Prognostic and diagnostic effects of high serum midkine levels in patients with hepatocellular carcinoma

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Abstract. Midkine (MK) is a soluble cytokine, and its serum levels strongly correspond to protein expression levels in tumors. The present study aimed to clarify the clinicopathological and prognostic significance of serum MK (s-MK) in patients with hepatocellular carcinoma (HCC). Serum samples were obtained before surgery from 123 patients with HCC who had undergone surgery between January 2012 and December 2020. The receiver operating characteristic curve revealed that the best cut-off value for s-MK in differentiating HCC from healthy cases was 426 pg/ml. The clinicopathological variables and overall survival of patients were compared between the s-MK-positive group and s-MK-negative group. The sensitivity, specificity and accuracy of s-MK were 82.1, 97.4 and 88.0%, respectively. An s-MK-positive status was significantly associated with the number of tumors (≥2). The positivity rate of s-MK was significantly higher compared with that of α-fetoprotein and protein-induced by vitamin K absence-II. In total, only 28% of the patients were positive for s-MK. The s-MK-positive group showed significantly worse overall survival compared with the s-MK-negative group. Moreover, multivariate analysis revealed that an s-MK-positive status was independently associated with poor prognosis. s-MK was useful in detecting early HCC. The findings of this study indicated that the s-MK-positive status is associated with the number of tumors and can act as an independent prognostic risk factor.

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Abbreviations: AFP, α-fetoprotein; HCC, hepatocellular carcinoma; MK, midkine; PIVKA-II, protein-induced by vitamin K absence-II

Key words: diagnosis, hepatocellular carcinoma, midkine, prognosis, tumor marker

Introduction

Midkine (MK) is a pleiotropic growth binding protein that is highly upregulated during embryogenesis, thereby playing a key role in neuronal differentiation (1,2). Furthermore, MK exhibits antiapoptotic and angiogenic activities and can lead to enhanced cell proliferation in tumors. Since MK is a soluble cytokine, its serum levels strongly correspond to protein expression levels in tumors (3). Serum MK (s-MK) has been proposed as a potential biomarker for different tumors, including hepatocellular carcinoma (HCC).

Serum α-fetoprotein (AFP) is the only diagnostic marker recommended in the HCC guidelines. However, its diagnostic performance is unsatisfactory, with low sensitivity and specificity. To improve the diagnosis of HCC, advances in biomarker detection techniques have led to the identification of several new biomarkers, such as autoantibodies and s-MK (4-6). s-MK, an emerging serum biomarker, activates several cell surface receptors to modulate various biological activities and is significantly increased in HCC (7). s-MK has been proposed as a promising serum biomarker for HCC diagnosis. Although several studies have estimated the diagnostic value of s-MK for HCC, the results are inconsistent (8-12). Precise clinicopathological analyses including AFP and protein induced by vitamin K absence-II (PIVKA-II) have not been published.

An s-MK-positive status has been reported to be associated with poor prognosis in some solid tumors, such as colorectal cancer and non-small cell lung cancer, but not in esophageal and gastric cancers (13-16). The correlation between an s-MK-positive status and prognosis of patients with HCC has not been published.

Therefore, this study aimed to clarify the clinicopathological and prognostic significance of an s-MK-positive status in patients with HCC.

Materials and methods

Patients. This study was registered as UMIN000014530. Serum samples were obtained before surgery from 123 patients with HCC who had undergone surgery at Omori Medical Center, Toho University School of Medicine, between January 2012 and December 2020. In total, 123 patients with histologically proven primary HCC were enrolled. The patient cohort

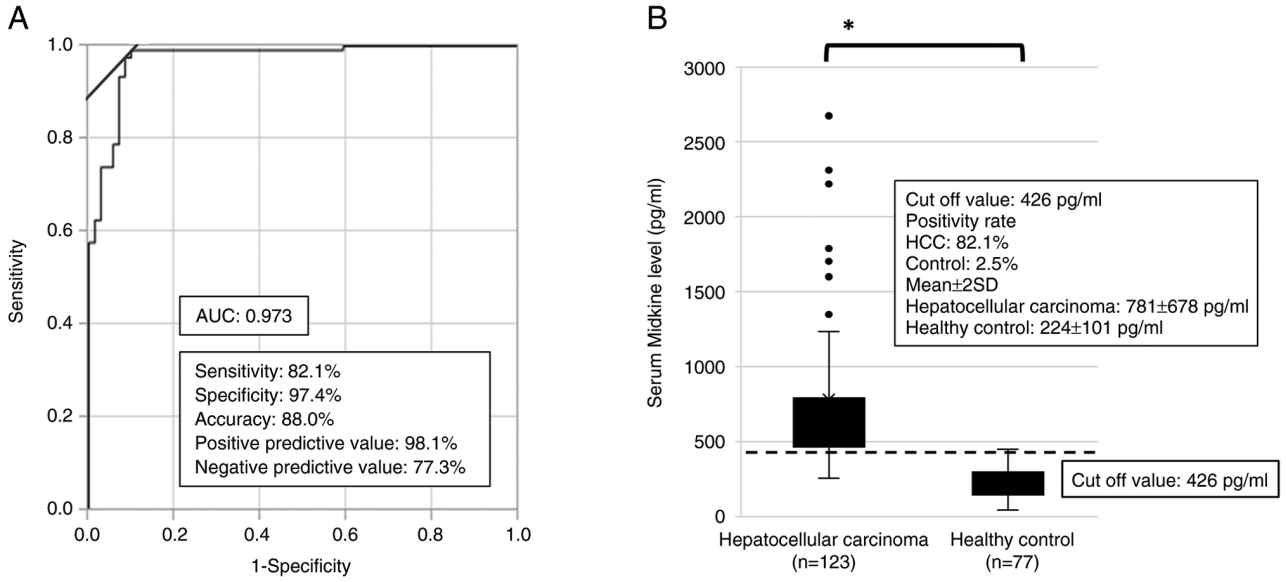

Figure 1. Receiver operating curve and a box-and-whisker plot for serum midkine. (A) Receiver operating characteristic curve showing the diagnostic performance of serum midkine for discriminating the hepatocellular carcinoma group from the healthy group. (B) Serum midkine expression is upregulated in the hepatocellular carcinoma group compared with the healthy group. Data are shown in a box-and-whisker plot (median, 25th, and 75th percentile, range, and extreme values outside the range). *P<0.05. AUC, area under the curve; HCC, hepatocellular carcinoma; SD, standard deviation.

consisted of 87 male (70.7%) and 36 female (29.3%) patients, with a median age of 69 (range, 40-85) years. To ensure complete absence of the influence of previous cancer, those with active coexisting cancer, i.e., synchronous coexisting cancer or metachronous cancer within 5 disease-free years, were excluded. The final HCC stage was assessed pathologically following the tumor-node-metastasis classification criteria of the eighth edition of the International Union against Cancer (17). Tumors associated with distant metastasis, including peritoneal dissemination, were considered unresectable. Hepatectomy was performed according to the treatment algorithm described in Japanese guidelines (18,19). The degree of liver damage is defined by the following factors: Ascites, serum total bilirubin level, serum albumin level, ICG R15, prothrombin activity value (20).

Data collection and serum biomarker analyses. Serum samples were obtained before surgery and stored at -80˚C until analysis. Serum samples of healthy controls, with no previous malignant disease and hepatitis B or C infection, were obtained from Biobank Japan. The average age of the control group (n=77) was 52 years, with a male-to-female ratio of 50:27.

Clinicopathological characteristics, AFP, and PIVKA-II were analyzed. Preoperative variables, pathological characteristics, postoperative status, and survival were entered into a spreadsheet and imported to a dedicated database. The prognostic value and clinical utility of s-MK for HCC diagnosis were estimated. Overall survival was calculated from the time of surgery until death or study conclusion.

Enzyme-linked immunosorbent assay kits for human MK (CDYELISA, Immuno-probe Ltd., Saitama, Japan) were used for detecting s-MK according to the manufacturer's protocol. The cutoff value for s-MK was fixed at 426 pg/ml based on the receiver operating characteristic curve (Fig. 1A).

Patients' clinicopathological variables, demographics, tumor characteristics, and overall survival were compared between the s-MK-positive group and s-MK-negative group. The cutoff values were 10.0 ng/ml and 40.0 mAU/ml for AFP and PIVKA-II, respectively, following the assay kit manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using JMP version 12 (SAS Institute, Cary, NC, USA). The comparison of s-MK levels in the HCC and healthy control groups was performed using unpaired t-test. A multiple comparison test of ANOVA was performed to compare the positivity rates of s-MK, AFP, and PIVKA-II according to TNM stages. We selected the Bonferroni post hoc test as multiple comparison test. Between-group comparisons of the clinicopathological variables were performed using Fisher's exact probability test. Overall survival was calculated using the Kaplan-Meier product limit estimate. Between-group differences in survival were compared using the log-rank test. Significant predictors were identified via univariate and multivariate analyses using Cox proportional hazard models, and hazard ratios with 95% confidence intervals (CIs) were calculated. A P value of <0.05 was considered statistically significant.

Results

Sensitivity and specificity of serum MK levels. Based on the ROC curve, the best cutoff point was determined to distinguish the HCC group using s-MK. The area under the curve for s-MK was 0.973 (95% CI 0.903-0.992) (Fig. 1A). According to the curve, the best cutoff value for s-MK in differentiating HCC from healthy cases was 426 pg/ml. At this value, the sensitivity, specificity, and accuracy were 82, 97, and 88%, respectively. The mean s-MK levels in the HCC and healthy control groups were 781±678 and 224±101 pg/ml, respectively (Fig. 1B, P<0.05).

Table I. Comparisons between serum midkine level according to clinicopathological factors and various biomarkers.

Variables	Groups	Number of patients (n=123)	Midkine level (median) (pg/ml)	P value[a]	No. of Midkine-positive patients (%)[b]	P-value[c]
Sex	Male	87	605 (452-786)	0.740	71 (81)	0.819
	Female	36	561 (486-937)		30 (83)	
Hepatitis B virus	Positive	24	561 (410-791)	0.268	16 (67)	0.038
	Negative	99	616 (486-791)		85 (85)	
Hepatitis C virus	Positive	57	654 (469-791)	0.504	49 (86)	0.297
	Negative	66	568 (461-792)		52 (79)	
Child-Pugh classification	A	118	588 (466-787)	0.034	96 (81)	0.155
	B	5	812 (731-901)		5 (100)	
Liver damage	A	99	616 (452-791)	0.652	81 (81)	0.861
	B	24	555 (492-792)		20 (83)	
Liver background	Normal	29	730 (452-953)	0.809	23 (79)	0.153
	CH	85	795 (481-787)		69 (81)	
	LC	9	721 (407-937)		9 (100)	
Tumor size, mm	<20	37	537 (486-687)	0.138	31 (83)	0.749
	≥20	86	616 (452-855)		70 (81)	
Tumor number	1	100	561 (446-730)	0.004	78 (78)	0.001
	2-	23	786 (574-1198)		23 (100)	
Differentiation	Well	18	795 (567-937)	0.597	18 (100)	0.054
	Moderate	101	726 (609-844)		79 (78)	
	Other	4	763 (415-1111)		4 (100)	
Microvascular invasion	Positive	53	771 (603-939)	0.763	41 (77)	0.233
	Negative	70	788 (614-962)		60 (85)	
Stage	I, II	63	554 (486-687)	0.056	53 (84)	0.550
	III, IV	60	696 (446-949)		48 (80)	
AFP, ng/ml	≤10	71	554 (452-738)	0.311	57 (80)	0.533
	>10	52	629 (511-799)		44 (84)	
PIVKA-II, mAU/ml	≤40	63	580 (452-873)	0.391	51 (81)	0.730
	>40	60	597 (486-731)		50 (83)	

[a]Mann-Whitney U test. [b]A serum midkine level of ≥421 pg/ml was the cut-off for Midkine-positive. [c]Fisher's exact probability test. SD, Standard deviation; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; CH, chronic hepatitis; LC, liver cirrhosis.

Comparison of clinicopathological characteristics between the s-MK-positive group and s-MK-negative group. Of the 123 patients enrolled, 101 (82%) were positive for s-MK (>426 pg/ml) (Table I). An s-MK-positive status was significantly associated with hepatitis B virus negativity and number of tumors (≥2) but not with the liver reserve or liver background.

Positivity rates of s-MK, AFP, and PIVKA-II according to TNM stages. The positivity rates of s-MK were significantly higher than those of AFP and PIVKA-II (P<0.05, Fig. 2A). In total, only 28% (34 of 123) of the patients were positive for s-MK. Among patients with stage I/II, only 33% (21 of 63) were positive for s-MK (Fig. 2B). Even among patients with stage III/IV, only 22% (13 of 60) were positive for s-MK (Fig. 2C).

Fig. 3A shows the positivity rates for s-MK, AFP, and PIVKA-II at each TNM stage. In stage I, the positivity rate for s-MK was significantly higher than that for AFP and PIVKA-II (83% vs. 31% vs. 31%, P<0.05). In stage II, the positivity rates for s-MK, AFP, and PIVKA-II were 86, 50, and 43%, respectively (P<0.05). In stage III, the positivity rates for s-MK, AFP, and PIVKA-II were 76, 39, and 63%, respectively (not significant). In stage IV, the positivity rates for s-MK, AFP, and PIVKA-II were 56, 78, and 56% (not significant), respectively.

The positivity rate for the combined use of s-MK and AFP + PIVKA-II was significantly higher than that for AFP + PIVKA-II (93% vs. 65%, P<0.05, Fig. 3B). In stage I, the positivity rate for the combined use of s-MK and AFP + PIVKA-II was significantly higher than that for AFP + PIVKA-II (94% vs. 51%, P<0.05). Moreover, in stage II, the positivity rate for the combined use of s-MK and AFP + PIVKA-II was significantly higher than that for AFP + PIVKA-II (100% vs. 79%, P<0.05). In stage III, the positivity rate for the combined use of s-MK and AFP + PIVKA-II

Table II. Univariate and multivariate analysis of risk factors for overall survival in 123 patients.

Variables	Groups	Univariate P-value[a]	Multivariate HR[b]	Multivariate 95% CI[c]	Multivariate P-value[d]
Hepatitis B virus	Positive/negative	0.128			
Hepatitis C virus	Positive/negative	0.339			
Child-Pugh classification	B/A	<0.001	2.007	0.526-7.556	0.298
Liver damage	B/A	0.029	2.100	0.738-5.248	0.153
Liver background	LC/CH/normal	0.599			
Tumor size, mm	≥20/<20	0.667			
Tumor number	≥2/1	0.072			
Differentiation	Well/moderate/other	0.614			
Microvascular invasion	Positive/negative	0.355			
AFP, ng/ml	>10/≤10	0.315			
PIVKA-II, mAU/ml	>40/≤40	<0.001	3.759	1.600-9.603	0.002
Serum midkine, pg/ml	>426/≤426	0.007	5.157	1.483-32.553	0.006

[a]Log-rank test. [b]Adjusted hazards ratio. [c]Adjusted 95% confidence interval. [d]Logistic regression analysis. AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; CI, confidence interval; CH, chronic hepatitis; LC, liver cirrhosis; HR, hazard ratio.

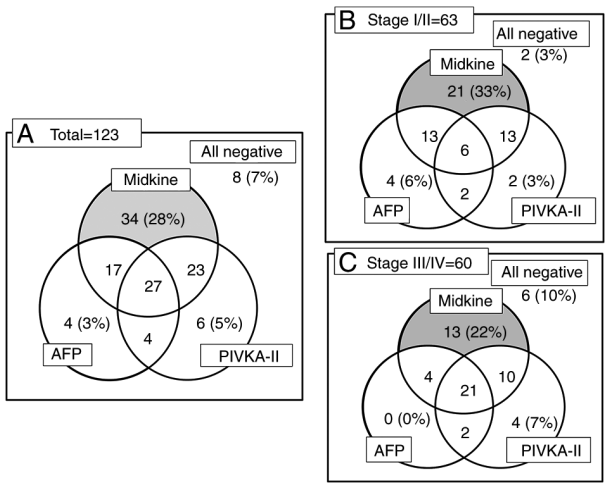

Figure 2. Relationship between positive serum tumor marker findings in patients with hepatocellular carcinoma. (A) All patients, (B) stage I/II patients and (C) stage III/IV patients. AFP, α-fetoprotein; PIVKA-II, protein-induced by vitamin K absence-II.

was significantly higher than that for AFP + PIVKA-II (88% vs. 67%, P<0.05).

Prognostic effect of s-MK, AFP, and PIVKA-II status on overall survival. The 5-year overall survival according to the s-MK, AFP, and PIVKA-II status is shown in Fig. 4. Although no significant difference was observed in the overall survival according to the AFP status (Fig. 4B, P=0.315), the s-MK-positive group showed significantly worse overall survival than the s-MK-negative group (Fig. 4A, P=0.007). Similarly, the PIVKA-II-positive group showed significantly poorer overall survival than the PIVKA-II-negative group (Fig. 4C, P<0.001).

Fig. 5 shows the comparison of overall survival at stages I/II and III/IV according to the s-MK, AFP, and PIVKA-II status. Regarding the prognostic effect of the s-MK status, the s-MK-positive group in stage I/II showed slightly worse overall survival than the s-MK-negative group (Fig. 5A, P=0.116). The s-MK-positive group in stage III/IV showed significantly worse overall survival than the s-MK-negative group (Fig. 5B, P=0.048). No significant difference was observed in the overall survival according to the AFP status (Fig. 5C and D, P=0.818, P=0.127). In contrast, a significant difference was observed in overall survival according to the PIVKA-II status (Fig. 5E and F, P=0.015, P=0.007).

Recurrence effect of s-MK status on recurrence-free survival. The 5-year recurrence-free survival according to the s-MK status is shown in Fig. 6. The s-MK-positive group showed significantly worse recurrence-free survival than the s-MK-negative group (Fig. 6A, P<0.001). The s-MK-positive group in stage I/II and III/IV showed significantly worse recurrence-free survival than the s-MK-negative group (Fig. 6B and C, P<0.001).

Univariate and multivariate analyses of overall survival. In the univariate analysis, the Child-Pugh classification (B), liver damage (B), PIVKA-II-positive status, and s-MK-positive status were significantly associated with poor prognosis (Table II). In the multivariate analysis, PIVKA-II-positive status (P=0.002; HR=3.759; 95% CI 1.600-9.603) and s-MK-positive status (P=0.006; HR=5.157; 95% CI 1.483-32.553) were independently associated with poor prognosis.

Discussion

The positivity rate for s-MK was 82% in patients with HCC. The positivity rate for the combined use of s-MK and AFP + PIVKA-II was significantly higher than that for AFP + PIVKA-II. An s-MK-positive status was associated

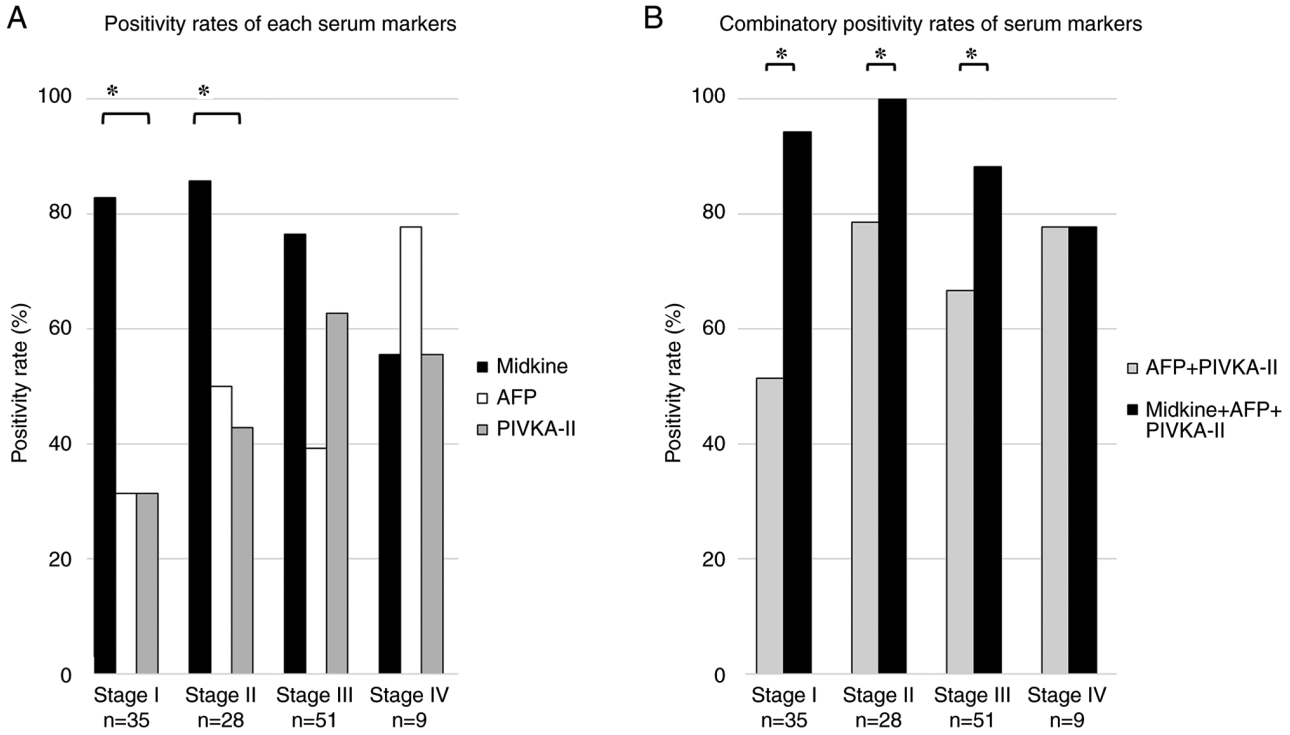

Figure 3. Positivity rates of serum tumor markers in hepatocellular carcinoma. (A) Comparison of the positivity rates of serum tumor markers. (B) Comparison of the positivity rates between AFP/PIVKA-II and AFP/PIVKA-II/Midkine. *P<0.05. AFP, α-fetoprotein; PIVKA, protein induced by vitamin K absence I.

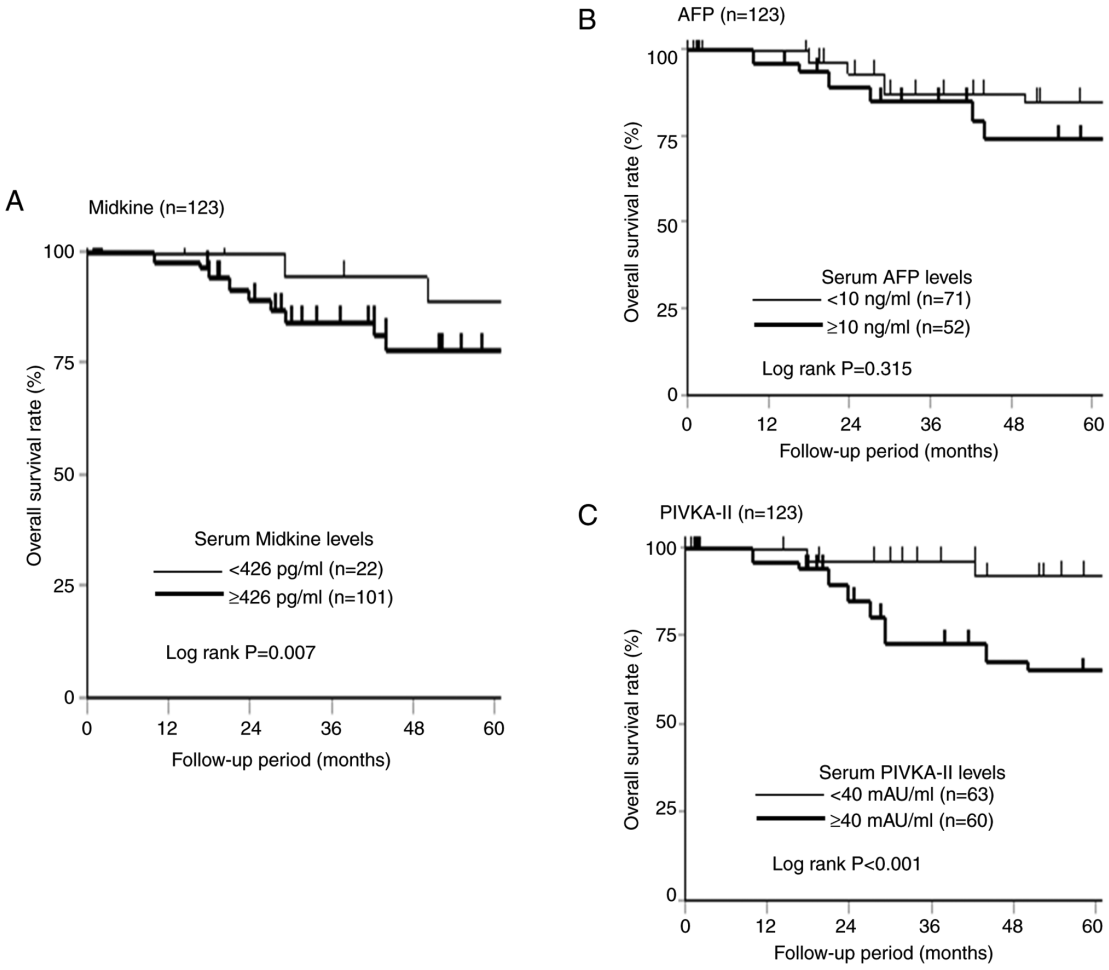

Figure 4. Overall survival for midkine, AFP and PIVKA-II. Comparison of overall survival between the (A) positive and negative midkine groups, (B) AFP groups and (C) PIVKA-II groups. AFP, α-fetoprotein; PIVKA, protein induced by vitamin K absence.

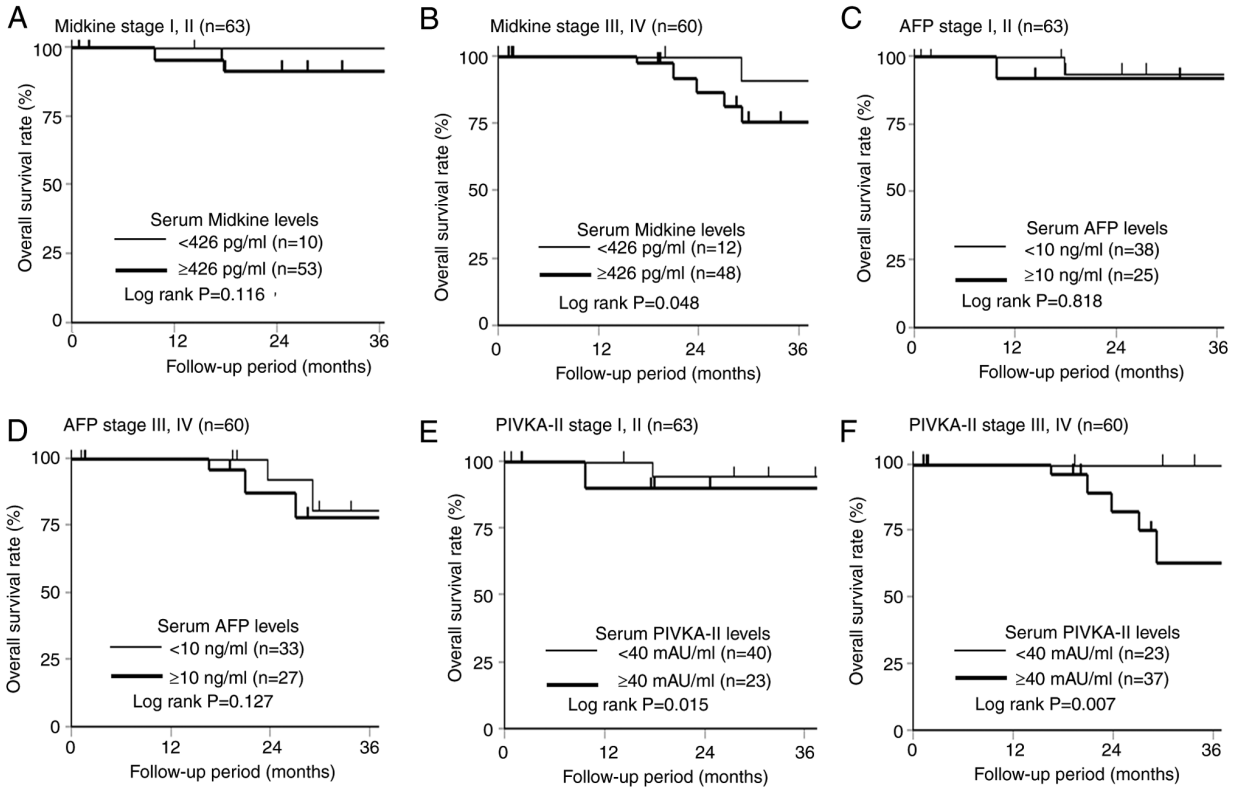

Figure 5. Overall survival for midkine, AFP and PIVKA-II at stages I/II and III/IV. Comparison of overall survival between the positive and negative midkine groups for (A) stage I/II, (B) midkine for stage III/IV, (C) AFP for stage I/II, (D) AFP for stage III/IV, (E) PIVKA-II for stage I/II and (F) PIVKA-II for stage III/IV. AFP, α-fetoprotein; PIVKA, protein-induced by vitamin K absence.

with the number of tumors. The s-MK-positive group showed poor overall survival.

An s-MK-positive rate was not associated with stage, and this tendency was similar to the pattern of serum autoantibodies, as previously reported (5,6). s-MK is induced not only by cancer but also by various factors such as inflammation and hemodynamics (21). At present, even in HCC, which has multistage carcinogenesis, the stage at which s-MK is induced is unclear. Shaheen *et al* reported that the s-MK level was significantly elevated in the HCC group compared with the healthy control group and liver cirrhosis group (22). These findings suggest that s-MK can be used to detect early-stage cancer follow up patients with cirrhosis.

In the present study, s-MK was associated with the number of tumors but not with liver background or tumor size. Among the 23 patients with multiple tumors, the positivity rates for s-MK, AFP, and PIVKA-II were 100, 69, and 43%, respectively. This may be because MK plays an important role in cell proliferation, survival, migration, angiogenesis, and carcinogenesis (23,24). Whether s-MK is a cause or a consequence of multiple tumors is unclear. However, given that an s-MK-positive status is a poor prognostic factor, an s-MK-positive status may reflect the biological grade of the tumor.

The prognostic effect of s-MK on various cancers was not consistent. In this study, we first evaluated the prognostic effect of s-MK on HCC. An s-MK-positive status was an independent risk factor for poor overall survival. The poor prognostic effect of an s-MK-positive status in HCC suggests the high biological malignancy of s-MK-positive HCC cells, given the lack of correlation between an s-MK-positive status and cirrhosis. MK-positive cancer cells have been reported to be associated with antiapoptotic function, and resistance to chemotherapy after HCC recurrence may contribute to poor prognosis (25). Considering that miRNA519d, an exosome derived from HCC, can inhibit apoptosis and distinguish between cirrhotic patients without HCC and cirrhotic patients with early-stage HCC, miRNA519d and s-MK may have a common mechanism (26). Considering the results of the IMbrave050 trial, patients with an s-MK positive status who are at a high risk of recurrence may be able to effectively prolong their recurrence-free survival by receiving adjuvant atezolizumab plus bevacizumab (27).

This study had some limitations. First, the sample size was not large enough. Assuming a 95% confidence level and a 5% confidence interval, we were unable to collect a sample size large enough for this study. Second, no data were available for evaluating the association between s-MK positivity and the immunoreactivity of cancer cells. Since several previous studies have reported that s-MK concentrations are significantly associated with immunoreactivity, MK expression in cancer cells may similarly be associated with s-MK (28,29). Third, we did not analyze the other cytokines, such as serum vascular endothelial growth factor (VEGF), in this study. Alzamzamy *et al* reported that in patients with HCV, serum VEGF and VEGF/PLT separately or in combination with AFP are reliable biomarkers for early and accurate HCC diagnosis (30). Furthermore, Mamdouh *et al* reported that the serum VEGF levels in patients with HCC and cirrhosis were significant compared with the control group (31). It is possible that s-MK, together with cytokines such as VEGF, will play a major role in the diagnosis of

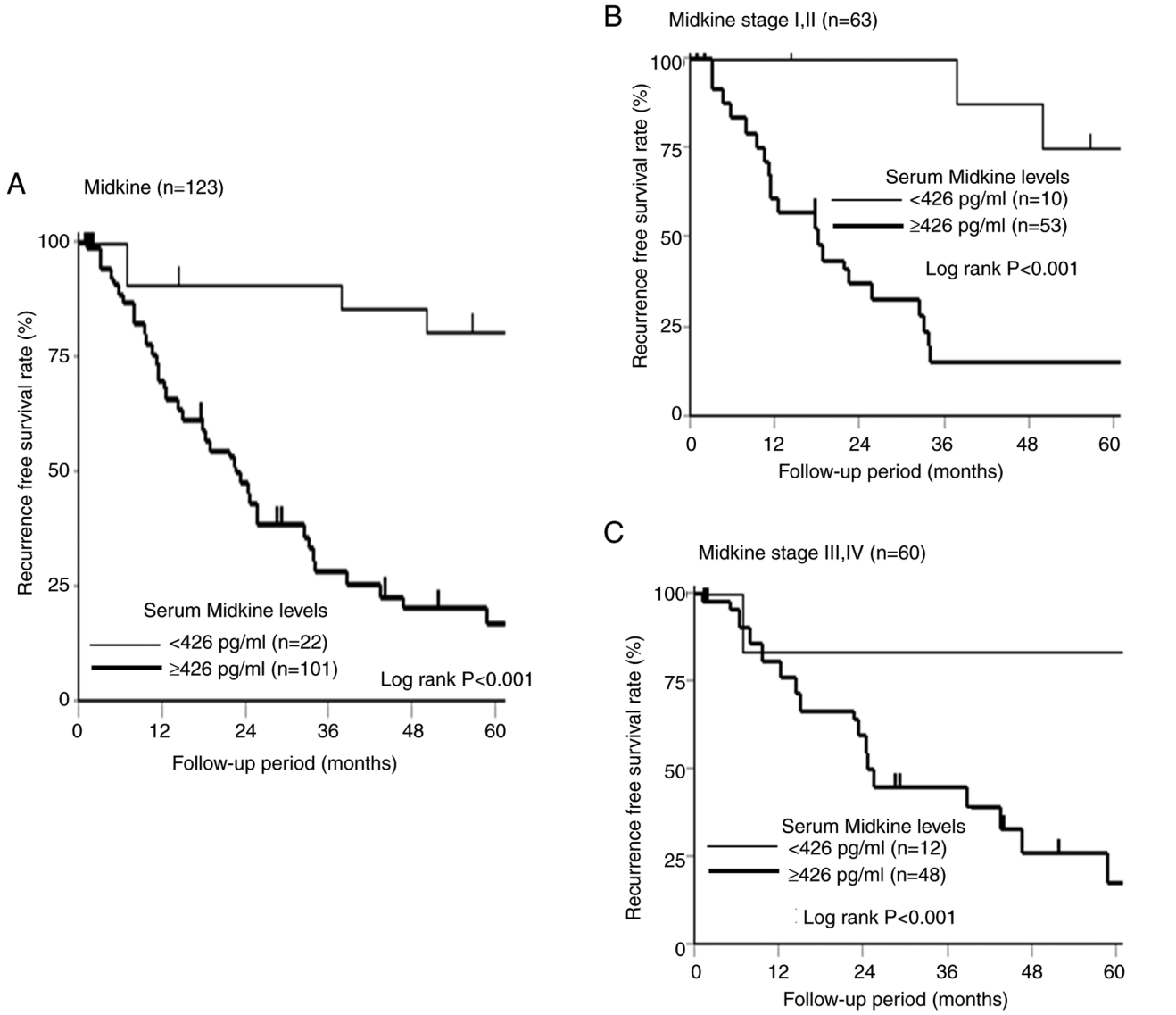

Figure 6. Recurrence-free survival for midkine at stages I/II and III/IV. Comparison of recurrence-free survival between the (A) positive and negative midkine groups for (B) stage I/II and (C) stage III/IV.

hepatocellular carcinoma in the future. Fourth, this study only focused on preoperative s-MK and had no data of postoperative monitoring. Therefore, we could not capture changes in s-MK levels before and after surgery. The s-MK level was reported to decrease significantly after surgery in esophageal cancer (28).

In conclusion, s-MK was a convenient and useful serum biomarker to detect HCC even in patients with stage I/II regardless of LC. An s-MK-positive status was associated with the number of tumors and was an independent prognostic risk factor. Considering the malignant potential of s-MK-positive HCC, more intensive follow-up is necessary after surgery.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

RO and HS confirmed the authenticity of all the raw data. RO conceptualized and designed the study, performed the statistical analysis and prepared the manuscript. YO, YK, TM, JI, KK, YM, YI and KF acquired the data. RO and YO performed the quality control of data and algorithms. RO, YO and HS analyzed and interpreted the data. RO and HS edited the manuscript. All authors reviewed the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All study participants provided consent for future analyses of their blood samples for research. The protocol for this study was

approved by the Ethics Committee of Toho University (approval nos. M22211, M21038_20197_19213 and A18103_A17052_A16035_A16001_26095_25024_24038_22047_22112). Patients provided written informed consent before enrolment. The study was registered in the UMIN Clinical Trials Registry (clinical trial no. UMIN000014530) and was conducted following the guidelines of the Declaration of Helsinki and the Japanese Ethical Guidelines for Clinical Research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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