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 tumorigenesis 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; Tumorigenesis

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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[5]. 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[6]. 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]. MK was found in pancreatic, gastric, Wilms’, colorectal, bile duct and breast tumors, but not in non-cancerous and normal tissues[11-14]. MK can promote Wilms’ tumor cell proliferation and tumor angiogenesis[7,10,13], inhibit tumor cell apoptosis, induce transformation of NIH3T3 cells, and protect patocellular carcinoma (HCC) cells
against TRAIL-mediated apoptosis. \(MK\) and \(tMK\) are correlated positively with metastasis of HCC, prostate carcinomas, Lewis lung carcinoma, gastric cancer, and gastrointestinal carcinomas. They can induce the transformation of SW-13 cells and shorten the latency of tumor formation in nude mice.

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. 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.

**MATERIALS AND METHODS**

**Plasmids construction**

Plasmids with \(MK\) and \(tMK\) cukaryotic expression were constructed \(2,3,5\) (Figure 1). In our previous work, we designed pMD18-T-MK and pMD18-T-\(tMK\) vector \(2,28\), and prepared the human \(MK\) and \(tMK\) DNA fragments by PCR using \(MK\)-\(f\) 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°C under 5% humidified CO\(_2\) and 100 \(\mu\)g/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 \(0.8 \times 10^5\) cells/well were grown overnight in 24-well plates. When the cells reached \(90%-95\%\) confluence, they were transfected with \(0.8\) \(\mu\)g of pcDNA3.1/\(MK\) or pcDNA3.1/\(tMK\) in serum-free medium using Lipofectamine 2000. After 4 h incubation at 37°C, 400 \(\mu\)L RPMI 1640 with 10% FBS was added. Stable transfectants were selected in the presence of 400 mg/L G418 (Amresco) 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 \(\mu\)g total RNA was reverse-transcribed using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, USA). Subsequently, 2 \(\mu\)L of the incubation mixture was used as the template for the following PCR using 2 \(\times\) 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°C), annealing (40 s at 55°C), and extension (30 s at 72°C). 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 \(\beta\)-actin signal using a digital imaging and analysis system (SmartSpec™ Plus, BIO-RAD, USA).

**Western blot analysis**

Cells (\(1 \times 10^5\)) were lysed in a buffer containing 50 mmol/L Tris-Cl, pH8.0, 150 mmol/L NaCl, 0.02% Na\(_2\)SO\(_4\), 1% SDS, 100 mg/L phenylmethylsulfonyl fluoride (PMSF) and 1 mg/L Aprotinin, 1% Triton. After centrifugation, cell lysates (75 \(\mu\)g/lane) were subjected to 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked for 1 h in PBSST (10 mmol/L Tris-Cl, 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), \(\beta\)-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 \(3 \times 10^3\) cells/well. After 4 h, the medium
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-tetrazolium]-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 (HynergyHT, 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 × 10^4 cells/mL was prepared in 0.7% (w/v) agar solution to 2x RPMI-1640 supplemented with 20% fetal calf serum at a 1:1 ratio. Stable transfectants or parental 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 mm³ was calculated by the formula: Volume = (width)^2 × length/2. Tumor growth rates were calculated by the formula: TGR = (V₁₄d-V₁₄d)/7d. 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. Endogenous peroxidase activity was suppressed by treatment with 3% H₂O₂ in methanol for 30 min. Sections were exposed to rabbit polyclonal anti-MK antibody (1:250, Boster) overnight at 4°C. 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
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 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 significantly higher than the control. Immunohistochemical staining for MK and tMK was presented in 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 vivo, respectively. To show that the upregulated MK and tMK
were exogenous in the transfected cells, we designed another pair of primers for MK-2 sequence (Table 1)\(^6\). 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\(^34\). 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\(^35\). 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, indicating that the idiographic effect of 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\(^36-38\). 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\(^39\).

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

Table 2 Frequency of tumorigenesis in nude mice

| Injected cells | No. of mice | No. of days to tumor detection (percent of tumorigenesis) |
|----------------|-------------|--------------------------------------------------------|
| 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) 6 (85.71) 7 (100)                             |
| BGC823/tMK     | 7           | 7 (100)                                               |

\(^{P}\) 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, \(P = 0.0489\). 14 \(P < 0.05\); 28 \(P < 0.01\).
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.

**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 (P < 0.05); D: Analysis of tumor growth rate (P < 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 (x 200). Arrows represent positive results of MK or tMK expressions.

**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. MK 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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