td>29 (96.6%)</td>
<td>95 (95.0%)</td>
</tr>
<tr>
<td>Combined MDK and AFP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.654;200</td>
<td>27 (90.0%)</td>
<td>93 (93.0%)</td>
</tr>
</tbody>
</table>

Note: HCCs in the learning cohort A (N=252). N (%): total number of positive patients with percentage according to different cutoff values presented above.

<sup>a</sup>: according to the BCLC (Barcelona Clinic Liver Cancer) staging system.

<sup>b</sup>: patients with AFP and/or MDK elevation in the serum were considered positive.
Evaluation of Midkine as a Diagnostic Serum Biomarker in Hepatocellular Carcinoma


Clin Cancer Res. Published OnlineFirst May 29, 2013.

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