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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.

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

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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% CO_2. The antibodies used were anti-MDK (R&D, MN, USA), anti-cyclin A, 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% H_2O_2 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–H_2O_2.

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′-GAAGGAGTTTGGAGCCGACT-3′ for the forward primer and 5′-TTCCCTTTCTTGGCTTTGG-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 $2^{-\Delta\Delta CT}$ 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′-GGAGCCGACUGCAAGUACATT-3′ and 5′-UGUACUUGCAGUCGGCUCCAA-3′. The negative control siRNA sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-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 5×10^3) 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