Original Article
TOPK promotes cell migration and is associated with poor prognosis in gastric cancer

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Abstract: T-LAK (lymphokine-activated killer T) cell-originated protein kinase (TOPK) is a MAPKK (mitogen-activated protein kinase kinase)-like protein kinase, which interacts with some tumor suppressor proteins and may play some important roles in the activation of T-LAK cells and signaling for the mediation of their cytotoxic functions against cancer cells. Here, in this study, a retrospective analysis of 79 resected gastric cancer patients was carried out to examine TOPK expression by immunohistochemistry. SGC7901 cell lines with depletion of endogenous TOPK were used to investigate if TOPK could promote cell migration. We found that the positive frequency of TOPK in cancer cells was 92.4%, whereas no TOPK expression was detected in non-cancerous mucosa. With increasing expression of TOPK, there was a significant increase in pT classification (P=0.0319) and pN classification (P=0.0337). In uni-variate analyses, AJCC stage (P<0.0001), pT classification (P<0.0001), pN classification (P<0.0001), differentiation (P=0.0268), peritoneal metastasis (P=0.0002) and gross features (P=0.0100) were significant prognostic factors. In multivariate analysis, AJCC stage was an independent predictor of survival (P<0.0001). Reductions of TOPK in SGC7901 cells were associated with decreases in cell invasion (P=0.0102) and migration (P=0.0278). Taken together, TOPK overexpression is associated with poor prognosis in GC. TOPK promotes gastric cancer cell migration.

Keywords: Gastric cancer, prognosis, TOPK, AJCC classification

Introduction

Gastric cancer (GC) is one of the most common cancers worldwide, and almost more than 40% of GC cases occur in China [1]. In spite of progress in the surgical treatment and chemotherapy, the prognosis of advanced GC patients remains poor [2]. Thus, it is important to investigate better biomarkers of GC for advances in treatment of this disease in the future.

T-LAK cell-originated protein kinase (TOPK), also known as PDZ-binding kinase (PBK), was isolated from a lymphokine-activated killer T (T-LAK) cell subtraction library [3]. TOPK is a MAPKK (mitogen-activated protein kinase kinase)-like protein kinase, which may play some important roles in the activation of T-LAK cells and signaling for the mediation of their cytotoxic functions against cancer cells [3]. Because it

functions as an enhancer of CDK1 (cyclin-dependent kinase 1)/cyclin B1-dependent phosphorylation of PRC1, TOPK has been presumed to be a promoter of cytokinesis [4]. Moreover, TOPK regulates cell-cycle by phosphorylating p97 and LGN/GPSM2 (Leu-Gly-Asn repeat-enriched protein/G-protein signaling modulator 2), which regulated spindle disassembly and formation respectively [5, 6]. In addition, TOPK has been shown to interact with some tumor suppressor proteins, including the human tumor suppressor hDlg (the human homologue of the *Drosophila Discs-large* (Dlg) tumor suppressor protein) [7] and p53 [8]. TOPK has been found overexpressed in highly proliferating normal tissues, fetal tissues and in several human cancers [9-16]. However, as far as we know, this is the first study evaluating the expression and prognostic significance of TOPK in GC. We aim to correlate TOPK expression

with clinicopathologic parameters and to assess its prognostic significance in GC.

Materials and methods

Tumor samples

The 101 paraffin-embedded specimens were collected from 79 GC patients who were surgically tumor-resected at the Third Affiliated Hospital of Harbin Medical University from January 2004 to December 2005, and were grouped as non-cancerous mucosa (n=22) and primary carcinoma (n=79). No patient was treated with preoperative chemotherapy or radiotherapy. According to the AJCC classification, there were 15 stage I patients, 20 stage II patients, 34 stage III patients, and 10 stage IV patients. All patients were followed up to August 2010 except those 56 patients who died before then. The follow-up time ranged from 1 to 79 months (mean 30.2 months). The pathological data were evaluated according to the 7th edition of the American Joint Committee on Cancer (AJCC) of GC. The Hospital Ethics Committee granted permission for this study.

Immunohistochemistry

The paraffin-embedded sections (4 μm) were deparaffinized and rehydrated through a series of xylene and ethanol rinses. Then, they were submerged in EDTA (pH 9) and exposed to microwave heating to retrieve their antigenicity. Endogenous peroxidase activity was blocked by incubating sections in 0.5% H_2O_2 solution for 30 min. After rinsing with phosphate-buffered saline (PBS), the sections were incubated with TOPK antibodies (Proteintech Group, 16110-1-AP, diluted at 1:100) at 4°C overnight. They were subsequently incubated with Streptavidin-horseradish peroxidase (HRP) complex for 30 min at room temperature. After visualization of the reaction with the diaminobenzidine (DAB) chromogen, all of them were counterstained with hematoxylin. Negative controls were composed of tissue sections incubated with PBS instead of the primary antibodies. Positive specimens exhibited cytoplasmic staining with little nuclear staining, while negative specimens exhibited no nuclear or cytoplasmic staining. The immunohistochemistry (IHC) score of each specimen was represented by the percentage of positive-stained cells: -, no or less than 5% cells stained; +, 6% to 30% of cells stained, + +,

31-50% of cells stained, + + +, 51%-75% of cells stained, and + + + +, more than 75% of cells stained.

Cells and reagents

Human gastric cancer cell lines SGC7901 were purchased from American Type Culture Collection and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Primary antibodies (dilutions) used include anti-TOPK (1:1000, Protein-tech Group, Wuhan, China), anti-β-actin (1:1000, Boster Bioengineering, Wuhan, China).

Transfection

50 nM of TOPK-specific siRNA were transfected into cells using lipofectamine 2000 reagent (Invitrogen). The TOPK-specific siRNA sequence (siTOPK: sense 5'-GGAGGAGAAUGGUGUU-AUU dTdT-3'; antisense 5'-AAUAACACCAUUC-UCCUCC dTdT-3') was purchased from Ribobio (Guangzhou, China). A scrambled siRNA sequence was obtained as a negative control. At forty-eight hours after transfection, cells were harvested and subjected to a migration assay and the expression of TOPK was determined by quantitative real-time PCR and Western blot analysis.

Quantitative real-time PCR (Q-RT-PCR)

Total cell RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and detected by Q-RT-PCR. A human GAPDH was used as an internal control. The primer sequences of TOPK and GAPDH were as follows: TOPK: 5'-TGAGGCTT-GTTACATTGGCACAG-3' (sense), 5'-AGCAGAAG-GACGATCTTTAGGGT-3' (antisense); GAPDH: 5'-GTGAAGGTCGGAGTCAACGG-3' (sense), 5'-CT-CCTGGAAGATGGTGATGGG-3' (antisense). The amount of TOPK mRNA expression is given by: $2^{-\Delta\Delta CT}$ and data are represented as mean ± s.d.

Western blot

Cell lysates were made by standard methods. 15 μl of each sample was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with buffer (Tris buffered saline [TBS], 0.1% Tween 20, and 5% non-fat dry milk) for

Table 1. Demographic data and survival in different stages of gastric cancer according to the AJCC classification

	Stage I (n=15)	Stage II (n=20)	Stage III (n=34)	Stage IV (n =10)	Total (n=79)
Gender					
Male	11	14	29	6	60
Female	4	6	5	4	19
Age (years)[a]	55.7 (10.4)	55.1 (11.6)	60.8 (10.6)	58.6 (11.99)	58.1 (11.1)
Follow-up period (months)[a]	57.9 (14.4)	45.3 (24.2)	14.9 (9.3)	10.4 (8.6)	30.2 (24.2)
Gross features					
Noninfiltrating	6	7	1	0	14
Infiltrating	9	13	33	10	65
Survival					
Yes	11	12	0	0	23
No	4	8	34	10	56

[a]Mean (standard deviation, SD).
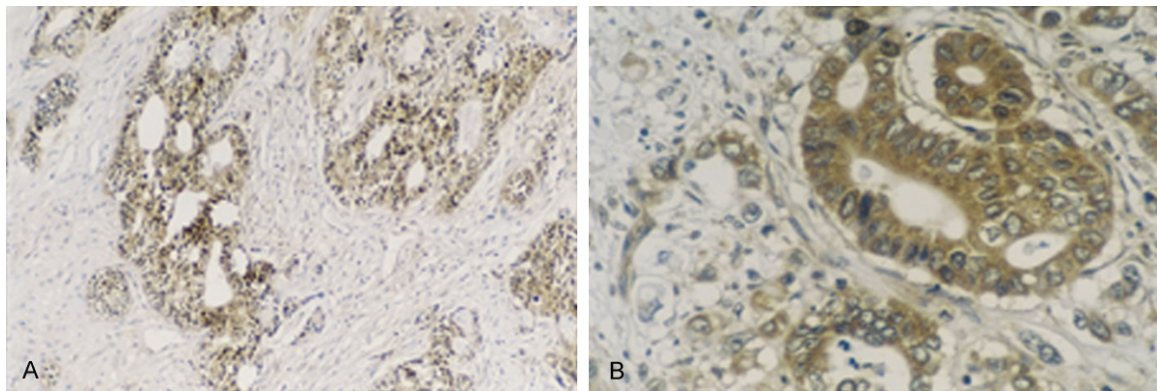
Figure 1. Immunohistochemical staining of gastric cancer: anti-TOPK [magnification, ×100 (A), ×400 (B)], the positive staining was mainly in the cytoplasm of GC cells besides little nuclear staining.

1 h at room temperature, followed by hybridization with the primary antibody overnight at 4°C. After washing three times with TBST, the membranes underwent hybridization with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing three times with TBST, signals were detected by chemiluminescence with the Western blotting luminal reagent (Santa Cruz, Biotechnology) according to the manufacturer's instructions.

Statistical analyses

The statistical analyses were carried out with Statistical Analysis Software version 9.1 for Windows. Cochran-Mantel-Harnszel (CMH) test and chi-square analysis were used to detect correlations between variables. The follow-up time was defined as the period from the operation date to the last visit. Survival analysis was performed by the Kaplan-Meier method, and the significance was determined by univariate log-rank test. The parameters that were significant in univariate analysis (P<0.05) were selected for the multivariate Cox regression model to assess the increment statistical power and independence of prognostic impact. All statistical tests were two-sided. A *P*-value <0.05 was considered statistically significant.

Results

TOPK is associated with invasiveness, metastasis and poor prognosis in gastric cancer

To determine the prognostic value of TOPK expression, its immunoreactivity was assessed using 101 specimens from 79 gastric cancer patients (**Table 1**). TOPK was detectable for scoring in GC tumor cells. The localization of

Table 2. Linear association between TOPK expression levels versus clinicopathological variables

Variables	Frequency (% of patients)					
	TOPK expression					
	- (n=6)	+ (n=4)	+ + (n=24)	+ + + (n=27)	+ + + + (n=18)	P value
Gross features						
Noninfiltrating	0 (0.00)	2 (14.29)	8 (57.14)	3 (21.43)	1 (7.14)	0.1635
Infiltrating	6 (9.23)	2 (3.08)	16 (24.62)	24 (36.92)	17 (26.15)	
Differentiation						
Poor/mucinous	6 (9.38)	2 (3.13)	18 (28.13)	23 (35.94)	15 (23.44)	0.8140
Well/moderate	0 (0.00)	2 (13.33)	6 (40.00)	4 (26.67)	3 (20.00)	
pT classification						
T1/2	2 (9.52)	2 (9.52)	10 (47.62)	5 (23.81)	2 (9.52)	0.0319
T3/4	4 (6.90)	2 (3.45)	14 (24.14)	22 (37.93)	16 (27.59)	
pN classification						
N0	3 (10.34)	1 (3.45)	13 (44.83)	10 (34.48)	2 (6.90)	0.0337
N1-N3	3 (6.00)	3 (6.00)	11 (22.00)	17 (34.00)	16 (32.00)	
Peritoneal metastasis						
Negative	6 (8.82)	3 (4.41)	21 (30.88)	22 (32.35)	16 (23.53)	0.6746
Positive	0 (0.00)	1 (9.09)	3 (27.27)	5 (45.45)	2 (18.18)	
AJCC stage						
I	2 (13.33)	0 (0.00)	9 (60.00)	4 (26.67)	0 (0.00)	0.1426
II	1 (5.00)	2 (10.00)	5 (25.00)	7 (35.00)	5 (25.00)	
III	3 (8.82)	1 (2.94)	4 (11.76)	14 (41.18)	12 (35.29)	
IV	0 (0.00)	1 (10.00)	6 (60.00)	2 (20.00)	1 (10.00)	

Table 3. Univariate analysis of prognostic markers in 79 patients with gastric cancer

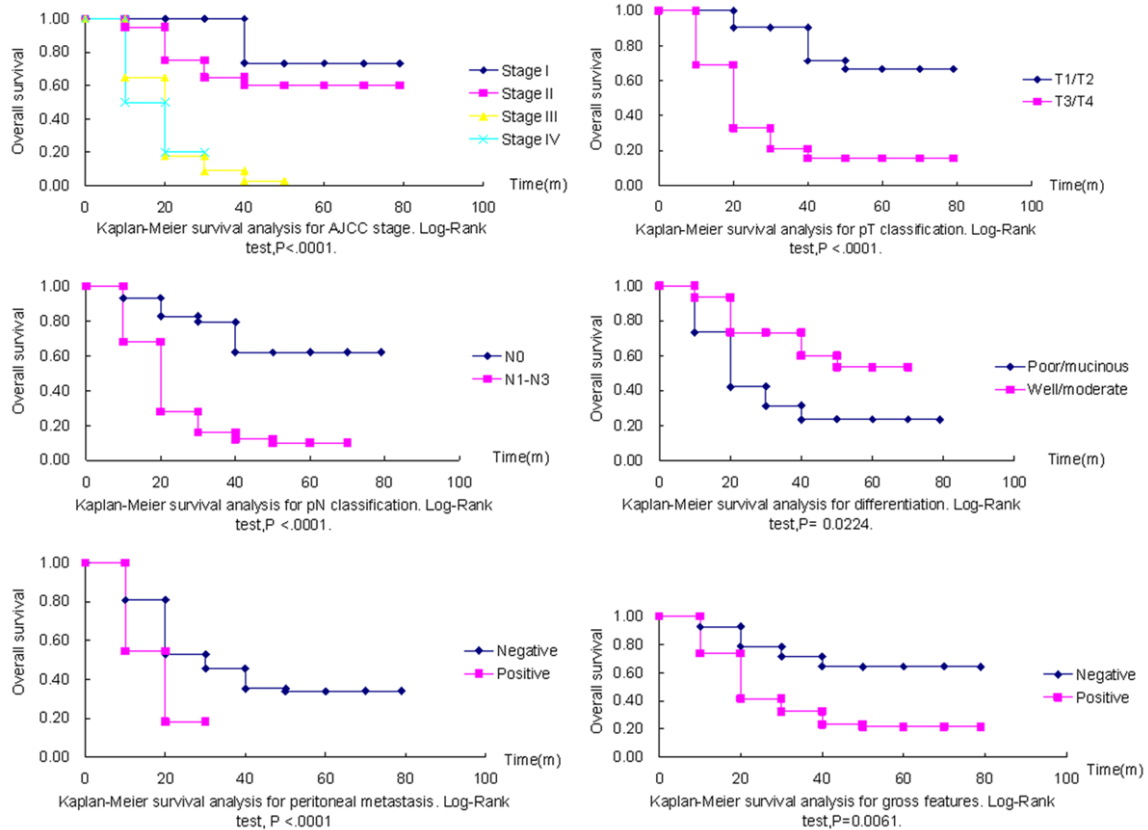| Variable	β	χ^2	P	HR	95% CI	
					Lower	Upper
TOPK	0.10256	0.6622	0.4158	1.108	0.865	1.418
pT classification	1.72288	17.6414	<.0001	5.601	2.507	12.514
pN classification	1.66036	22.9267	<.0001	5.261	2.666	10.381
AJCC stage	1.16801	45.4364	<.0001	3.216	2.290	4.516
Differentiation	-0.89937	4.9039	0.0268	0.407	0.184	0.902
Peritoneal metastasis	1.29536	13.6801	0.0002	3.652	1.838	7.256
Gross features	1.21123	6.6301	0.0100	3.358	1.335	8.442

HR hazard ratio, CI confidence interval.

TOPK staining was mainly in the cytoplasm of tumor cells besides little nuclear staining (**Figure 1**). The positive frequency of TOPK in cancer cells was 92.4%, whereas no TOPK expression was detected in non-cancerous mucosa. TOPK expression increased significantly from non-cancerous mucosa to cancer. The correlations between the expression levels of TOPK and the clinicopathological variables are summarized in **Table 2**. With increasing TOPK expression, there was a significant increase in pT classification (P=0.0319) and pN classification (P= 0.0337). There was no correlation between TO-PK expression and other clinicopathological variables in **Table 2**, such as gross features, differentiation, peritoneal metastasis and AJCC stage. The univariate analysis demonstrated that AJCC stage (P<0.0001), pT classification (P<0.0001), pN classification (P<0.0001), differentiation (P=0.0268), peritoneal metastasis (P=0.0002) and gross features (P=0.0100) had an impact on overall survivals (**Table 3**). TOPK IHC score in tumor cells was not significantly associated with survival rate of the patients (P=0.4158). The Kaplan-Meier curves for correlations of clinical outcomes with the parameters are shown in **Figure 2**. The multivariate Cox regression analysis revealed that AJCC stage significantly affected the overall survivals was an independent predictor of sur-

Figure 2. Kaplan-Meier survival curves for clinicopathological factors.

Table 4. Multivariate Cox regression analysis of prognostic markers in 79 patients with gastric cancer (Backward stepwise model)

Variable	β	χ^2	P	HR	95% CI	
					Lower	Upper
AJCC stage	1.16801	45.4364	<.0001	3.216	2.290	4.516
I						
II+III+IV						

HR hazard ratio, CI confidence interval.

vival (**Table 4**). Taken together, our data show that overexpression of TOPK is associated with invasiveness, metastasis and poor prognosis in gastric cancer.

SiRNA-mediated knockdown of TOPK suppresses gastric cancer cell migration

To further confirm that TOPK expression correlates with the invasiveness and metastasis of gastric cancer, we examined whether TOPK-specific siRNAs could affect cell migration in SGC7901 cells. We transiently transfected a scrambled control siRNA (Neg.) and a siRNA directed against TOPK into SGC7901 cells and tested the effect of TOPK on cell migration. As expected, reductions of TOPK protein in SGC7901 cells were associated with decreases in cell invasion (P=0.0102) and migration (P=0.0278). Endogenous TOPK was suppressed in SGC-7901 cells, which led to a great decrease in cellular migration as compared with the vehicle-treated or scrambled control cells (**Figure 3**).

Discussion

The overexpression of TOPK has been observed in highly proliferating normal tissues, fetal tissues and in a variety of human cancers [9-17]. However, the expression and prognostic significance of TOPK in GC has not been evaluated. This study presents the first evidence to assess the expression and prognostic impact of TOPK in human GC. Wei et al. [18] found that TOPK
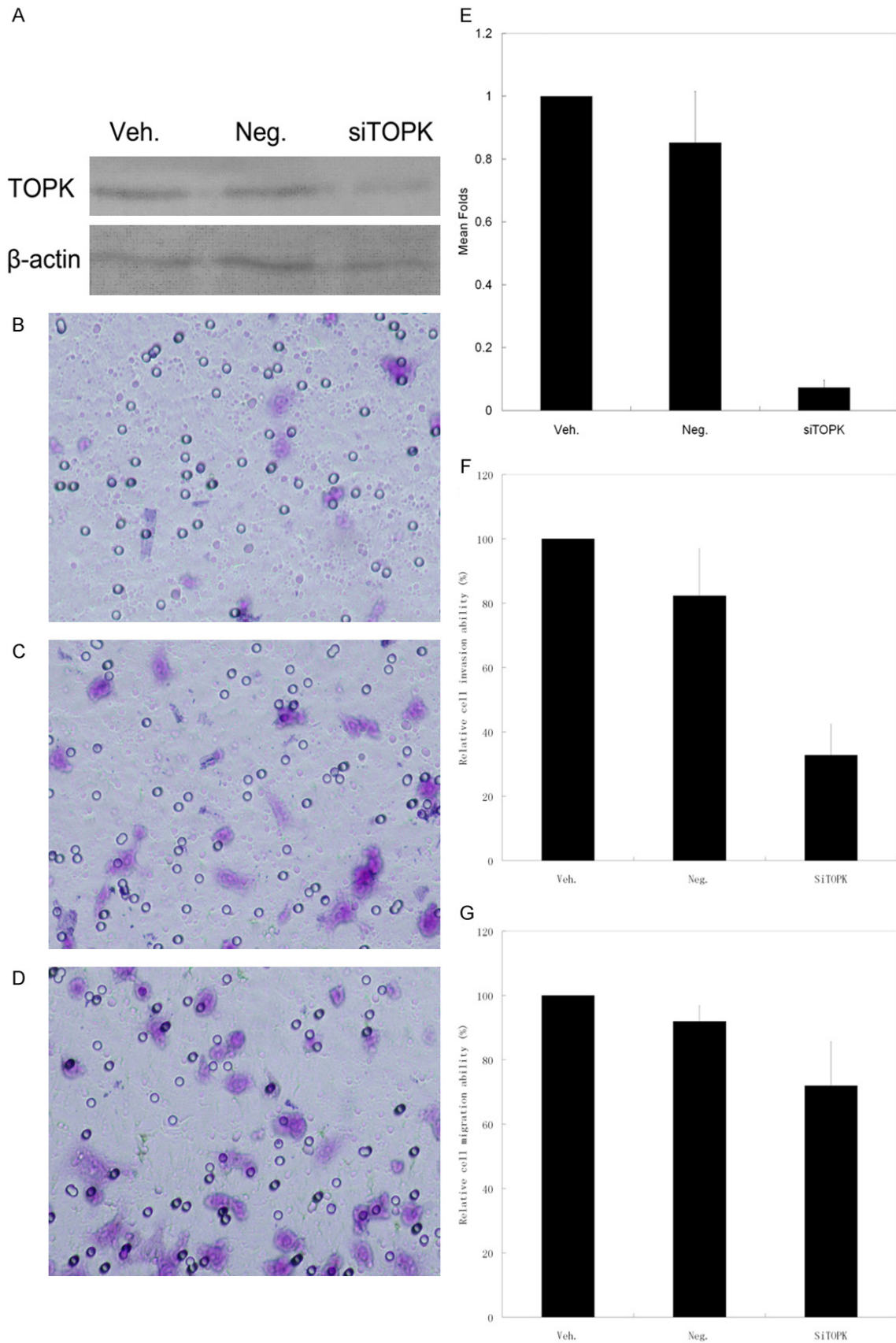
Figure 3. Knockdown of TOPK suppresses gastric cancer cell migration. SGC7901 cells were transiently transfected without (vehicle; Veh.) or with a scrambled siRNA (Neg.) or a TOPK-specific siRNA (siTOPK). Forty-eight hours after transfection, one portion of cells was harvested for detection of β-actin and TOPK expression by Western blot (A) and Q-RT-PCR analysis (E). The other portions of the cells were incubated in Matrigel-coated or uncoated transwell inserts for another 24 hours. The diagrams of invasion assay (F) and migration assay (G) are representative of relative migration fold as compared with vehicle control. Invasion assay in SGC7901 cells (×200): siTOPK (B), Neg. (C), Veh. (D) All assays were performed in triplicate, independently repeated at least three times.

was overexpressed in lung adenocarcinoma specimens, which was correlated with the metastatic capability of tumors and predicted poor prognosis in patients with stage I lung adenocarcinoma. Park et al. [16] reported that TOPK was upregulated in breast cancer cells and knocking down the expression of TOPK led to growth suppression of breast cancer cell lines. By immunohistochemistry we found that non-cancerous mucosa had no detectable TOPK, whereas the positive frequency of TOPK in cancer cells was 92.4%. TOPK expression increased significantly from non-cancerous mucosa to cancer. This result is consistent with the idea that TOPK is a mitotic kinase and upregulated in vigorously proliferating cells during mitosis [7, 11, 14, 16]. At an early stage of mitosis, protein phosphatase 1 alpha (PP1α) is inactivated through phosphorylation at Thr320 by CDK1/cyclin B1 complex, which resulted in the activation of TOPK by autophosphorylation [19, 20]. Then, induced by CDK1 inactivation, the restored PP1α gradually dephosphorylates and inactivates TOPK to the steady level [11, 19]. Nandi et al. [21] found that the regulation of TOPK is mostly due to binding of cell cycle-specific transcription factors E2F and CREB (cAMP-response element binding protein)/ATF to E2F (-146 bp) and CREB/ATF (-312 bp) binding sites within the TOPK promoter. However, due to diversified ways of regulating TOPK expression [7, 11, 17, 22], there are likely various mechanisms causing activation and overexpression of TOPK in gastric cancer.

TOPK has been reported earlier to be associated with invasion, metastasis and prognosis of various cancers [9, 18, 23]. He et al. [24] found that TOPK frequently expressed in cholangiocarcinoma but never expressed in hepatocellular carcinoma and the low expression of TOPK was linked to an unfavorable prognosis in patients with cholangiocarcinoma. Zlobec et al. [9] showed that TOPK expression combined with *KRAS* or *BRAF* mutations was related to poor prognosis in sporadic colorectal cancer. Wei et al. [18] demonstrated that TOPK promot-

ed migration, invasion, and clonogenic activity of lung cancer cells. Here, we presented supportive data that TOPK overexpression was positively correlated with pT classification and pN classification, which were prognosticators for poor survival of GC. In addition, by inhibiting expression of TOPK, we have demonstrated that TOPK can promote gastric cancer cell migration. These results also agree with previous studies showing that TOPK overexpression in stage I lung adenocarcinoma could be a prognostic predictor of reduced overall survival and time to recurrence [18]. Shih et al. [23] showed that TOPK might stimulate cell migration through the PI3K (phosphatidylinositol-3-kinase)/PTEN (phosphatase and tensin homolog)/AKT (protein kinase B)-dependent signaling pathway to facilitate metastasis of lung cancer cells. Other investigators showed that TOPK functioned as a regulator of p38 MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinases) and ERK (extracellular signal-regulated kinases)/MAPK signaling pathway, which regulated cell proliferation, oncogenesis, differentiation, apoptosis, invasion and migration [3, 17, 25-29]. Ayllón et al. [30] observed that TOPK mediated cell motility in response to growth factor stimulation by activating p38 MAPK and DNA damage response through histone H2AX phosphorylation, which facilitated the recruitment of repair factors to the sites of damage [31]. This observation was supported by the findings that TOPK phosphorylated H2AX thereby contributing to the prevention of arsenite (As^{3+})-induced apoptosis in RPMI7951 melanoma cells [13]. Oh et al. [25] demonstrated that TOPK, which was an upstream regulator of UVB (ultraviolet B)-induced JNK activation, might activate JNK1 by directly phosphorylating JNK1 and synergistically interacting with the JIP1 (JNK-interacting protein 1) scaffolding complex to mediate H-Ras-induced cell transformation in skin cancers. Zhu et al. [17] found that TOPK phosphorylated ERK2 and vice versa, which promoted a positive feedback loop to increase kinase activity of both proteins thereby inducing trans-

formation in colorectal cancer. Interestingly, studies of ER (estrogen receptor)-positive T47D breast cancer cells with PI3K mutations indicated that TOPK might play a role in mediating PI3K/Akt-sensitive MEK-independent ERK activation [32]. Besides, Hu et al. [33] reported that TOPK overexpression promoted tumor cell progression and resistance to chemotherapy-induced apoptosis through interference with the DNA-binding domain of p53 and consequent reductions in the cell-cycle regulatory proteins including p21.

This article shows that TOPK can be an excellent biomarker for gastric cancer and is correlated with invasiveness, metastasis and poor prognosis in patients with GC. Due to TOPK is involved in the growth, invasiveness, migration and DNA damage repair pathways of cancer cells, it may be a potential novel target for the development of antineoplastic therapeutics and to be used in combination with DNA damaging drugs [13, 30].

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Disclosure of conflict of interest

None.

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