

ESOPHAGEAL CANCER

Expression of midkine and its clinical significance in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of midkine in esophageal squamous cell carcinoma (ESCC) and analyze its relationship with clinicopathological features.

METHODS: RT-PCR and immunocytochemical staining were used to detect the expression of midkine mRNA and protein in EC109 cells, respectively. Then the expression of midkine in 66 cases of ESCC samples were detected by immunohistochemistry using monoclonal antibodies against human midkine.

RESULTS: Midkine was expressed in EC109 cell by RT-PCR and immunocytochemistry. The immunoreactivity was detected in 56.1% (37/66) of the ESCC samples. The expression of midkine was found in cytoplasm of tumor cells. Notably, the intensity of midkine was stronger at the area abundant in vessels and the invading border of the tumors. Midkine was more intensely expressed in well differentiated tumors (76.9%) than in moderately and poorly differentiated tumors (43.1% and 41.2%, respectively) ($P < 0.05$). There was no statistically significant correlation between midkine expression and gender, age, clinical stage, lymph node metastasis or survival in ESCC.

CONCLUSION: Midkine is overexpressed in ESCC. It may play a role in tumor angiogenesis and invasion. The expression of midkine is correlated with tumor cell differentiation in ESCC. The more poorly tumor cells differentiate, the weaker midkine expresses.

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INTRODUCTION

Midkine is a novel heparin-binding growth factor, originally identified as the product of a retinoic acid-responsive gene. Midkine and pleiotrophin (PTN; also called HB-GAM) are the only members of a family distinct from other heparin-binding growth factor families^[1,2]. In the last few years, midkine has been found to be overexpressed in various human malignant tumors, such as esophageal^[3-5], gastric^[4,5], colorectal^[4-6], liver^[4,7,8], lung^[9], thyroid^[10], urinary bladder^[11] and prostate carcinomas^[12], as well as neuroblastomas^[13] and astrocytomas^[14,15]. Especially in neuroblastomas^[13] and bladder carcinomas^[11], the level of midkine expression correlates negatively with the patients' prognosis. Furthermore, it has also been reported that midkine is extensively expressed in the early stages of carcinogenesis^[6,12]. All of these studies suggested that midkine may play an important role in carcinogenesis, development and metastasis of tumors, and that it could serve as a novel tumor marker.

In the present study, we investigated midkine expression in esophageal squamous cell carcinoma (ESCC). First, we detected midkine mRNA and midkine protein expression in EC109 cells (a cell line of well differentiated human esophageal squamous cell carcinoma) by means of RT-PCR and immunocytochemical staining, respectively. Then we examined midkine expression in 66 cases of ESCC tissues by immunohistochemical staining using monoclonal antibody against human midkine, and analyzed the correlation between midkine expression and clinicopathological features.

MATERIALS AND METHODS

Cell lines

EC109 cells, a well differentiated human ESCC cell line, established by National Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College

in 1976, and generously supplied by Department of Immunology, the School of Oncology, Peking University, were maintained in RPMI1640 with 100 mL/L heat-inactivated fetal bovine serum in humidified 50 mL/L CO₂ atmosphere at 37 °C.

RT-PCR

Total RNA was extracted from EC109 cells with a single method using the Trizol reagent according to the manufacturer's protocol. Complementary DNA (cDNA) of EC109 cells was synthesized from 5 µg of total RNA using M-MLV reverse transcriptase. PCR was performed in a total of 20 µL reaction mixture, comprising Taq DNA polymerase, dNTPs, buffer, 10 µmol/L of each gene-specific forward and backward primers and 2 µL of cDNA. β -actin mRNA levels were used as internal controls. The sequences of PCR primers are as follows: for midkine, 5' AAAGAATTCGAGATGCAGCACCGAGG 3' (forward) and 5' AACTCGAGCCAGGCTTGGCGTCTAGTC3' (backward); for β -actin, 5' AACTGGGACGACATGGAGAA 3' (forward) and 5' GGTAGTCAGTCAGGTCCC3' (backward). Expected RT-PCR product sizes were 477 bp for midkine and 318 bp for β -actin. PCR conditions consisted of an initial denaturation step for 5 min at 94 °C, followed by 35 cycles of amplification (denaturation for 30 s at 94 °C, annealing for 30 s at 62.5 °C, and extension for 30 s at 72 °C) and a final extension for 10 min at 72 °C. Then 7 µL of PCR product was electrophoresed on 15 g/L agarose gels to visualize cDNA products.

Immunocytochemical staining of EC109 cells

Dako EnVision system was used for immunocytochemical staining of midkine. EC109 cells were fixed with acetone/methanol (1 : 1, V/V). Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxide for 10 min at room temperature. After washed with PBS, the slides were blocked with 50 mL/L horse serum for 1 h at 37 °C, followed by incubation with mouse anti-human midkine antibody overnight at 4 °C. After washed with PBS, the slides were incubated with EnVision for 1.5 h at 37 °C and then further washed for 3 times with PBS. After that the sections were reacted with DAB for 5 to 7 min to allow visualization. Finally, the slides were counterstained with hematoxylin, dehydrated, and evaluated under a light microscope.

Patients

Sixty-six specimens of ESCC were obtained from patients who underwent surgery at Beijing Cancer Hospital from Aug. 1998 to Dec. 2000. None of the patients received chemo- or radiotherapy prior to resection. These patients included 56 males and 10 females, aged from 38 to 76 years with a median age of 57.5 years. All the cases had been pathologically proven to be ESCC, including 26 well, 23 moderately and 17 poorly differentiated tumors; and 35 metastatic lymph node specimens.

Immunohistochemical staining of tumor tissues

Mouse monoclonal antibody against human midkine was prepared by our laboratory^[16]. Secondary antibody

of EnVision kit was purchased from Dako Inc. The sections were stained using standard EnVision, peroxidase method. Four-micrometer-thick tissue sections were dewaxed with xylene and rehydrated with graded ethanol, then briefly immersed in water. Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxide for 10 min at room temperature. Then heat-mediated antigen retrieval was performed by heating the sections (immersed in 0.01 mol/L citrate buffer, pH 6.0) in a microwave oven for 10 min. After washed with PBS, the slides were blocked with 50 mL/L milk to prevent nonspecific binding for 1 h at 37 °C, followed by incubation with mouse anti-human midkine antibody overnight at 4 °C. After washed with PBS, the slides were incubated with EnVision for 1.5 h at 37 °C and then further washed for 3 times with PBS. Finally, the sections were reacted with DAB for 5 to 7 min to allow visualization, and counterstained with hematoxylin, dehydrated, and evaluated under a light microscope. The sections known to be midkine positive were stained under the same conditions as above and served as positive controls. For negative controls, sections were processed as above but the primary antibody was replaced by PBS. Anti-midkine immunoreactivity was confined primarily to the cytoplasm. One hundred cells from 5 randomly selected representative fields ($\times 400$) of each section were counted. It was considered positive when all immunoreactivity levels were more than 10% with anti-midkine antibody.

Statistical analysis

The χ^2 test was used to examine the differences of midkine expression between groups, and Cox regression was employed to analyze the correlation between midkine expression and patients' post-operation survival time by SPSS 11.0 software. $P < 0.05$ was considered as statistically significant.

RESULTS

Midkine expression in EC109 cells

Midkine mRNA transcription was detected in EC109 cells by means of RT-PCR (Figure 1). After amplification of RT-PCR, we observed that there were two strands located in the gaps from 400 bp to 500 bp and from 300 bp to 400 bp, respectively. The sizes of RT-PCR product indicated by strands were conformed with expected RT-PCR product sizes of 477 bp for midkine and 318 bp for β -actin. The positive signals of midkine protein immunocytochemical staining were located in cytoplasm. Expression of midkine protein was observed in EC109 cells (Figure 2).

Expression of midkine protein in ESCC tissues

Midkine expression was confined primarily to the cytoplasm, shown as brown granules (Figure 3A). Midkine was expressed in 37 of 66 ESCCs (56.1%), while it was present at a very low level in just a few normal esophageal epithelia adjacent to tumor tissues. Midkine positive tumor cells were distributed in foci, and the intensity of midkine was stronger at the area abundant in vessels and the invading border of tumors (Figure 3B). In addition, Midkine ex-

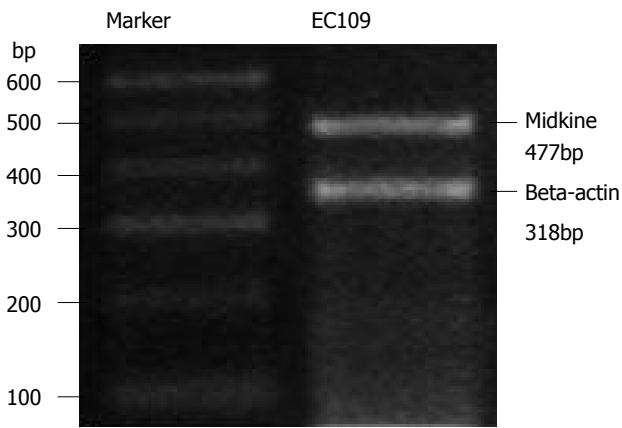


Figure 1 Amplification of midkine mRNA in EC109 cells by RT-PCR.

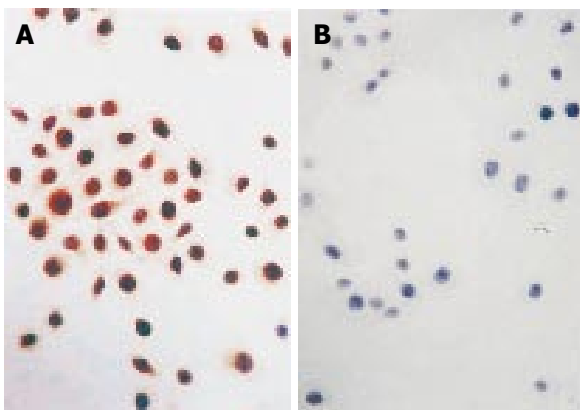


Figure 2 Midkine expression in EC109 cells (Immunoperoxidase method $\times 400$). A: Positive in EC109 cells; B: Negative control.

pression was also found in epithelial cells and smooth muscle cells of vessels in tumor stromal elements.

Correlation between midkine protein expression and clinicopathological features

Midkine was expressed in 20 of 26 well differentiated ESCCs (76.9%), more than that in moderately (10/23, 43.1%) and poorly (7/17, 41.2%) differentiated tumors ($P < 0.05$). There was no statistically significant correlation between midkine expression and gender, age, clinical stage, lymph node metastasis or post-operation survival time in ESCC ($P > 0.05$, Table 1).

DISCUSSION

Many studies have shown that growth factors not only promote tissue proliferation but also induce malignant transformation, and they play important roles in the development of neoplasm^[17]. Over-expression of growth factors has been found in many human tumors. Midkine, a novel heparin-binding growth factor, is strongly expressed in mid-gestational period. While in normal adult tissues, its expression is highly restricted. It is normally highly expressed in small bowel epithelium, moderately expressed in the thyroid and lowly expressed in lung, stomach, colon and kidney^[18]. In the last few years, Midkine has been found

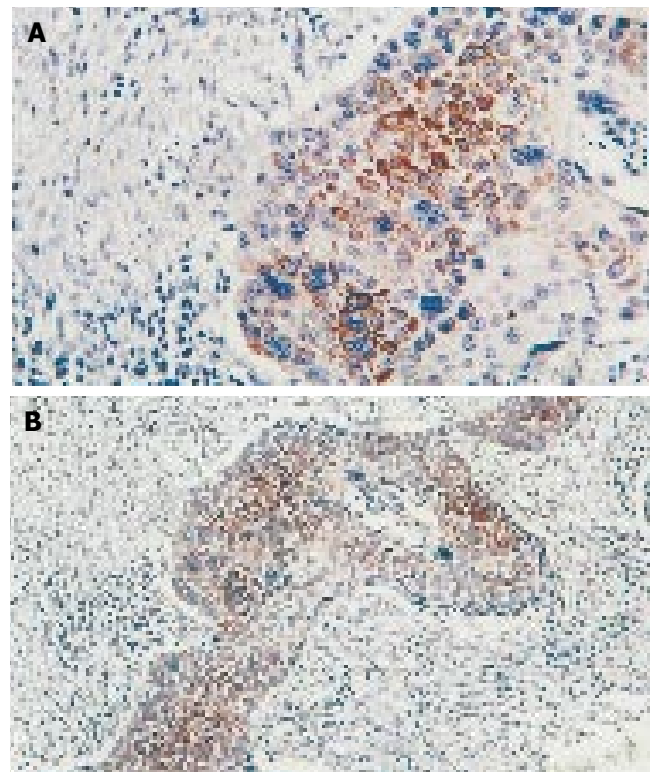


Figure 3 Midkine expression in ESCC (Immunoperoxidase method). A: In cytoplasm ($\times 400$); B: Stronger at the area abundant in vessels ($\times 200$).

to be over-expressed in various human malignant tumors, such as esophageal^[3-5], gastric^[4,5], colorectal^[4-6], liver^[4,7,8], lung^[9], thyroid^[10], urinary bladder^[11] and prostate carcinomas^[12], as well as neuroblastomas^[13] and astro-cytomas^[14,15]. Especially in neuroblastomas^[13] and bladder carcinomas^[11], the level of midkine expression correlates negatively with the patients' prognosis. It has also been reported that midkine is extensively expressed in the early stages of carcinogenesis^[6,12]. Further studies showed that midkine had several cancer-related activities. Midkine could transform NIH3T3 cells^[19] and enhanced the plasminogen activator/plasmin levels in bovine endothelial cells^[20]. It also promotes cell growth^[21,22], cell survival^[23] and migration of various cells such as neutrophils and macrophages^[24]. These biological activities of midkine supported the possible involvement of midkine in carcinogenesis and tumor advancement.

To further investigate the role of midkine in carcinogenesis and tumor advancement, we examined midkine expression in ESCC. First, we found that midkine was expressed in EC109 cell by means of RT-PCR and immunocytochemistry. Further, we examined midkine expression in 66 ESCC samples using immunohistochemistry. Our data showed that midkine was over-expressed in ESCC with the positive rate of 56.1% (37/66), while it was present at a very low level in just a few normal esophageal epithelium adjacent to tumor tissues. And the intensity of midkine was stronger at the area abundant in vessels and the invading border of tumors. Midkine expression was also found in epithelial cells and smooth muscle cells of vessels in tumor stromal tissues. These characteristics of distribution were confirmed in Kato's study^[10], suggesting

Table 1 Intensity of midkine expression in relation to clinicopathological variables in esophageal squamous cell carcinoma

Variable		<i>n</i>	Positive <i>n</i>	Negative <i>n</i>	Positive rate (%)	χ^2 value	<i>P</i> value
Gender	Male	56	31	25	55.4	0.074	0.785
	Female	10	6	4	60.0		
Age (yr)	60	35	15	20	57.1	0.035	0.851
	≥60	31	17	14	54.8		
Differentiation ^a	Well	26	20	6	76.9	7.906	0.019
	Moderately	23	10	13	43.5		
	Poor	17	7	10	41.2		
TNM Classification	I-II	15	16	7	69.6	2.669	0.102
	III-IV	13	21	22	48.8		
Lymph node metastasis	Positive	35	18	17	51.4	0.651	0.420
	Negative	31	19	12	61.3		

^a*P*<0.05 as compared between well differentiated and moderately or poorly differentiated tumors.

that midkine may play a role in tumor angiogenesis and invasion. It has been demonstrated that midkine could promote the endothelial cells growth and it is a novel molecular mediator in tumor angiogenesis^[25].

Our study also showed that the expression of midkine was correlated with tumor cell differentiation in ESCC. Midkine was more intensively expressed in well differentiated tumors (76.9%) than in moderately (43.1%) and poorly (41.2%) differentiated tumors (*P*<0.05). The phenomenon that the more poorly tumor cells differentiated, the weaker midkine was expressed was also observed in other studies on midkine expression in esophageal cancer and vulvar tumors^[27,28].

In summary, our study showed that midkine was over-expressed in ESCC. It may play a role in tumor angiogenesis and invasion. The expression of midkine was correlated with tumor cell differentiation in ESCC: the more poorly tumor cells differentiated, the weaker midkine was expressed. Since midkine is a secretory protein, midkine level in blood can be detected when it over-expresses in tumors. It has been reported that an elevated serum midkine level can be detected in many malignant tumors, and serum midkine level decreases after the removal of tumors^[27,28]. In addition, the elevated midkine level even can be detected in urine^[29]. These studies suggest that midkine could serve as a tumor marker which can be conveniently detected.

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