Article

The Expression and Location of Midkine in Gastric Carcinomas of Chinese Patients

Yaling Huang[1], Guochun Cao[1], Hui Wang[1], Qingling Wang[1] and Yayi Hou[1, 2]

Midkine (MK), a heparin-binding growth factor, can regulate cell growth, survival and differentiation. MK is expressed at high levels in a variety of human carcinomas. Recently, the urine and serum MK concentration was analyzed in gastric cancer patient. However, the association of the cytokine mRNA expression with the categorical clinicopathological variables of the tumors and the location of its protein expression in the tumor tissues are still elusive. MK mRNA expression from the surgically resected specimens of healthy gastric tissues (9 cases), gastric cancer tissues and the matched non-cancerous tissues adjacent to the cancer (37 cases) were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR. Immunohistochemical analysis was performed to locate MK in gastric cancer. The expression of MK mRNA in gastric cancer was much higher in tumor tissues than that in the non-cancerous tissues and control tissue samples. And its expression was significantly associated with the pTNM stage and distant metastasis, but not with the differentiation grade, tumor size and nodal involvement. MK protein was ubiquitous in the tumor, especially in the adenoid part of tumors. In addition, it was found in the cytoplasm of tumor cells and highly concentrated in nucleus and nucleolus. The expression level and location of MK in gastric tumor tissues of Chinese Patients may be related to the tumor genesis and progression. Further study is necessary on the mechanism of MK in gastric tumorigenesis and tumor growth. *Cellular & Molecular Immunology*. 2007;4(2):135-140.

Key Words: midkine, gastric cancer, RT-PCR, real-time PCR, immunohistochemical analysis

Introduction

In China, gastric cancer is one of the most common malignancies with the highest mortality. It is reported that 20 in every 100,000 persons on average die of gastric cancer each year in the whole country. Therefore, it is crucial to elucidate the molecular mechanisms of tumorigenesis before we can finally develop new and more effective therapeutic strategies.

Midkine (MK) is a heparin-binding growth/differentiation factor first found in 1988 (1). Studies showed that MK was expressed at high levels in a variety of human carcinomas (2-6). Its expression increased with advancing stages of human astrocytomas and urinary bladder carcinomas, and was significantly linked to the prognosis (4, 7). Excitingly, recent studies showed that the MK concentrations in urine and serum of gastric cancer patients were associated with gastric cancer progression stages (8, 9). So we tried to study whether MK expression was high in Chinese gastric cancer and closely associated with the development and deterioration of tumors.

In this study, we assayed 37 gastric carcinoma tissues, the corresponding non-cancerous tissues, and 9 healthy gastric tissues from patients without any gastropathy by gastroscope as control. Reverse transcription-PCR (RT-PCR) and real-time PCR assay were used for the qualitative and quantitative analysis of MK. The association between MK expression and the categorical clinicopathological variables of the tumors was analyzed. In addition, we assessed the localization of MK protein in gastric cancer using immunohistochemistry.

Materials and Methods

Tissue sources

The tumor samples (n = 37) and the matched non-cancerous samples adjacent to the cancer (n = 37) were surgically resected from 37 patients with gastric cancer. The mean age of the patients was 56 and the ratio of male/female was 3:1. The 9 biopsy specimens resected from patients without any gastropathy by gastroscope were also obtained as a source of

[1]Immunology and Reproduction Biology Lab, Medical School & State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China;

[2]Corresponding to: Dr. Yayi Hou, Immunology and Reproductive Biology Lab, School of Medicine, Nanjing University, Nanjing 210093, China. Tel: +86-25-8368-6341, Fax: +86-25-8368-6341, E-mail: yayihou@niu.edu.cn

Received March 30, 2007. Accepted April 19, 2007.

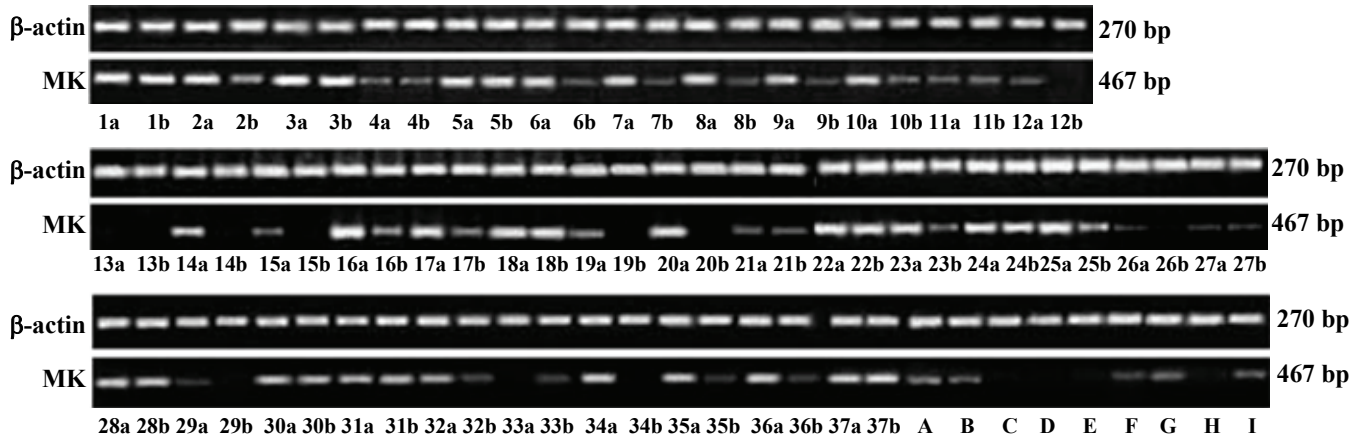

Figure 1. Ethidium bromide stained agrose gel containing PCR products. Lanes 1-37, results of the tissues from 37 patients. Lanes A-I, gastric tissue for control. a, gastric cancerous tissues; b, the corresponding non-cancerous tissues.

normal tissues for control. All samples including biopsy specimens were obtained at Jiangsu Cancer Hospital, China from 2004 to 2005, confirmed and approved by the Research Ethical Committee of Jiangsu Cancer Hospital, China about the legitimacy. These tissues were immediately frozen in liquid nitrogen and kept at -70°C for RNA isolation or freezing microtomy (LeicaCM1900). None of these patients received preoperative treatment and informed consents were obtained from all of the patients.

RNA isolation

Total cellular RNA was extracted from about 100 mg of frozen tissues using TRIzol Reagent (Ferment, Inc., Nanjing, Jiangsu) according to the manufacturer's instructions. The concentration and purity of the isolated total RNA were determined with the spectrophotometer; OD_{260}/OD_{280} = 1.7-2.0 was taken as the range of pure RNA.

First strand cDNA synthesis

With 1 μg total RNA as template the first strand of cDNA of each species was synthesized using RT Kit Reagent (Ferment, Inc., Nanjing, Jiangsu) according to the manufacturer's instructions.

Polymerase chain reaction (PCR)

Human β-actin gene was chosen as reference gene. The oligonucleotides used for amplification were as follows: MK forward primer: 5'-ATG CAG CAC CGA GGC TTC CT-3' and MK reverse primer: 5'-ATC CAG GCT TGG CGT CGT CTA GT-3', β-actin forward primer: 5'-CCA CGA AAC TAC CTT CAA CTC C-3', and reverse primer: 5'-TCA TAC TCC TGC TTG CTG ATC C-3'. The conditions for PCR were as follows: pre-denaturation at 94°C for 30 s, 35 cycles of denaturation (94°C for 30 s), re-annealing (53°C for 30 s), and extension (72°C for 40 s), then kept at 72°C for 5 min. Finally 5 μl PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide staining. All assays were repeated at least twice and found to be reproducible. The final results were analyzed with SAS software (version 8.0; SAS Institute Inc, Cary, NC) to test the differentiation among the 3 groups of tissues, Wilcoxon signed rank test were used where appropriate. $p < 0.05$ was considered to be a significant value.

Real-time PCR

Human PBGD (porphobilinogen deaminase) gene was chosen as inner reference. The primers for MK PCR (forward primer: 5'-ATG CAG CAC CGA GGC TTC CT-3', reverse primer: 5'-ATC CAG GCT TGG CGT CGT CTA GT-3') and PBGD PCR (forward primer: 5'-GGC AAT GCG GCT GCA A-3', reverse primer: 5'-GGG TAC CCA CGC GAA TCA C-3') were designed by Primer Express software. cDNA (2 μl) was added to the system of PCR using the SYBR Green PCR Kit Reagents (Applied Biosystems Inc., USA). The conditions for PCR were as follows: 50°C 2 min Uracil-N-Glycosylase was initiated to get rid of carryover pollution, pre-denaturation at 95 °C for 10 min, and then 40 cycles of denaturation (95°C for 15 s), re-annealing (59°C for 30 s), and extension (72°C for 30 s). After the reaction, results were given automatically by ABI PRISM® 7000 software and the dissociation curve was checked for its specificity. Using the healthy samples as calibrator, data were analyzed according to $2^{-\triangle\triangle CT}$ relative quantitation method. At last, SAS software (version 8.0; SAS Institute Inc., Cary, NC) was used to test the differentiation among the 3 groups of tissues and the association between MK expression and the categorical clinicopathological variables of the tumors. To compare variables of interest, Fisher's exact test, χ^2 test, and Wilcoxon test were used where appropriate. $p < 0.05$ was considered to be a significant value.

Immunohistochemical analysis

Each specimen taken from -70°C was embedded and cut into a 6 μm section. The dried sections were fixed by neutral buffered formalin (NBF) for 30 min. After quenching endogenous peroxidase activity for 30 min in pure carbinol

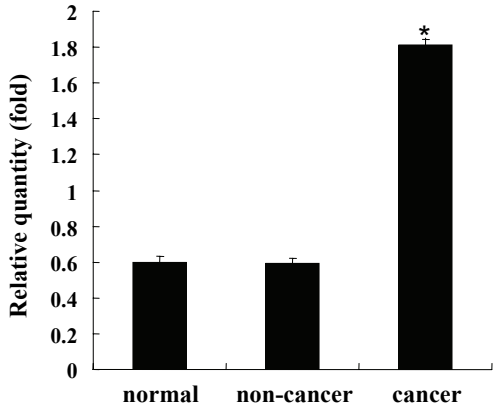

Figure 2. Densitometric measurement of MK expression levels among normal, non-cancer and cancer groups. Relative quantity was shown as the MK/β-actin. The results are presented as mean ± SE. *$p < 0.01$.

containing 0.6% (v/v) hydrogen peroxide, non-specific binding was blocked by treatment with 5% (v/v) bovine serum albumin (BSA) for 20 min. The sections were incubated at 37°C for 1 h in a moist chamber with affinity purified rabbit anti-MK polyclonal antibodies (produced in our lab). After washed in 0.02 M phosphate buffer saline (PBS) pH 7.4, the sections were incubated at room temperature for 20 min with biotinylated goat anti-rabbit immunoglobulin (Boster Ltd. Wuhan, Hubei). After washed in PBS, the sections were incubated again for 20 min with Streptavidin-Biotin Complex (Boster Ltd. Wuhan, Hubei) according to the manufacturer's instructions. The excess complexes were then washed off in PBS and MK was visualized by incubating the sections in DAB solution (Boster Ltd. Wuhan, Hubei). Negative control sections were incubated with PBS instead of anti-MK antibodies.

Results

Reverse transcription-polymerase chain reaction (RT-PCR)
MK expression in surgically resected cancerous (n = 37), the corresponding non-cancerous tissues adjacent to the cancer (n = 37) and 9 samples of healthy gastric tissues were examined by the RT-PCR assay (Figure 1). As the results showed, MK was detectable in 35 of the total 37 cancerous samples, 27 of the total 37 non-cancerous samples and 5 of the total 9 control samples.

With the Quantity One 4.5.0 Software (BIO-RAD, USA), the PCR products were quantified by densitometric measurement and the relative quantity of MK to β-actin was obtained by MK/β-actin. Using the Wilcoxon signed rank test, we found that there was significant difference between cancerous group and the control group ($p < 0.01$) or the

Table 2. Associations between MK expression and the categorical clinicopathological variables of the tumors

Stage[a]	n	Low expression[b]	high expression[b]	χ^2	p
pT Stage[c]					
pT1-2	9	4	5		
pT3	15	6	9		
pT4	13	5	8	0.08	0.960
pN Stage[c]					
pN0	11	6	5		
pN1	13	5	8		
pN2-3	13	4	9	4.67	0.097
pM Stage[c]					
pM0	23	13	10		
pM1	14	2	12	6.44	0.011
pTNM Stage[c]					
I-II	12	8	4		
III	10	4	6		
IV	15	3	12	6.02	0.049
Grade of differentiation[c]					
well	10	5	5		
moderate	13	6	7		
poor	14	4	10	1.37	0.503

[a]Tumor staging was performed according to the Tumor-Node-Metastasis (TNM) staging system by the International Union Against Cancer (UICC) (19).
[b]$2^{-\triangle\triangle Ct}$ stands for the relative quantity, 2.01 (the median of the $2^{-\triangle\triangle Ct}$ of the cancer samples) was used as a cutoff value. $2^{-\triangle\triangle Ct} < 2.01$, indicated low expression; $2^{-\triangle\triangle Ct} > 2.01$, indicated high expression.
[c]p, T, N and M stand for pathological, tumor, node and metastasis, respectively.

Table 1. MK expression of cancerous, non-cancerous and control groups by using real-time PCR

Group	n	Average of Ct		Median of $2^{-\triangle\triangle Ct}$	p^a	p^b
		MK	PBGD			
Control	9	26.66 ± 1.30	33.40 ± 3.42	1.00		
Non-cancer	37	26.74 ± 2.41	32.82 ± 3.65	1.23	0.250	
Cancer	37	25.95 ± 3.17	31.63 ± 3.70	2.01	0.034	0.027

[a]Compared with the control, Wilcoxon signed rank test.
[b]Compared between cancer and non-cancer, Wilcoxon signed rank test (paired comparison).

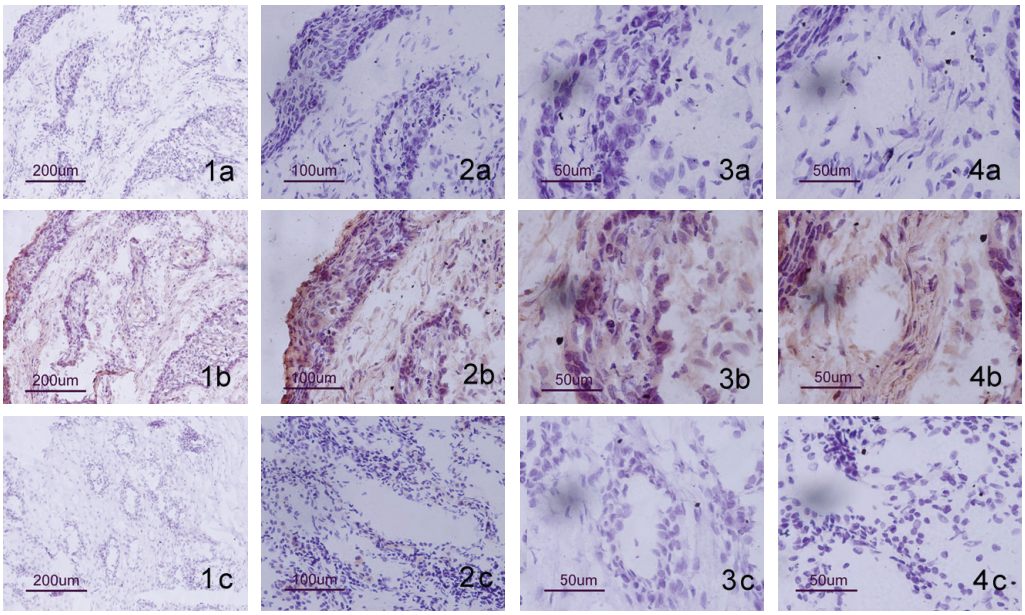

Figure 3. Immunohistochemical staining of human gastric cancerous tissues with rabbit anti-MK antibodies. (1a-4a) Results of negative control incubated with PBS in place of anti-MK antibodies were shown under different microscopic fields. (1b-4b) Staining results of the cancerous tissue incubated with anti-MK antibody were shown under different microscopic fields. Cancer cells were stained in the cytoplasm, nucleus and nucleolus. (1c-4c) Staining results of the normal tissue incubated with anti-MK antibody were shown under different microscopic fields, no positive result was obtained.

non-cancerous group ($p < 0.01$), but no difference between the non-cancerous group and the control group ($p = 0.86$) (Figure 2). Additionally, the expression in nearly 1/3 of the non-cancerous tissues was found as high as the corresponding tumor tissues compared with the control tissues.

Real-time PCR

The relative quantitation analysis verified the results of RT-PCR (Table 1). With χ^2 test, MK expression was found to be associated with the pTNM stage ($p = 0.011$) and distant metastasis ($p = 0.049$), but not with the size ($p = 0.960$), nodal involvement ($p = 0.097$) and differentiation grade ($p = 0.503$) (Table 2). The dissociation curves of MK and PBGD PCR products were consistent, confirming that there were no primer-dimer and non-specific PCR products.

Immunohistochemical analysis

To clarify the location of MK in gastric cancer tissues, 37 tumor tissues and 9 control tissues were assayed by immunohistochemical analysis. The results showed 34 of 37 cancer samples presented the specific brown-yellow color as positive results and all the control cases were negative. The validity of the staining was confirmed by negative control sections which were stained with PBS in place of anti-MK antibodies (Figure 3). At the cellular level, the cytoplasm of all the cancer cells in 34 positive cases was immunoreactive with anti-MK antibodies, and 23 of the 34 positive cases had the nucleus and nucleolus immunoreactivity. MK was also found to concentrate in the adenoid cells and around the vesicles in all the tissues with such components (Figure 4).

Discussion

For the high expression of MK in gastric cancer and its close relationship with the state of cancer, which was generally consistent with the previous studies, we drew two conclusions. In the first place, MK might be applied as a useful marker for diagnosis. Previous studies had shown the phenomena that urine and serum MK concentrations were elevated in gastric cancer patients, and reflected the progression stage, which postulated an easier and feasible way for diagnosis. By interpreting with the MK expression of cancer itself, our studies reasoned the phenomena and confirmed the postulation. The other conclusion was that MK might play an important role in the tumorigenesis of gastric cancer. Recent researches proved that MK could boost the growth of cells (10, 11), advance the genesis of blood vessels (11-13), accelerate fibrinolysis and have the function of chemotaxis (14-16), which were essential to the development and progression of cancer. All these gave evidence that MK might stimulate and prompt the tumor.

In the process of RT-PCR experiments, the MK expression in nearly 1/3 of the non-cancerous tissues was found as high as the corresponding tumor tissues compared with the control tissues. We investigated which pTNM stages these samples were in. Interestingly, they were almost in stage IV and few in stage III. Considering that cancer in advanced stages was aggravated, we suspected that these

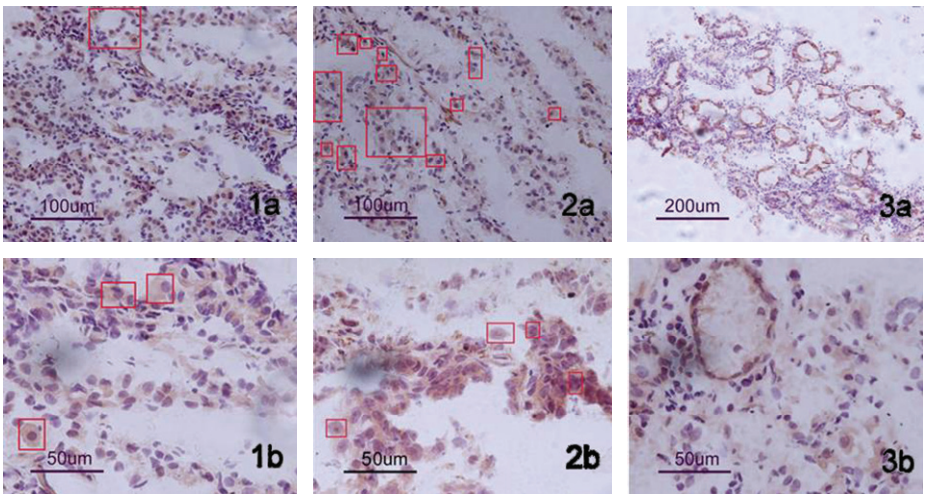

Figure 4. The location analysis of MK in human gastric cancerous cells. (1a & 1b) Positive results of different amplifications show that MK located in cytoplasm; (2a & 2b) Positive results of different amplifications show that MK located in nucleus and nucleolus; (3a & 3b) Positive results of different amplifications show that MK concentrated in adenoid cells and vesicles. The cells in red frames are the typical cells.

corresponding non-cancerous tissues were under the way of cancerization or preliminary cancerization. At the same time, the high expression of MK in these non-cancerous samples indicated that MK might contribute to the gastric tumor development. Furthermore, through statistic analysis of RT-PCR and real-time PCR results, MK expression in gastric tumor group was found significantly higher than the corresponding non-cancerous group or the control group and associated with the pTNM stage and pM-category. All these substantiated our previous suspicion that MK might contribute to the gastric tumor development. In addition, the location of MK protein in gastric cancer gave clue to us that we could use the gastric adenoid carcinoma cell line to study the effect of MK on gastric tumorigenesis and tumor progress.

However, due to the difficulty of collecting gastric samples including both the tumor tissues and control tissues, in the study we only assayed 37 gastric cancerous tissues, 37 of the matched corresponding non-cancerous tissues and 9 healthy gastric tissues. As the assayed result of these samples showed, MK expression failed to be associated with the pT-category and nodal involvement, unlike the pTNM stage and pM-category. On the one hand, such phenomenon might be due to the limited number of samples. On the other hand, we hypothesized that the function of MK might be more correlated to the distant metastasis through vasculature than the lymphatic system and direct extension. Thus, to clarify the problem more samples need to be collected and assayed in future research in order to eliminate possible affecting factors such as gender, age, region, and race.

MK protein was found especially in the adenoid part of tumors and highly concentrated in nucleus and nucleolus. The previous studies showed that MK could boost the growth of cells, advance the genesis of blood vessels and so on as mentioned above. These actions might be achieved *via* its anti-apoptotic activities (17, 18). So we thought that MK was expressed and excreted by the tumor cells, acted to them or transmitted by blood vessels to distant tissues and functioned as a cytokine through modulation of the gene expression in target cell nucleus. Yet, there was no report about the delivery pathway of MK and its excretion phenomenon of MK in cancer. So further studies are still needed to elucidate the precise mechanism and MK.

In conclusion, the expression level and location of MK in Chinese gastric tumor tissues might be related to the tumor genesis and progression.

Acknowledgement

This work was supported by the Research Foundation from the Ministry of Education of China (Grant No.02111).

References

1. 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 mid-gestation period of mouse embryogenesis. Biochem Biophys Res Commun. 1988;151:1312-1318.
2. Garver RIJ, Radford DM, Donis-Keller H, Wick MR, Milner PG. Midkine and pleiotrophin expression in normal and malignant breast tissue. Cancer. 1994;74:1584-1590.
3. Aridome K, Tsutsui J, Takao S, et al. Increased midkine gene expression in human gastrointestinal cancers. Jpn J Cancer Res. 1995;86:655-661.
4. 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;56:2515-2518.
5. Koide N, Hada H, Shinji T, et al. Expression of the midkine gene in human hepatocellular carcinomas. Hepatogastroenterology.

1999;46:3189-3196.
6. 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;79:179-184.
7. Mishima K, Asai A, Kadomatsu K, et al. Increased expression of midkine during the progression of human astrocytomas. Neurosci Lett. 1997:233:29-32.
8. Ikematsu S, Okamoto K, Yoshida Y, et al. High levels of urinary midkine in various cancer patients. Biochem Biophys Res Commun. 2003;306:329-332.
9. Obata Y, Kikuchi S, Lin Y, et al. Serum midkine concentrations and gastric cancer. Cancer Sci. 2005;96:54-56.
10. Kadomatsu K, Hagihara M, Akhter S, Fan QW, Muramatsu H, Muramatsu T. Midkine induces the transformation of NIH3T3 cells. Br J Cancer. 1997;75:354-359.
11. 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;61:8486-8491.
12. Laaroubi K, Delbe J, Vacherot F, et al. Mitogenic and *in vitro* angiogenic activity of human recombinant heparin affin regulatory peptide. Growth Factors. 1994;10:89-98.
13. Choudhuri R, Zhang HT, Donnini S, Ziche M, Bicknell R. An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. Cancer Res. 1997;57:1814-1819.
14. Kojima S, Muramatsu H, Amanuma H, Muramatsu T. Midkine enhances fibrinolytic activity of bovine endothelial cells. J Biol Chem. 1995;270:9590-9596.
15. Qi M, Ikematsu S, Maeda N, et al. Haptotactic migration induced by midkine: Involvement of proteintyrosine phosphatase, mitogen-activated protein kinase and phosphatidylinositol 3-kinase. J Biol Chem. 2001;276:15868-15875.
16. Maeda N, Ichihara-Tanaka K, Kimura T, Kadomatsu K, Muramatsu T, Noda M. A receptor-like protein-tyrosine phosphatase PTPζ/RPTPb binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPζ. J Biol Chem. 1999;274:12474-12479.
17. Muramatsu H, Zou K, Sakaguchi N, Ikematsu S, Sakuma S, Muramatsu T. LDL receptor-related protein as a component of the midkine receptor. Biochem Biophys Res Commun. 2000; 270:936-941.
18. Shibata Y, Muramatsu T, Hirai M, et al. Nuclear targeting by the growth factor midkine. Mol Cell Biol. 2002;22:6788-6796.
19. Sobin LH, Wittekind Ch, eds. UICC: TNM Classification of Malignant Tumors, 6th edition, New York, Wiley-Liss, 2002.