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Evaluation of Midkine as a Diagnostic Serum Biomarker in Hepatocellular Carcinoma

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Abstract

Purpose: To evaluate the value of serum midkine (MDK) as a diagnostic biomarker in hepatocellular carcinoma, 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 hepatocellular carcinomas or liver cirrhosis. Serum MDK levels were detected by ELISA in 933 participants including hepatocellular carcinomas and hospital controls from different medical centers. Sensitivities and specificities of serum MDK in diagnosing

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No potential conflicts of interest were disclosed.

hepatocellular carcinoma according to AFP level and Barcelona Clinic Liver Cancer (BCLC) stage were analyzed.

Results: MDK levels were significantly elevated in hepatocellular carcinoma tissues as well as serum samples. The sensitivity of serum MDK for hepatocellular carcinoma 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 hepatocellular carcinomas from different controls: In those AFP-negative hepatocellular carcinomas, the sensitivity could reach as high as 89.2%. Moreover, receiver operating characteristic (ROC) curve analysis also showed that serum MDK had a better performance compared with AFP in distinguishing early-stage hepatocellular carcinomas as well as small hepatocellular carcinomas. Even in very early-stage hepatocellular carcinomas, MDK showed an obviously higher sensitivity compared with AFP (80% vs. 40%). Furthermore, serum MDK level was significantly decreased in patients with hepatocellular carcinomas after curative resection and re-elevated when tumor relapse occurred.

Conclusions: Serum MDK is significantly elevated in most hepatocellular carcinomas, 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 hepatocellular carcinomas.

Introduction

Liver cancer is the fifth most common cancer but the second leading cause of cancer-related death in men worldwide (half of these cases and deaths are estimated to occur in China), and hepatocellular carcinoma represents the major histologic 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 hepatocellular carcinoma 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% to 20% of patients are currently eligible for potentially curative therapies at the time of diagnosis (4, 5), most of the patients with hepatocellular carcinomas are diagnosed at an advanced stage and their prognosis remain very dismal (6). Thus, early detection and diagnosis of hepatocellular carcinomas still present the best chance for successful treatments and improved outcomes (7).

Alpha-fetoprotein (AFP) has been widely used as a serologic diagnostic tumor marker for hepatocellular carcinomas. However, serum AFP is elevated in only about 33% to 65% of small hepatocellular carcinomas and nonspecific elevation of serum AFP has been found in 15% to 58% of patients with chronic hepatitis and 11% to 47% of liver cirrhosis (8), thus there is a debate about the roles of AFP in early diagnosis and, particularly, surveillance of hepatocellular carcinomas. Many alternative novel biomarkers, such as AFP-L3, DCP, GP73, and GPC3, have been investigated (9–12); however, their diagnostic values about early hepatocellular carcinomas remain controversial (13, 14). Thus, it is necessary to identify new serologic biomarkers with both sufficient sensitivity and specificity to detect hepatocellular carcinomas at an early stage and/or with negative AFP.

Midkine (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 (15). In our previous gene expression profiling study, MDK was identified as 1 of the 5 important potential novel biomarkers for early detection of hepatocellular carcinomas (16). In addition, 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 hepatocellular carcinomas (17–19). However, the diagnostic value of serum MDK for hepatocellular carcinomas, 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; and HepG2 and Hep3B were from American Type Culture Collection. They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO₂.

Tissue specimens

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

Serum samples and study design

Three independent cohorts with a total of 933 participants including 388 hepatocellular carcinomas and 545 different controls were enrolled in this study from different medical centers in 2 countries (Fig. 1). Learning set one (cohort A) consisted of 707 serum samples: 252 hepatocellular carcinomas 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 1 year, only those patients without liver tumor were included. The clinical and biochemical information of non-hepatocellular carcinoma liver cirrhosis from cohort A are shown in Supplementary Table S2. Most of the patients with hepatocellular carcinomas 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 hepatocellular carcinoma 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 described (20); independent validation set (cohort C): consisting of 86 hepatocellular carcinomas at early stage (BCLC-0/A) and 40 HBV-related liver cirrhosis from Shanghai, China. The clinicopathologic characteristics of the participants from above three cohorts are presented in Supplementary Tables S3 and S4. All of the pathological diagnoses were confirmed by 2 experienced pathologists after surgery or liver biopsy. Among 252 patients with hepatocellular carcinomas 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 hepatocellular carcinomas was conducted in multiplex assay and were divided into 2 study phases (Fig. 1). In phase 1 (characterization) study, serum MDK was detected and the diagnostic performance was evaluated preliminary in 2 separate cohorts (cohort A and B) with different make up from 2 countries. Meanwhile, 36 randomly selected patients with hepatocellular carcinomas 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 hepatocellular carcinomas were evaluated for the monitoring role of serum MDK in response to curative resection and early recurrence. In phase 2 (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 $3,000 \times g$ for 10 minutes. 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 (TMA) and immunohistochemistry (IHC) were constructed and conducted as described previously (ref. 21; see details in the Appendix). IHC evaluation was determined independently by 3 pathologists without prior knowledge of the patients' information. The mean percentage value of the 2 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).

ELISAs

Serum MDK concentrations were determined by ELISA using a commercial kit (BioVendor, LLC). The assay was conducted 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 conducted using SPSS 17.0 and MedCalc software. The significance level is 0.05. The χ^2 or Fisher's exact tests were used to analyze the categorical data. The quantitative variables were analyzed by the Student *t* test or the Mann–Whitney *U* test. Pearson correlation test was used to investigate the correlation between 2 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 hepatocellular carcinomas were obtained through the *K*-fold cross-validation method (see details in Appendix). Pairwise comparison of receiver operating characteristic (ROC) curves were produced for the 2 variables (AFP and MDK) to investigate their capability to distinguish between hepatocellular carcinomas and non-hepatocellular carcinomas (23). Logistic regression model including both AFP and MDK as covariate was also fitted to combine diagnose information of 2 biomarkers (detailed in Appendix).

Results

Overexpression of MDK in hepatocellular carcinoma tissues

To evaluate the role of MDK in hepatocellular carcinoma development, we first investigated the expression of MDK in hepatocellular carcinoma cell lines, tumor tissues, and paired serum samples. Elevated expression of MDK was observed in hepatocellular carcinoma 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 of 88) of the hepatocellular carcinomas, which was significantly higher than that of peritumor liver tissues (12 of 88; 14%; $P < 0.001$) and cancer-free cirrhotic liver tissues (2 of 17; 12%; $P < 0.001$; Supplementary Table S5), and the positive staining of MDK in hepatocellular carcinoma tissues was mostly confined to the cytoplasm of hepatocellular carcinoma 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 hepatocellular carcinomas compared with their noncancerous tissues (Fig. 2C). Moreover, the correlation between MDK protein expression levels in 61 paired hepatocellular carcinoma tissues and serum samples was also analyzed. We noticed that MDK expression in tumor tissues positively correlated with the serum samples ($r = 0.670$, $P < 0.0001$), 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 ($P < 0.001$; Fig. 2D).

Serum MDK Level is elevated in hepatocellular carcinomas

To investigate the role of MDK as a tumor marker for hepatocellular carcinomas, serum MDK levels were first analyzed by ELISA in cohort A: 252 HBV-related hepatocellular carcinomas and 455 hospital controls from Shanghai, China. As shown in Fig. 2E, the median serum MDK level in hepatocellular carcinomas (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, $P < 0.0001$) and patients with different types of liver diseases [0.739 ng/ml (range, 0.483–1.231, $P < 0.05$) in patients with benign liver tumors; 0.265 ng/mL (range,

0.093–0.540, $P < 0.0001$) in patients with liver cirrhosis]. More importantly, the median serum MDK level in hepatocellular carcinomas was also significantly higher than that in patients with gastrointestinal malignant tumors (0.470 ng/mL; range, 0.296–0.633, $P = 0.0001$).

Although, serum AFP levels were found to be significantly associated with aggressive clinicopathologic features such as poorly tumor differentiation ($P = 0.019$), microvascular invasion ($P < 0.001$), larger tumor size ($P = 0.002$), and advanced tumor stage ($P = 0.002$), no significant correlation was found between serum MDK levels and the clinicopathologic parameters mentioned above (Supplementary Table S6). In addition, elevated AFP levels were correlated with poor overall survival (OS; $P = 0.002$) and early time to tumor recurrence (TTR; $P = 0.008$) in patients with hepatocellular carcinomas 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 Table S7). We also tested serum MDK levels in another cohort (cohort B; HCV-related hepatocellular carcinomas and non-cancer liver disease from Egypt) and it was also found to be significantly elevated in hepatocellular carcinomas compared with the controls ($P = 0.001$; Supplementary Fig. S3).

Better diagnostic performance of serum MDK compared with AFP in hepatocellular carcinomas

We next analyzed the ROC curves to evaluate the sensitivity and specificity of serum MDK for hepatocellular carcinoma 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; $P < 0.001$). The sensitivities and specificities at various cutoff 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 hepatocellular carcinoma diagnosis at different cutoff 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 cutoff values of serum MDK were obtained through the analysis of K cross-validation. To reduce variability, multiple rounds of cross-validation were conducted, and the final results were averaged over the rounds randomly (Supplementary Table S9). The optimal cutoff value of MDK according to the 5-fold cross-validation analysis was 0.654 ng/mL, which was used in the following study; While 20 ng/mL, the currently recommended clinical cutoff value was used for AFP. At the cutoff value of 0.654 ng/mL, the sensitivity of MDK for hepatocellular carcinoma diagnosis was 86.9%, which was much higher than that of AFP (51.9%). Meanwhile, the distribution pattern of AFP and MDK in hepatocellular carcinomas, healthy controls, as well as liver cirrhosis is shown in Fig. 3B. Nonspecific elevation of serum AFP was found in 36.4% (47 of 129) of patients with liver cirrhosis, which was strikingly higher than 13.2% (17 of 129) of MDK using the cutoff value mentioned above. Positive predictive value (PPV) and negative predictive value (NPV) for

identifying hepatocellular carcinomas through this cutoff value according to different prevalence are presented in Supplementary Fig. S5.

In addition, patients with advanced-stage hepatocellular carcinomas (BCLC B/C) had significantly higher AFP positive rate than that of early-stage tumors (BCLC 0/A; $P=0.021$); 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 ($r=0.0443$, $P=0.483$; Supplementary Fig. S6); even in those with negative AFP (<20 ng/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 hepatocellular carcinomas with negative AFP and at early stage

To further evaluate the diagnostic performance of MDK in early detection and diagnosis of hepatocellular carcinomas, we next focused on a subset of patients with negative AFP and early-stage hepatocellular carcinomas in cohort A. BCLC stage system, the currently widely accepted prognostic classification system for hepatocellular carcinomas (24, 25), was adopted in our study. ROC curve analysis suggested that serum MDK had a better performance compared with AFP for distinguishing early-stage hepatocellular carcinomas as well as small hepatocellular carcinomas (tumor size <5 cm) from non-hepatocellular carcinoma controls including liver cirrhosis (Fig. 4A and B). In detecting early-stage hepatocellular carcinomas (BCLC 0/A), the sensitivity of MDK was much higher than that of AFP (87.1% vs. 46.7%); even in very early-stage hepatocellular carcinomas (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 hepatocellular carcinomas (Table 1) and TNM early-stage hepatocellular carcinomas (see details in Appendix). More importantly, the diagnostic performance of serum MDK was also carefully investigated in AFP-negative (<20 ng/mL) hepatocellular carcinomas. We noticed that serum MDK had an outstanding performance for distinguishing AFP-negative hepatocellular carcinomas from non-hepatocellular carcinoma 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 hepatocellular carcinomas.

Validation of the early diagnostic values of MDK for hepatocellular carcinomas in another independent cohort

To further assess the robustness of the serum MDK level as a novel early diagnostic marker in hepatocellular carcinomas, we validated externally in another independent cohort of 86 hepatocellular carcinomas with early-stage (BCLC-0/A) and 40 patients with liver cirrhosis blindly (the validation cohort; Fig. 1). The serum MDK level (1.093 ng/mL; range, 0.813–1.780) of hepatocellular carcinomas was also significantly increased compared with that of liver cirrhosis ($P<0.001$), which was quite similar to that of the early-stage hepatocellular carcinomas derived from the learning cohort ($P=0.295$; Supplementary Fig. S7). No significant correlation between serum MDK levels and clinicopathologic parameters as well

as disease recurrence was found (Supplementary Tables S10). Using the same cutoff value of 0.654 ng/mL, the sensitivity of MDK for hepatocellular carcinoma 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 hepatocellular carcinomas

The postoperative dynamic changes of serum MDK levels were monitored in 36 hepatocellular carcinomas 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 ($P < 0.001$), 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 ($r = 0.984$; $P < 0.001$) was found between the baseline MDK levels before the first operation and that at time of tumor recurrence (Fig. 5B and C).

Notably, in 2 patients 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 postoperation tumor recurrence in patients with hepatocellular carcinomas.

Discussion

It has been estimated that 70% to 90% of patients with hepatocellular carcinomas have an established background of chronic liver disease or cirrhosis, the major causes of which are HBV or HCV infection (26, 27). More than 50% (340,000 cases) of all hepatocellular carcinomas worldwide are associated with HBV infection and near 30% (195,000 cases) are HCV infection-related (28, 29). Once cirrhosis is established, the annual risk of developing hepatocellular carcinoma is estimated to be as high as 3% to 4% (30). Screening for hepatocellular carcinoma using serum AFP or combined with ultrasonography in these high-risk populations is still the essential way for detection and diagnosis of hepatocellular carcinomas at an earlier stage, when curative therapies are likely to be more successful (31, 32).

On the basis of the specific screening requirements for hepatocellular carcinomas in the context of chronic liver disease and cirrhosis and the general criteria for an adequate screening test to detect disease (33–35), an ideal tumor marker for screening, early detection, and diagnosis of hepatocellular carcinomas should have the following characteristics: (i) is a secreted protein that can be detected in serum or urine which will facilitate patients examination with minimal invasiveness to them; (ii) has a high sensitivity which is elevated in most of hepatocellular carcinomas including those at an early stage; (iii) has a high specificity which can differentiate hepatocellular carcinomas from other chronic liver diseases/cirrhosis and benign liver tumors; (iv) can reflect the tumor treatment response, such as postoperative therapeutic efficacy and relapse monitoring; and (v) has a

good repeatability and cost-effectiveness. Currently, AFP is still the only widely used serologic tumor marker in screening and diagnosing hepatocellular carcinomas. However, serum AFP has only a sensitivity of 39% to 65% and a specificity of 76% to 94% for the presence of hepatocellular carcinomas, and it shows a tumor burden-dependent manner which hinders its clinical use (36). Although tremendous efforts have been applied to identify improved hepatocellular carcinoma biomarkers such as AFP-L3, DCP, GPC3, and GP73, to date, none has been shown to be superior to AFP in clinical performance (9–12). Therefore, an additional biomarker favoring early detection and diagnosis of hepatocellular carcinomas is still urgently needed.

In this study, the circulation levels of MDK, a small secreted protein which was identified as 1 of the 5 novel candidate diagnostic biomarkers for hepatocellular carcinomas in our previous gene expression profiling study, was analyzed in 388 patients with hepatocellular carcinomas 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 patients with hepatocellular carcinomas compared with the controls ($P < 0.001$). At the “optimal” cutoff value of 0.654 ng/mL which was generated from a 5-fold cross-validation analysis, only 13.2% (17 of 129) of patients with liver cirrhosis exceeded the threshold; in contrast, 36.4% (47 of 129) of these patients were above the cutoff value of AFP (20 ng/mL). In addition, ROC curves showed a higher classification power of MDK with respect to AFP among healthy controls, liver cirrhosis, and hepatocellular carcinomas. These indicate that MDK is a novel marker with a lower false-positive rate in diagnosing and differentiating hepatocellular carcinomas 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 2 markers was found. Even in those AFP-negative hepatocellular carcinomas, 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%; ref. 11). These indicate that MDK may become a novel diagnostic tumor marker superior to AFP for hepatocellular carcinomas.

Despite the variation of practice pattern worldwide, surgical resection, liver transplantation and ablative therapies are the currently therapeutic options with curative intent for patients with hepatocellular carcinomas (37). The 5-year survival rate after curative treatment for patients with early-stage hepatocellular carcinoma is more than 50%, whereas the 5-year survival rate for patients with advanced-stage disease remains very dismal (<5%; ref. 37). Therefore, early detection and diagnosis of hepatocellular carcinomas 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 hepatocellular carcinomas and those with cirrhosis, AFP was found to be the most accurate diagnostic marker for hepatocellular carcinomas 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 hepatocellular carcinomas (38). However, its sensitivity decreased dramatically with the elevated cutoff value. In our present study, MDK showed a superior

diagnostic performance than AFP in those hepatocellular carcinomas with early stage (BCLC 0/A; sensitivity, 87.1% vs. 46.7%), which was also obviously higher than other reported markers such as DCP (61%) and GPC3 (56.3%; refs. 39, 40). 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. In addition, the combination of MDK and AFP further significantly improved the detection rate of very early hepatocellular carcinoma from 80% to more than 96.6%, which was much higher than the simultaneous use of GPC3 and AFP (from 56.3% to 75%; ref. 40). Thus, combination of MDK and AFP may be a promising strategy for early diagnosis of hepatocellular carcinomas 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 hepatocellular carcinomas that different types of hepatocellular carcinomas 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 hepatocellular carcinomas.

Monitoring response to therapies and tumor recurrence is another important role of tumor marker. In our present study, radical resection of hepatocellular carcinomas 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 hepatocellular carcinoma recurrence that warrants additional investigation.

Although we tested preliminarily the serum MDK in another independent cohort of HCV-related liver diseases including hepatocellular carcinomas from Egypt and found it was significantly elevated in hepatocellular carcinomas compared with the controls ($P = 0.001$), most of these hepatocellular carcinomas 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 clinicopathologic parameters as well as the value of MDK in detection of HCV-related hepatocellular carcinomas were not analyzed in this study, which need to be further investigated later.

In the present study, we show that serum MDK may serve as a novel diagnostic tumor marker for the detection of hepatocellular carcinomas, particularly for those with negative AFP and/or at an early stage. However, most of the hepatocellular carcinomas 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 Research Network (EDRN; ref. 41). Further validation using larger cohort of serum hepatocellular carcinoma samples with hepatitis B and hepatitis C infectious liver disease, nonalcoholic fatty liver disease (NAFLD), and alcohol-induced liver disease (ALD) from multiple centers in a prospective, randomized controlled trial is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Early diagnosis still represents the best chance for successful treatments and improved outcomes of patients with hepatocellular carcinoma. It is necessary to identify new serologic biomarkers with both sufficient sensitivity and specificity to detect hepatocellular carcinomas at an early stage. In our previous study, midkine (MDK) was identified as one of the candidate biomarkers for hepatocellular carcinomas. In this study, we used a total of 933 participants including hepatocellular carcinomas and hospital controls from different medical centers to further investigate the diagnostic value of MDK in clinical practice for hepatocellular carcinomas. This is, by far, the largest study on the diagnostic role of serum MDK in hepatocellular carcinomas, and our findings suggest that serum MDK may serve as a novel diagnostic marker in early detection of hepatocellular carcinomas especially for those with negative alpha-fetoprotein and/or at an early stage. Moreover, monitoring of serum MDK after surgery is useful in evaluation of treatment response and early recurrence of hepatocellular carcinomas.

Figure 1.

Distribution of patients eligible for diagnosis and early detection of hepatocellular carcinomas (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). HCC, hepatocellular carcinoma.

Figure 2.

Upregulation of MDK in tumor tissues and corresponding serum samples in hepatocellular carcinomas. A, expression of MDK in 7 established hepatoma cell lines and 2 human normal liver cell lines. B, MDK expression detected by immunohistochemical staining in tissue microarrays of liver cirrhosis and hepatocellular carcinomas. Overview of the tissue microarrays (up) and 2 representative cylinders (down;a). HBV-induced liver cirrhosis, positive staining for MDK was shown in bile duct (arrow; b). Strong positive (c) and negative (d) expression of MDK in hepatocellular carcinoma tissues. Bar, 50 μ m. C,

representative Western blotting showing the expression of MDK protein in tumor tissue (T) and paired peritumortissue(N) from 6 patients with hepatocellular carcinomas. D, relationship between serum MDK and MDK immunoreactivity in 61 patients with hepatocellular carcinomas. E, comparison of serum MDK levels between the learning set of patients with hepatocellular carcinoma and different controls. Serum MDK levels of patients with hepatocellular carcinomas are significantly higher than that of different controls (black dots represent outliers).

Figure 3.

Comparison of the diagnostic performance of serum MDK and AFP for hepatocellular carcinomas. A, sensitivities and specificities of MDK and AFP for hepatocellular carcinoma diagnosis was compared through the analyses of ROC curves in the learning cohort A ($n = 707$). 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 hepatocellular carcinomas, patients with liver cirrhosis (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 patients with hepatocellular carcinomas. D, similar positive rate (present above the bar) of serum MDK (using the cut off value 0.654 ng/mL) was observed in patients with hepatocellular carcinoma with different AFP levels.

Figure 4.

The performance of MDK and AFP for detecting early-stage hepatocellular carcinomas, small hepatocellular carcinomas, and AFP-negative hepatocellular carcinomas in the learning cohort A. A, ROC curves for cases and controls assigned to early-stage hepatocellular carcinomas (BCLC-0/A) versus non-hepatocellular carcinoma controls (left) and liver cirrhosis (LC, middle); the relative expression pattern of serum MDK in early-stage hepatocellular carcinomas and liver cirrhosis with different etiology (right). B, comparison of the ROC curves of AFP and MDK for distinguishing small hepatocellular carcinomas (tumorsize < 5cm) from liver cirrhosis (middle) and other non-hepatocellular carcinoma controls (left); the relative expression pattern of serum MDK in small hepatocellular carcinomas and liver cirrhosis with different etiology was shown (right). C, ROC curve

evaluating those with AFP-negative hepatocellular carcinomas and cirrhosis (middle) and non-hepatocellular carcinoma controls (left); the relative expression pattern of serum MDK in AFP-negative hepatocellular carcinomas as well patients with LC (right).

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Figure 5.

The role of MDK in evaluating therapy response and surveillance of hepatocellular carcinomas after curative resection. A, at the fourth week after curative resection, the serum MDK levels of most patients with hepatocellular carcinomas 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 ($r = 0.984$; $P < 0.0001$) was found between MDK levels before the first operation and after recurrence. Gray lines represent 0.654 ng/mL.

Table 1. Sensitivity of MDK and AFP in patients with hepatocellular carcinomas according to different tumor size and BCLC stage

Cutoff value	(ng/mL)	Tumor size			BCLC stage ^a	
		2 cm n (%)	2–5 cm n (%)	>5 cm n (%)	0-A n (%)	B-C n (%)
MDK	0.654	24 (80.0%)	88 (88.0%)	107 (87.7%)	108 (87.1%)	113 (88.3%)
AFP	20	12 (40.0%)	45 (45.0%)	74 (60.6%)	58 (46.7%)	73 (57.0%)
AFP-2	200	6 (20.0%)	29 (29.0%)	59 (48.4%)	35 (28.2%)	59 (46.1%)
Combined MDK and AFP ^b	0.654;20	29 (96.6%)	95 (95.0%)	115 (94.3%)	119 (95.9%)	123 (96.1%)
Combined MDK and AFP	0.654; 200	27 (90.0%)	93 (93.0%)	113 (92.6%)	115 (92.7%)	120 (93.7%)

NOTE: Hepatocellular carcinomas in the learning cohort A (N= 252), n (%), total number of positive patients with percentage according to different cutoff values presented above.

^a According to the BCLC (Barcelona Clinic Liver Cancer) staging system.

^b Patients with AFP and/or MDK elevation in the serum were considered positive.