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BASIC RESEARCH

Over-expressed and truncated midkines promote proliferation of BGC823 cells *in vitro* and tumor growth *in vivo*

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Abstract

AIM: To determine whether midkine (*MK*) and its truncated form (*tMK*) contribute to gastric tumorigenesis using *in vitro* and *in vivo* models.

METHODS: Human *MK* and *tMK* plasmids were constructed and expressed in BGC823 (a gastric adenocarcinoma cell line) to investigate the effect of over-expressed *MK* or *tMK* on cell growth and turmorigenesis in nude mice.

RESULTS: The growth of *MK*-transfected or *tMK*-transfected cells was significantly increased compared with that of the control cells, and *tMK*-transfected cells grew more rapidly than *MK*-transfected cells. The number of colony formation of the cells transfected with *MK* or *tMK* gene was larger than the control cells. In nude mice injected with *MK*-transfected or *tMK*-transfected cells, visible tumor was observed earlier and the tumor tissues were larger in size and weight than in control animals that were injected with cells without the transfection of either genes.

CONCLUSION: Over-expressed *MK* or *tMK* can promote human gastric cancer cell growth *in vitro* and *in vivo*, and *tMK* has greater effect than *MK*. *tMK* may be a more promising gene therapeutic target compared with *MK* for treatment of malignant tumors.

Key words: Midkine; Truncated midkine; Gastric cancer; BGC823; Tumorigenisis

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INTRODUCTION

Midkine (*MK*), a heparin-binding growth factor, was discovered through screening for factors that mediate retinoic acid-induced cell differentiation by Kadomatsu in 1988[1]. *MK* is a cysteine- and basic amino acid-rich protein, which is composed of two domains, i.e., N- and C-terminal half domains. The two domains are linked by a flexible linker region. Although the precise relationship between structural features and biological activities remains to be elucidated, it is interesting that only the C-terminal half domain of *MK* retains biological activities[2,3]. *MK* gene maps to band 11p11.2[4] and consists of five exons. Exon 1 does not encode amino acid sequence. Exon 2 encodes the hydrophobic leader sequence, which constitutes the beginning of gene translation. The signal peptide cleavage site lies toward the 3' end of exon 2[2]. A truncated form of *MK* (*tMK*), which lacks exon 3 encoding the N-terminal half, was found in pancreatic carcinoma cell lines by Kaname in 1996[5]. Recently two novel truncations of the *MK*, *tMKB* and *tMKC*, were found in a number of tumor cell lines, including A549 cells (lung adenocarcinoma), SGC-7901 cells (gastric cancer), 8910 cells (ovarian tumor) and MG-63 cells (osteosarcoma)[6].

Many evidences showed that *MK* is expressed at higher levels in various tumors, such as digestive, lung, liver and breast cancers, neuroblastoma and Wilms' tumor[7-10]. *tMK* was found in pancreatic, gastric, Wilms', colorectal, bile duct and breast tumors, but not in non-cancerous and normal tissues[5,11-14]. *MK* can promote Wilms' tumor cell proliferation and tumor angiogenesis[7,10,15], inhibit tumor cell apoptosis, induce transformation of NIH3T3 cells, and protect patocellular carcinoma (HCC) cells

against TRAIL-mediated apoptosis[16-19]. *MK* and *tMK* are correlated positively with metastasis of HCC, prostate carcinomas, Lewis lung carcinoma, gastric cancer[20-23] and gastrointestinal carcinomas[24]. They can induce the transformation of SW-13 cells and shorten the latency of tumor formation in nude mice[25].

Our previous study also showed that *MK* highly expressed in gastric cancer tissues of Chinese patients, and the expressions of *MK* mRNA and protein were both associated with the clinical stage and distant metastasis of gastric cancer[26]. Therefore, it is necessary to determine the roles of *MK* and *tMK* in both tumorigenesis and tumor development in gastric cancer. BGC823 cell is a poorly differentiated gastric adenocarcinoma cell line and is an idea in vitro model for studying the tumorigenic activity. In the present study, we obtained human *MK* and *tMK* cDNA from gastric carcinoma tissues, constructed *MK* or *tMK* over expression plasmids (Figure 1), and then transfected the plasmids into BGC823 cell to study the effect of *MK* and *tMK* on tumorous characteristics *in vitro* and *in vivo*.

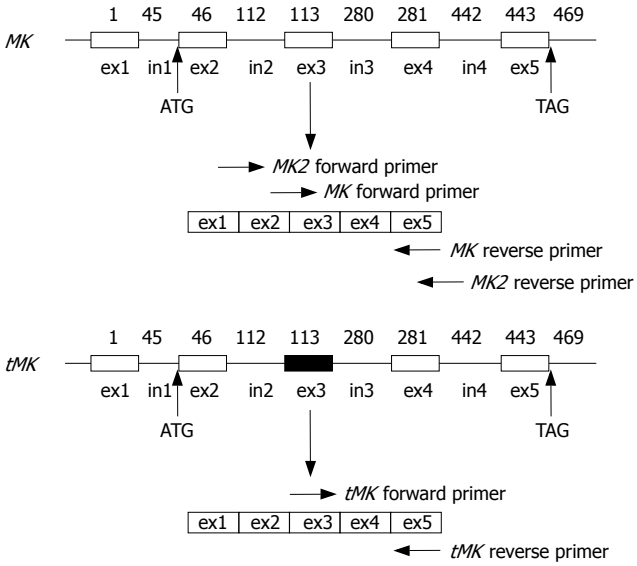

Figure 1 Illustration of *MK* and *tMK* gene DNA structures. Box: Exon (ex); Line: Intron (in); Shaded box; Truncated portion; ATG: Start site; TAG: Terminal site; Numeric figures: Nucleotide position of the mRNA transcript. Arrowheads indicate the sites of primer complemented with *MK* or *tMK* mRNA.

MATERIALS AND METHODS

Plasmids construction

Plasmids with *MK* and *tMK* eukaryotic expression were constructed[2,5,13] (Figure 1). In our previous work, we designed pMD18-T-*MK* and pMD18-T-*tMK* vector[27,28], and prepared the human *MK* and *tMK* DNA fragments by PCR using *MK-1* and *tMK* primers, (Table 1). The products of PCR digested with *Hind*Ⅲ and *Eco*R Ⅰ were inserted into the eukaryotic expression plasmid vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA), which resulted in the formation of pcDNA3.1/*MK* and pcDNA3.1/*tMK*. The resultant recombinant plasmids were characterized by detailed restriction digestion (Figure 2).

Cell culture and transfection

BGC823, a poorly differentiated gastric adenocarcinoma cell line, was cultured in RPMI medium 1640 (Gibco/BRL) supplemented with 10% fetal calf serum (Si Ji Qing, China) at 37℃ under 5% humidified CO_2 and 100 μg/mL each of streptomycin and penicillin G (Amresco, USA). The plasmid was transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, approximately 0.8×10^5 cells/well were grown overnight in 24-well plates. When the cells reached 90%-95% confluence, they were transfected with 0.8 μg of pcDNA3.1/*MK* or pcDNA3.1/*tMK* or pcDNA3.1 in serum-free medium using Lipofectamine 2000. After 4 h incubation at 37℃, 400 μL RPMI 1640 with 10% FBS was added. Stable transfectants were selected in the presence of 400 mg/L G418 (Amersco) during 2 wk of culture.

RNA extraction and RT-PCR

Total RNA was extracted using the TaKaRa RNAiso Reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA concentrations were quantified by spectrophotometer at 260 nm. One μg total RNA was reverse-transcribed using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, USA). Subsequently, 2 μL of the incubation mixture was used as the template for the following PCR using 2 × Taq enzyme mix kit (Tian Gen, China). Primers were synthesized by Bioasia (Shanghai, China) and are listed in Table 1. PCR was carried out for 28 or 30 cycles of denaturation (30 s at 94℃), annealing (40 s at 55℃), and extension (30 s at 72℃). The PCR products were then detected on 1% agarose gel containing 0.5 mg/L ethidium bromide. The gel was put on an UV-transilluminator and photographed. The *MK* signal was measured by a densitometer and standardized against the β-actin signal using a digital imaging and analysis system (SmartSpec™ Plus, BIO-RAD, USA).

Western blot analysis

Cells (1×10^7) were lysed in a buffer containing 50 mmol/L Tris-Cl, pH8.0, 150 mmol/L NaCl, 0.02% NaN_3, 0.1% SDS, 100 mg/L phenylmethylsulfonyl fluoride (PMSF) and 1 mg/L Aprotinin, 1% Triton. After centrifugation, cell lysates (75 μg/lane) were subjected to 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked for 1 h in PBST (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween-20) containing 2% nonfat dried milk. Antibodies specific for *MK* (1:400, BA1263, Boster, China), β-actin (1:400, BA0410, Boster) and HRP-conjugated goat anti-rabbit secondary antibody (1:2000, BA1054, Boster) were used. Protein bands were detected by the enhanced chemiluminescence (ECL) reaction (Kibbutz Beit Haemek, Israel).

Proliferation analysis

Cell viability was assessed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan). Briefly, BGC823 cells transfected with pcDNA3.1/*MK*, pcDNA3.1/*tMK*, or pcDNA3.1 and parental BGC823 cells were plated onto 96-well plates in RPMI 1640 supplemented with 10% FBS at a density of 3×10^3 cells/well. After 4 h, the medium

Table 1 Primers used in this study

Primers	Sequence 5'-3'	Reference	Excepted size (bp)	Cycles of PCR
MK-1 sense	AAAAAAGCTTATGAAAAAGAAAGATAAGGTGAAGAAG	Paul *et al*, 2001[13]	389	28
MK-1 antisense	AAAAGAATTCCTAGTCCTTTCCCTTCCCT			
tMK sense	AAAAAAGCTTATGAAAAAGAAAGCCGACTG		221	28
tMK antisense	AAAAGAATTCCTAGTCCTTTCCCTTCCCT			
MK-2 sense	ATGCAGCACCGAGGCTTCCT	Kaname *et al*, 1996[5]	447	30
MK-2 antisense	ATCCAGGCTTGGCGTCTAGT		279	
β-actin sense	CCACGAAACTACCTTCAACTC		270	28
β-actin antisense	TCATACTCCTGCTGCTTGCTGATCC			

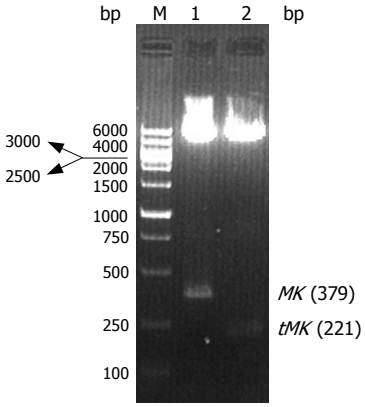

Figure 2 Restriction digestions of recombinant plasmids. M: Wide range DNA marker 100-6000 (TaKaRa); Lane 1: pcDNA3.1/*MK*; Lane 2: pcDNA3.1/*tMK*.

was changed to serum-free medium, and the cells were cultured ⩽ 2 d. Ten microliter of a solution containing 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl) - 2H-5-tetrazolio]-1, 3-benzene disulfonate sodium salt (WST-8) was added to each well. Following incubation of an additional 4 h, the absorbance was measured at 450 nm with a multi-detection microplate reader (Hynergy™ HT, BIO-TEK, USA).

Colony formation in soft agar

To perform the soft agar assay, a base layer of 0.5% (w/v) agar was prepared by adding autoclaved 1% (w/v) agar solution to 2x RPMI-1640 supplemented with 20% fetal calf serum at a 1:1 ratio. Stable transfectants or parental cell suspension containing 2×10^3 cells were prepared in a 1:1 mixture of 0.7% (w/v) agar solution and 2x RPMI -1640 supplemented with 20% FCS. Cell suspension was added to the top of the base layer, allowed to solidify, and the plate was incubated at 37℃ in a humidified 5% CO_2. The plates were incubated for 10-15 d. The number of colonies was determined by direct counting under microscopy. Counts were expressed as number of colonies per plate on average from three independent experiments.

Wound healing assay

The transfected BGC823 cells with pcDNA3.1/*MK*, pcDNA3.1/*MK* or pcDNA3.1 and parental cells were plated onto 6-well plates in RPMI 1640 supplemented with 10% FBS at a density of 2×10^5 cells/well. After 4 h, the medium was changed to serum-free medium. After 24 h, a plastic cell scraper was used to make an approximate 0.6 mm gap on the cell monolayer. Migration was quantitated by determining the distance between the cell edges at 0, 24 h and 48 h at the four marked locations on each well, using an inverted microscope with a scale in the eyepiece[29]. The results of the four readings from each well were averaged. Experiments were repeated three times.

Tumorigenicity study in vivo

Female BALB/c nude mice (5-6 wk old) were obtained from Vital River Lab Animal Co, Ltd, Beijing Laboratory Animal Research Center (Beijing, China). Cultured cells were harvested by trypsinization, washed and suspended in PBS at 10^7 cells/mL. One hundred μL cell suspensions were injected subcutaneously into the flank of female nude mice (seven mice per cell line). Tumor diameters were measured on d 14, 21 and 28, and tumor volume in mm^3 was calculated by the formula: Volume = $(\text{width})^2 \times \text{length}/2$. Tumor growth rates were calculated by the formula: TGR = $(V_{28th}-V_{21th})/7$ d. Data were presented as mean ± SE. Twenty-eight days after injection, nude mice were sacrificed, and the tumors were removed, photographed and weighed.

Immunohistochemistry

Immunostaining was performed on 6-μm tissue sections using strept-avidin-biotin staining kit (Boster). For antigen retrieval, slides were heated by microwave in 0.01 mol/L Tri-sodium citrate buffer. Nonspecific binding sites were blocked with 5% BSA for 30 min and endogenous peroxidase activity was suppressed by treatment with 3% H_2O_2 in methanol for 30 min. Sections were exposed to rabbit polyclonal anti-*MK* antibody (1:250, Boster) overnight at 4℃. 3,3-diamino-enzidine was used as chromogen (Boster). Counterstaining was done with hematoxylin. Negative control sections were incubated with PBS instead of anti-*MK* antibodies. In each step, samples were washed with PBS.

Statistical analysis

Results were presented as mean ± SE. Statistical significance between groups was analyzed by one-way ANOVA followed with the Student-Newman-Keuls multiple comparison tests. A P value of < 0.05 was considered significant. Frequency of tumorigenesis in nude mice was calculated by Fisher's exact test.

RESULTS

Expression of MK and tMK

To evaluate the roles of *MK* and *tMK* in gastric tumorigenesis, we used transfection assay to obtain a

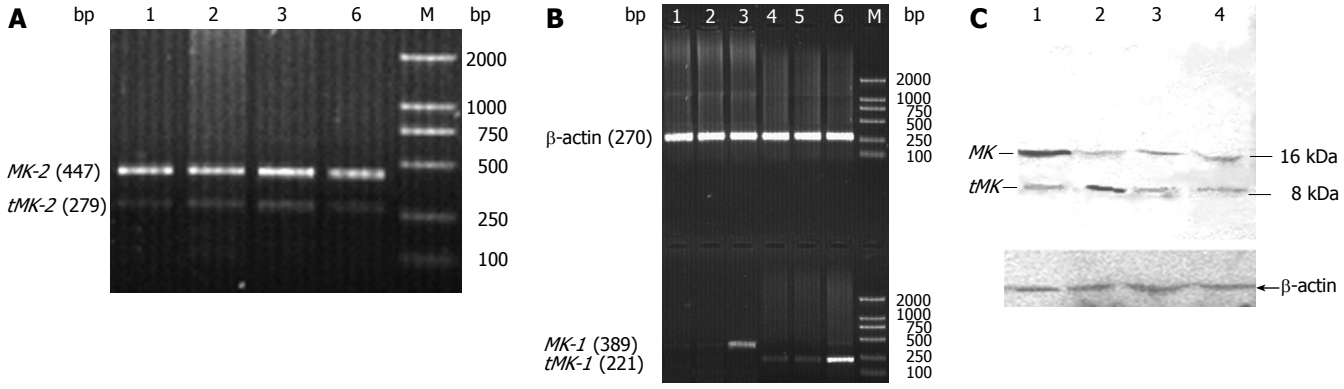

Figure 3 RT-PCR (**A**, **B**) and Western blotting (**C**) analysis of the expression of *MK* or *tMK* in BGC823 after transfection. **A** and **B**, M: DNA molecular weight standards, DL2000 (TaKaRa); Lane 1 and 4: BGC823; Lane 2 and 5: BGC823/vector; Lane 3: BGC823/*MK*; Lane 6: BGC823/*tMK*. **C**, Lane 1: BGC823/*MK*; Lane 2: BGC823/*tMK*; Lane 3: BGC823/vector; Lane 4: BGC823.

MK or *tMK* over-expressed gastric cell line. RT-PCR and Western blotting were performed to determine *MK* or *tMK* expression level in the transfected gastric carcinoma cells. Compared with the parental cells and pcDNA3.1 transfected cells, transfection of BGC823 cells with pcDNA3.1/*MK* or pcDNA3.1/*tMK* resulted in significant enhancement of *MK* or *tMK* expression in BGC823 cells. These results indicated that transfection of pcDNA3.1/*MK* and pcDNA3.1/*tMK* was successful (Figure 3B and C).

Effect of over-expression of MK or tMK on BGC823 cells

To determine whether over-expression of *MK* and *tMK* could affect the BGC823 cell growth, cell proliferation activity was detected using Cell Counting Kit. The transfection of pcDNA3.1/*MK* or pcDNA3.1/*tMK* to BGC823 significantly increased the proliferation of BGC823 cells compared with the control. This showed that over-expressed *MK* or *tMK* could accelerate the cellular proliferation at 12 h, 24 h, 36 h and 48 h. Moreover, *tMK* exhibited stronger stimulatory effect than *MK* (Figure 4A). No difference between BGC823/vector and BGC823 was detected (Figure 4A). Furthermore, colony-forming assay was conducted in BGC823, BGC823/vector, BGC823/*MK* and BGC823/*tMK* (Figure 4B and C). The results showed that the colony number of BGC823/*MK* and BGC823/*tMK* cells was increased by 2- to 3-fold compared with BGC823 and BGC823/vector (Figure 4C). In addition, the wound healing assay also showed that over-expressed *MK* or *tMK* could induce significant migration of the cell at 24 h and 48 h, about 1.5-fold over BGC823 and BGC823/vector cells, and *tMK* showed stronger effect than *MK* (Figure 4D). These results demonstrated that over-expression of *MK* and *tMK* significantly enhanced the malignant state and invasive ability of BGC823 cells.

Tumor growth promoted by MK or tMK in vivo

As the over-expression of *MK* or *tMK* significantly changed the behavior of BGC823 cells *in vitro*, it is necessary to analyze the tumorigenicity of the stable transfectant *in vivo*. The time and frequency of visible tumor in nude mice treated with BGC823, BGC823/vector, BGC823/*MK* and BGC823/*tMK*, respectively, are presented in Table 2. Tumor was clearly observed in most BGC823/*MK*- and all BGC823/*tMK*-injected mice at d 7, whereas visible tumor formed in about half of BGC823/vector and BGC823 injected mice until d 14. Furthermore, tumor diameters and volume were subsequently measured at d 14, 21 and 28. The results showed that tumor volumes of mice injected with BGC823/*MK* or BGC823/*tMK* cells were significantly larger than the control at d 21 and 28 (Figure 5C). Tumor growth rate (TGR) from d 21 to 28 showed that the TGR of nude mice injected with BGC823/*MK* or BGC823/*tMK* was significantly higher than the control mice (Figure 5D). At d 28 after inoculation, the tumors were removed, photographed and weighed. The tumor in mice injected with BGC823/*MK* and BGC823/*tMK* cells was 2-fold of that of the control (Figure 5B), and tumors in two mice injected with BGC823/*tMK* cells had erosive appearance (Figure 5A). Apparently, BGC823/*MK* or BGC823/*tMK* transfected cells could multiply and grow earlier and more rapidly than the BGC823 and BGC823/vector control cells in nude mice.

Immunohistochemical analysis

To detect whether BGC823/*MK*- or BGC823/*tMK*-transfected cells can stably express *MK* or *tMK* in nude mice for an extended period and the association between tumor growth and *MK* or *tMK* protein levels, immunohistochemical staining was conducted. *MK* was detected in cytoplasm and nucleus of tumor cells from different treatment groups of mice. The number and density of the positive points in tumor tissues induced with BGC823/*MK* and BGC823/*tMK* cells were evidently higher than the cells treated with BGC823 and BGC823/vector (Figure 6).

DISCUSSION

To determine whether *MK* and *tMK* contribute to gastric tumorigenesis and tumor development, BGC823 cells that over-expressed *MK* and *tMK* genes, and nude mice inoculated with the BGC823 cells over-expressing either *MK* or *tMK* were used as model systems *in vitro* and *in vivio*, respectively. To show that the upregulated *MK* and *tMK*

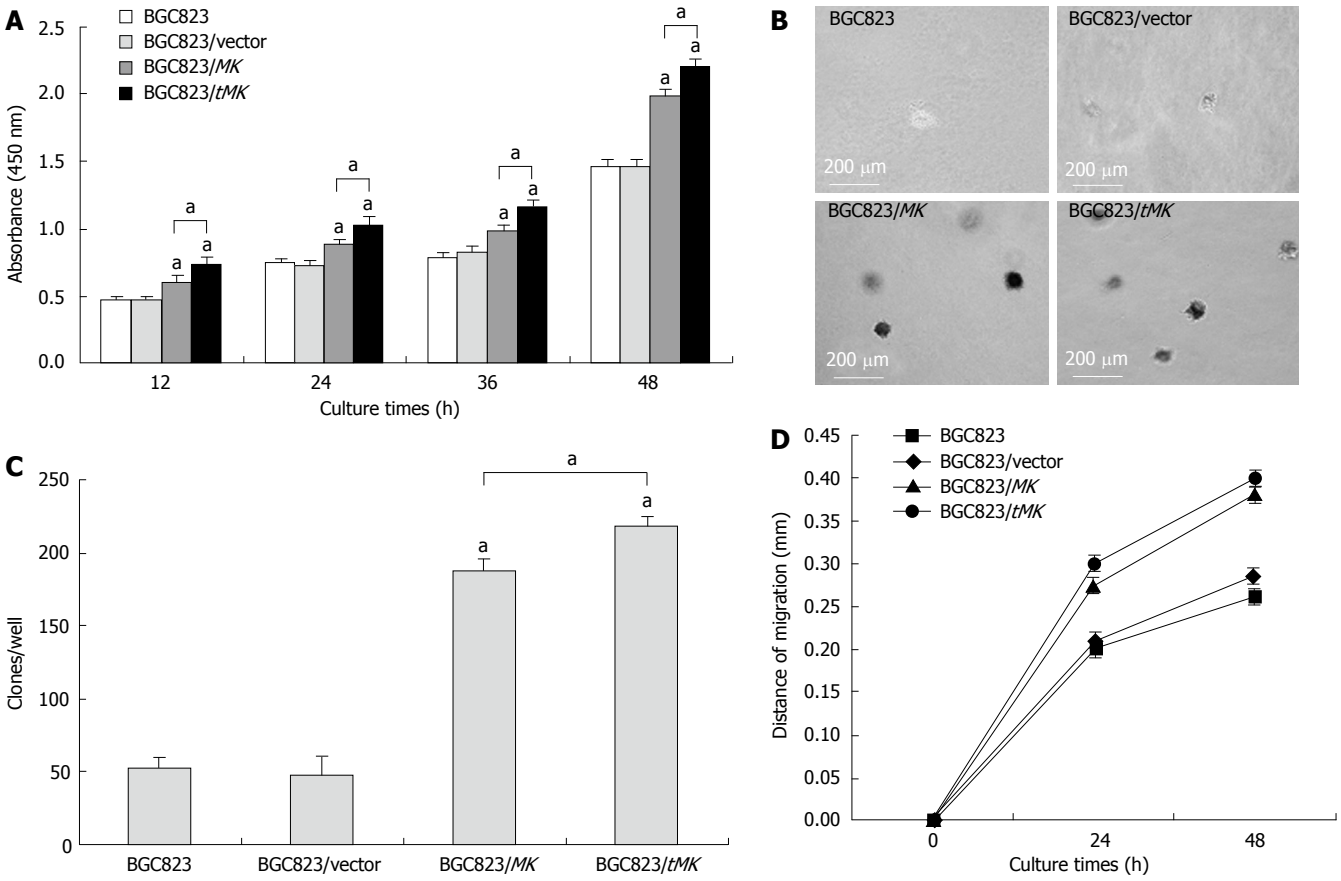

Figure 4 Effects of over-expressed *MK* or *tMK* on BGC823 cells *in vitro*. **A**: The cell proliferation determined by Cell Counting Kit ($P < 0.05$); **B**: Colony formation in soft agar observed under light microscope; **C**: Comparison of colony numbers. **D**: Analysis of cell migration.

were exogenous in the transfected cells, we designed another pair of primers for *MK-2* sequence (Table 1)[5]. The forward primer of *MK-2* was complemented with the start section of exon 2, and the reverse primer was complemented with exon 5 and several base pairs of 3'-untranslated regions. *tMK* lacks exon 3, so *MK* (448 bp) and *tMK* (296 bp) DNA were obtained at the same time by RT-PCR using primers for *MK-2*. There was no significant difference in the expression of *MK* and *tMK* between transfected cells and parental cells. The state in those cells transfected with or without *MK* and *tMK* genes can imitate *MK* and *tMK* expression from initial to metastatic stages of tumor formation.

Previous studies showed that the over-expression of *MK* in S462 cell (malignant peripheral nerve sheath tumor cell line) could increase the cell viability and protect the cells from apoptosis under serum deprivation, but did not induce the proliferation of S462 cells to promote xenograft tumor growth in nude mice[16]. *MK* and *tMK* can induce the transformation of SW-13 cells (adrenal carcinoma cell line) and shorten the latency of tumor formation in nude mice, but SW-13/*MK* and SW-13/*tMK* showed no difference in tumor growth rate from the control[25]. However in our study, the growth of BGC823 cells which over-expressed *MK* and *tMK*, was increased significantly compared with the control cells. The tumor formation time was shortened in nude mice injected with BGC823/*tMK* or BGC823/*MK* cells. Tumor growth rate of was significantly higher than the control, and tumor volume and weight were higher than the control, indicating that the idiographic effect of

Table 2 Frequency of tumorigenesis in nude mice

Injected cells	No. of mice	No. of days to tumor detection (percent of tumorigenesis)			
		7	14	21	28
BGC823	7	0 (0.00)	3 (42.86)	6 (85.71)	7 (100)
BGC823/vector	7	0 (0.00)	4 (57.14)	7 (100)	
BGC823/*MK*	7	5 (71.43)[a]	6 (85.71)[a]	7 (100)	
BGC823/*tMK*	7	7 (100)[b]			

P value was calculated by Fisher's exact test. 7 d: BGC823/MK *vs* BGC823 or BGC823/vector, $P = 0.0105$; BGC823/*tMK vs* BGC823 or BGC823/vector, $P = 0.0003$. 14 d: BGC823/*MK vs* BGC823, $P = 0.0174$; BGC823/*MK vs* BGC823/vector, $P = 0.0489$. [a]$P < 0.05$; [b]$P < 0.01$.

MK and *tMK* on tumorigenesis and tumor development may be related to types of tumors.

MK and *tMK* are heparin-binding growth factors. They play fundamental roles in the regulation of cell differentiation and development. Their aberrant expressions are usually associated with tumorigenesis[30-33]. In our study, *tMK*, which was only found in cancer tissues, had stronger effects than *MK* on tumor cell proliferation, and tumors from two mice injected with BGC823/*tMK* cells had erosive appearance. This result was in agreement with the previous studies. The differential activities of *MK* and *tMK* in promoting tumor proliferation may be attributed to the difference of the tertiary structure between these two proteins[34].

In conclusion, over-expressed *MK* or *tMK* could promote tumor development of human gastric cancer

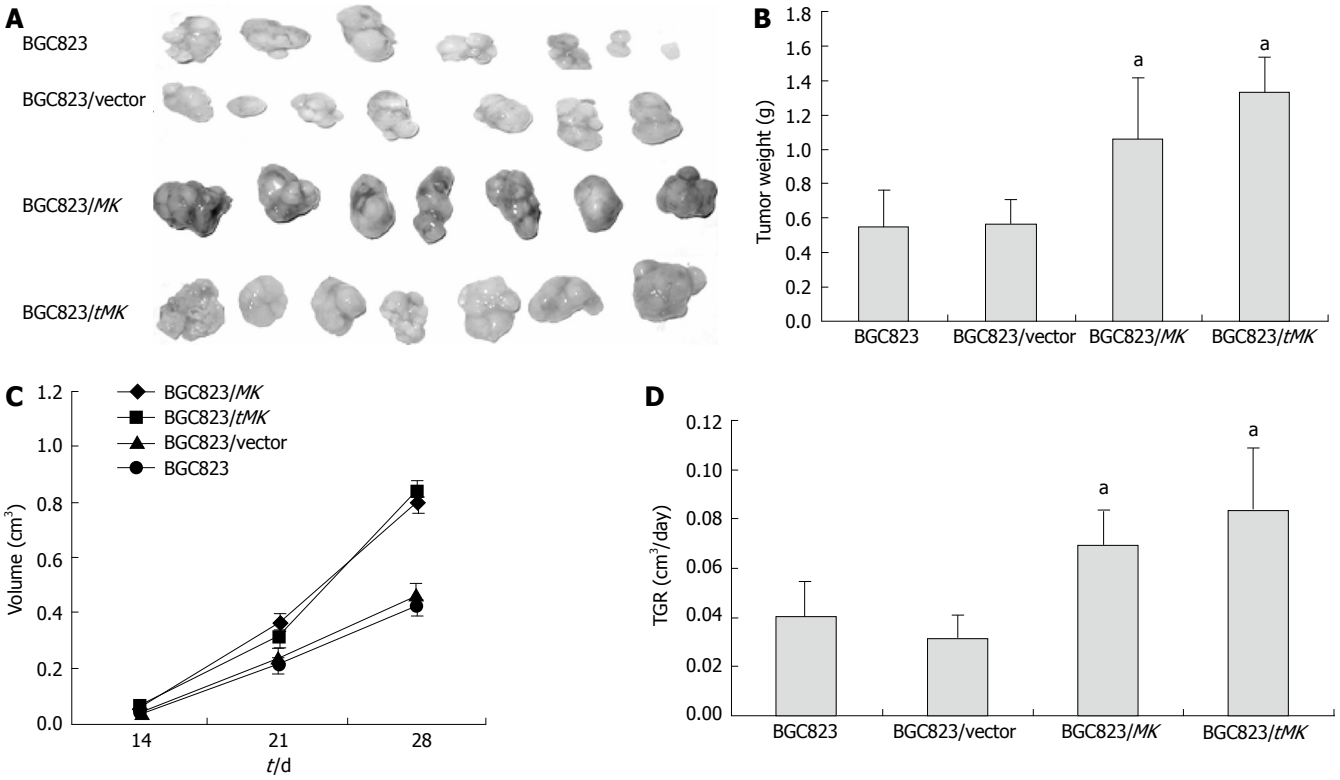

Figure 5 Promotion of tumorigensis of *MK*- or *tMK*- transfected cells *in vivo*. **A**: Photograph of tumor size; **B**: Comparison of tumor weight ($P < 0.05$); **C**: Measure of tumor volume ($^aP < 0.05$); **D**: Analysis of tumor growth rate ($^aP < 0.05$).

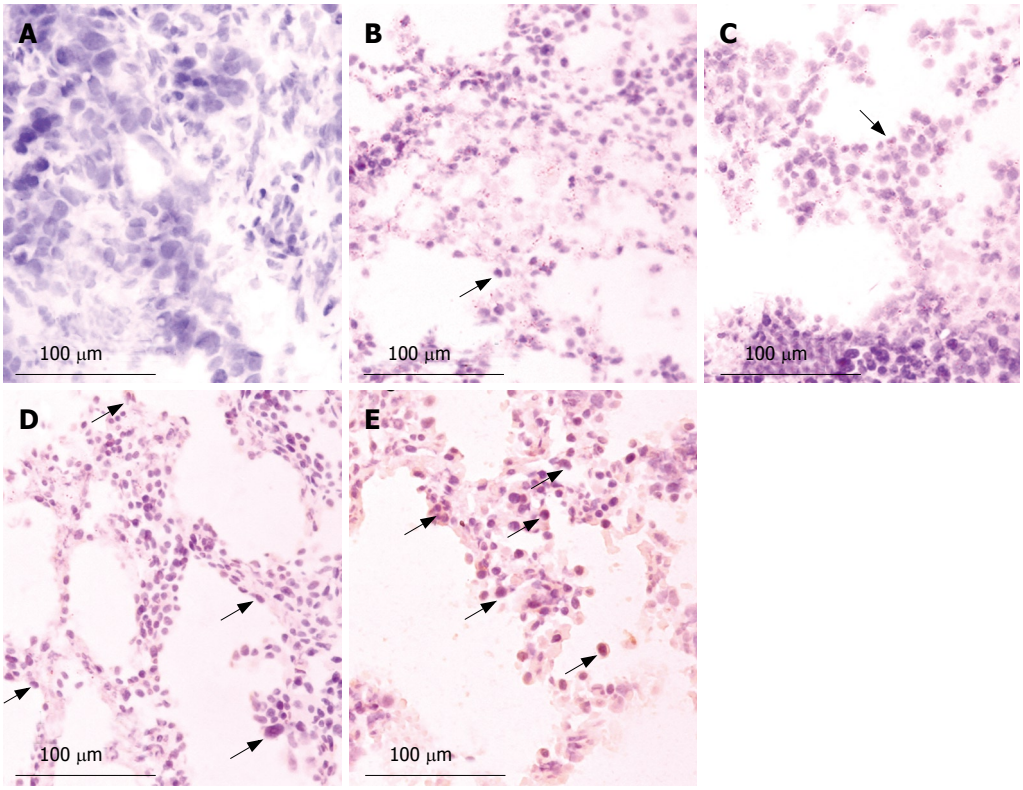

Figure 6 Immunohistochemical staining of tissues for *MK* and *tMK* with rabbit polyclonal anti-*MK* antibody. **A**: Negative control sections; **B**: Tumor tissue from BGC823 injected mice; **C**: Tumor tissue from BGC823/vector injected mice; **D**: Tumor tissue from BGC823/MK injected mice; **E**: Tumor tissue from BGC823/*tMK* injected mice (× 200). Arrows represent positive results of *MK* or *tMK* expressions.

and tumorigenesis *in vitro* and *in vivo*. *tMK* had greater effect than *MK* in promoting the tumor formation. *tMK* might become a more promising gene therapeutic target compared with *MK* for treatment of tumors.

COMMENTS

Background

Midkine (*MK*), a heparin-binding growth factor, and its truncated form (*tMK*), were found expressing at higher levels in various tumors, and involve the growth and metastasis of some carcinomas. The expressions of *MK* mRNA and the protein

are both associated with the clinical stage and distant metastasis of gastric cancer in the Chinese patients. But few studies were conducted on the roles of *MK* and *tMK* in both tumorigenesis and tumor development in gastric cancer. In this article, the effect of *MK* and *tMK* on the growth and metastasis of BGC823 (a poorly differentiated gastric adenocarcinoma cell line), and tumorigenesis in nude mice was investigated.

Research frontiers

Many studies of *MK* and *tMK* expression in various tumors including gastric cancer, have been reported. It has been found that *MK* can promote Wilms' tumor cell proliferation and tumor angiogenesis, inhibit tumor cell apoptosis, induce transformation of NIH3T3 cells, and protect hepatocellular carcinoma cells against TRAIL-mediated apoptosis. However, there has been no investigation about the effect of *MK* and *tMK* on the characteristics of gastric carcinoma.

Innovations and breakthroughs

This article suggests that over-expressed *MK* and *tMK* can promote BGC823 cell growth, colony formation, wound healing and tumorigenesis in nude mice. *tMK* had greater effect than *MK*, and it might become a promising gene therapeutic target for treatment of malignant tumors.

Applications

This observation might be of potential value in gene therapy for gastric cancer.

Peer review

The manuscript describes that over-expressed *MK* and *tMK* can promote BGC823 cell growth, colony formation, wound healing and tumorigenesis in nude mice. The results were found important for *MK* and *tMK* as gene therapeutic target in gastric cancer.

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