

Mixed lineage kinase-4 promotes gastric carcinoma tumorigenesis through suppression of the c-Jun N-terminal kinase signaling pathway

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Abstract. Mixed lineage kinase-4 (MLK-4) is an important member of the mixed-lineage family of kinases that regulates the extracellular signal-regulated kinases and c-Jun N-terminal kinase (JNK) signaling pathways. The functions and mechanisms of MLK-4 in cancer initiation and progression have not been well understood. The present study investigated the expression, function and regulatory mechanism of MLK-4 in gastric carcinoma cells. Biochemical data indicated that normal MLK-4 was downregulated, which exerted dominant negative effects on gastric carcinoma cell viability, migration and invasion. The experimental data demonstrated that MLK-4 supplement abrogated activity of these mutants and induced inhibitory effects on gastric carcinoma cell viability, migration and invasion *in vitro* and *in vivo*. In addition, to determine the regulatory mechanism of MLK-4, its signaling pathway was assessed in gastric carcinoma cancer cells by regulating MLK-4. The present observations indicated that restoring MLK-4 activity by supplemental MLK-4 reduced gastric carcinoma cell colony formation *in vitro* and suppressed tumor viability, migration and invasion *in vivo*. The results of the present study indicated that MLK-4 may be a potential protein for targeting gastric carcinoma by suppressing kinases, which may lead to reduction of JNK signaling and enhance therapeutic efficacy in gastric carcinoma.

Introduction

Gastric cancer is one of the most common human malignancies among all clinical types of cancer and remains the second

leading cause of cancer-related death worldwide (1). Statistics have indicated that more than 70% of new cases and mortalities of gastric cancer occur in developing countries (2). Gastric cancer has demonstrated a higher morbidity and mortality rate than other carcinomas of the digestive system (3,4). Previous research has indicated that the 5-year survival rate was <80% (5). Resistance to apoptosis of gastric cancer cells in patients with gastric cancer has previously been reported, and various reports have demonstrated that apoptosis resistance of gastric cancer was inevitable in cancer progression (6,7). At present, resistance to apoptosis has become the greatest challenge in cancer therapy due to fierce resistance of tumor cells though various kinds of molecular mechanisms (8-10). In addition, despite the great progress made in the treatment of gastric cancer, patients often miss the opportunity for a surgical cure as the cancer has already developed into the advanced stage by the time of diagnosis, which leads to a reduced overall survival for gastric cancer patients (11). A previous study has suggested that targeted therapies for advanced gastric cancer are efficient for patients with gastric cancer (12). Therefore, exploring more efficient targeted molecular therapies has attracted increasing interest by researchers and clinicians in the field of cancer research and clinical therapy.

Mixed-lineage kinases (MLKs) are a class of serine-threonine kinases that belong to the superfamily of mitogen-activated protein kinase kinases (MAP3Ks) and are believed to control multiple intracellular signaling pathways in cells (13). All MLK family members are characterized by a signature Tyr kinase domain and a Ser/Thr label in the catalytic domain in the amino-terminal SRC-homology domain, as well as a Cdc42/Rac-interactive binding motif and a leucine-zipper region (14). Proline is abundant in the carboxyl terminus, with different forms in different members of the family among all MLKs, which suggests that this region serves in different and essential regulatory functions in cells (15). Previous research has indicated that MLK-4 is activated in colorectal cancer, where it synergistically cooperates with activated Ras signaling to drive tumorigenesis (16). Therefore, we assumed that MLK-4 may have an important role in gastric carcinoma tumorigenesis.

MLK-4 is the second most frequently mutated protein kinase and has been identified in microsatellites in various

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human tumor cells (17). However, the function and pathobiological importance of MLK-4 is not fully understood. MLK-4 is an important member of the MLK family that regulates the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling pathways (18). In addition, a study by Kim *et al* (19) indicated that serine/threonine kinase MLK-4 determined mesenchymal identity in glioma stem cells through nuclear factor (NF)- κ B signaling. However, previous research has also reported that MLK-4 regulated the JNK, p38, and ERK signaling pathways in colorectal cancer cells (20). The function of MLK-4 has not been well elucidated and the important implications of MLK-4 in the apoptosis, development and treatment are equivocal in gastric carcinoma.

The present study demonstrated that MLK-4 was overexpressed in gastric cancer cells and tumors. The results indicated that neutralizing MLK-4 expression using anti-MLK-4 antibody decreased viability, self-renewal, motility, metastasis, invasion and radioresistance of gastric cancer cells through modulation of the JNK signaling pathway. The present results also demonstrated that MLK-4 induced JNK activation through regulation of mitogen-activated protein kinase kinase (MKK)4 and JNK kinase (JNKK)2/MKK7 phosphorylation, which may be involved in gastric cancer cell apoptosis in response to anti-cancer drug treatments. Collectively, the present results suggested that MLK-4 serves as an upstream regulator in the JNK signaling pathway and may be a potential molecular target for gastric cancer therapy.

Materials and methods

Cells and reagents. Gastric tumor cell lines, HGC-27 and BGC-823, and human gastric mucosa epithelial cells, GES-1, were purchased from the American Type Culture Collection (Manassas, VA, USA). All tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). GES-1 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS; both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). All cells were cultured in a 37°C humidified atmosphere of 5% CO₂.

MTT cytotoxicity and colony formation assays. HGC-27 and BGC-823 cells (1x10⁶) were incubated with MLK-4 (2 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 96-well plates for 96 h at 37°C in triplicate, and phosphate-buffered saline (PBS) was added instead of MLK-4 as a control. Subsequently, 20 μ l MTT (5 mg/ml) in PBS solution was added to each well and the cells were further incubated for 4 h at 37°C. Following this, all medium was removed and 100 μ l dimethyl sulfoxide was added into the wells to solubilize the crystals. The optical density was measured by a Bio-Rad (ELISA) reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm. In addition, HGC-27 and BGC-823 cells (1x10⁵/well) in 6 well plates cultured in DMEM with 10% fetal bovine serum were transfected with 50 nM small interfering (si)RNA-MLK-4 (5'-CAUCUACGAUCCGACUAUU-3') using Lipofectamine[®] 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's

protocol and colony formation was observed in a 6 day culture. Cells were further analyzed 48 h after transfection.

ELISA. The affinity of MLK-4 for MLK receptor was determined by the Human MLK4 ELISA kit (cat. no. EH10132; Wuhan Fine Biotech Co., Ltd., Wuhan, China). The procedures were performed according to the manufacturer's protocol. The final results were recorded at 450 nm on an ELISA plate reader (Spectra Max 190; Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell invasion and migration assays. MLK-knockdown HGC-27 and BGC-823 cells (1x10⁵) were cultured in Eagle's minimal essential medium with 5% FCS. For migration assays, HGC-27 and BGC-823 cells were transfected with 50 nM siRNA-MLK-4 and incubated for in the upper chamber for 96 h at 37°C using a control Transwell insert (BD Biosciences, Franklin Lakes, NJ, USA). Eagle's minimal essential medium with 5% FCS was added to the upper and lower chambers. For invasion assays, HGC-27 and BGC-823 cells were suspended at a density of 1x10⁵ cells in 500 μ l of serum-free DMEM. The cells were treated with 50 nM siRNA-MLK-4 for 48 h at 37°C and then subjected to the tops of BD BioCoat Matrigel invasion chambers (BD Biosciences), according to the manufacturer's protocol. The cells were then washed with PBS, fixed with 4% paraformaldehyde for 20 min at 37°C and stained with Giemsa stain at 37°C for 20 min. The number of tumor cells that had invaded and migrated were counted in at least three randomly stained fields using a fluorescence microscope for every membrane (BZ-9000; Keyence Corporation, Osaka, Japan).

Targeted deletion of the MLK-4 locus in gastric tumor cells. Disruption of MLK-4 exon 1 in gastric tumor HGC-27 and BGC-823 cells was conducted to knockdown MLK-4 according to a previous study (21). The purpose clones were screened after a 12-day growth period under 0.4 mg/ml geneticin (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, the clones were propagated for 10 generations. Homologous recombination clones were screened and confirmed using locus-specific polymerase chain reaction (PCR).

Overexpression of MLK-4 in gastric tumor cells. Human MLK-4 cDNA plasmids (2.5 μ g) were transfected into 293T cells (1x10⁶; both Cell Biology Laboratory, Zhejiang Chinese Medical University, Zhejiang, China) for 48 h to generate a lentivirus using Lipofectamine 2000 according to the manufacturer's protocol. The viral supernatant was subsequently collected and used to infect the HGC-27 and BGC-823 cells using Lipofectamine 2000 and the MLK-4 lentivirus (5 μ g). Further analysis was performed 72 h post-transfection.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was obtained from HGC-27, BGC-823 and GES-1 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. For DNase treatment, 2 units of DNase I polymerase (Invitrogen; Thermo Fisher Scientific, Inc.) were used per μ g of total RNA at 37°C for 30 min. Approximately 5 μ g RNA for each sample was reverse transcribed using an oligo-(dT) primer and M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA,

USA) according to the manufacturer's protocol. qPCR analysis was performed in a final volume of 10 μ l, which contained 5 μ l SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc.), 1 μ l cDNA (1:50 dilution) and 2 μ l forward and reverse primers (1 mM) with the ABI Prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). All forward and reverse primers (MLK-4, sense 5'-ACGACGATCATATCGAGACA-3' and antisense 5'-CGAGATGACGAGGATTGCAG-3'; β -actin, sense 5'-GTGGGGCGCC CAGGCACCA-3' and antisense 5'-CTCCTTAATGTCACG CACGATTT-3') were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). PCR cycling was performed under the following conditions: 94°C for 30 sec, and 45 cycles of 95°C for 5 sec, 54°C for 10 sec and 72°C for 10 sec. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (22). The results were expressed as the n-fold of the control. β -actin was used as the endogenous control.

Mutation screening by semi-quantification RT-PCR analysis. Total RNA from cultured cells was extracted with RNeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. A volume of 20 μ l DNase (Invitrogen; Thermo Fisher Scientific, Inc.) was added to remove the genomic DNA according to the manufacturer's protocol. Following this, a total of 1 μ g RNA was reverse transcribed into cDNA using a High Capacity cDNA reverse transcription kit (Qiagen Sciences, Inc.) at 37°C for 30 min. cDNA (10 ng) was subjected to qPCR using a SYBR Green Master Mix system (Bio-Rad Laboratories, Inc.) using the primers (Takara Biotechnology Co., Ltd., Dalian, China) in Table I. The PCR conditions included an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 2 min and a final elongation step at 72°C for 10 min. PCR products were mixed with an equal volume of gel-loading buffer (95% formamide, 20 mM EDTA and 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 5 min and immediately placed on ice for 5 min. The samples (1 μ g) were loaded directly onto 8% polyacrylamide gels and run for 8 h at room temperature and 40 W in a solution of 0.5X TBE (Takara Biotechnology Co., Ltd.). Bands were visualized using 1 μ g/ μ l ethidium bromide, which was added to the gel. GAPDH was used as an internal control to normalize gene expression. The relative gene expression levels were calculated using Quantity-One software (version 1.0; Bio-Rad Laboratories, Inc.) and the $2^{-\Delta\Delta Cq}$ method (23). All experiments were repeated ≥ 3 times.

Co-immunoprecipitation assay. HGC-27 (1×10^6) cells grown in DMEM supplemented with 10% FCS were transfected with 5 μ g MLK-4 vector (Shanghai Zeye Biotechnology Co., Ltd., Shanghai, China) using Lipofectamine 2000. After 24 h, the cells were chilled to 4°C and lysed by incubating for 15 min in lysis buffer (Sigma-Aldrich; Merck KGaA). Nuclei were removed by centrifugation at 10,000 x g for 15 min at 37°C. Flag- or V5-tagged proteins were purified from the cell lysate by immunoprecipitation using EZ-Magna ChIP kit (cat. no. 17-409; EMD Millipore, Billerica, MA, USA) according to manufacturer's protocol. The immunoprecipitates were subjected to western blotting analysis with different antibodies.

Table I. Sequences of primers pairs mixed lineage kinase-4 used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Sequence
H261Q	F: 5'-ACGACAGCCATATCGAGACA-3' R: 5'-ACAGTCCTCCTTCATTTCAGT-3'
G291E	F: 5'-TGGATGTATGAAGGGTTGAA-3' R: 5'-GAAAATATAAGGGGGCAGAT-3'
A293E	F: 5'-TCTCCCCTGTAAACCCTAAC-3' R: 5'-GATGGAGGACAAGGGTATGC-3'
W296E	F: 5'-TCTCCCCTGTAAACCCTAAC-3' R: 5'-GCCAGCCGGCTTTTACAAT-3'
R338H	F: 5'-TCTCCCCTGTAAACCCTAAC-3' R: 5'-GCCAGCCGGCTTTTACAAT-3'
Wild type	F: 5'-GAGGGCAGAATCATCACGAAGT-3' R: 5'-GGTGAGCATTATCACCCAGAA-3'
β -actin	F: 5'-AGAAAATCTGGCACCACACC-3' R: 5'-TAGCACAGCCTGGATAGCAA-3'

F, forward; R, reverse.

Colony formation assay. MLK-4-silenced HGC-27 and BGC-823 cells were cultured for 5 days and transferred to 6-well plates at a density of 500 cells/well. After 7 days of culturing, the cells were fixed with 4% polyformaldehyde (Sigma-Aldrich; Merck KGaA) and then stained with diluted Giemsa stain (1:20) for 20 min at 37°C. Following the rinsing of the cells with distilled water, colonies of cells were detected by a BZ-9000 fluorescence microscope.

Western blotting. HGC-27 and BGC-823 cells were homogenized in lysate buffer containing protease-inhibitor (Sigma-Aldrich; Merck KGaA) and were centrifuged at 6,000 x g (4°C) for 10 min. Subsequently, the supernatant was used for analysis of protein levels. Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, Inc.). SDS-PAGE assays were performed, as previously described (24). Protein samples (20 μ g/lane) were resolved by 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk for 1 h at 37°C and subsequently incubated with primary antibodies: MLK-4 (1:1,000; cat. no. ab93798), Bcl-2 (1:1,000; cat. no. ab692), P53 (1:1,000; cat. no. ab26), Bax (1:1,000; cat. no. ab32503), MMP-3 (1:1,000; cat. no. ab53015), CT-1 (1:500; cat. no. ab34710), Fibronectin (1:1,000; cat. no. ab6328), PPAR- γ (1:1,000; cat. no. ab45036), STAT-3 (1:1,000; cat. no. ab119352), NF- κ Bp65 (1:1,000; cat. no. ab16502) and β -actin (1:1,000; cat. no. ab124721) (all Abcam, Cambridge, UK) for 12 h at 4°C. Following this, membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG mAb (cat. no. PV-6001; OriGene Technologies, Inc., Beijing, China) for 1 h at 37°C. Following the washing of the membranes with 0.1% Tween 20 in Tris-buffer solution, the membranes were developed using a chemiluminescence assay system (Roche Diagnostics, Basel, Switzerland) and exposed

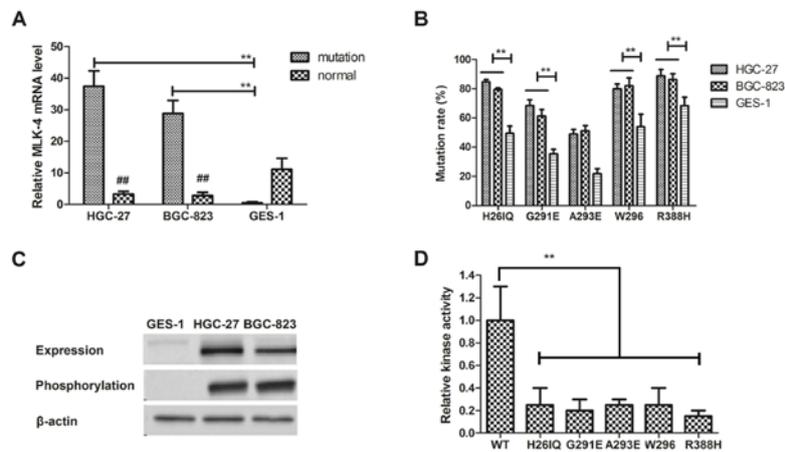


Figure 1. Expression and mutation of MLK-4 in gastric cancer cells. (A) Expression of normal or mutated MLK-4 mRNA in gastric cancer cell lines (HGC-27 and BGC-823) and normal gastric mucosa epithelial cells (GES-1). (B) Analysis of mutation rate (H261Q, G291E, A293E, W296 and R388H) of MLK-4 within the kinase catalytic domain in HGC-27 and BGC-823 cells. (C) Analysis of mitogen-activated protein kinase kinase-4 phosphorylation level in HGC-27 and BGC-823 cells with GES-1 as a control. (D) Analysis of kinase activity of MLK-4 mutations in the c-Jun N-terminal kinase signaling pathway in gastric cancer cells. Data are presented as the mean \pm standard error of the mean of three independent experiments. ** $P < 0.01$. ## $P < 0.01$ vs. the normal GES-1 group. MLK-4, mixed lineage kinase-4; WT, wild type.

to Kodak exposure films (Kodak, Rochester, NY, USA). Densitometric quantification of the western blotting data was performed using Quantity-One software (version 1.0; Bio-Rad Laboratories, Inc.).

Animal study. All animal procedures were approved by the Ethics Committee of the First Affiliated Hospital, Shihezi University School of Medicine (Shihezi, China). A total of 60 immunocompromised CD1-nude athymic male mice (6-8 weeks old; 30-35 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were housed in a temperature-controlled facility at $23 \pm 1^\circ\text{C}$ with a relative humidity of $50 \pm 5\%$ and 12 h light/dark cycle with free access to food and water. MLK-4 knockdown HGC-27 or HGC-27 cells (1×10^7) were injected subcutaneously in posterior flanks of 30 mice. Gastric tumor diameters were measured every 2 days by using a caliper. Tumor volumes were calculated according to a previous study (25).

Statistical analysis. All data are presented as the mean \pm standard error of the mean of triplicate experiments. All data were analyzed using SPSS Statistics software (version 19.0; IBM Corp., Armonk, NY, USA). Unpaired data were compared using Student's *t*-tests and comparisons of data between multiple groups were analyzed using one-way analysis of variance followed by Dunnett's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression and function of MLK-4 in gastric cancer cells. In order to analyze MLK-4 expression levels, RT-qPCR and western blotting were used to evaluate the expression level of mutational MLK-4 in gastric cancer cells. The results in Fig. 1A demonstrated that the expression level of mutated MLK-4 was significantly higher in gastric cancer cell lines, HGC-27 and BGC-823, compared with normal GES-1 cells

($P < 0.01$). The results in Fig. 1B revealed that H261Q, G291E, A293E, W296 and R388H had higher mutation rates located within the kinase catalytic domain compared with normal gastric GES-1 cells. The results in Fig. 1C indicated that these mutations (H261Q, G291E, A293E, W296 and R388H) markedly decreased MKK4 phosphorylation levels. The results in Fig. 1D demonstrated that MLK-4 functions as an oncogene with gain-of-function mutations in gastric cancer cells. Overall, the data suggested that the MLK-4 mutations located within the kinase domain in gastric cancer cells.

Reintroduction of MLK-4 decreases cell migration and tumor viability in vitro. To confirm the binding receptor of MLK-4, an ELISA system was used to determine the intracellular binding receptor of MLK-4. The results in Fig. 2A demonstrated that MLK-4 interacted with the Toll-like receptor (TLR)-4 intracellular domain in HGC-27 and BGC-823 cells. In addition, co-immunoprecipitation by Flag-labeled TLR-4 with V5-tagged MLK-4 was performed to confirm the interaction between MLK-4 and TLR-4 in human gastric mucosa epithelial GES-1 cells. Flag-labeled death receptor-4 (DR-4) was used as a control for TLR-4. The results in Fig. 2B demonstrated that MLK-4 co-precipitated with Flag-TLR-4, but not with Flag-DR-4 for homogenates of HGC-27 or BGC-823 cells. In addition, the inhibitory effects of MLK-4 on gastric cancer cell lines, HGC-27 and BGC-823, were analyzed. As demonstrated in Fig. 2C, the viability of HGC-27 and BGC-823 cells was significantly inhibited in the MLK-4-treated group compared with control cells ($P < 0.01$). The migration and invasion assays (Fig. 2D and E) indicated that the migratory and invasive abilities of gastric cancer cells were significantly suppressed following MLK-4 treatment compared with the control cells ($P < 0.01$). These data suggested that MLK-4 had an important role in suppressing viability, migration and apoptosis-resistance in gastric cancer cells.

Inhibition of the JNK signaling pathway regulated by MLK-4 in gastric cancer cells. To evaluate the biological mechanism

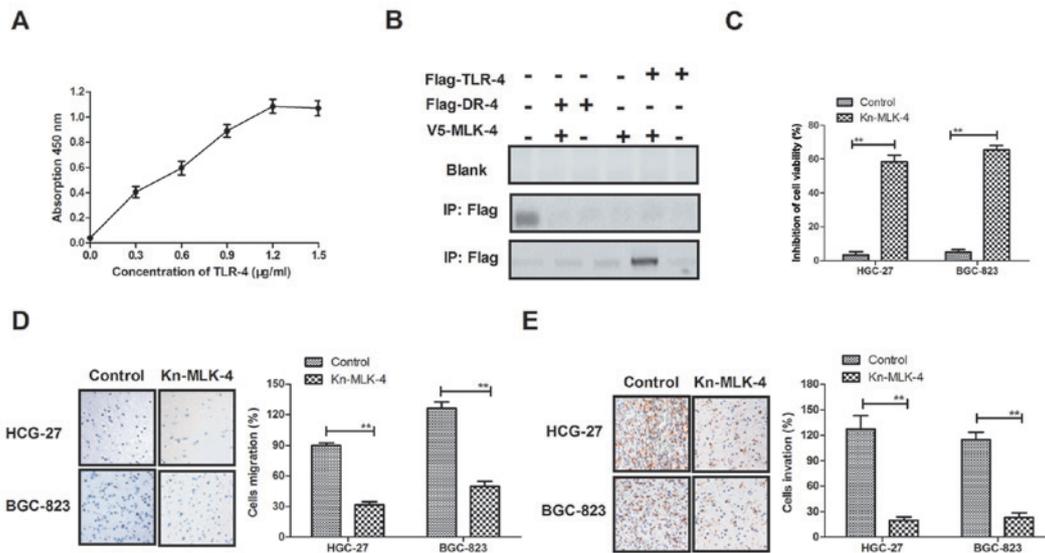


Figure 2. Knockdown of MLK-4 inhibits cell viability and migration *in vitro*. (A) ELISA analysis of the receptor of MLK-4. Data are presented as the mean \pm standard error of the mean of three independent experiments (B) Analysis of the affinity between MLK-4 and TLR-4 by co-immunoprecipitation. (C) Analysis of inhibitory cell viability effects of MLK-4 on HGC-27 and BGC-823 cells. (D) Analysis of inhibitory migration effects of MLK-4 on HGC-27 and BGC-823 cells. (E) Analysis of inhibitory invasion effects of MLK-4 on HGC-27 and BGC-823 cells. Data are presented as the mean \pm standard error of the mean of three independent experiments. ** $P < 0.01$ as indicated. MLK-4, mixed lineage kinase-4; TLR-4, Toll-like receptor 4; -, empty vector; IP, immunoprecipitated; DR-4, death receptor-4.

of MLK-4 loss-of-function mutations in gastric cancer cells, JNK expression and phosphorylation in MLK-4 knockdown in HGC-27 and BGC-823 cells were analyzed. The results in Fig. 3A demonstrated that MLK-4 knockdown markedly decreased JNK expression and phosphorylation in HGC-27 and BGC-823 cells compared with the control. P53, peroxisome proliferator (PPAR)- γ , signal transducer and activator of transcription (STAT)-3 and nuclear factor (NF)- κ B expression levels in MLK-4-silenced gastric tumor cells were also analyzed. As demonstrated in Fig. 3B, upregulation of MLK-4 expression markedly increased P53, PPAR- γ , STAT-3 and NF- κ B expression levels in gastric tumor cells. Subsequently, apoptosis-related gene expression was analyzed in gastric tumor cells overexpressing MLK-4. The results in Fig. 3C indicated that expression levels of B-cell lymphoma (Bcl)-2 and Bcl-2-associated X protein were downregulated in gastric tumor cells overexpressing MLK-4. Furthermore, migration-related protein expression in gastric tumor cells was evaluated. It was demonstrated that matrix metalloproteinase-3, collagen type I and fibronectin expression levels were upregulated following MLK-4 treatment (Fig. 3D). Collectively, these data suggested an inhibitory role of MLK-4 on the TLR-4-mediated JNK signaling pathway.

Gene silencing or genetic inactivation of mutational MLK-4 blocks the tumorigenic properties of cancer cells. The regulatory effects of MLK-4 on transcriptional downregulation in gastric cancer were investigated. The results in Fig. 4A demonstrated that knockdown of MLK-4 expression in gastric cancer cells significantly decreased the number of colonies formed compared with control cells ($P < 0.01$). It was observed that cell viability with wild type MLK-4 was significantly higher compared with MLK-4-knockdown cells ($P < 0.01$; Fig. 4B). In addition, it was demonstrated that downregulation of MLK-4 markedly decreased MLK-4 and vinculin protein expression levels in tumors (Fig. 4C). Furthermore, the present study

demonstrated the inhibitory effects of reduced or abrogated mutational MLK-4 expression or injected normal MLK-4 on tumor-bearing mice *in vivo*. The MLK-4 knockdown HGC-27 cells and the parental control were injected into immunocompromised mice. Notably, it was observed that tumor volume in mice with MLK-4 knockdown was significantly reduced compared with those injected with control HGC-27 cells at day 24, 30, 36 and 42 ($P < 0.01$; Fig. 4D). These results suggested that knockdown of MLK-4 may contribute to the treatment of gastric cancer.

Discussion

The aim of the present study was to investigate the function of MLK-4 in gastric carcinoma growth and tumorigenesis. A study by Martini *et al* (16) suggested that MLK-4 was frequently mutated in colon cancer, which has an important role in cancer tumorigenesis, local invasion and long distance metastasis. Therefore, understanding the role of MLK-4 is essential for tumor therapy in human tumorigenesis and metastasis. The present study investigated the expression and mutation of MLK-4 in gastric cancer cells and subcutaneous tumors, as well as the normal function of MLK-4. Results demonstrated that H261Q, G291E, A293E, W296 and R388H mutations occur at a high rate and all of them are located within the kinase catalytic domain, which is critical for kinase activity of DFG and HRD motifs (26). These findings were consistent with previous research (26,27). The mutational locations in the kinase catalytic domain affected the biological function of MLK-4 and influence protein catalytic function, which provided the impetus to cancer cell viability and invasion. The *in vivo* experiments confirmed our hypothesis and indicated that MLK-4 mutation contributed to the formation of subcutaneous tumors, which suggested that mutated MLK-4 may serve as a potential target in cancer therapy.

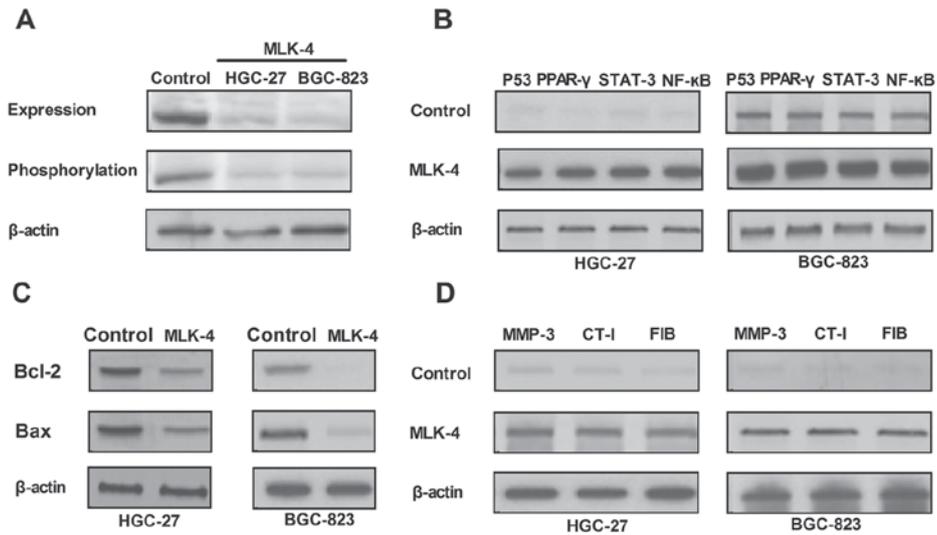


Figure 3. Regulation of JNK signaling pathway induced by MLK-4 in gastric cancer cells. (A) Expression and phosphorylation levels of JNK in HGC-27 and BGC-823 cells following transduction with MLK-4. (B) Upregulation of P53, PPAR- γ , STAT-3 and NF- κ B expression in MLK-4-transduct gastric tumor cells. (C) Downregulation of Bcl-2 and Bax in gastric tumor cells overexpressing MLK-4. (D) Expression levels of MMP-3, CT-I and FIB following MLK-4 treatment. MLK-4, mixed lineage kinase-4; JNK, c-Jun N-terminal kinase; PPAR- γ , peroxisome proliferator- γ ; STAT-3, signal transducer and activator of transcription-3; NF- κ B, nuclear factor- κ B; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; MMP-3, matrix metalloproteinase-3; CT-I, collagen type I; FIB, fibronectin.

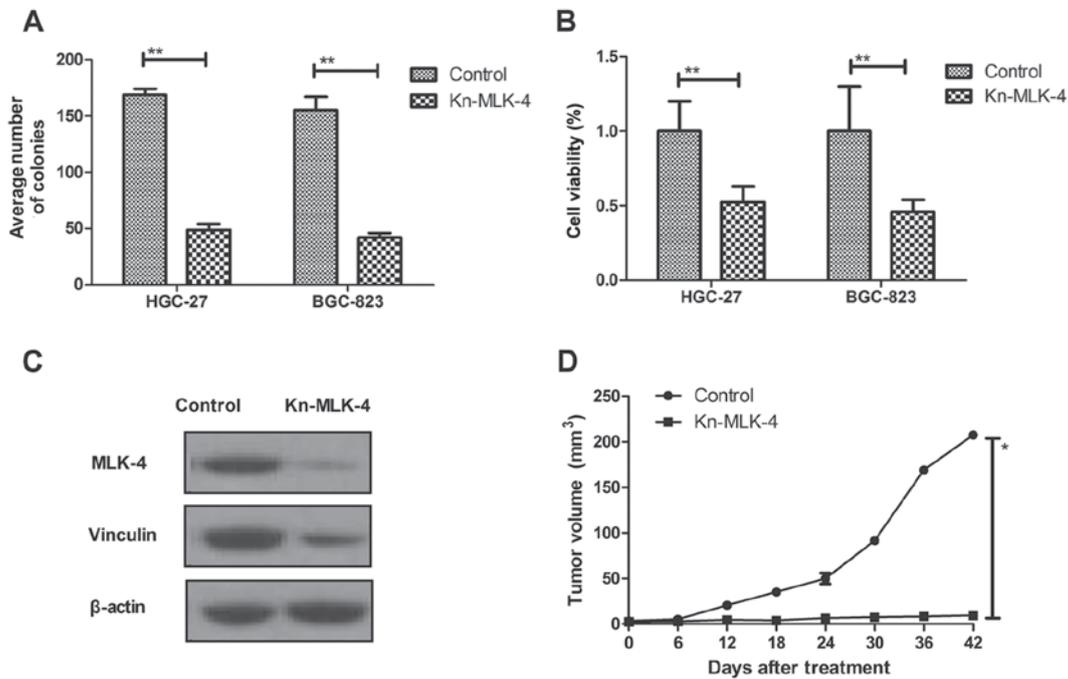


Figure 4. Inhibition of tumorigenic properties of MLK-4 by knockdown of mutational MLK-4. (A) Effect of knockdown of MLK-4 on clone formation of human gastric cancer cells. (B) Cell viability gastric cancer cells with MLK4 knockdown. Data are presented as the mean \pm standard error of the mean of three independent experiments (C) Improvement of the MLK-4-transforming potential in MLK-4 heterozygous mutation cells. (D) Effect of MLK-4 knockdown cells on tumor volume in a xenograft murine model. Data are presented as the mean \pm standard error of the mean of three independent experiments. * $P < 0.05$ at day 24, 30, 36 and 42. ** $P < 0.01$ at days 24, 30, 36 and 42. MLK-4, mixed lineage kinase-4; Kn, knockdown.

MLKs are a class of serine/threonine protein kinases that regulate JNK and p38 mitogen-activated protein kinase (MAPK) signaling pathways in cells (28). MLK-4 is a member of the MLK family of kinases and is an understudied protein kinase; however, understanding of this protein is essential to gain insight into the role of this kinase in tumorigenesis (29). A previous report indicated that MLK1-4 have regulatory

effects on MAP3Ks and may regulate the activities MKK3/6 and MKK4/7, which further induces JNK and p38 signaling pathway activation (28). In addition, previous research has also identified that MAPK/ERK kinase (MEK) function, endowed by MLK-1-4, are able to directly activate and reactivate the MEK/ERK pathway in a kinase-dependent manner in the presence of RAF inhibitors (13). Therefore,

MLK-4 serves as a direct activator of the JNK, p38 MAPK and MEK/ERK signaling pathways in cancer cells. The results of the present study focused on the JNK signaling pathway regulated by MLK-4 and indicated that silencing mutational MLK-4 expression significantly inhibited tumor cell viability, promoted apoptotic sensibility and prevented the growth of subcutaneous tumors.

Apoptosis resistance is the most serious obstacle in cancer clinical treatment (30,31). Decreasing apoptosis resistance of cancer cells and/or tumors will be beneficial for patients undergoing oncotherapy in clinics. MLK-4 is associated with apoptosis resistance in different cancer cells (32). MLK-4 is involved in the regulation of apoptosis in mammalian cells, and presents a potential molecular target in the treatment of human cancer with higher MLK-4 mutation rates. A study by Wang *et al* (33) indicated that proliferation, survival, migration, invasion and apoptosis of estrogen receptor-positive breast cancer cells were highly associated with MLK-4 activities, and suggested that silencing MLK-4 activities may serve as a novel target therapy for breast cancer. A study by Müller *et al* (34) demonstrated that MLK was able to protect granule cells against colchicine-induced apoptosis through the JNK/MLK signaling pathway. In addition, MLKs have been evidenced as a physiological element of nerve growth factor induction of the JNK signaling pathway, which suggests that MLKs may be potential therapeutic targets in the majority of apoptosis-related diseases (35). Furthermore, essential roles of MLKs in the tumor necrosis factor-induced programmed necrosis pathway has been indicated in tumor cells (36). However, the apoptosis resistance function of MLK-4 in cancer cells is seldom reported and the signaling pathway remains unclear.

The present study aimed to understand the molecular mechanisms of MLK-4 in regulation of protein kinase-based apoptosis resistance in the context of gastric tumor tissue. Apoptosis-related gene expression in the JNK signaling pathway was investigated *in vivo*. The results indicated that knockdown of mutational MLK-4 was beneficial for blocking subcutaneous tumor formation in gastric tumor-bearing mice as MLK-4 mutation promoted gastric carcinoma tumorigenesis and normal MLK-4 expression was downregulated (37). Therefore, MLK-4 may be a potential biomarker and may be used to diagnose and provide prognostic information for gastric cancer.

In conclusion, the present study provided insight on genetic mutation and the normal function of MLK-4 in gastric cancer cells and tissues. Activation of the JNK signaling pathway through mutation of MLK-4 induced metastasis and apoptosis-resistance in gastric cancer. Due to the present molecular study of gastric cancer, MLK-4 may serve as a potential molecular target for the treatment of gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YX, JN and DL performed the experiments and organized the data. JH, LQ and XP assisted in the analysis of data. YX wrote the manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Ethics Committee of the First Affiliated Hospital, Shihezi University School of Medicine (Shihezi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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