Preoperative serum midkine concentration is a prognostic marker for esophageal squamous cell carcinoma

Hideaki Shimada,[1] Yoshihiro Nabeya,[1] Masatoshi Tagawa,[2] Shin-ichi Okazumi,[1] Hisahiro Matsubara,[1] Kenji Kadomatsu,[3] Takashi Muramatsu,[3] Shinya Ikematsu,[4] Sadatoshi Sakuma[5] and Takenori Ochiai[1]

[1]Department of Academic Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, [2]Division of Pathology, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260-8717, [3]Department of Biochemistry, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, [4]Research & Development Section II, Pharmaceuticals Development, Meiji Milk Products Co., Ltd., 540 Narita, Odawara 250-0862 and [5]Cell Signals Co., Ltd., 75-1 Onomachi, Tsurumi-ku, Yokohama 230-0045

(Received January 14, 2003/Revised April 2, 2003/2nd Revised April 25, 2003/Accepted April 25, 2003)

High preoperative serum midkine concentration is associated with poor survival in patients with esophageal cancer, even after radical surgery, and thus may have prognostic value. Midkine (MK), a heparin-binding growth factor, is expressed in numerous cancer tissues, and serum MK (S-MK) concentrations are increased in patients with various neoplasms. The aim of this study is to evaluate the clinical significance of S-MK in patients with esophageal squamous cell cancer (SCC). S-MK was measured by enzyme-linked immunosorbent assay in 135 healthy controls, 16 patients with benign esophageal disease, and 93 patients with primary esophageal SCC before surgery. The serum concentrations of carcinoembryonic antigen (CEA), SCC antigen (SCC-Ag), and cytokeratin 19 fragment (CYFRA21-1) were also evaluated. All patients with esophageal SCC underwent radical esophagectomy. Tumor MK expression was assessed by immunohistochemistry in 14 fresh tumor specimens. To determine whether S-MK is of value as a prognostic factor, the authors conducted a survival analysis using Cox's proportional hazards model. S-MK values in patients with esophageal SCC were significantly higher than those in healthy controls (417±342 pg/ml vs. 154±76 pg/ml, $P<0.001$). Using 300 pg/ml as the cut-off value (representing the mean +2 standard deviations of the S-MK of healthy controls), 61% of patients with esophageal SCC were classified as positive. MK expression by the tumor was significantly associated with high level of S-MK. High S-MK (≥300 pg/ml) was associated with tumor size, immunoreactivity and poor survival. Multivariate analysis indicated that S-MK was an independent prognostic factor. S-MK may be a useful tumor marker for esophageal SCC. Increased preoperative S-MK in patients with esophageal SCC is associated with poor survival. (Cancer Sci 2003; 94: 628–632)

Midkine (MK) is a heparin-binding growth factor, which was first reported as the product of a retinoic acid-responsive gene.[1)] It plays a role in neuronal survival and differentiation[2, 3)] and is also expressed at higher concentrations in various malignant tumors than in the adjacent normal tissue,[4–10)] even in the early stages of disease.[11, 12)] In neuroblastomas, glioblastomas, and urinary bladder cancers, MK protein expression was correlated with poor prognosis.[7, 13)] Because MK is a secreted protein, serum MK (S-MK) concentrations would be expected to increase when tumor tissues express abundant MK.

Recently, determination of MK expression by measuring S-MK in patients with cancer has been made possible by the development of enzyme-linked immunosorbent assays.[14–16)] Although MK protein expression was reported to be associated with poor prognosis in some types of cancer,[7, 13)] the clinicopathological significance of S-MK in patients with malignant tumors has not been examined. Esophageal carcinoma is one of the most lethal malignant tumors in the gastrointestinal carcinoma family. The aggressive behavior of this tumor is often associated with rapid growth and systemic spread of the disease.[17)] Serum concentrations of some types of growth factors are useful to predict treatment response and patients' outcome in esophageal squamous cell carcinoma (SCC).[18, 19)] These serum markers may complement the clinical impact of TNM staging. The purpose of this study was therefore to determine both the clinicopathological and prognostic significance of S-MK in these patients. To this end, we measured preoperative S-MK in 93 patients with primary esophageal SCC.

Patients and Methods

Patients and healthy controls. Ninety-three patients with primary SCC of the esophagus who have been treated by radical esophagectomy without any preoperative therapy, between 1997 and 2000, at the Department of Academic Surgery, Chiba University Hospital were investigated in this study. They consisted of 76 males (82%) and 17 females (18%) with a median age of 65 years (range 40–87 years) and were classified according to the TNM/UICC guidelines[20)] as follows: 15 stage I; 28 stage II; 25 stage III; and 25 stage IV. All patients with M1 showed no organ metastasis, but had distant lymph node metastasis. No patient had received blood transfusion, radiotherapy, or chemotherapy during the 6 months preceding this study. All patients with esophageal SCC underwent radical esophagectomy with three-field lymphadenectomy,[21)] defined as macroscopically complete removal of the tumor. For the entire population, the median follow-up period was 36 months (range 19 to 69 months).

One hundred and thirty-five healthy individuals and 16 patients with benign esophageal disease were also investigated as controls. Patient recruitment and sample collection were performed within the guidelines of protocols approved by the institutional review boards. Informed consent was obtained from all subjects. Healthy individuals consisted of 94 males (70%) and 41 females (30%) with a median age of 34 years (range, 21–75 years) and without evidence of comorbid disease (e.g., esophagitis, liver dysfunction, diabetes, etc.) as previously described.[16)] Patients with benign esophageal disease consisted of 12 males and 4 females with a median age of 59 years (range, 28–78 years) who served as non-cancer controls. This group included cases of achalasia (4 cases), reflux esophagitis (4 cases), benign stenosis (4 cases), leiomyoma (3 cases), and papilloma (1 case).

Serum sampling and enzyme immunoassay for S-MK. Sera were ob-

E-mail: hshimada@med.m.chiba-u.ac.jp

tained before surgery by venipuncture and immediately centrifuged at 3000*g* for 5 min. Sera were obtained again from 19 sero-positive patients 4 weeks after surgery. The serum was frozen at −80°C until the assay was performed. Repeated thawing and freezing of samples was avoided. S-MK was measured with solid-phase human MK immunoassay ELISA kit systems as previously described.[16] As a standard method, 50 μl of rabbit anti-human MK antibody at a concentration of 5.5 μg/ml in phosphate-buffered saline (PBS) was adsorbed onto the wells of microtiter plates (Polysorp plates, Nunc, Naperville, IL) for 16 h at room temperature. After washing with 1% Tween 20 in PBS, the wells were blocked with 150 μl of 0.5% bovine serum albumin in PBS for 16 h at 37°C. Control human MK samples or serum samples (10 μl each) were mixed with 100 μl of 50 m*M* Tris HCl (pH 8.4), 0.5 *M* KCl, 0.5% bovine serum albumin, 0.01% Microcide I (aMReSCO, Solon, OH) containing peroxidase-labeled chicken anti-human MK antibody (0.1 μg/ml). Aliquots of 50 μl of this mixture were added to the wells of microtiter plates prepared as described above. After incubation for 1 h at room temperature, the wells were washed five times with 1% Tween 20 in PBS. Aliquots of 100 μl of a substrate solution (tetramethylbenzidine at 0.5 mg/ml in Dako S1600, Dako, Kyoto) were added to the wells and the plates were incubated for 30 min at room temperature. The reaction was stopped by adding 100 μl of 2 *N* sulfuric acid, and $A_{450}$was detected using a multiplate reader (Model 3550, BioRad, Tokyo).

Immunohistochemistry (IHC). To determine the relationship between MK protein expression and S-MK, IHC analysis was performed using fresh tumor specimens which were surgically obtained from 14 patients with esophageal SCC.[9] The samples were fixed with 4% paraformaldehyde/phosphate-buffered saline at 4°C. Sections of 4–5 μm thickness were incubated with 10% skimmed milk for 20 min, and then with affinity-purified rabbit anti-MK antibody for 60 min. The sections were sequentially reacted with 0.3% H_2O_2/methanol, biotinylated anti-rabbit IgG, avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine, then washing with PBS after each reaction. The sections were then counter-stained with hematoxylin.

For microscopic analysis, the entire field of each section was examined, regardless of whether it stained positive. At least 500 carcinoma cells in each specimen were examined and positive staining was determined when more than 10% of the cells stained positive. The evaluation of IHC was conducted by a single investigator without prior knowledge of the S-MK or clinicopathological data.

Carcinoembryonic antigen (CEA), CYFRA21-1 and SCC antigen (SCC-Ag) assays. Serum CEA and CYFRA21-1 concentrations were measured with Enzymun-TEST CEA and Enzymun-TEST CYFRA21-1 (Boehringer Mannheim, Mannheim, Germany), respectively. SCC-Ag concentrations were measured in serum with an immunoradiometric assay kit (Dynabott, Tokyo). The cut-off values for serum CEA, CYFRA21-1 and SCC-Ag concentrations were 4.6 ng/ml, 3.5 ng/ml and 1.5 ng/ml, respectively, according to the manufacturer's instructions. The specificity at these cut-off values is 95%.

Statistical analyses. Data are expressed as mean±standard deviation. Comparisons between unpaired groups were made using the Mann-Whitney *U* test. Comparisons between paired groups were made using the paired *t* test. Survival probabilities were calculated by the product limit method of Kaplan and Meier. Differences between groups were tested using the log-rank test. Fisher's exact probability test was applied to determine the significance of differences between two groups. The influence of each clinicopathological variable on survival was assessed by means of Cox's proportional hazards model. All statistical analyses were carried out using the Stat View program (SAS Institute, Inc., Cary, NC), and *P* values <0.05 were considered to be statistically significant.

Results

S-MK and clinicopathologic features of esophageal SCC. The S-MK values in patients with esophageal SCC were significantly higher than those in patients with benign esophageal disease or healthy controls (417±342 pg/ml vs. 183±73 pg/ml or 154±76 pg/ml, $P<0.001$) (Fig. 1). Non-parametric approximation and univariate analyses indicated a cut-off concentration of 306 pg/ml for the 95% confidence interval. Changing the cut-off concentration to the more practical value of 300 pg/ml resulted in an increase of positive rate of S-MK to 61% in esophageal cancer with a specificity of 96.3%. The positive rate increased slightly at each stage as follows: 53% in stage I; 54% in stage II; 60% in stage III; and 76% in stage IV. Although no correlation was found between S-MK and gender, age, depth of invasion, nodal involvement, or distant metastasis, S-MK was significantly higher in large tumors than small tumors (Table 1).

S-MK and conventional tumor markers. In order to assess the comparative value of S-MK in detecting esophageal cancer, we also examined the sensitivity of three conventional tumor markers,

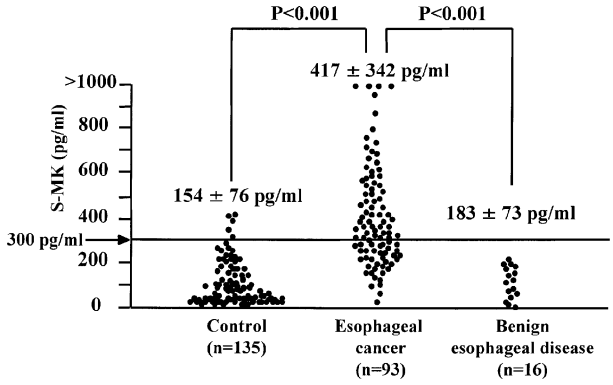

Fig. 1. The value and standard deviation of S-MK in patients with esophageal squamous cell carcinoma, benign esophageal disease and healthy controls. Statistical significance was determined by means of the Mann-Whitney *U* test.

Table 1. Relationship between S-MK and the clinicopathological variables in 93 patients with esophageal squamous cell carcinoma

Variables		Number of patients	S-MK (pg/ml)	*P*[1]
Gender	Male	76	388±237	0.401
	Female	17	547±627	
Age	<65	56	382±200	0.981
	≥65	37	471±485	
Tumor size	<50 mm	42	361±248	0.049
	≥50 mm	51	464±402	
Tumor depth	T1	23	461±566	0.441
	T2–T4	70	403±233	
N factor	N0	31	457±484	0.804
	N1	62	397±248	
M factor	M0	68	408±372	0.172
	M1	25	443±253	

1) According to the Mann-Whitney *U* test.

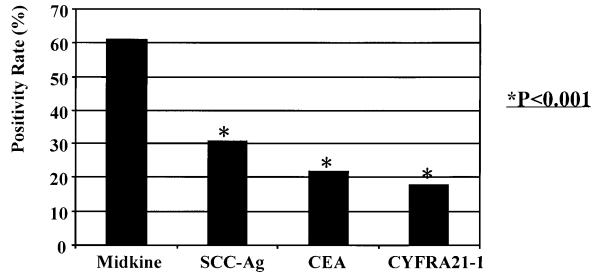

Fig. 2. Positive rate of S-MK and conventional tumor markers (SCC-Ag, CEA and CYFRA21-1) in 93 patients with esophageal squamous cell carcinoma. Statistical significance was determined by means of Fisher's exact test.

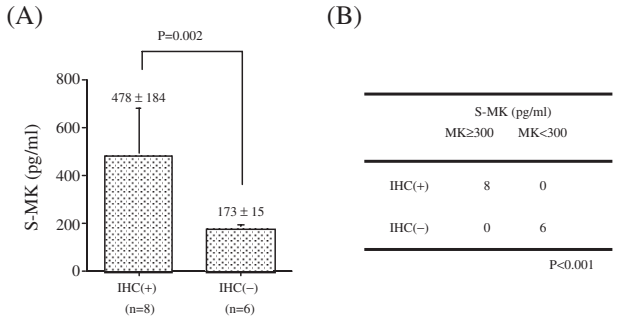

Fig. 3. Midkine immunoreactivity and S-MK in 14 patients with esophageal squamous cell carcinoma. (A) The mean S-MK and standard deviation are shown for the immunohistochemistry-positive and -negative groups. Statistical significance was determined by means of the Mann-Whitney *U* test. (B) Association of S-MK with immunoreactivity. Statistical significance was determined by means of Fisher's exact test.

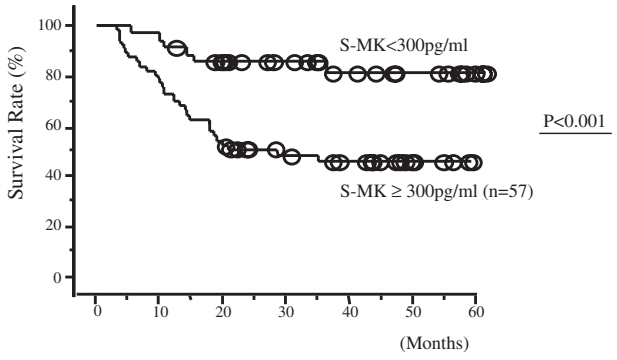

Fig. 4. Survival curves and pretreatment S-MK of 93 patients with esophageal squamous cell carcinoma. The low S-MK group includes patients with S-MK <300 pg/ml (n=36). The high S-MK group includes patients with S-MK ≥300 pg/ml (n=57). Circles depict survivors. Statistical significance was determined by means of the log-rank test.

CEA, SCC-Ag, and CYFRA21-1 (Fig. 2). The positive rate of S-MK was significantly higher than any of these conventional markers: S-MK, 61%; SCC-Ag, 31%; CEA, 22%; and CYFRA21-1, 18% (P<0.001).

Relationship between S-MK and MK immunoreactivity. IHC staining for MK was carried out to analyze the correlation between MK protein expression and S-MK (Fig. 3). Among 93 resected tumors, 14 tumors were analyzed and 8 tumors were MK IHC-positive. The mean S-MK in patients with IHC-positive tumors was significantly higher than that in patients with IHC-negative tumors (478±184 pg/ml vs. 173±15 pg/ml, Fig. 3A). High S-MK (≥300 pg/ml) was significantly associated with tumors expressing MK (Fig. 3B).

Monitoring of S-MK after surgery. The effects of surgical removal of tumors on S-MK were examined in 19 randomly selected patients out of 57 who were positive for S-MK before surgery. S-MK significantly decreased below 300 pg/ml in all patients 4 weeks after surgery (436±85 pg/ml vs. 179±55 pg/ml, P<0.001). Among 57 patients who were positive for S-MK before surgery, 30 patients died due to recurrent disease, 9 patients were alive with recurrent disease and 18 were alive without recurrent disease. Among a total of 39 patients who showed recurrent disease, blood samples were available from 16 patients 1 to 6 months before diagnosis of recurrence, and 11 of 16 patients showed increased levels of S-MK.

Relationship between S-MK and survival. The 5-year survival rate in the high S-MK group (≥300 pg/ml, n=57) was significantly worse than that in the low S-MK group (<300 pg/ml, n=36), 81.2% vs. 46.2%, respectively (log-rank test) (Fig. 4). The prognostic value of S-MK and other clinicopathological factors, such as gender, age, tumor size, and TNM factors, were assessed by univariate analysis (Table 2). When S-MK, tumor size and TNM factors were assessed by multivariate analysis using Cox's proportional hazards model, S-MK was identified as an independent prognostic factor (Table 3). A value of 300 pg/ml or more for S-MK yielded a relative risk of cancer death of 3.82, with 95% confidence limits raging from 1.54 to 9.43.

Table 2. Univariate analysis of patients with esophageal squamous cell carcinoma

Variables		Number of patients	5-year survival rate	$P^{1)}$
Gender	Male	76	62.3	0.150
	Female	17	46.3	
Age	<65	56	64.1	0.607
	≥65	37	68.0	
Tumor size	<50 mm	43	69.7	0.048
	≥50 mm	50	51.1	
Tumor depth	T1	23	87.0	0.006
	T2–T4	70	50.5	
N factor	N0	31	82.5	0.001
	N1	62	47.8	
M factor	M0	68	66.8	0.042
	M1	25	37.2	
S-MK	<300 pg/ml	36	81.2	<0.001
	≥300 pg/ml	57	46.2	

1) According to the log-rank test.

Table 3. Determination of risk factors affecting the survival rate of patients with esophageal squamous cell carcinoma by multivariate analysis

Variables		P value	Hazard ratio	95.0% CI
Tumor size	<50 mm vs. ≥50 mm	0.774	1.29	0.54–2.28
T1 vs. T2–T4		0.094	2.83	0.84–9.52
N0 vs. N1		0.019	3.42	1.21–9.52
M0 vs. M1		0.762	1.12	0.54–2.28
S-MK	<300 pg/ml vs. ≥300 pg/ml	0.004	3.82	1.54–9.43

Discussion

In the current study, S-MK was significantly elevated in patients with esophageal SCC as compared with healthy controls and patients with benign esophageal diseases. In addition, S-MK was associated with MK protein expression in cancer cells and poor survival. The sensitivity of S-MK to detect esophageal SCC was 61% at a cut-off value of 300 pg/ml, with a specificity of 96.3%. In this report, 19 patients with high S-MK were reexamined 4 weeks after surgery and, although the number of patients was small, each demonstrated a significant decrease in S-MK after surgery. This behavior is consistent with what would be expected of a tumor marker.

Although 500 pg/ml was used as a cut-off value in the previous report, we decided to use 300 pg/ml as a more practical concentration, based on the distribution of values.[16] The specificity of this cut-off value was 96.3%, being comparable to that of other conventional tumor markers. If 500 pg/ml is applied to this study, the sensitivity of S-MK decreases to about 30%, which is almost identical to that of SCC-Ag. In this case, although the specificity of S-MK is 100%, the specificity of SCC-Ag is 95%. We have to increase the cut-off value of SCC-Ag to 2.0 ng/ml to reach 100% specificity. In this case, the sensitivity of SCC-Ag decreases to about 20%. The S-MK values of the patients with esophageal carcinoma in the present series were relatively low compared to those of a previous series.[16] Because all sero-positive patients, reexamined 4 weeks after surgery, became sero-negative when the cut-off concentration was set at 300 pg/ml, this cut-off concentration may be appropriate in the clinical setting.

Recently, MK has been shown to have anti-apoptotic activity in embryonic neurons[2, 22] and in Wilm's tumor cells.[23] MK also promotes migration of various cells such as macrophages,[24] osteoblasts[25] and neutrophils.[26] Overexpression of MK leads to enhanced tumor growth and vascular density.[26] These functions may enhance survival and invasiveness of tumor cells and suggest that a high S-MK reflects a resistance to chemotherapy and rapid tumor growth, resulting in a poor prognosis for the patient. In the present series, the survival rate in the high S-MK group was significantly worse than that in the low S-MK group. Moreover, S-MK had an independent prognostic value in multivariate analysis. Although the median follow-up period was relatively short, it is well known that patients with advanced esophageal SCC frequently die within 3 years of surgery. We considered that a 3-year follow-up period would be sufficient for identifying a group at high-risk for recurrent disease among patients with esophageal SCC. Indeed, among stage II patients (n=28), none of 12 patients with low S-MK developed recurrent disease and 5 of 16 patients with high S-MK developed recurrent tumors (data not shown). Although a larger series of patients will be needed to confirm the association between S-MK and prognosis, it is of interest that S-MK may complement the prognostic value of TNM staging. Because ELISA is a quick and easy assay to perform, S-MK may prove to be a useful clinical tumor marker to predict prognosis. SCC-Ag is also a possible prognostic biomarker.[27] However, because SCC-Ag level was strongly associated with tumor progression, SCC-Ag was not an independent prognostic factor in the current series (data not shown).

It may also serve as a useful marker for residual microscopic tumor cells after surgery, or for recurrent disease. Among a total of 39 patients who showed recurrent disease, blood samples were available from 16 before diagnosis of recurrence, and 11 of these 16 patients showed increased level of S-MK. Although the number of patients was small, more than a half of patients with recurrent disease, who were positive for S-MK before surgery, were positive for S-MK before diagnosis. This may support the clinical significance of S-MK as a tumor marker. However, further evaluation in a larger study with appropriate follow-up, and comparison with other modalities currently in use, remains desirable.

The prognosis for patients with node-positive esophageal carcinoma is generally very bad, and nodal status is currently believed to be one of the strongest prognostic indicators for esophageal cancer among several clinicopathological variables. However, 22% of node-positive patients are still alive after more than 5 years and 44% of node-negative patients die due to recurrent disease within 5 years.[28] To assist in the application of adjuvant treatment, we have searched for some prognostic factor other than pathological factors to differentiate patients at high risk for recurrence. Because the level of S-MK independent of tumor progression, S-MK might be a good indicator to select high-risk patients.

An unexpected finding in the present study was the lack of correlation between S-MK and tumor progression. If the tumor itself secretes MK protein, what is the explanation for this finding? We suggest that MK may play a key role only in carcinogenesis, but not in tumor progression. Therefore, the MK protein expression level could decline during tumor progression. A larger volume of MK-positive tumor during tumor progression could be counterbalanced by a decrease in the amount of MK protein secreted.

In conclusion, preoperative S-MK is concluded to be a prognostic marker for esophageal SCC and may be useful as a tumor marker.

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (#12671200 and Advanced Medicine Development Project).

1. Kadomatsu K, Hagihara M, Akhter S, Fan QW, Muramatsu H, Muramatsu T. Midkine induces the transformation of NI-H3T3 cells. *Br J Cancer* 1997; **75**: 354–9.
2. Michikawa M, Xu RY, Muramatsu H, Muramatsu T, Kim SU. Midkine is a mediator of retinoic acid-induced neuronal differentiation of embryonal carcinoma cells. *Biochem Biophys Res Commun* 1993; **192**: 1312–8.
3. Muramatsu T. Midkine (MK), the product of a retinoic acid responsive gene, and pleiotrophin constitute a new protein family regulating growth and differentiation (review). *Int J Dev Biol* 1993; **37**: 183–8.
4. Tsutsui J, Kadomatsu K, Matsubara S, Nakagawara A, Hamanoue M, Takao S, Shimazu H, Ohi Y, Muramatsu T. A new family of heparin-binding growth/differentiation factors: increased midkine expression in Wilms' tumor and other human carcinomas. *Cancer Res* 1993; **153**: 1281–5.
5. Garver RI, Chan CS, Milner PG. Reciprocal expression of pleiotrophin and midkine in normal versus malignant lung tissues. *Am J Respir Cell Mol Biol* 1993; **9**: 463–6.
6. Garver RI, Radford DM, Donis-Keller H, Wick MR, Milner PG. Midkine and pleiotrophin expression in normal and malignant breast tissue. *Cancer* 1994; **74**: 1584–90.
7. Nakagawara A, Milbrandt J, Muramatsu T, Deuel TF, Zhao H, Cnaan A, Brodeur GM. Differential expression of pleiotrophin and midkine in advanced neuroblastomas. *Cancer Res* 1995; **55**: 1792–7.
8. Aridome K, Tsutsui J, Takao S, Kadomatsu K, Ozawa M, Aikou T, Muramatsu T. Increased midkine gene expression in human gastrointestinal cancers. *Jpn J Cancer Res* 1995; **86**: 655–61.
9. Miyauchi M, Shimada H, Kadomatsu K, Muramatsu T, Matsubara S, Ikematsu S, Takenaga K, Asano T, Ochiai T, Sakiyama S, Tagawa M. Frequent expression of midkine gene in esophageal cancer suggests a potential usage of its promoter for suicide gene therapy. *Jpn J Cancer Res* 1999; **90**: 469–75.
10. Koide N, Hada H, Shinji T, Ujike K, Hirasaki S, Yumoto Y, Hanafusa T, Kadomatsu K, Uramatsu H, Muramatsu T, Ysuji T. Expression of the midkine gene in human hepatocellular carcinomas. *Hepatogastroenterology* 1999; **46**: 3189–96.
11. Ye C, Qi M, Fan QW, Ito K, Akiyama S, Kasai Y, Matsuyama M, Muramatsu T, Kadomatsu K. Expression of midkine in the early stage of carcinogenesis in human colorectal cancer. *Br J Cancer* 1999; **79**: 179–84.
12. Konishi N, Nakamura M, Nakaoka S, Hiasa Y, Cho M, Uemura H, Hirao Y,

Muramatsu T, Kadomatsu K. Immunohistochemical analysis of midkine expression in human prostate carcinoma. *Oncology* 1999; **57**: 253–7.
13. Mishima K, Asai A, Kadomatsu K, Ino Y, Nomura K, Narita Y, Muramatsu T, Kirino T. Increased expression of midkine during the progression of human astrocytomas. *Neurosci Lett* 1997; **233**: 29–32.
14. Muramatsu H, Song XJ, Koide N, Hada H, Tsuji T, Kadomatsu K, Inui T, Kimura T, Sakakibara S, Muramatsu T. Enzyme-linked immunoassay for midkine, and its application to evaluation of midkine concentrations in developing mouse brain and sera from patients with hepatocellular carcinomas. *J Biochem* 1996; **119**: 1171–5.
15. Song XJ, Muramatsu H, Aridome K, Aikou T, Koide N, Tsuji T, Muramatsu T. The serum concentration of midkine, a heparin-binding growth factor, as a tumor marker. *Biomed Res* 1997; **18**: 375–81.
16. Ikematsu S, Yano A, Aridome K, Kikuchi M, Kumai H, Nagano H, Okamoto K, Oda M, Sakuma S, Aikou T, Muramatsu H, Kadomatsu K, Muramatsu T. Serum midkine concentrations are increased in patients with various types of carcinomas. *Br J Cancer* 2000; **83**: 701–6.
17. Daly JM, Karnell LH, Menck HR. National cancer data base report on esophageal carcinoma. *Cancer* 1996; **78**: 1820–8.
18. Shimada H, Takeda A, Nabeya Y, Okazumi SI, Matsubara H, Funami Y, Hayashi H, Gunji Y, Kobayashi S, Suzuki T, Ochiai T. Clinical signifcance of serum vascular endothelial growth factor in esophageal cancer patients. *Cancer* 2001; **92**: 663–9.
19. Shimada H, Takeda A, Nabeya Y, Okazumi SI, Matsubara H, Funami Y, Hayashi H, Gunji Y, Kobayashi S, Suzuki T, Ochiai T. Prognostic significance of serum thymidine phosphorylase in the patients with esophageal squamous cell carcinoma. *Cancer* 2002; **94**: 1947–54.
20. Sobin LH, Wittekind CH, editors. UICC TNM classification of malignant tumors. 5th ed. New York: John Wiley & Sons Inc; 1997.
21. Isono K, Ochiai T. Recent advances of treatment of cancer of the esophagus (review). *Ann Cancer Res Ther* 1992; **1**: 9–16.
22. Qi M, Ikematsu S, Ichihara-Tanaka K, Sakuma S, Muramatsu T, Kadomatsu K. Midkine rescues Wilms' tumor cells from cisplatin-induced apoptosis: regulation of Bcl-2 expression by Midkine. *J Biochem* 2000; **127**: 269–77.
23. Takada T, Toriyama K, Muramatsu H, Song XJ, Torii S, Muramatsu T. Midkine, a retinoic acid-inducible heparin-binding cytokine in inflammatory responses: chemotactic activity to neutrophils and association with inflammatory synovitis. *J Biochem* 1997; **122**: 453–8.
24. Horiba M, Kadomatsu K, Nakamura E, Muramatsu H, Ikematsu S, Sakuma S, Hayashi K, Yuzawa Y, Matsuo S, Kuzuya M, Kaname T, Hirai M, Saito H, Muramatsu T. Neointima formation in a restenosis model is suppressed in midkine-deficient mice. *J Clin Invest* 2000; **105**: 489–95.
25. Qi M, Ikematsu S, Maeda N, Ichihara-Tanaka K, Sakuma S, Noda M, Muramatsu T, Kadomatsu K. Haptotactic migration induced by midkine: involvement of protein-tyrosine phosphatase zeta, mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *J Biol Chem* 2001; **276**: 15868–75.
26. Choudhuri R, Zhang HT, Donnini S, Ziche M, Bicknell R. An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. *Cancer Res* 1997; **57**: 1814–9.
27. Vassilakopoulos T, Troupis T, Sotiropoulou C, Zacharatos P, Katsaounou P, Parthenis D, Noussia O, Troupis G, Papiris S, Kittas C, Roussos C, Zakynthinos S, Gorgoulis V. Diagnostic and prognostic significance of squamous cell carcinoma antigen in non-small cell lung cancer. *Lung Cancer* 2001; **32**: 137–44.
28. The Japanese Society for Esophageal Diseases. Comprehensive registry of esophageal cancer in Japan (1998, 1999) & long-term results of esophagectomy in Japan (1988–1997). 3rd ed. Tokyo, Japan.