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Full Length Research Paper

Detection of serum midkine levels in cancer patients using rabbit anti-human midkine monoclonal antibodies

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Midkine (MK) is a heparin-binding growth factor and was found to be highly expressed in many types of human carcinomas. MK may become a novel tumor marker. In this study, we used the rabbit specific antibodies against human MK to establish a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) of MK system and applied it to detect serum MK levels in different types of cancer patients. The standard curve, precision and recovery rate were tested, respectively, and serum MK concentration of 102 cancers patients and 102 normal individuals were detected using this method. The detection range of this method was 0.2 to 10 ng/ml (R^2 = 0.97). The average intra and intro-assay coefficients of variation (CVs) were 3.6 and 7.9%, respectively. The average recovery rate was 89.9% when some standard antigens were added into the serum. The medians (25th and 75th percentiles) of serum MK levels were 1.35 ng/ml (0.96 and 1.64) in cancer patients and 0.30 ng/ml (0.23 and 0.38) in the controls; the MK levels of the patients were significantly higher than those of the controls (P<0.05). Moreover, 87.2% of the patients showed more than 0.6 ng/ml levels of MK. Serum MK could serve as a general tumor marker with a good potential for clinical application.

Key words: Midkine, rabbit monoclonal antibody, sandwich ELISA, tumor marker.

INTRODUCTION

Midkine (MK) is a heparin-binding growth factor found as the product of a retinoic acid-responsive gene (Kadomatsu et al., 1988). It has 45% sequence identity to pleiotrophin [(PTN)/heparin-binding growth-associated molecule (HB-GAM)], and together, these molecules comprise a family of heparin-binding growth factors (Tomomura et al., 1990). MK has been implicated in diverse activities, for example, it enhances angiogenic activities of tumour cells (Choudhui et al., 1997), it promotes cell survival and cell migration, and is deeply involved in cancer progression. It is highly expressed at both mRNA and protein levels in many human carcinomas, such as breast, lung, esophageal, stomach, colorectal, liver, ovary, urinary bladder and prostate carcinomas, glioblastomas, neuroblastomas and Wilms' tumours (Muramatsu et al., 1996; O'Brien et al., 1996; Takada et al., 1997). Furthermore, MK is a secreted protein; serum MK levels are expected to increase when tumors tissues express abundant MK. So, it is probable that MK would be a marker for diagnostic tumors through the detection of the levels of MK in the patients' serum. In this study, we employed sandwich ELISA to analyze serum samples from 102 cancer patients and 102 controls. It was suggested that sandwich ELISA of MK would likely be applied to assist in the diagnosis of various tumors and that is has enormous clinical marker.

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Abbreviations: CVs, Coefficients of variation; **ELISA,** enzyme-linked immunosorbent assay; **HB-GAM**, heparin-binding growth-associated molecule; **MK,** midkine**; PTN**, pleiotrophin.

# These authors contributed equally to this work.

MATERIALS AND METHODS

MK monoclonal antibodies

The rabbit anti-human midkine monoclonal antibodies were preserved in our laboratory. As described previously, prokaryotic expressed MK protein were immunize into rabbits to prepare monoclonal antibodies against human MK by the rabbit hybridoma

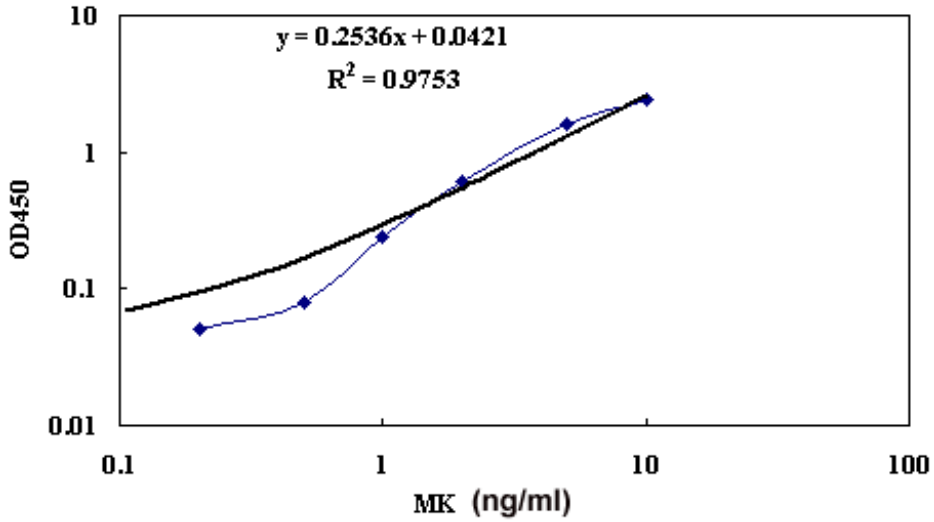

Figure 1. Standard curve of sandwich ELISA using recombinant MK. MK protein was measured by a sandwich ELISA using purified anti-MK antibody.

technique, then hybridoma cell line secreting anti-MK mAb was obtained and the monoclonal antibodies were purified (Yao et al., 2011).

Serum samples

Sera were obtained from blood collected from the cancer patients and normal individuals. The patients with inflammatory condition such as diabetes mellitus, rheumatoid arthritis and hepatitis were excluded. Informed consent was obtained from patients and normal individuals.

Cancer patients (n = 102; male = 79; female = 23; ages 31 to 85; average value = 58.6) and normal individuals (n = 102; male = 79; female = 23; ages 20 to 81; average value = 50.3) were used. 3 ml of sera were immediately frozen and kept at -80°C until the assay.

Construction and application of ELISA for detection of serum MK levels

Each well of a 96-well microplate was coated with 100 µl of goat anti-human MK poly antibody (R&D, Minneapolis, USA) diluted to 1.25, 2.5, 5, 7.5 and 10 µg/ml, respectively, then was incubated for 30 min at 37°C followed by overnight incubation at 4°C. After washing wells with PBS containing 0.05% Tween 20 (PBST), 300 µl of 1% bovine serum albumin in PBST was added to each well to block non-specific binding sites. After 1 h incubation at room temperature, wells were washed three times with PBST, 100 µl of 5 ng/ml purify MK protein was added and incubated for 1 h at room temperature. Then, wells were washed three times with PBST; incubated with 100 µl of rabbit anti-human mAb of MK for 1 h at room temperature. The wells were again washed, and the HRP conjugated IgG (1:1000) was added for 1 h at room temperature, and finally, incubated with a substrate solution (100 µl TMB) for 10 min. The reaction was stopped by adding 100 µl of 2 N sulphuric acid, and OD_{450} was detected using a multiplate reader.

Accurate sample concentrations of human MK were determined by comparing specific absorbances with those obtained from the standards plotted on a standard curve. Serum from a healthy donor was used as a negative control. The ELISA was subsequently used in a blinded study to measure MK protein in the serum from cancer patients.

Statistics

Data were analyzed with SPSS software 12.0. As the serum MK levels of patients were non normal distribution, the data were presented as median with percentile values. Difference between the two groups was evaluated by non-parametric Mann-Whitney U test. Correlation was examined by Spearman's correlation coefficient. Differences were considered to be statistical significant when the *P* value was less than 0.05.

RESULTS AND DISCUSSION

Establishment of sandwich ELISA of MK

A sandwich ELISA was developed using the anti-human MK ploy and monoclonal antibody. The standard curve using purified recombinant MK is shown in Figure 1, with sandwich ELISA. In this ELISA, dose-dependent increase in the absorbance at 450 nm enables the measuring of MK ranging between 0.2 and 10 ng/ml. The intra- and inter-assay variabilities of the ELISA curve for MK were used to show the precision of this method. The intra-assay variability was given by the average of nine replicated wells in 1 microplate. The interassay variability was given by the average of eight replicated microplates at various times. The intra- and inter-assay coefficients of variation (CVs) were 4.16, 3.12, 3.52 and 4.25, 9.77 and 8.29%, respectively (Table 1), which showed that it was feasible to apply this method to detect MK in various tumor serum. The accuracy of MK sandwich ELISA was evaluated with recovery rate analysis. The average recovery rate of MK in the serum was 89.9%. The

Table 1. Coefficients of variation of MK level by sandwich ELISA assay.

Sample ID	Intra-assay (n = 8)	CV (%)	Inter-assay (n = 5)	CV (%)
	Mean ±SD (ng/ml)		Mean ±SD (ng/ml)	
1	1.68±0.07	4.16	0.94±0.04	4.25
2	1.28±0.04	3.12	1.33±0.13	9.77
3	4.54±0.16	3.52	4.34±0.36	8.29

n represents the number of replications.

Table 2. Precision of MK from serum by sandwich ELISA assay.

Midkine (ng/ml)	Measure (ng/ml)	Recovery rate (%)
1.01	0.92	91.1
1.76	1.53	86.9
2.51	2.13	84.9
2.87	2.94	92.4
3.62	3.29	90.9
4.37	4.06	92.9

Table 3. Application of sandwich ELISA for the detection of MK in various tumors serum.

Tumor	Number	Male/female	Year (mean)	MK (ng/ml) (25th and 75th percentiles medians)	*P*
Lung carcinoma	13	12/1	63.1	0.93 (0.52 and 1.56)	< 0.05
Gastric carcinoma	40	10/30	58.5	1.43 (1.19 and 1.77)	< 0.05
Esophageal carcinoma	12	12/0	63.5	1.23 (0.97 and 1.46)	< 0.05
Colon carcinoma	19	12/7	58.5	1.26 (0.96 and 1.47)	< 0.05
Hepatocellular carcinoma	11	9/2	49.2	1.23 (0.56 and 1.38)	< 0.05
Pancreatic carcinoma	7	4/3	55.4	1.48 (0.92 and 2.64)	< 0.05
Patients	102	79/23	58.6	1.35 (0.96 and 1.64)	< 0.05
Controls	102	79/23	50.3	0.30 (0.23 and 0.38)	

samples' recovery rates are given in Table 2.

Application of ELISA to detect serum MK levels in cancer patients

The median (25th and 75th percentiles) of serum MK levels of the 102 control individuals was 0.30 ng/ml (0.23 and 0.38), and no case had the value above 0.6 ng/ml. The median (25th and 75th percentiles) of serum MK levels of 102 cancer patients was 1.35 ng/ml (0.96 and 1.64). The serum MK levels of the cancer patients were higher than those of the controls ($P < 0.05$). Furthermore, the serum MK levels of 102 cancer patients in the six types of tumors showed a profile that was significantly different from those of the normal controls ($P < 0.05$) (Table 3). 87.2%of the cancer patients had a serum MK value higher than 0.6 ng/ml. Recently, it is was demonstrated that MK expressed in carcinomatous tissues and secreted into the bloodstream, leads to an increase in serum MK level in cancer patients, and did not show specification for a particular tissue. So, it is suggested that MK would likely be a novel marker for diagnosing tumors.

The ELISA for MK reported previously (Muramatsu et al.,1996) employed just polyconal anti-MK antibodies to detect MK level. In this study, we raised rabbit monoclonal antibodies against human MK to develop a sandwich immunoassay for the detection of levels of MK in the serum. More importantly, the ELISA was advantageous in that it uses an ordinary colorimetric detection system that is more convenient for clinical use. Indeed, a combination of two anti-human MK antibodies exhibited a much higher sensitivity in the detection of human MK than rabbit antibodies alone. Using this procedure, we analyzed sera from a large number of patients and found that serum MK levels were elevated in most of the cancer patients examined. The ratio of patients with increased serum MK levels to a total of cancer patients examined agreed with that of patients with

increased MK expression in tumor tissues reported previously (Kato et al., 2000a, b; Koide et al., 1999; Obata et al., 2005; Ren et al., 2006; Shimada et al., 2003). When the cutoff value was 0.6 ng/ml, the sensitivity and specificity of this assay was comparable to or higher than those of previous methods for the detection of serum MK (Ikematsu et al, 2000; Shimada et al., 2003a,b).

Future studies are required to show whether the determination of serum MK through sandwich ELISA can serve as a marker in the detection of carcinomas at the early stages.

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