Midkine and its clinical significance in endometrial carcinoma

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Midkine (MK) is a secreted heparin-binding growth factor. Several types of human cancer have increased MK expression with elevated serum levels. The purpose of this study was to determine whether MK was expressed in endometrial carcinoma and to evaluate the clinicopathological significance of serum MK in patients with endometrial carcinoma. Immunohistochemical expression of MK was evaluated in 85 endometrial carcinoma samples and 33 controls. MK expression was significantly higher in the carcinomas than in normal endometrium ($P < 0.001$). Interestingly, MK expression was highest at the margins of invasion and low in the superficial areas of the tumor samples. Using ELISA, we compared serum MK concentration in 120 endometrial carcinoma patients with the concentration in 46 patients with benign gynecologic tumors. Serum MK value in patients with cancer was significantly higher than that in the patients with benign diseases ($P = 0.01$). Patients with positive lymph node metastasis or recurrence, or cancer death, had a higher serum MK level ($P = 0.008$, $P = 0.009$, respectively). In conclusion, MK immunoreactivity in endometrial carcinoma is significantly higher than in normal endometrium. Additionally, preoperative serum MK levels are significantly correlated with prognosis and the presence of lymph node metastasis. Thus, MK may be a useful serum biomarker for identifying high risk patients of endometrial carcinoma. (*Cancer Sci* 2008; 99: 1125–1130)

Endometrial carcinoma is one of the most common female pelvic malignancies worldwide, and its incidence has recently increased in Japan.[1,2] As approximately 80% of endometrial carcinomas are diagnosed at an early stage when surgery is curative, they carry a better prognosis than other cancers. However, advanced or recurrent cases tend to respond poorly to conventional treatments such as radiation, chemotherapy, or hormonal therapy, and as a result carry a poor prognosis. Identification of additional prognostic markers could help detect patients at a high risk of relapse or death from the disease.

Clinical, biological, and epidemiological findings all suggest that prolonged or unopposed estrogenic stimulation increases the risk of type I endometrial carcinoma. The initiation and progression of type I endometrial carcinoma, however, are poorly understood at a molecular level. We previously studied the gene expression profile of endometrioid adenocarcinoma, and identified 24 genes that had at least a 1.5-fold increased expression in both well (grade 1) and poorly (grade 3) differentiated endometrioid adenocarcinoma compared to normal endometrium (unpublished data). MK was identified as one of the up-regulated genes. Though MK expression has been reported in many human cancers, it has not been studied in endometrial carcinoma. Therefore, we focused our subsequent experiments on the actions of MK.

MK is a secreted, heparin-binding growth factor. It is a 13-kDa protein rich in basic amino acids and cysteine.[3,4] MK is highly expressed in the mid-gestational period during embryogenesis, and is involved in tooth, lung, kidney, and bone development. In the adult, MK has a very restricted pattern of expression. The highest transcript levels are in the intestine with low levels in the cerebellum, thyroid, kidney, bladder, lung alveoli, colon, stomach, and spleen.[5] The pathophysiological effects of MK include the oncogenic transformation of fibroblasts, antiapoptotic activity, and angiogenic activity.[6–8] MK mRNA levels and protein expression are frequently elevated in various human carcinomas of the breast, lung, esophagus, colon, ovary, urinary bladder, and prostate; and glioblastomas, neuroblastomas, and Wilms' tumor.[9–18] Furthermore, MK concentrations in serum are also elevated in various carcinomas.[19–22] To our knowledge, however, no study has focused on the clinicopathological significance of MK expression in human endometrial carcinoma. The purpose of this study was to determine whether MK was expressed in endometrial carcinoma, and whether differences existed between the expression level in cancer and levels in benign gynecologic conditions. We also explored whether correlations existed between MK expression and clinicopathological features.

Materials and Methods

Tissue and serum samples. Eighty-five endometrioid endometrial carcinomas (37 well differentiated, 25 moderately differentiated, 23 poorly differentiated; 55 stage I, 16 stage II, 11 stage III, three stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan for immunohistochemical analysis. The controls were selected from patients who underwent hysterectomy for benign gynecologic diseases without any personal cancer history from April 1996 to March 2004. The median follow-up time for patients whose samples were examined immunohistochemically was 60 months (range, 2–148 months). The disease-free and overall survival times of the patients were calculated from the time of initial surgery to recurrence or death, or the date of last contact. The survival times of patients still alive or lost to follow-up were censored in December 2004. Serum samples were obtained from 120 patients with endometrial carcinoma (66 well differentiated, 16 moderately differentiated, 12 poorly differentiated, 26 other histological type; 80 stage I, 11 stage II, 17 stage III, 12 stage IV) and from 45 patients with non-malignant gynecologic diseases at Tohoku University Hospital from April 2002 to January 2007. None of the patients examined had received radiation, hormonal therapy, or chemotherapy prior to surgery. The median follow-up time for the patients whose serum was tested for MK was 91 months (range, 1–166 months). The

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survival times of patients still alive or lost to follow-up were censored in August 2007. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine.

Total RNA extraction from endometrial tissues and cDNA synthesis. All tumor and normal specimens were frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from normal endometrium and carcinoma tissues, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A reverse transcription kit, SuperScript III RT (Invitrogen, Carlsbad, CA, USA), was used for the synthesis of cDNA.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). Real-time PCR was carried out using the LightCycler System (Roche Diagnostics, Mannheim, Germany). cDNAs of known concentrations for target genes and the housekeeping gene, ribosomal protein L13a (RPL13A) were used to generate standard curves for determining the quantity of target cDNA transcripts. The mRNA level in each case was represented as a ratio with RPL13A.[23] The PCR thermal profile for MK was: initial denaturation at 95°C for 10 min followed by 32 amplification cycles of denaturation at 95°C for 10 s, annealing at 68°C for 10 s, and elongation at 72°C for 12 s; and for RPL13A, initial denaturation at 95°C for 10 min followed by 30 amplification cycles of denaturation at 95°C for 12 s, annealing at 68°C for 10 s, and elongation at 72°C for 12 s.

The primer sequences used in our study were: 5′-CCA AGA CCA AAG CAA AGG-3 and 5′-GGC AGG GCA TGA TTG ATT-3′ for MK; 5′-CCT GGA GGA GAA GAG GAA AGA GA-3′ and 5′-TTG AGG ACC TCT GTG TAT TTG TCA A-3′ for RPL13A.

Immunohistochemistry. After deparaffinization and rehydration in graded alcohol, antigen retrieval for MK immunostaining was done by heating the sections in a 600-W microwave for 20 min in 10 mM trisodium citrate buffer, pH 7.0. The sections were then blocked with normal goat serum for 30 min at room temperature, followed by incubation with chicken antihuman MK antibody (given by K.K.) overnight at 4°C. The dilution of the primary antibody used in this study was 1/250. The slides were incubated in 99.7% methanol containing 0.3% hydrogen peroxide at room temperature for 30 min to inhibit endogenous peroxidase. They were then incubated with biotin-conjugated rabbit antichicken IgG (ICN Pharmaceuticals, Aurora, OH, USA) at room temperature for 30 min, followed by incubation with peroxidase-conjugated streptavidin for 30 min at room temperature, using a Histofine Kit (Nichirei, Tokyo, Japan). The antigen-antibody complex was visualized with a 3, 3′-diaminobenzidine solution (1 mmol/L 3, 3′-diaminobenzidine, 50 mmol/L Tris-HCl [pH 7.6], 0.006% H2O2) and counter-stained with hematoxylin. Serous adenocarcinoma of the ovary was employed as a positive control for MK immunostaining.[13] The primary antibody was replaced with phosphate-buffered saline (PBS) as a negative control. Samples were considered negative if none of the cells stained for MK. Very weak positive was defined as less than 5% staining, weak positive as 5–25% staining, moderate positive as 25–50% staining, and strong positive as more than 50% staining. Slides were then numerically scored based on immunoreactivity. A score of 0 was negative, 1 very weak, 2 weak, 3 moderate, and 4 strong positive.

ELISA for human MK. An ELISA for human MK was performed as described previously.[22] Briefly, human MK was produced using Pichia pastoris GS115 by transfection with a human MK expression vector, which was constructed into pHIL-D4 (Invitrogen). This yeast-produced human MK was used to immunize rabbits and chickens to raise antibodies. The rabbit antihuman MK antibody (50 mL of 5.5 mg/mL in 50 mM Tris HCl [pH 8.2], 0.15 M NaCl, 0.1% NaN_3) was coated onto the wells of microtiter plates (Polysorpplates; Nunc, Rochester, NY, USA) for 20 h at room temperature. After washing with 0.05% Tween 20 in PBS, the wells were blocked with 300 mL of 0.1% casein, 0.01% Microcide I (aMReSCO) in PBS for 20 h at 37°C. Plasma samples (10 mL each) were mixed with 100 mL of 50 mM Tris HCl (pH 8.4), 0.5 M KCl, 0.1% casein, 0.5% bovine serum albumin, 0.01% Microcide I, and 0.1 mg/mL peroxidase-labeled chicken antihuman MK antibody. Aliquots of 50 mL of this mixture were added to wells prepared as described above, and subjected to chromogenic detection at OD450 using tetramethylbenzidine as the substrate. This ELISA system shows linearity from 0 to 4 ng/mL of MK, and there is no crossreaction with Pleiotrophin.[22]

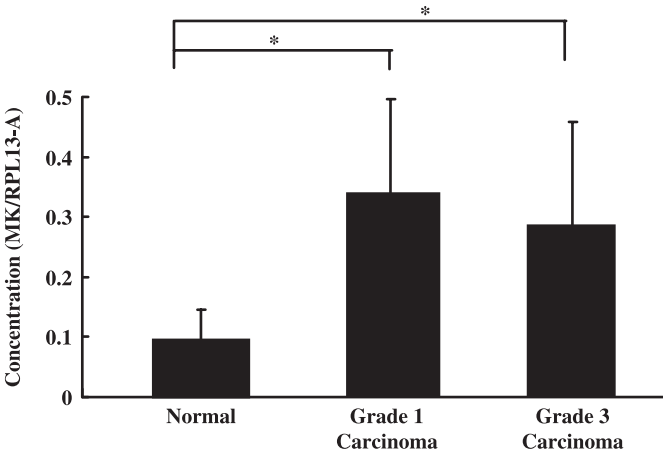

Fig. 1. Midkine (MK) mRNA expression levels in normal endometrial tissues and endometrial carcinoma tissues measured by reverse transcription–polymerase chain reaction (RT-PCR). MK mRNA expression levels in carcinoma tissues were significantly higher than in normal endometrial tissues ($P < 0.001$, Mann–Whitney test).

Statistical analysis. mRNA levels and serum concentrations of MK were compared using the Mann–Whitney test. Immunoreactivities for MK were compared using a Student's *t*-test. *P*-values less than 0.05 were considered significant.

Results

MK was expressed at higher levels in endometrioid adenocarcinoma tissues than in normal endometrium samples. To validate the microarray-based MK expression difference, we performed real-time RT-PCR using cDNA from 10 normal endometrium specimens and 20 carcinoma specimens; 10 were grade 1 and 10 were grade 3. The quantitative mRNA expression levels of MK were significantly higher in the endometrioid adenocarcinomas than in normal endometrium samples. However, there was no difference in the expression level between grade 1 and grade 3 (Fig. 1).

We then confirmed the high expression of MK in carcinoma tissues not only at the mRNA level but also at the protein level by immunohistochemical staining. The intensity of MK immunostaining in tissues is summarized in Table 1. As shown in Figure 2, MK protein was predominantly expressed in the epithelial cytoplasm with little nuclear expression. Positive staining for MK was scarcely detected in the stroma. In both normal proliferative and secretory phase endometrium samples, MK expression in the basal layer was significantly stronger than in the functional layer or endometrial stroma ($P < 0.001$, *t*-test) (Table 1 and Fig. 2c–f). No significant difference in protein expression was detected between the endometrial stroma and the functional layer in either the proliferative or the secretory phase. MK immunoreactivity at the basal layer tended to be stronger in

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Table 1. Midkine protein expression in normal and endometrial cancer tissues by immunohistochemistry (mean ± SD of immunostaining score)

Normal	*n*	Endometrial stroma	Functionalis	Basalis	
Total	33	0.41 ± 0.56	0.62 ± 0.89	*1.72 ± 1.17*	*P* < 0.001*
Proliferative	21	0.35 ± 0.61	0.62 ± 0.86	1.35 ± 1.18	
Secretory	12	0.50 ± 0.52	0.67 ± 0.98	2.17 ± 0.94	
Carcinoma	*n*	Endometrial stroma	Superficial area	Invasive area	
Total	85	0.38 ± 0.56	0.81 ± 0.78	*2.66 ± 0.79*	
G1	37	0.41 ± 0.55	1.00 ± 0.77	2.69 ± 0.82	
G2	25	0.32 ± 0.56	0.60 ± 0.71	2.56 ± 0.77	
G3	23	0.39 ± 0.58	0.75 ± 0.79	2.74 ± 0.81	

**P*-value, *t*-test.

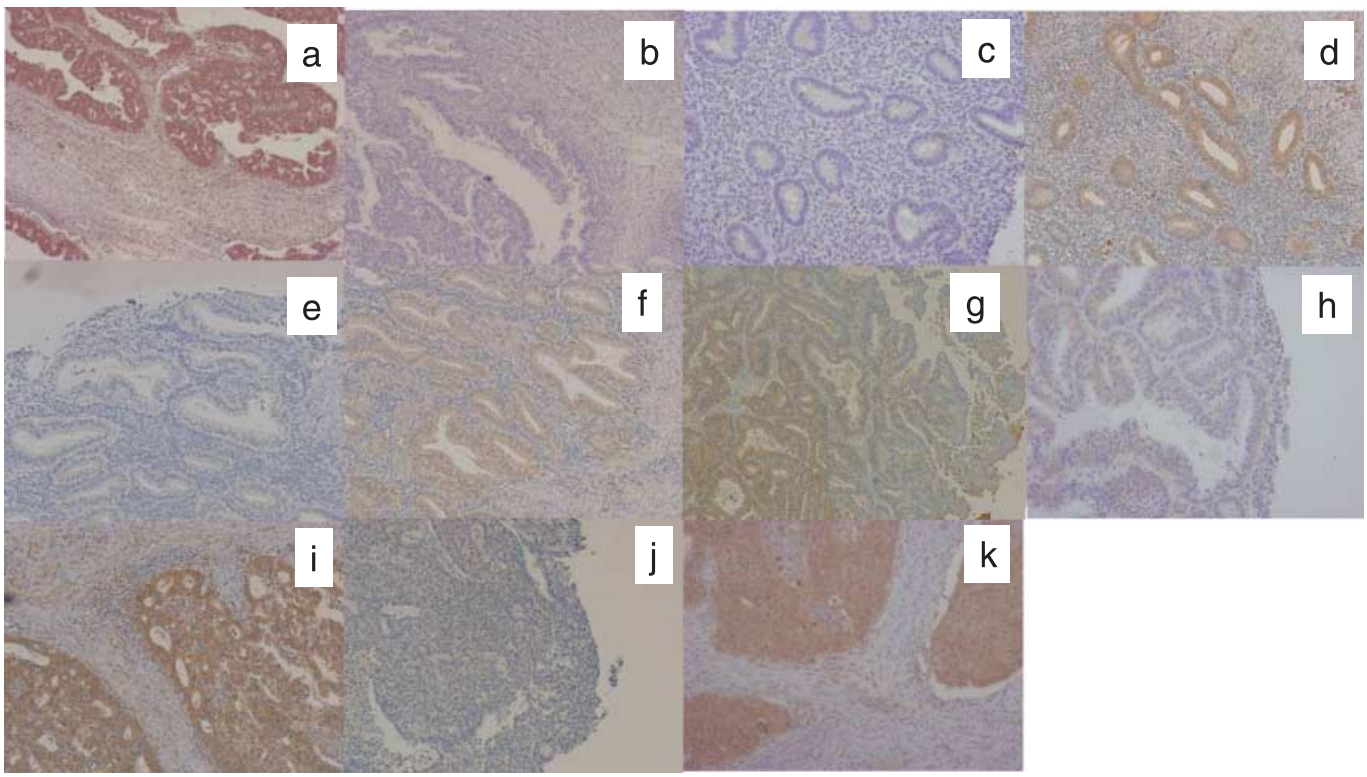

Fig. 2. Representative panels of immunohistochemical staining with anti-midkine (MK) protein antibody. (a) Positive control, (b) negative control, (c) proliferative phase (functionalis), (d) proliferative phase (basalis), (e) secretory phase (functionalis), (f) secretory phase (basalis), (g) transitional area of endometrial carcinoma grade 1, (h) superficial area of endometrial carcinoma grade 1, (i) invasive area of endometrial carcinoma grade 1, (j) superficial area of endometrial carcinoma grade3, (k) invasive area of endometrial carcinoma grade 3.

the secretory phase than in the proliferative phase ($P = 0.09$, *t*-test). Interestingly, MK expression was strongest at the margins of invasion and low in the superficial layers of the tumor samples (Fig. 2g–j). MK expression was significantly higher in the carcinomas than in the basal area of the normal endometrium ($P < 0.001$, *t*-test) (Table 1). No statistical correlation was detected between grade 1 and grade 3 endometrioid adenocarcinoma. MK immunoreactivity was not associated with any clinicopathological features including histological grade, depth of myometrial invasion, the presence of lymph node metastasis, or prognosis.

Serum MK protein concentration was higher in patients with endometrial carcinoma than in patients with benign gynecologic diseases. We measured serum MK protein concentrations with ELISA. Serum MK values for the patients with endometrial carcinoma was significantly higher than those for patients with benign gynecologic diseases ($P = 0.01$, Mann–Whitney test). The data suggest that MK protein is not only expressed in cancer tissues but also secreted into the sera at higher levels in endometrial carcinoma patients. To test whether the serum MK level could be used to discriminate endometrial carcinoma from benign disease, we set various cut-off values and classified the cases based on their MK values. Serum MK level had a high false negative ratio, thereby limiting its use in clinical applications.

A higher serum MK protein concentration was correlated with the presence of lymph node metastases and prognosis of endometrial carcinomas. We calculated the mean serum MK concentrations of cancer patients categorized by clinicopathological features. Results of the associations between clinicopathological parameters and serum MK levels are summarized in Table 2. Serum MK concentration was not associated with age, histological grade, or lymphovascular invasion. Although serum MK had a tendency to be lower in stage I–II or no myometrial

Table 2. Serum midkine (MK) levels and clinicopathological factors in endometrial carcinomas

Clinicopathological factors		*N* (%)	MK concentrations (Mean ± SD)	*P**-values
Age	50 =	25 (21)	104 ± 253	0.111
	50 <	95 (79)	81 ± 113	
Histological grade	Grade1	66 (55)	82 ± 169	0.455
	Grade2	16 (13)	64 ± 97	
	Grade3	12 (10)	144 ± 112	
	Others	26 (22)	76 ± 143	
Stage	I–II	91 (76)	71 ± 157	0.054
	III–IV	29 (24)	133 ± 159	
Myometrial invasion	None	19 (16)	46 ± 76	0.074
	< 1/2	58 (48)	79 ± 178	
	= 1/2	40 (33)	100 ± 130	
	Unknown	5 (4)	183 ± 201	
Lymphovascular invasion	Negative	83 (69)	75 ± 153	0.720
	Positive	35 (29)	90 ± 139	
	Unknown	2 (2)	400 ± 33	
Lymph node metastasis	Negative	103 (86)	73 ± 142	0.008
	Positive	5 (4)	253 ± 246	
	Unknown	12 (10)	131 ± 161	
Prognosis	Non-recurrence	102 (85)	71 ± 142	0.009
	Recurrence or death	18 (15)	172 ± 184	

**P*-value, Mann–Whitney test.

invasion, the difference was not statistically significant ($P = 0.054$, $P = 0.072$). Interestingly, the patient group with positive lymph node metastasis had a higher level of serum MK ($P = 0.008$, Mann–Whitney test). Patients with recurrence or cancer related death had significantly higher serum levels of MK protein than those without recurrence ($P = 0.009$).

Discussion

This is the first report showing that mRNA levels and protein expression of MK in endometrial carcinoma are significantly higher than in normal endometrium. Additionally, serum MK levels in endometrial carcinoma patients were significantly elevated relative to levels in patients with benign gynecologic diseases. Although MK is overexpressed in various human malignant tumors, its effects on tumor growth and progression are not fully understood. Growth of mouse colorectal carcinoma cells is inhibited by antisense midkine oligo DNA.[24] Transfection of the breast carcinoma line MCF-7 with MK accelerates tumor growth and increases tumor vascularity after cell implantation in nude mice.[25] MK also rescues Wilms' tumor cells from cisplatin-induced apoptosis.[26] These effects are likely mediated by signaling via phosphatidylinositol-3-kinase and mitogen-activated kinase.[27] Taken together these biological data support the hypothesis that MK plays an important role in oncogenesis and tumor progression.

Despite the increased MK immunoreactivity in endometrial carcinomas, there was no relationship between immunoreactivity and clinicopathological features. This was surprising since high MK immunoreactivity significantly correlates with worse clinical outcome of neuroblastomas,[17] urinary bladder cancer,[14] gastrointestinal stromal tumor,[28] oral squamous cell carcinomas,[29] and pancreatic cancer.[30] Interestingly, in esophageal carcinoma, MK is more intensely expressed in well-differentiated tumors than in poorly differentiated tumors.[11] A noteworthy immunohistochemical finding in this study was that the intensity of MK protein expression was not the same across different areas within a single tissue sample. MK expression in normal endometrium was higher in the basalis than in the functionalis. It was highly expressed at the margin of invasion but not in the superficial areas of the cancer specimens. To confirm that these findings were not due to the unequal localization of antibody, endometrial biopsy samples from cancer patients were also immunostained. These superficial specimens all demonstrated weak expression (data not shown). The MK immunohistochemical findings in normal endometrium were inconsistent with the previously reported pathophysiological effects of MK. MK is involved in angiogenesis and antiapoptosis. Microvessel density in normal endometrium, however, is not significantly different between the functionalis and basalis,[31] and apoptotic cells are equally distributed on each layer.[32] Donoghue *et al*. reported that lymphatic vessel density (LVD) is higher in the basalis than in the functionalis across the menstrual cycle.[31] In this study, the distribution of lymphatic vessels is consistent with the diversity of MK immunoreactivity across the menstrual cycle. Rogers *et al*. suggested that unknown lymphangiogenic growth factors may be involved in normal endometrium, since no difference is observed in immunostaining intensity for the vascular endothelial growth factor (VEGF)-C or VEGF-D between the functionalis and basalis.[33] We speculate that MK would be a candidate molecule for lymphangiogenesis in normal endometrium. In endometrial adenocarcinoma, the peritumoral LVD is higher compared with the LVD within the tumor and in normal endometrium, which also correspond to MK immunoreactivity. These observations suggest a role for MK in lymphangiogenesis in endometrial adenocarcinoma.

Since MK is a secretory protein, it could potentially be used to screen for and monitor the progression of endometrial carcinoma in a manner similar to cancer antigen (CA)–125 for ovarian cancer. An elevated serum MK level is detected in more than 80% of human adult carcinomas, and its level decreases when the tumor is resected.[19] A high serum MK level is associated with higher stage and disease progression in gastric cancer,[21] with tumor size in esophageal cancer,[20] and with progression in neuroblastoma.[22] As shown in Figure 3, serum MK was significantly elevated in patients with endometrial carcinoma compared with patients with non-malignant gynecologic diseases ($P = 0.014$). Regarding the relationship between serum MK concentration and clinicopathological features in patients with endometrial carcinoma, statistical differences were seen in both lymph node metastasis and prognosis. Our observations are consistent with another recent study in esophageal carcinoma.[34] In

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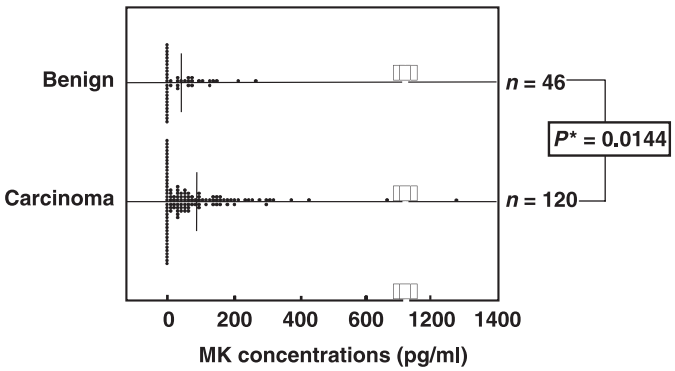

Fig. 3. Serum midkine (MK) protein concentrations from patients with benign gynecologic tumors or endometrial carcinoma. MK protein levels were measured by ELISA. The serum concentration for the carcinoma patients was significantly higher than that for the benign patients ($P = 0.014$, Mann–Whitney test).

esophageal squamous cell carcinoma, serum MK is a good marker of lymph node metastasis that correlates with serum levels of VEGF-C. Lymph node metastasis is a critical prognostic factor in endometrial carcinoma, and myometrial invasion and histological grade are correlated strongly with lymph node metastasis.[35,36] Thus, preoperative serum MK levels might prove to be useful for selecting high risk patients or predicting prognosis.

In conclusion, MK immunoreactivity in endometrial carcinoma is significantly higher than in normal endometrium. Additionally, preoperative serum MK levels are significantly correlated with prognosis and the presence of lymph node metastasis. Further, larger, prospective studies with longer follow-up periods are needed to fully understand the role of MK in endometrial carcinoma carcinogenesis.

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References

1 Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007; **57**: 43–66.
2 Jobo T, Kanai T, Kuramoto H. Epidemiology of endometrial carcinoma. *Nippon Rinsho* 2004; **62** (Suppl. 10): 243–7.
3 Kadomatsu K, Tomomura M, Muramatsu T. cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in midgestation period of mouse embryogenesis. *Biochem Biophys Res Commun* 1988; **151**: 1312–8.
4 Tomomura M, Kadomatsu K, Nakamoto M *et al*. A retinoic acid responsive gene, MK, produces a secreted protein with heparin binding activity. *Biochem Biophys Res Commun* 1990; **171**: 603–9.
5 Tsutsui J, Uehara K, Kadomatsu K, Matsubara S, Muramatsu T. A new family of heparin-binding factors: strong conservation of midkine (MK) sequences between the human and the mouse. *Biochem Biophys Res Commun* 1991 (Apr 30); **176** (2): 792–7.
6 Kadomatsu K, Hagihara M, Akhter S, Fan Q-W, Muramatsu H, Muramatsu T. Midkine induces the transformation of NIH3T3 cells. *Br J Cancer* 1997; **75**: 354–9.
7 Owada K, Sanjo N, Kobayashi T *et al*. Midkine inhibits caspase-dependent apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase in cultured neurons. *J Neurochem* 1999; **73**: 2084–92.
8 Sumi Y, Muramatsu H, Takei Y, Hata K, Ueda M, Muramatsu T. Midkine, a heparin-binding growth factor, promotes growth and glycosaminoglycan synthesis of endothelial cells through its action on smooth muscle cells in an artificial blood vessel model. *J Cell Sci* 2002; **115**: 2659–67.
9 Garver RI Jr, 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.
10 Garver RI Jr, Radford DM, Donis-Keller H, Wick MR, Milner PG. Midkine and pleiotrophin expression in normal and malignant breast tissue. *Cancer* 1994; **74**: 1584–90.
11 Ren YJ, Zhang QY. Expression of midkine and its clinical significance in esophageal squamous cell carcinoma. *World J Gastroenterol* 2006; **12**: 2006–10.
12 Ye C, Qi M, Fan QW *et al*. Expression of midkine in the early stage of carcinogenesis in human colorectal cancer. *Br J Cancer* 1999; **79**: 179–84.
13 Nakanishi T, Kadomatsu K, Okamoto T, Tomoda Y, Muramatsu T. Expression of midkine and pleiotropin in ovarian tumors. *Obstet Gynecol* 1997; **90**: 285–90.
14 O'Brien T, Cranston D, Fuggle S, Bicknell R, Harris AL. The angiogenic factor midkine is expressed in bladder cancer, and overexpression correlates with a poor outcome in patients with invasive cancers. *Cancer Res* 1996; **56**: 2515–18.
15 Konishi N, Nakamura M, Nakaoka S *et al*. Immunohistochemical analysis of midkine expression in human prostate carcinoma. *Oncology* 1999; **57**: 253–7.
16 Mishima K, Asai A, Kadomatsu K *et al*. Increased expression of midkine during the progression of human astrocytomas. *Neurosci Lett* 1997; **233**: 29–32.
17 Nakagawara A, Milbrandt J, Muramatsu T *et al*. Differential expression of pleiotrophin and midkine in advanced neuroblastomas. *Cancer Res* 1995; **55**: 1792–7.
18 Tsutsui J, Kadomatsu K, Matsubara S *et al*. A new family of heparin-binding growth differentiation factors: increased midkine expression in Wilms' tumor and other human carcinomas. *Cancer Res* 1993; **53**: 1281–5.
19 Ikematsu S, Yano A, Aridome K *et al*. T. Serum midkine levels are increased in patients with various types of carcinomas. *Br J Cancer* 2000; **83**: 701–6.
20 Shimada H, Nabeya Y, Tagawa M *et al*. Preoperative serum midkine concentration is a prognostic marker for esophageal squamous cell carcinoma. *Cancer Sci* 2003; **94**: 628–32.
21 Obata Y, Kikuchi S, Lin Y, Yagyu K, Muramatsu T, Kumai H, Tokyo Research Group on Prevention of Gastric Cancer. Serum midkine concentrations and gastric cancer. *Cancer Sci* 2005; **96**: 54–6.
22 Ikematsu S, Nakagawara A, Nakamura Y *et al*. Correlation of elevated level of blood midkine with poor prognostic factors of human neuroblastomas. *Br J Cancer* 2003; **88**: 1522–6.
23 Vandesompele J, De Preter K, Pattyn F *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Bio* 2002; **3**: RESEARCH0034.
24 Takei Y, Kadomatsu K, Matsuo S *et al*. Antisense oligodeoxynucleotide targeted to Midkine, a heparin-binding growth factor, suppresses tumorigenicity of mouse rectal carcinoma cells. *Cancer Res* 2001; **61**: 8486–91.
25 Choudhuri R, Zhang HT, Donnini S, Ziche M, Bicknell R. An angiogenic role for the neurokines midkine and pleiotrophin in tumourigenesis. *Cancer Res* 1997; **57**: 1814–9.
26 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 (Tokyo)* 2000; **127**: 269–77.
27 Qi M, Ikematsu S, Maeda N *et al*. 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.
28 Kaifi JT, Fiegel HC, Rafnsdottir SL *et al*. Midkine as a prognostic marker for gastrointestinal stromal tumors. *J Cancer Res Clin Oncol* 2007; **133**: 431–5.
29 Ruan M, Ji T, Wu Z, Zhou J, Zhang C. Evaluation of expression of midkine in oral squamous cell carcinoma and its correlation with tumour angiogenesis. *Int J Oral Maxillofac Surg* 2007; **36**: 159–64.
30 Maeda S, Shinchi H, Kurahara H *et al*. Clinical significance of midkine expression in pancreatic head carcinoma. *Br J Cancer* 2007; **97**: 405–11.

31 Donoghue JF, Lederman FL, Susil BJ, Rogers PA. Lymphangiogenesis of normal endometrium and endometrial adenocarcinoma. *Human Reprod* 2007; **22**: 1705–13.
32 Tao XJ, Tilly KI, Maravei DV *et al*. Differential expression of members of the bcl-2 gene family in proliferative and secretory Human endometrium: glandular epithelial cell apoptosis is associated with increased expression of bax. *J Clin Endocrinol Metab* 1997; **82**: 2738–46.
33 Rogers PAW, Donoghue JF, Girling JE. *et al*. *Endometrial Lymphangiogensis, Placenta* 2007, doi:10.1016/j.placenta.2007/09.009.
34 Krzystek-Korpacka M, Matusiewicz M, Diakowska D *et al*. Serum midkine depends on lymph node involvement and correlates with circulating VEGF-C in oesophageal squamous cell carcinoma. *Biomarkers* 2007; **12**: 403–13.
35 Creasmann WT, Morrow CP, Bundy BN, Homesley HD, Graham JE, Heller PB. Surgical pathologic spread patterns of endometrial cancer. A Gynecol Oncol Group Study. *Cancer* 1987; **60**: 2035–41.
36 Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. *Lancet* 2005; **366**: 491–5051. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007.

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