Midkine positively regulates the proliferation of human gastric cancer cells

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Abstract

Midkine (MDK), a heparin-binding growth factor, modulates the proliferation and migration of various cells, is often highly expressed in many malignant tumors, and may act as an oncoprotein. We found that MDK is overexpressed in clinical human gastric cancer tissues relative to its expression in adjacent noncancerous tissues. To further investigate the biological activities of MDK in gastric cancer, we introduced the MDK gene into human SGC7901 gastric cancer cells, where it contributed to the proliferation of SGC7901 cells in vitro and in vivo. Conversely, the knockdown of MDK expression by siRNA resulted in significantly reduced proliferation of BGC823 cells. Our study also shows that MDK activates both the Akt and ERK1/2 pathways and upregulates the expression of several cell-cycle-related proteins, including cyclin A, cyclin D1, Cdk2, Cdk4, and Cdk6, which in part explains the contribution of MDK to gastric cancer cell survival and growth. These results demonstrate that MDK contributes to gastric cancer cell proliferation and suggest that it plays an important role in the development of human gastric cancer.

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1. Introduction

Gastric cancer is one of the most common malignancies in the world, particularly in eastern Asian countries such as China, Korea, and Japan [1]. Despite advances in its diagnosis and treatment, the prognosis for advanced gastric cancer is poor, with a five-year survival rate of less than 10%. In recent years, much evidence has clearly demonstrated that multiple genetic changes are responsible for the development and progression of gastric cancer. Changes in specific genes involved in gastric cancer play important roles in cell adhesion, signal transduction, cell differentiation, tumor development and metastasis [2–4]. However, the detailed mechanisms of the molecular genetic changes that contribute to the malignant phenotype of gastric carcinoma remain unknown.

Numerous growth factors and their downstream signaling systems are involved in the development, progression, and dissemination of cancer [5]. Midkine is a heparin-binding growth factor, the expression of which is generally low or undetectable in adults, whereas it is high in various human cancers, including esophageal, gastric, urinary bladder, pancreatic, colorectal, breast, and lung carcinomas, neuroblastoma, and Wilms’ tumor [6–9]. Recent studies of gastric cancer have shown that urinary MDK and serum MDK levels are elevated in cancer patients and are associated with disease progression [10,11], and that MDK mRNA and protein levels are both associated with the clinical stages and distant metastases in Chinese patients [12]. These data suggest that MDK contributes to the oncogenesis and progression of gastric cancer, and can be used as a cancer biomarker and therapy target.

Several biological functions of MDK are thought to contribute to tumorigenesis and tumor progression. It promotes the proliferation [13,14], survival [15–18], and tumorigenicity [19,20] of different cells and stimulates...
angiogenesis [21,22]. Some researchers have reported that MDK stimulates neuron cell and tumor cell growth via diverse paths. For instance, MDK induces the growth of ameloblastoma through the mitogen-activated protein kinase (MAPK) and Akt pathways [23]; it significantly enhanced STS cell growth via the Src and extracellular-signal-regulated kinase (ERK) pathways [24]; it stimulated G401 cell proliferation through a cell-surface receptor, which in turn activated the JAK/STAT pathways [25]; and recombinant midkine induced STAT3 tyrosine phosphorylation in a time- and dose-dependent manner and stimulated the proliferation of postconfluent 3T3-L1 cells [26]. However, the relationship between gastric cancer cell proliferation and MDK has rarely been examined. Recently, Qing showed that MDK promotes human gastric cancer cell growth [27], whereas little is known about the molecular mechanism underlying its proliferative effects. Therefore, we investigated the biological activities of MDK and illustrate the possible signal transduction pathways by which MDK acts in human gastric cancer.

2. Materials and methods

2.1. Tissue specimens

Tissue specimens of gastric cancer were obtained from the First Hospital of China Medical University between October 1997 and October 2007. None of the patients had received preoperative radiation therapy or chemotherapy. Data on the sex, age, tumor size, histological type of the neoplasm, and tumor–node–metastasis (TNM) stage were obtained from surgical and pathological records, with the patients’ consent.

2.2. Cell culture and reagents

Human SGC7901 and BGC823 gastric cancer cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 U/mL) at 37 °C under an atmosphere of 95% air and 5% CO₂. The antibodies used were anti-MDK (R&D, MN, USA), anti-cyclin D1, anti-Cdk2, anti-Cdk4, anti-Cdk6, and anti-tubulin (Santa Cruz Biotechnology, CA, USA). Anti-phosphoAkt (Ser-473), anti-Akt, anti-phosphoERK, and anti-ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Immunohistochemistry

For the immunohistochemical detection of MDK, 4 μm histological sections were dewaxed with xylene and rehydrated through a graded series of alcohol. The sections were then boiled for 10 min in 0.01 M citrate buffer and cooled for 30 min at room temperature to expose the antigenic epitopes. Endogenous peroxidase activity was quenched by incubation in 0.3% H₂O₂ in methanol for 30 min. Nonspecific binding was blocked by incubating the slides with rabbit serum for 30 min at room temperature. The sections were incubated with anti-MDK antibody (1:100) overnight at 4 °C, then with biotinylated anti-goat secondary antibody (1:200) for 1 h. The sections were then exposed to a streptavidin–peroxidase reaction system, and developed with diaminobenzidine–H₂O₂.

2.4. RNA extraction, cDNA synthesis, and quantitative real-time PCR

RNAiso (Takara, Japan) was used to extract the cellular RNA. Total RNA (1 μg) was converted into first-strand cDNA with SuperScript II Reverse Transcriptase Kit (Invitrogen, CA, USA). The oligonucleotides used to amplify the MDK cDNA were 5’-GAAGGAGTTTGGACCGCAGT-3’ for the forward primer and 5’-TTCCCTTTCTTGCTTTG-3’ for the reverse primer (Takara, Japan). Real-time reverse transcription (RT)–PCR was performed in triplicate for each sample using the SYBR Green PCR Kit Reagents (Takara, Japan). The cycling conditions for the PCR were: 95 °C for 10 s, and 45 cycles of 95 °C for 10 s, 53 °C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as the internal standard, and the data were analyzed with the 2⁻ΔΔCT relative quantitation method.

2.5. Stable transfection

The pLXSN or pLXSN–MDK plasmid was transfected into packed GP293 cells with Lipofectamine™ 2000 reagent (Invitrogen, CA, USA). After 48 h, 1.5 mL of virus supernatant from various plasmids was added to 80% confluent SGC7901 cells, which were incubated at 37 °C for 24 h, and then screened with G418 (400 mg/L). Monoclonal cells were selected and cultured further. The clones were screened for MDK expression with RT–PCR and western blot. One empty-vector-transfected control clone (vector) and two clones that expressed different levels of MDK (clone 7 and clone 11) were selected for further experiments.

2.6. Transient knockdown of MDK expression

Chemically synthesized MDK siRNA and negative control siRNA were purchased from GenePharma (Shanghai, China) for the transient knockdown of MDK expression. The nucleotide sequences of the MDK siRNA were 5’-GGAGCGACUGCAAGUACATT-3’ and 5’-UGUACUUGACGU CCUCUCAA-3’. The negative control siRNA sequences were 5’-UUUCUGGACUGUCAGUUTT-3’ and 5’-ACUG ACACGUUUCGGAGAT-3’.

BGC823 cells at 30–50% confluence were transfected with either MDK siRNA or control siRNA using Lipofectamine™ 2000 reagent (Invitrogen). After siRNA had been transfected for 48 h, the total RNA and protein of the cells were extracted and quantified.

2.7. MTT proliferation assay

The capacity for cellular proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (approximately 5 × 10⁴) were seeded into 96-well culture plates for 24, 48, 72, or 96 h. The cells were then incubated with 20 μL of MTT (5 mg/mL) for 4 h at 37 °C and 200 μL of DMSO was added to
solubilize the crystals for 20 min at room temperature. The optical density was determined with a spectrophotometer (Bio-Rad, USA) at a wavelength of 570 nm. The experiment was repeated three times in triplicate.

2.8. Growth curves

Equal numbers (approximately $4 \times 10^4$) of each stable cell line were seeded into six-well plates. The cells were harvested and counted every day after seeding. Each point on the curve is the average of three duplicates.

2.9. Anchorage-independent growth assay

Cells ($8 \times 10^2$) were suspended in 1.5 mL of 0.3% agarose with RPMI 1640 medium containing 10% FBS and plated into six-well plates on the top of an existing layer of 0.6% agarose prepared with the same medium. Each cell line was tested in triplicate. The plates were incubated at 37°C in a 5% CO$_2$ incubator. After three weeks, cell colonies >0.1 mm in diameter were counted under a microscope.

2.10. Cell extracts and western blotting analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 2 μg/mL aprotinin) on ice, then quantified with the Lowry method. Samples (50 μg) of the cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in TBST buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) at room temperature for 2 h and incubated at 4°C overnight with the indicated primary antibodies. After the membranes had been washed with TBST buffer, they were reacted with the appropriate horseradish–peroxidase-conjugated secondary antibody for 30 min at room temperature. After the membranes had been extensively washed with TBST buffer, the proteins were visualized with enhanced chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

2.11. In vivo tumor model

Six-week-old female athymic nude mice were subcutaneously injected into the right armpit region with $2 \times 10^6$ cells in 0.1 mL of PBS. Three groups of mice ($n = 7$) were tested. Group 1 was injected with SGC7901 cells stably transfected with pLXSN; group 2 was injected with clone 7 stably transfected with pLXSN–MDK; and group 3 was injected with clone 11 stably transfected with pLXSN–MDK. The tumor size was measured every two days with calipers. The tumor volume was calculated with the formula: $(L \times W^2)/2$, where $L$ is the length and $W$ the width of the tumor. After the mice were killed at three weeks, the weights of the tumors were measured.

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Fig. 1. (A) Representative image of immunohistochemical staining of gastric cancer tissue (original magnification, 400×). Compared with noncancer tissue, cancer tissue exhibited positive immunostaining for MDK. (B) Real-time quantitative PCR assay. MDK mRNA expression in tumor tissue was strongly upregulated compared with that in matched normal mucosa.
2.12. Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed with Student’s t-test or the \( \chi^2 \) test (SPSS 13.0 software). \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. MDK expression in clinical cancer specimens and adjacent noncancerous tissues

We initiated our study by investigating the levels of MDK expression in human malignant gastric tissues and adjacent noncancerous tissues using immunohistochemistry. Our results show that 54% of gastric cancer tissues (55 of 102 patients) stained positively for MDK, which is significantly higher than the 28% (5 of 18 patients) of adjacent noncancerous tissues positive for MDK (\( P < 0.05; \) Fig. 1A).

Next, we analyzed MDK mRNA expression by real-time quantitative RT–PCR in 19 pairs of tumor tissue and matched normal mucosa. In 11 of 19 patients (57.9%), MDK expression was upregulated in the tumor tissue, two fold higher than the expression in the matched normal mucosa (\( P < 0.05; \) Fig. 1B), which supports the immunohistochemical results.

3.2. MDK transfection of SGC7901 cells

The SGC7901 cell line was stably transfected with human MDK cDNA. Cells transfected with the empty vector were used as the control. We screened 15 individual SGC7901–MDK clones for MDK transcript levels. Two SGC7901–MDK clones (clone 7 and clone 11) showed MDK expression levels over 10-fold higher than those of the control cells (Fig. 2A). The MDK mRNA levels of clone 7 were 1.5-fold higher than those of clone 11. Western blotting verified the results of real-time PCR (Fig. 2B).

3.3. Transient knockdown of MDK expression in BGC823 cells

To further confirm the function of MDK, we also transfected BGC823 cells with different concentration of MDK siRNA. The two target interference groups all had a significant efficiency in gene silencing. The mRNA level was decreased by 46.9% in 10 nM siRNA group and 74.8% in 20 nM siRNA group compared to control (non-target siRNA group), respectively (Fig. 2C). Western Blot verified the results of real-time PCR (Fig. 2D).

3.4. MDK contributes to SGC7901 cell growth

The anchorage-dependent growth rates of clone 7, clone 11, and vector-transfected cells were tested by MTT assay (Fig. 3A) and the Trypan blue exclusion method (Fig. 3B). Significant enhancement of growth was induced by MDK in clone 7 and clone 11. To determine whether the facilitated growth of SGC7901 cells induced by MDK was anchorage-independent, the cells were plated on soft agar. After three weeks, the cells stably transfected with MDK formed significantly more clones on soft agar than did the vector-transfected control cells (Fig. 3C). These data indicate that MDK plays an essential role in the anchorage-dependent and anchorage-independent growth of SGC7901 cells.

3.5. MDK knockdown by siRNA inhibited the proliferation of BGC823 cells

We then examined the effects of MDK knockdown on cell proliferation. As shown in Fig. 3D, the MDK knockdown cells proliferated significantly more slowly than control cells.
3.6. MDK affects the activation of ERK1/2 and Akt pathways

As has been shown in other cell systems, growth factors contribute to proliferation through their downstream signaling pathways, of which the mitogen-activated protein kinase (MEK)/ERK and phosphoinositide 3-kinase (PI3 K)/Akt are among the most important. Therefore, we investigated the activation of these pathways by monitoring their phosphorylation status after MDK transfection and knockdown. We found that MDK did not affect total ERK and Akt protein levels. However, ERK and Akt phosphorylation was clearly increased in MDK over-expressed SGC7901 cells (clone 7 and clone 11) (Fig 4A), and decreased in MDK hypo-expressed BGC823 cells (Fig. 4B). Taken together, these results indicate that not only the PI3 K/Akt signaling pathway but also the ERK signaling pathway play predominant roles in regulating the MDK-induced proliferation.

3.7. MDK regulates the expression of cell-cycle proteins

To verify that the MDK-stimulated cells were in a proliferative state, we investigated the expression levels of the cell-cycle regulatory proteins cyclin A, cyclin D1, Cdk2, Cdk4, and Cdk6, which participate in the progression of the cell cycle. The expression levels of these proteins coincided with the MDK levels, suggesting that MDK is the upstream molecular of these proteins (Fig. 4C and D).

3.8. MDK contributes to the proliferation of SGC7901 cells in vivo

Because MDK confers a proliferative capacity upon SGC7901 cells in vitro, its effect in vivo was investigated. A vector-transfected control cell line (vector) or MDK-transfected cells (clone 7 or clone 11) were injected separately into three groups of nude mice. The tumor volumes were measured every two days until the mice were killed on day 21.

Fig. 3. Effect of MDK on the proliferation of gastric cancer cells. (A) Stable transfection of MDK contributed to the proliferation of SGC7901 cells. MTT proliferation assay. \( P < 0.01 \). (B) Growth curve constructed with the Trypan blue exclusion method. \( P < 0.01 \). (C) Anchorage-independent growth assessed with a colony-formation assay. \( P < 0.01 \). (D) Transient knockdown of MDK expression inhibited the proliferation of BGC823 cells. MTT proliferation assay. \( P < 0.01 \).

4. Discussion

The identification and characterization of genes that are differentially expressed in gastric cancer tissues and adjacent noncancerous tissues provide important information that extends our understanding of the mechanisms responsible for carcinogenesis. In a previous study, MDK expression was higher in human gastrointestinal cancers than in the corresponding noncancerous tissues and normal tissues [28]. Our research examined a greater number of clinical specimens and demonstrated that MDK is expressed more highly in carcinoma tissues, suggesting that MDK plays an oncogenic role in the development of gastric cancer. Because MDK boosts the growth of various cells [23,24], we established two MDK-overexpressing cell lines...
that express different levels of MDK. Consistent with our hypothesis, MDK enhanced SGC7901 cell growth in vitro. We then transiently transfected an siRNA that targeted MDK, which markedly inhibited the proliferation of BGC823 cells, further confirming our conclusion. Because oncogenic proteins often enhance the anchorage-independent growth of tumor cells in soft agar [29], the control and stably MDK-transfected cell lines were also tested for anchorage-independent growth. Overexpressed MDK facilitated the anchorage-independent growth of SGC7901 cells in soft agar, suggesting that MDK is an oncoprotein that functions in gastric carcinogenesis. This was further supported by the finding that the overexpression of MDK facilitated tumor formation and growth in nude mice.

Growth factors and cytokines provide both mitogenic and anti-apoptotic signals to cells and therefore play an important role in maintaining the homeostatic balance between cell proliferation and apoptosis [30]. The ability to induce cellular proliferation often correlates with the ability to promote cell survival. Two signaling cascades have emerged as major players in the mitogenic and anti-apoptotic responses in many cells: MEK/ERK and PI3 K pathways. Akt/PKB is a serine/threonine protein kinase that functions as a critical regulator of cell survival, proliferation, and apoptosis [31]. ERK1/2 is activated by growth factors and plays an important role in the regulation of cell proliferation and cell differentiation [32].

In our study, both Akt and ERK phosphorylation were obviously upregulated in MDK overexpressing cells and downregulated in MDK hypo-expressing cells, suggesting both signaling pathways were activated by MDK and contributed to its proliferative activity. These results are consistent with studies of ameloblastoma and soft-tissue sarcoma [23,24]. Much research has focused on the fact that MDK exerts an anti-apoptotic effect by influencing the Bcl-2 family proteins and the caspase cascade [22,33,34]. The PI3 K/Akt and Raf/MEK/ERK pathways can phosphorylate the proapoptotic protein Bad, a downstream cellular target of these pathways, at serine 136 and serine 112, causing its dissociation from Bcl-2, which allows these anti-apoptotic proteins to prevent the mitochondrial events required for apoptosis [35,36]. In this context, our results may also explain the anti-apoptotic action of MDK.

Mammalian cell proliferation is governed by the cell-cycle machinery. Cell-cycle progression is a tightly controlled series of events that are positively regulated by cyclin-dependent kinases (CDKs) and their cyclin-regulatory subunits [37]. The effect of Akt on cell proliferation has been suggested to involve its action on cell-cycle proteins such as p27, p21, and cyclin D [38]. Therefore, we asked whether the expression of cell-cycle proteins is altered by MDK. In this study, we have shown that five cell-cycle-related proteins (cyclin A, cyclin D1, Cdk2,
Cdk4, and Cdk6) are upregulated in cells transfected with MDK and their increased expression correlated with the expression of MDK. Conversely, they were downregulated by knocking down of MDK. Among these proteins, cyclin D1 is required for cell-cycle progression to the G0/G1 phase and can induce genetic instability and genomic DNA amplification, resulting in the transformation of mammalian cells [39]. The overexpression of cyclin D1 leads to abnormal cellular proliferation, which underlies the process of tumorigenesis. Together with its cdk partner, Cdk4(6), cyclin D1 is likely to play a major role in phosphorylating the retinoblastoma protein, thereby orchestrating the progression through G1 and into S phase, whereas cyclin A can bind Cdk2 and is required for DNA replication in S phase, as well as in the initiation of mitosis (M phase) [40]. We conclude that MDK exerts its proliferative action by facilitating cell-cycle progression. The PI3 K/Akt pathway is a strong activator of cyclin D1, and regulates cyclin D1 via the phosphorylation of glycogen synthase kinase 3 (GSK3) [41]. On the other hand, the sustained activation of ERK1/2 is necessary for G1 to S phase progression and is associated with the induction of positive regulators of the cell cycle [42]. In this study, we found that the activation of the Akt and ERK1/2 pathways is accompanied by the upregulation of positive cell-cycle regulatory proteins, from which we infer that the increase in these proteins is affected by the activation of both signaling pathways.

In conclusion, we have demonstrated that midkine, which is upregulated in human gastric cancer tissues, promotes the proliferation of human gastric cancer cells. Akt and ERK1/2 signaling have been shown to be involved in MDK-induced cell proliferation. We have also suggested for the first time that MDK promotes cell growth partly through cell-cycle regulatory proteins such as cyclin A, cyclin D1, Cdk2, Cdk4, and Cdk6. These observations provide new insight into the biological functions of MDK and clarify the molecular mechanisms by which MDK stimulates the proliferation of gastric cancer cells. Our results also strongly indicate that MDK is an oncogene involved in the development of gastric cancer and also a promising therapeutic target for cancer treatment.

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