TOPK Promotes Metastasis of Esophageal Squamous Cell Carcinoma by Activating Src/GSK3β/STAT3 Signal Pathway though γ-catenin

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Abstract
Background Esophageal squamous cell carcinoma (ESCC) is a deadly disease with the poor prognosis in the world. The distal metastasis is the most death reason of ESCC. It is needed to have a comprehensive understanding of the molecular mechanism of metastasis to increase the free survive rate. T-LAK cell-originated protein kinase (TOPK) which is a MAPKK-like kinase takes an vital role in many physical and pathophysiological progress. However, the function of TOPK in ESCC metastasis was unclear. Methods Tissue array was used to evaluate the relationship between TOPK and ESCC patient with lymph node metastasis. Wound healing assay, transwell assay and lung metastasis mice model were assessed for the role of TOPK in the migration of ESCC cells in vitro and in vivo respectively. Protein kinase array, MS and molecular modeling were carried out to find the relational pathways and target protein of TOPK. Even, immune-fluorescence and western blot were performed to evaluate the mechanism of TOPK. Results We found that the high level of TOPK was correlated with the aggressive phenotype in ESCC tissues. Knocking down TOPK inhibited the invasion and migration of ESCC cells. We also verified that TOPK inhibitor HI-TOPK-032 inhibited the lung metastasis in ESCC cell exnograft model. Even more, molecular investigation indicated that TOPK promoted the invasion of ESCC cells by activing Src/GSK3β/STAT3, ERK pathway by binding with γ-catenin. Conclusion These findings reveal that TOPK was sincerely related with ESCC cell metastasis and TOPK promoted the invasion of ESCC cells by activing Src/GSK3β/STAT3, ERK pathway. This means that TOPK may be a potential molecular target for ESCC in clinic.

Background
Esophageal cancer is the sixth leading cause of cancer death worldwide (1). Esophageal squamous cell carcinoma (ESCC) was the main histological type and account for about 80% of esophageal cancer in China. Most patients of ESCC are diagnosed at advanced stages of the disease due to lack of alarming symptoms at an early stage. Lack of effective clinical approaches for earlier diagnosis and therapy of ESCC results in poor prognosis and a low 5-year survival rate which was about 10%-15% (2). What is more, ESCC frequently metastasizes to the liver, lungs, regional lymph nodes, bone and adrenal glands (3). Wide-spread genomic alterations have been detected in ESCC by using high-
throughout sequencing technologies (4). These alterations corrupt in diverse cell populations, signaling factors, and structural molecules that interact with tumor cells and support all stages of tumorigenesis. Therefore, identifying the effective gene as therapeutic target remains urgently needed to understand the molecular mechanisms of ESCC tumorigenesis and metastasis.

LAK cell-originated protein kinase (TOPK) is a serine-threonine kinase which involves in many physiological and pathological progresses, such as cell cycle (5), apoptosis (6), cancer cell proliferation (7) and cell invasion (8). There is accumulating evidence that TOPK is an oncogenic kinase which highly expressed in several types of human cancers that included lung cancer, colon cancer and ovary cancer and so on and correlates with more aggressive phenotype (9, 10). However, the underlying mechanism that leads to this aggressive phenotype in ESCC is still unclear.

In this report, we examine the molecular mechanisms of TOPK in the ESCC metastasis. Through systematic analyses, we found that the high level of TOPK was correlated with the aggressive phenotype in ESCC. Knocking down TOPK and TOPK inhibitor HI-TOPK–032 inhibited the invasion and migration of esophageal cell line in vitro. HI-TOPK–032 also inhibited the lung metastasis of ESCC cell exnograft model in vivo. Even more, molecular investigation indicated that TOPK can prompt the invasion of ESCC cells through Src/GSK3β/STAT3 signal pathway by binding with γ-catenin.

Methods

Chemical reagents

Chemical reagents, including Tris, NaCl, and SDS, for molecular biology and buffer preparation were purchased from Sigma-Aldrich. Antibodies for western blot analysis were from Cell Signaling Technology, Abcam, or Santa Cruz Biotechnology. The Human Phospho-Kinase Arrays were obtained from R&D Systems. 1640, DMEM and MEM medium were all purchased from BI (Bioind, Israel), FBS was purchased from Gibico. TOPK inhibitor HI-TOPK–032 was a present from Prof Zigang Dong.

Human ESCC tissue array

Tissue microarray was brought from Shanghai Outdo Biotech Co., Ltd. Clinical stage (AJCC 7.0) which included I, II, III, IV stages and TNM score information were available in patients’ clinical data which consisted of 10 cases of ESCC with matched adjacent normal tissue and negative lymph node(N0), 19
cases of N1, 10 cases of N2 and 8 cases of N3 ESCC and positive lymph node points. Immunohistochemical staining was performed for evaluating the expression of TOPK in the ESCC metastasis tissue. The antibody of TOPK (1:50) was applied to the slides and incubated at 4°C overnight. The slides were washed and then the slides were cultured with second antibody, then detected by DAB. The grading of positive cells were observed by microscope (20X) and analyzed by Image J software.

Cell culture
ESCC cell lines KYSE450 (Catalogue number: XY-H600), KYSE510(Catalogue number: AD0604), KYSE140 (Catalogue number: AD0602),KYSE70 (Catalogue number: XY-H601) and KYSE30(Catalogue number: AD0601) were purchased Shanghai Xinyu Biological Technology Co., Ltd.(Shanghai, China). KYSE450 cell lines were maintained in Dulbecco’s modified Eagle's medium (DMEM, BI, USA) supplemented with 10% FBS (Bioind, Israel). Other ESCC cell lines were maintained in 1640 medium with 10% FBS, 100 μg/mL of penicillin, and 100 units/mL of streptomycin at 37°C with 5% CO₂.

Western blot
Cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM Na₃VO₄, and complete EDTA-free protease inhibitor (Roche)] for 20 min on ice and clarified by centrifugation. The supernatants were subjected to 10% SDS-PAGE, and separated proteins were blotted on polyvinylidene difluoride membranes (Millipore, USA). Signals were developed by ECL western blotting detection reagent (GE, USA) and detected using an LAS-1000UVmini image analyzer(GE USA).

Lentiviral infections
The lentiviral TOPK shTOPK #1 (ATTAGTGCATACAGAGAAGAGTT) and shTOP #2 (GTCTGTGTCTTGCTATGGAAT) constructs were presented from the Prof Zigang Dong. Lentiviruses were produced by co-transfecting the shRNA expressing vector with psPAX2 and pMD2.G constructs into 293T cells using jetPrime reagent. Viral supernatants were harvested, and used to infect cells with 8 μg/mL polybrene. Cells were selected by using 2 μg/mL puromycin to established stable KYSE510 and KYSE30TOPK knocking down cell lines.
Wound-healing migration assay

For wound-healing migration assays, shMock, shTOPK #1 and shTOPK #2 of KYSE510 and KYSE30 cells were seeded on 6-well plates at a density of 80% in culture medium, and incubated for 24 h. The confluent monolayer was then scratched with a fine pipette tip, and then the plate was washed twice with phosphate buffered saline (PBS) and then we removed cell debris and isolated cells. The cells were then incubated with the complete growth medium. When the cells migrated to the injured blank area, photographs were taken at 0, 24 and 48 h after the wound, respectively.

Transwell migration assay

Transwell migration assay was performed by using transwell inserts for a 24-well plate containing 8 µm pores (Millipore). The upper chamber membrane was coated with 100 µL 200 µg/mL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C for 1 h before adding cells. 1x10^5 KYSE510 shMock, shTOPK #1, shTOPK #2 and KYSE30 shMock, shTOPK #1, shTOPK #2 cells were plated in culture medium without serum or growth factors in the upper chamber, and medium supplemented with 20% FBS as a chemo-attractant was used in the lower chamber. After incubating for 24 h, the cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were fixed with methanol for 10min, and stained with 0.1% crystal violet, and then counted in 5 randomly selected microscope fields per well.

Protein kinase array

Analysis of phosphorylated proteins by human phospho-kinase arrays was conducted following the manufacturer’s instructions. Briefly, cell lysates (500 µg) of KYSE510 shMock, shTOPK#1 and shTOPK#2 were collected and incubated with each array at 4°C overnight on a rocking platform shaker. In the next day, the cell lysate was removed and arrays were washed 3 times with washing buffer. Arrays were incubated with the second antibody solution for 2 hours at room temperature and washed 3 times with washing buffer. The HRP solution was added to the array and incubated on a rocking shaker for another 30 min. The array was washed 3 times with washing buffer, and protein spots were visualized using a chem-iluminescence detection kit. The density of each duplicated array
spot was assessed using the Image J computer program (v.1.37v, NIH), and the density was calculated by subtracting the background and PBS negative control. The fold change was obtained by comparing shMock samples (indicated as a value of 1).

**Surface plasmon resonance**

GST-tagged TOPK proteins were purified from BL21 bacteria using glutathione-Sepharose 4B beads. GST antibody was immobilized onto the CM5 chip by amine coupling, according to the manufacturer’s instructions (GE). TOPK kinase (Signalchem, T14-10G-10) and purified GST-TOPK protein (>90%) was captured by GST antibody of CM5 chip. And then KYSE510 cell lysis was introduced into the CM5 chip at a flow rate of 10 μL/min for 5 minutes with running buffer for one cycle, and recycle the captured protein (1x Kinase buffer, pH7.2). The control group was treated without the TOPK kinase or GST-TOPK protein. The whole procedures were completed by Bicore T200 (GE, USA).

*Nanoflow LC-ESI-MS/MS*

The captured protein in TOPK kinase group, TOPK protein group (TOPK protein was purified by GST tag) and negative group (KYSE510 lysis buffer) by SPR was analyzed by mass spectrometric (MS). In the sample with final concentration of 10 mmol/L, reduction after 1 h of DTT37 DEGC, final concentration 30mmol/L iodoacetamide at room temperature to avoid light, 45min for alkylated modification. Finally, with 25 mmol/L ammonium hydrogen carbonate dilution of the sample enable urea concentration below 2 mol/L, with mass ratio of 1: 50 sequencing grade trypsin and incubated at 37°C for 14h. R3 self-loading desalting column, 100%ACN, 0.1% trifluoroacetic acid, the sample (2 times), 0.1% TFA, 1%TFA, with 30% ACN, 60% CAN elution, 30% ACN and 60% ACN elution solution combined with freeze dried samples with a final concentration of 0.1% formic acid after re-dissolution by LC-ESI-MS/MS analysis. The mobile phase includes solution A (0.1% formic acid 99.9%H2O), solution B (0.1% formic acid, 99.9%ACN), sample volume 10 L, continuous gradient elution, flow rate of 200nL/min. Analytical column gradient elution settings for the LTQ Orbitrap elite and mass spectrometric parameters setting mode of cation, CID collision model, resolution 120000, mass range for 350-1800, before top 15 ionic strength for MS/MS analysis. Using a thermo proteome Discoverer1.4.0.288 software analysis mascot database search, database uniprot
-human, enzyme for trypsin-P, maximum missed cleavage is 2, the precursor mass tolerance is 10ppm, the fragment mass tolerance for 0.5Da, fixed modifications: Carbamidomethyl(c); variable modification: Gln->pyro-Glu (N-term q), acetyl (protein N-term) and oxidation (M) phosphorylation of dynamic modification more the Phospho(st) and Phospho (y). Peptide confidence is high FDR<0.01.

**Molecular modeling**

We wanted to further confirm whether TOPK binds with γ-catenin, we perform the in silico docking using the Schrödinger Suite 2016 software. The TOPK and γ-catenin crystal structure was derived from the Protein Data Bank (11) (PDB ID:5J0A and 3IFQ) and prepared under standard procedures of the Protein Preparation Wizard (Schrödinger Suite 2012). TOPK and γ-catenin protein-protein docking was performed using protein docking server with interactive molecular graphics program (12), and we selected the best configuration to represent the binding mode.

**Immunofluorescence assay**

Immunofluorescence studies used TOPK primary antibodies from Santa Cruz™ and γ-catenin primary antibodies from Abcam™. Fluorescein-conjugated affinity pure goat anti-mouse IgG and rhodamine (TRITC)-conjugated affinity pure goat anti-rabbit IgG secondary antibodies were added for 1h. Coverslips were mounted with DAPI (Santa CruzTM), and image captured and analyzed with high-throughput confocal microscopy (IN Cell Analyzer 6000, GE).

**Co-immunoprecipitation**

For co-immunoprecipitation studies, HEK293 cells were transfected pcDNA3-HA-TOPK and/or pDEST-myc-γ-catenin at 4 μg by using jetPRIME reagent (Polyplus- transfection® SA, France) for 72 h. The protein was isolated in CHAPS buffer (as previously described) supplemented with protease inhibitors cocktails (Rhoche). Lysates were centrifuged for 30 minutes at 14,500 g, and 500μg protein in 500 μl was incubated with HA antibody for overnight at 4°C. The next day, 40 μl of immobilized Protein A/G agrose (sc-2004, Sata Cruz) were incubated with these proteins for 4 h at 4°C, the collected protein complexes were washed 3 times with cold PBS, and eluted by boiling in loading buffer at 95°C, 2 min on ice, then myc protein was resolved by SDS-PAGE and analyzed by Western blot.
Lung metastasis in ESCC cell xenograft mouse models

pcDNA3.1-GFP vector was transferred to KYSE510 cells, then stable GFP-KYSE510 cells were established by G418 and evaluated by IVIS® Lumina III In Vivo Imaging System. Then 2x10⁶ GFP-KYSE510 cells were injected by tail vein of BALB/c nude mice which were bought from Vital River, Beijing, China and were treated with solvent as control group (n = 12, 6 femal and 6 male) and HI-TOPK-032 (n = 13, 7 femal and 6 male) as treatment group respectively. 4–5 mice were kept in a pathogen-free environment with light controlled rooms (12 h cycles) and provided with food and water adlibitum. HI-TOPK-032 was dissolved in 5% DMSO-PBS and was given to each mouse in treatment group once daily at 10 mg/kg (i.p., 100µL per mouse). After two weeks, in vivo imaging was collected and the fluorescence of GFP was evaluated as before (13). After euthanasia by breathing CO₂, lung tumor tissues were used to certify the level change of singal pathways. The study was approved by the Ethics Committee of Basic Medical College of Zhengzhou University.

Statistical analysis

All data are expressed as mean ± standard error (SEM) and all comparisons between samples were evaluated using the two-tailed non-parametric Mann-Whitney test, one-way or two-way ANOVA with Bonferroni post hoc testing. P values were obtained from the tests and described in the Figure legends. Statistical significance is denoted by * for P ≤ 0.05, ** for P ≤ 0.01, and *** for P ≤ 0.001.

Results

TOPK expression positively correlates with tumor metastasis of ESCC patients

To investigate the clinical significance of TOPK expression in ESCC metastasis, the expression of TOPK was analyzed in 49 human ESCC patients with or without the lymph node metastasis. The positive expression of TOPK was mainly in the cytoplasm and/or cell nucleus with different intensity brown staining accompanied with the different lymph node metastasis (Fig 1A). We further analyzed the relationship between TOPK expression and the lymph node metastasis, the results indicated the
expression of TOPK was significantly higher in ESCC patients with lymph node metastasis group (N1 and N2–3 group) compared with no lymph node metastasis group (N0 group). The expression difference between them is statistically significant, \( P < 0.001 \) (Fig 1B). In addition, the expression of TOPK was different in a panel of ESCC cell lines, the expression of TOPK in KYSE510, KYSE140, KYSE30 cells was higher than the level in KYSE450, and KYSE70 was low (Figure 1C-D).

**Down regulated TOPK attenuates migration and invasion of ESCC cells**

Then we established TOPK knockdown stable cell line by transferring shTOPK#1 and shTOPK#2 in KYSE510 (Figure 2A) and KYSE30 (Figure 2B). Knocking down TOPK inhibited KYSE510 (Figure 2C) and KYSE30 (Figure 2D) cell migration by wound-healing migration assay. The result of transwell migration assay indicated that the invasion of KYSE510 (Figure 2E) and KYSE30 (Figure 2F) was decreased by down-regulating TOPK expression. To confirm whether TOPK is a target in the metastasis of ESCC, a specific TOPK inhibitor HI-TOPK–032 which inhibits TOPK kinase activity was treated in KYSE510 and KYSE30 cells. The results indicated that HI-TOPK-032 inhibited the cell migration in KYSE510 (Fig 3A) and KYSE30 (Fig 3B) cell lines. The invasion and mobility of KYSE510 (Fig 3C) and KYSE30 (Fig 3D) cell were decreased and dose dependent after treating HI-TOPK-032 for 24 h. These data suggested that TOPK was sincerely related with ESCC cell mobility.

**Src/GSK3β/STAT3 and ERK signaling pathways are strongly inhibited by knocking down TOPK or treatment with HI-TOPK–032**

To further explore the mechanism of TOPK function in ESCC metastasis, the human phospho-kinase array (ARY003B, R&D) was used to screen the signal transduction pathways that changed after knocking down TOPK in KYSE510 cells. The results showed that several signaling pathways were affected in the KYSE510 shTOPK#1 and shTOPK#2 cells compared with shMock cell (Figure 4A). The level of STAT3 Y705 phosphorylation dramatically decreased to 0.35 and 0.11 in KYSE510 shTOPK#1 and shTOPK#2 cells respectively. Src Y419 phosphorylation decreased to 0.71 and 0.65, GSK3αβ kinase S21/S9 phosphorylation decreased to 0.65 and 0.66, and ERK1/2 T202/Y204 phosphorylation decreased to 0.81 and 0.79 in each of the two shTOPK cell lines (Fig 4A). These data increased that TOPK might be a crossroad of different signal pathways and play a complicated role in the ECSS
progression. To further verify human phospho-kinase array data, the same cell lysates was tested for western blot analysis. The results revealed the phosphorylation level of STAT3 Y705, GSK3β S21, Src Y419 and ERK1/2 T202/Y204 were strongly inhibited after knocking down TOPK in KYSE510 and KYSE30 cells (Fig. 4B), which are consistent with the array data. To further confirm the effects of TOPK on these pathways, KYSE510 and KYSE30 cells were treated with different doses of HI-TOPK-032 for 24h. Western blotting results indicated p-TOPK, STAT3, Src/GSK3β and ERK signal pathway were inhibited in a dose-dependent manner (Fig4 C). In summary, our data supports that TOPK may promote the invasion and migration of ESCC cells by moderating more signal pathways which included Src/GSK3β/STAT3 and ERK signaling pathways. This indicated that TOPK may be as a crosstalk center of signal pathways in ESCC metastasis.

**TOPK interacts with multi-proteins and binding with γ-Catenin to increased invasion and migration of ECSS cell lines**

To better understand the mechanism of TOPK in ESCC, we conducted surface plasmon resonance (SPR) and MS to screen TOPK target proteins. We chose the proteins which have a score above 25 in MS analysis. There are 53 and 39 proteins in TOPK kinase group and protein group respectively (Fig 5A). TOPK kinase group only and TOPK protein alone included 39 and 25 proteins respectively, 14 proteins were in the both groups. Among the 14 both proteins, Plakophilin–1 with score 81.95, Dermcidin with score 65.91 and EEF1A protein with 41.6 were screening by the relationship with cell migration and invasion (Fig 5B). What is more, γ-Catenin was the highest score 184.85 among the migration related proteins (Fig 6A). Molecular modeling was also performed in order to understand the detailed binding interactions between TOPK and γ-Catenin (Fig 6B). To verify whether or not TOPK can bind with γ-Catenin, we transfected pcDNA3-HA-TOPK and/or pDEST-myc-γ-Catenin into HEK293 cell for 72h and did immunoprecipitation with anti-HA antibody. γ-Catenin was found to interact with TOPK (Fig 6C). Immunofluorescence analysis indicated that knocking down TOPK would induce the expression of γ-Catenin in KYSE510 and KYSE30 cells (Fig 6D). Even more, HI-TOPK-032 increased the
expression of γ-Catenin with dose dependent in KYSE30 cells (Fig 6E). Collectively, these data demonstrated that TOPK maybe interaction with some proteins including γ-Catenin, Plakophilin-1, Dermcidin and EEF1A to promote the migration and invasion of ESCC cells. 

**TOPK inhibitor HI-TOPK–032 suppresses ESCC lung metastasis in a KYSE510 cell xenograft mouse model**

Next, we set out to detect the effect of TOPK in ESCC metastasis in vivo. Results of an athymic nude xenograft mouse model showed that HI-TOPK–032 apparently suppressed KYSE510 cell lung metastasis with no weight changes (Fig 4A-C). Western blot results indicated that HI-TOPK–032 inhibits the expression of p-TOPK and the activation of Src, GSK3β and ERK signal pathway. Taken together, knocking down TOPK and HI-TOPK–032 exerts an inhibitory effect on KYSE510 xenograft lung metastasis by suppressing Src, GSK3β and ERK signal transduction pathway in vivo.

**Discussion**

Metastasis which is a complex, multi-stage process is the most lethal hallmark of ESCC (14). Tumor metastasis involves enhanced cell motility, transited in lymphatic and blood vessels, and finally the spread from the primary tumor to surrounding tissues and to distant organs. Clinically, tumor metastasis is, by and large, incurable and is the primary cause of cancer morbidity and mortality. Over 50% of ESCC-associated deaths are caused by distal metastasis which is associated with poor prognosis and to the lymph nodes, lung, liver, bones, adrenal glands, and brain(15). Therefore, identification of critical regulators and signaling pathways that drive invasion and metastasis in ESCC will facilitate development of new treatment strategies.

TOPK, firstly discovered in lymphokine-activated killer T cells, is a branch of MEK1/2 and MKK7 from phylogenetic tree(16, 17). More reports has revealed that a significant relationship exists between TOPK expression and poor prognosis in numerous cancers, for instance, leukemia (18), breast cancer (19, 20), ovarian cancer (21), cervical cancer (22), lung cancer (23), colon cancer (24) and oral cancer (9) and so on. What’s more, high expression of TOPK has been linked to tumor aggressiveness, invasion and metastatic spread(25). Indeed, recent reports indicate TOPK has garnered interest as a cancer-specific biomarker and biochemical target with the potential to enhance cancer therapy whilst
causing minimal harm to normal tissues (26). However, to date, TOPK function in ESCC metastasis have not been systematically reported and is needed to clarified. In our report, TOPK expression was higher in N1-2 and N3 than N0 of ESCC tissues. Knocking down TOPK inhibits the migration and invasion of ESCC cells and TOPK inhibitor HI-TOPK-032 which targets p-TOPK apparently inhibits ESCC cells’ motility in vitro and lung metastasis in vivo. These data indicated that TOPK overexpression was sincerely related with the ESCC patients’ metastasis in clinical and might be regarded as one of the most valuable biomarker in ESCC. Therefore, studies targeting the mechanism of TOPK as a therapeutic strategy in ESCC are worth conducting.

More and more data indicated that abnormal expression of TOPK in many cancers triggers excessive activation of a series of signaling pathways, such as the ERK1/2, p38, c-Jun, and NF (nuclear factor)-κB pathways (27). In our report, ERK signal pathway was activated by TOPK kinase, that was consistant with other reports. Our data also indicated that TOPK may be a center which moderates more signal pathways included Src, GSK3β and STAT3 signal pathway in the process of ESCC metastasis. Shegan Gao et al (28) reported higher GSK3β expression was associated with poorer differentiation, higher metastasis rates, and worse prognosis of ESCC. They also certified that STAT3 is a target of GSK3β by cancer phospho-antibody array and proved that higher GSK3β expression promotes ESCC progression through STAT3 in vitro and in vivo. These indicated GSK3-STAT3 signaling could be a potential therapeutic target for ESCC treatment. On the other hand, Src is overexpression and functionally relevant to the progression of human ESCC. These indicated that Src might be a useful molecular target for ESCC prognosis and treatment (29). What’s more, some researches have demonstrated that a Src-mediated Y216 GSK–3β phosphorylation and activation leading to prostate cancer cell motility, proliferation, micrometastasis and progression in prostate cancer (30). These indicated that GSK–3 activity can be modulated by Src kinases and Src-GSK–3 pathway may be using for prostate cancer therapy by screening pharmacological inhibitors. Here, we found that down-regulated TOPK could decreased the phospho level of Src, GSK3β, STAT3 and ERK proteins in ESCC. We also first reported that Src/GSK3β/STAT3 signal pathway was moderated by TOPK and was involved in the invasion process of ESCC in vitro and vivo. These means TOPK may be a vital signal pathway center to
promote the progression of ESCC. Therefore, TOPK as therapeutic target may have unexcepted effections by inhibiting more signal pathways in ESCC.

To certify the direct target of TOPK kinase in the progression of ESCC, we screened TOPK binding proteins by performing SPR and MS between the TOPK protein and TOPK kinase group in KYSE510 cell. We found that more proteins might bind with TOPK kinase, for instance, Plakophilin-1, Dermcidin, EEF1A protein. Among these binding proteins, γ-catenin has the highest score of MS results. Therefore, we focused on the relationship between TOPK and γ-catenin. It is well known that γ-catenin (also known as junction plakoglobin) is a desmosomal protein and may play a central role in desmosome organization and is required for effective intermediate filament anchorage to desmosomes. γ-catenin was involved in cell invasion and metastasis of human oral squamous cell cancer, lung cancer, and bladder cancer (31). Some researches reported γ-catenin is a tumor suppressor in ESCC and γ-catenin expression levels had an impact on the survival curve. These mean that low γ-catenin is associated with lymph node metastasis and may serve as a prognostic marker in human ESCC (32). This is responded with that TOPK expression increasing induced the lymph node metastasis from N0 to N3. Our results reported that knocking down TOPK and HI-TOPK-032 can rescue the expression of γ-catenin in ESCC cells. Even more, TOPK binds with γ-catenin directly, this resulted in the dissociation of desmoplakin, which is an important prerequisite for cell movement during wound healing. Therefore, these data mean that down regulating TOPK kinase may increase and stabilize γ-catenin to decrease the cell adhesion and motility.

In addition, similar to its homolog β-catenin, γ-catenin is also involved in several cell signaling cascades during the processes of cell motility, cell proliferation, and apoptosis. The biology of the desmosome-like junction a versatile anchoring junction and signal transducer in the seminiferous epithelium(33). All these processes involve the interaction of cytoplasmic γ-catenin with various kinases and the inhibition of Wnt–β-catenin signaling by nuclear γ-catenin. It is reported that γ-catenin but not Desmoplakin silencing caused activation of p38MAPK dependent keratin filament collapse and cell dissociation(34). However, the signaling mechanism(s) by which γ-catenin exerts its tumour suppressor activity in ESCC cells remains unclear. Recent data proved that γ-catenin knockout
cells exhibited an increase in activated Src and γ-catenin regulated cell motility through distinct Src and Rho GTPase-dependent pathways (35). Many desmosomal proteins such as γ-catenin and plakophilin are also found in the nucleus, even in nondesmosome-bearing cells where they appear to participate in the regulation of gene expression. Therefore, we have enough data to believe that decreased γ-catenin may be the main reason for activating Src signal pathways in ESCC metastasis. In a word, in our reports, there may be one model that TOPK promotes the metastasis of ESCC cells (Fig8): TOPK binds with γ-catenin, then deregulated γ-catenin actives the Src/GSK3β/STAT3 signal pathway, then increased the cell invasion and movability to increase the ESCC metastasis. In the same time, TOPK kinase also active ERK signal pathway to cooperate with above. Interestingly, TOPK inhibitor HI-TOPK–032 can inhibit the ESCC cell metastasis by above signal pathway. However, more researches targeting the detailed mechanism that TOPK promotes ESCC metastasis were needed in the future.

Conclusions
TOPK was sincerely related with ESCC metastasis and was regarded as one of the most valuable molecular therapeutic target for ESCC in clinic. It is proved that TOPK promotes metastasis of ESCC cells by activating Src/GSK3β/STAT3 signal pathway though γ-catenin. It may be the novel mechanism of ESCC invasion.

Declarations
Ethics approval and consent to participate
The study was approved and supervised by the research ethics committee of Zhengzhou University, Zhengzhou, China. All animal experiments were performed in accordance with the animal experimental guidelines of Zhengzhou University.

Consent for publication
Not applicable.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
YJ, JZ, JZ, ZL and XC conducted experiments; HC and YQ did molecular modeling, KL ZD and YJ designed the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations
ESCC: Esophageal squamous cell carcinomas; TOPK: T-LAK cell-originated protein kinase; SPR: surface plasmon resonance; MS: mass spectrometric; IHC: Immunohistochemistry staining; SEM: mean ± standard error; STAT3: Signal transducer and activator of transcription 3

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Figures
TOPK expression positively correlates with tumor metastasis of ESCC patients. a The IHC staining of TOPK in ESCC with different lymph node. Left: TOPK expression in ESCC N0 tissues (n=10), Middle: TOPK expression in ESCC N1-2 tissues (n=19), Right: TOPK expression in ESCC N3 tissues (n=18). Scale bar: 200μm (upper) and 50μm (down). b IHC analysis of TOPK expression in N0, N1-2 and N3 ESCC tissues. c Expression of TOPK in different ESCC cell lines by western blot. d Relative expression of TOPK in different ESCC cell lines compared with TE1 cell line. **P<0.01, ***P<0.001 indicates a significant compared with N0 group.
Knocking down TOPK attenuates migration and invasion of ESCC cells. a and b Knocking
down TOPK expression in KYSE510 (a) and KYSE30 cell (b) by lentiviral infections. c and d
Cell migration was observed for 0, 24 and 48h in the stable TOPK knockdown of KYSE510
shTOPK (c) and KYSE30 shTOPK (d) by wound healing migration assay respectively. e and f
KYSE510 (e) and KYSE30 (f) cell invasion was measured by transwell migration assay after
knocking down TOPK respectively. ***P<0.001 indicates a significant compared with shMock
group.
TOPK inhibitor HI-TOPK-032 attenuates migration and invasion of ESCC cells a and b.

KYSE510 cell (a) was treated with HI-TOPK-032 0, 0.5, 1, 5 and 10 μM and KYSE30 cell (b) was treated at 0, 0.25, 0.5, 1 and 1.5 μM is for 0, 24, 48h by wound healing migration assay.
and d After KYSE510 cell (c) and KYSE30 cell (d) was treated with different concentrate HI-TOPK-032, they were seed on the upper lay of tranwell house. Then the transferred cell number was counted in transwell migration assay.

Figure 4

Src/GSK3β/STAT3 and ERK signaling pathways are strongly inhibited by knocking down TOPK and HI-TOPK-032 a KYSE510 shMock, shTOPK#1 and shTOPK#2 cells were harvested and TOPK level was determined by western blot analysis. Protein extracts (500 μg) were used for phospho-MAPK array analysis. Array spots were visualized using an ECL kit (Fig A left). The
density of each duplicated array spot (Fig A right) was measured as described in Materials and Methods. The graph shows the fold change in phosphorylation of Src, GSK3β, STAT3, ERK compared with shMock (i.e., value of 1). Data are shown as an average of duplicate samples. b Cell lysates (30 μg) from KYSE510 and KYSE30 shMock, shTOPK#1 and shTOPK#2 cells were subjected to 10% SDS-PAGE. Protein bands were visualized by using a chemiluminescence detection kit after hybridization with a horseradish peroxidase–conjugated secondary antibody. c Western blot analysis of total and phosphate Src, GSK3β, STAT3, ERK and TOPK in KYSE510 and KYSE30 cells which were treated with different H1TOPK-032 for 24h.
Src/GSK3β/STAT3 and ERK signaling pathways are strongly inhibited by knocking down TOPK and HI-TOPK-032 a KYSE510 shMock, shTOPK#1 and shTOPK#2 cells were harvested and TOPK level was determined by western blot analysis. Protein extracts (500 μg) were used for phospho-MAPK array analysis. Array spots were visualized using an ECL kit (Fig A left). The density of each duplicated array spot (Fig A right) was measured as described in Materials and Methods. The graph shows the fold change in phosphorylation of Src, GSK3β, STAT3,
ERK compared with shMock (i.e., value of 1). Data are shown as an average of duplicate samples. b Cell lysates (30 μg) from KYSE510 and KYSE30 shMock, shTOPK#1 and shTOPK#2 cells were subjected to 10% SDS-PAGE. Protein bands were visualized by using a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody. c Western blot analysis of total and phosphate Src, GSK3β, STAT3, ERK and TOPK in KYSE510 and KYSE30 cells which were treated with different HI-TOPK-032 for 24h.
TOPK interacts with γ-catenin

- The secondary mass spectrogram of γ-catenin which binds with TOPK kinase
- Representative snapshot of TOPK-γ-catenin binding complex structure
derived from MD simulations. TOPK and γ-catenin are represented as cartoon, and colored in cyan and rainbow, respectively. c TOPK binds with γ-catenin in vitro. pcDNA3- HA-TOPK and pDEST-myc-YBX1 were co-transfected into HEK293 cells for 48h, immunoprecipitated with a HA antibody, and then probed with agarose HA, respectively. The expression of Myc was tested by Western blot. d Immunofluorescence analysis indicated that knocking down TOPK would induce over expression of γ-Catenin in KYSE510 and KYSE30 cells. e TOPK inhibitor HI-TOPK-032 increased the expression of γ-Catenin with dose dependent in KYSE30 cells
HI-TOPK-032 suppresses lung metastasis in KYSE510 cell xenograft model. a Representative photographs for lung metastasis in control and HI-TOPK-032 group are shown. b Treatment with HI-TOPK-032 significantly suppresses KYSE510 cell lung metastasis compared with the vehicle-treated group. The asterisks (***p < 0.001) indicate a significant decrease in GFP Radant efficiency compared to control. c HI-TOPK-032 has no effect on the body weight of mice. d Western blot analysis was used to determine the levels of γ-Catenin and the phosphorylation of TOPK, Src, GSK3β, and ERK in lung tumors treated with HI-TOPK-032 compared with control group.
Schematic model of TOPK promotes the metastasis of ESCC cells. TOPK binds with γ-catenin, then deregulated γ-catenin actives the Src/GSK3β/STAT3 signal pathway, and TOPK kinase also active ERK and Src/GSK3β/STAT3 signal pathway, then through both pathways increased the cell invasion and movability to increase the ESCC metastasis. Further, TOPK inhibitor HI-TOPK-032 which inhibits TOPK kinase and inhibits the actived Src/GSK3β/STAT3 and ERK signal pathway then leads to decrease the ESCC metastasis.

Supplementary Files
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