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RESEARCH

TROAP promotes esophageal squamous cell carcinoma progression via the PI3K/AKT pathway

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Abstract

Purpose Trophinin-associated protein (TROAP) plays a crucial role in various human cancers. However, its involvement in esophageal squamous cell carcinoma (ESCC) remains unclear. This study aimed to explore the clinical significance and biological function of TROAP in ESCC.

Methods The expression and clinical relevance of TROAP in ESCC were analyze using GEO and TCGA databases. TROAP expression in ESCC samples was further validated by qRT-PCR, western blotting, and immunohistochemistry. In vitro and in vivo experiments were performed to assess TROAP's role in ESCC progression. RNA-seq analysis followed by western blotting and pathway-specific activator were conducted to explore the underlying mechanism.

Results TROAP was found to be overexpressed in ESCC and was positively correlated with higher histological grade and advanced clinical stage. Overexpression of TROAP promoted the proliferation, migration, and invasion of ESCC cells in vitro, whereas knockdown of TROAP suppressed ESCC progression both in vitro and in vivo. Mechanistically, TROAP facilitated ESCC progression by activating PI3K/AKT signaling pathway.

Conclusion This study revealed that TROAP promotes ESCC progression via activating PI3K/AKT pathway, suggesting that TROAP might be a promising therapeutic target for ESCC.

Keywords TROAP · Esophageal squamous cell carcinoma · Malignant phenotype · PI3K/AKT

Introduction

Esophageal cancer ranks as the seventh most prevalent malignancy and the sixth leading cause of cancer-related mortality worldwide (Sung et al. 2021), with over half of the global esophageal squamous cell carcinoma (ESCC) cases occurring in China (Han et al. 2024). Despite recent advances in therapeutic approaches such as chemo-radiotherapy and immunotherapy, their clinical efficacy remains limited by drug resistance and adverse effects (Yang et al. 2020). Therefore, it is an urgent need to gain comprehensive understanding of ESCC pathogenesis and to identify novel therapeutic targets for ESCC.

Trophinin-associated protein (TROAP), also known as tastin, is a critical component for the function of trophinin as a cell adhesion molecule (Suzuki et al. 1998).

Beyond its role in cell adhesion, TROAP also contributes to cell proliferation by regulating key mitotic processes, such as bipolar spindle assembly and centrosome integrity—both of which are essential for accurate cell division

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(Yang et al. 2008). Recent studies reported that TROAP plays an important role in several solid tumors, including lung cancer, gastric cancer and colorectal cancer (Jing et al. 2018; Xu et al. 2023; Ye and Lv 2018). For instance, TROAP is significantly upregulated in gastric cancer and has been shown to promote cell proliferation, migration and invasion (Jing et al. 2018). Similarly, elevated TROAP expression closely correlates with poor prognosis in lung cancer (Xu et al. 2023). Nevertheless, the clinical relevance and biological function of TROAP in ESCC have not been clarified yet.

In this study, we combined bioinformatics analyses with experimental validation, and found that TROAP is significantly upregulated in ESCC tissues compared to adjacent normal tissues. Subsequent in vivo and in vitro functional assays revealed that TROAP promotes the progression of ESCC. Furthermore, RNA sequencing and additional experiments indicated that the oncogenic role of TROAP is mediated through activation of the PI3K/AKT signaling pathway. These findings suggest TROAP may serve as a potential therapeutic target for ESCC.

Materials and methods

Data collection

Clinical data and gene expression profiles of patients with ESCC were obtained from the GEO database (GSE53625; $n = 179$). Expression data were normalized using the normalizeBetweenArrays function. Patients were divided into TROAP-high group and TROAP-low group based on median TROAP expression level. Differential gene expression analysis between the two groups was performed using the "limma" package(Ritchie et al. 2015), with a significance threshold of $p < 0.05$ to identify differentially expressed genes (DEGs). Detailed bioinformatics procedures are described in the Supplementary Methods. Additionally, ESCC patients with complete clinical annotations ($n = 85$) were retrieved from TCGA for further analysis of the association between TROAP expression and clinical parameters.

Tumor tissue and cell lines

The study protocol was approved by medical ethics committee of Shanghai Ruijin Hospital. Eight pairs of primary ESCC tissues and matched adjacent normal tissues were obtained from patients undergoing surgical resection at the hospital between March and July 2023. All patients provided written informed consent prior to surgery and had not received chemotherapy or radiotherapy before tissue collection. Tissue samples were snap-frozen and stored at −80 °C until use.

The human normal esophageal epithelial cell line Het-1 A and ESCC cell lines KYSE450 and KYSE150 were obtained from the National Collection of Authenticated Cell Culture in the Chinese Academy of Sciences (Shanghai, China). The cells were identified and characterized as previously described(Jian et al. 2022). Cells were maintained in DMEM (Procell, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, and cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

RNA extraction and qRTPCR assays

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized through reverse transcription using ABScript Neo RT Master Mix for qPCR with gDNA Remover (ABclonal, Wuhan, China). The qPCR was conducted with SYBR Green Master Mix (YEASEN, Shanghai, China) to detect the expression levels of TROAP. Relative mRNA expression was quantified using $2^{-\Delta\Delta CT}$ method, with GAPDH serving as the internal control. The primer sequences are provided in Table S1.

Immunohistochemistry (IHC)

Paraffin-embedded tissue Sect. (5 μm) were dehydrated through a graded ethanol series and underwent antigen retrieval. Sections were incubated overnight at 4 °C with rabbit anti-TROAP antibody (1:150; Proteintech, Wuhan, China). Conjugated antibodies were detected by adding biotinylated anti-rabbit secondary antibodies for 1 h at room temperature. DAB (diaminobenzidine) was used as the chromogen, followed by counterstaining with hematoxylin. Slides were rinsed, dried, and sealed.

Lentivirus infection and knockdown-rescue in ESCC cells

Short hairpin RNAs (shRNAs) targeting the TROAP (Table S2) were cloned in to pLKO.1 vectors. The coding sequence of TROAP was cloned into the PCDH lentiviral expression vector for rescue or overexpression experiments. For lentivirus production, 293T cells were co-transfected with pLKO.1, pLKO.1-shRNA, PCDH, or PCDH-TROAP together with the packaging plasmids psPAX2 and pMD2.G. After 48 h, viral supernatants were collected, filtered through a 0.45 μm filter, and used to infect KYSE450 and KYSE150

cells in the presence of 10 μg/mL polybrene. Following 48 h of infection, cells were cultured in fresh medium and selected with 5 μg/mL puromycin for 7 days. For rescue experiments, TROAP-knockdown cells were transfected with the PCDH-TROAP plasmid for 48 h. TROAP expression levels were confirmed by western blotting.

Western blotting (WB)

Whole cell proteins were extracted from ESCC cells lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with phosphorylase and protease inhibitors. Protein samples were loaded onto SDS-PAGE gels, separated by electrophoresis, and electro-transferred onto PVDF membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% skim milk powder dissolved in TBST solution for 2 h at room temperature. After washing with TBST, membranes were incubated with specific primary antibodies overnight at 4 °C. Following additional TBST washes, the membranes were incubated with secondary antibodies for 1 h, and the signals were detected using enhanced chemiluminescence assays. The antibodies and reagents used are listed in Table S3.

Cell counting Kit-8 (CCK-8) assay

KYSE450 and KYSE150 cells were seeded in 96-well plates at a density of 4×10^3 cells per well. At 0, 24, 48 and 72 h, 10 μL of Cell Counting Kit-8 (CCK-8; CYTOCH, Shanghai, China) solution was added to each well and incubated for 1 h at 37 °C. Absorbance at 450 nm was measured using a microplate reader (Tecan, Switzerland) to assess cell viability and proliferation.

Colony formation assay

KYSE450 and KYSE150 cells were seeded in 6-well plates at a density of 1,000 cells per well and cultured in DMEM supplemented with 10% FBS. The medium was refreshed every three days. After 14 days, colonies were fixed with 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet (Beyotime, Shanghai, China) for 30 min. Wells were washed three times with Phosphate Buffered saline (PBS), air-dried, and photographed.

Cellular migration and invasion assays

Cell migration and invasion assays were performed using transwell chambers (Corning, USA). For the migration assay, 5×10^4 cells suspended in 150 μL of serum-free DMEM were seeded into the upper chamber, and 600 μL of DMEM containing 20% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells that had migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. For cell invasion, 1×10^5 cells suspended in serum-free DMEM were seeded into the upper chamber pre-coated with Matrigel (BD Bioscience, USA). After 48 h, invaded cells were fixed and stained as described above. Stained cells were subsequently destained with 33% acetic acid, and the absorbance of the eluate was measured at 570 nm using a microplate reader. Absorbance values were used as an indirect measure of cell migration and invasion (Chen et al. 2023).

Cell cycle analysis

Cell cycle analysis was performed using the cell cycle and apoptosis analysis kit (Beyotime, Shanghai, China). Cells were washed twice with cold PBS and fixed in 70% ethanol at −20 °C overnight. After fixation, cells were washed with cold PBS and stained with 500 μL of propidium iodide (PI) staining solution containing 200 μg/mL RNase A and 50 μg/mL PI for 30 min at 37 °C. Flow cytometric analysis was performed using CytoFLEX flow cytometer (Beckman Coulter, Inc.).

RNA-seq

Total RNA was extracted from KYSE450 cells (NC and ShTROAP groups) using the Universal RNA Extraction CZ Kit (RNC643, ONREW) according to the manufacturer's instructions. RNA quantity was analyzed using Qubit 4.0 (Invitrogen), and its quality was assessed by denaturing agarose gel electrophoresis. RNA libraries were prepared using the VVAHTS® Universal V8 RNA-seq Library Prep Kit for Illumina (NR605-0, Vazyme) and sequenced using the Illumina NovaSeq 6000 platform with a 150 bp paired-end sequencing strategy. Enrichment of mRNA, library construction, sequencing, and data analysis were performed by Shanghai Xu Ran Biotechnology Co., Ltd (http://www.xuran gene.com). DEGs were identified using DESeq2 with a threshold of $|\log_2 \text{fold change}| > 0.5$ and $p < 0.05$. The GO and KEGG analyses are described in the Supplementary Methods.

Xenografted tumor model

Six male BALB/c nude mice (4–5 weeks old) were purchased from GemPharmatech Co., Ltd. (Jiangsu, China) and housed under specific pathogen-free (SPF) conditions at 23 ± 3 °C with 60–75% relative humidity. Mice were anaesthetized via intraperitoneal injection of 50 μL pentobarbital sodium

(20 mg/kg), KYSE150 NC cells (1×10^7) or shTROAP cells (1×10^7) were subcutaneously injected into the right flank. Tumour dimensions were measured every 4 days using a vernier caliper, and tumour volume was calculated using the formula: (length × width²)/2. At the study endpoint (28 days post-injection), mice were euthanized by intraperitoneal injection of 150 µL pentobarbital sodium (20 mg/kg). The mouse's heart and breathing stopped, the pupils dilated, and the tumors were excised and weighed. All experimental protocols involving animals were approved by the Animal Care Committee of Shanghai Ruijin Hospital.

Statistical analysis

Statistical analyses were performed using R software (version 4.3.1) and GraphPad Prism 8. Data are presented as mean ± standard deviation. Differences between continuous variables were assessed using Student's t-test, one-way analysis of variance (ANOVA), or the Wilcoxon rank-sum test, as appropriate. Pan-cancer expression levels of TROAP were obtained from the TCGA database via GEPIA2 (http://gepia2.cancer-pku.cn/) (Tang et al. 2017). Disease Ontology analysis was conducted using Coexpedia(http://www.coexpedia.org/) (Yang et al. 2017). Overall survival (OS) curves were generated using the Kaplan–Meier method and compared using the log-rank test (Li et al. 2021b). $p < 0.05$ was considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: no significance).

Results

Bioinformatic analysis reveals upregulation of TROAP and its potential oncogenic role in ESCC

First, we analyzed TROAP expression pattern across various tumor types using TCGA dataset. Compared to adjacent normal tissues, TROAP was upregulated in multiple human cancers (Fig. S1A). Disease Ontology analysis revealed that dysregulation of TROAP is implicated in carcinogenesis, particularly in squamous cell carcinoma (Fig. S1B). To specifically assess TROAP expression in ESCC, we analyzed transcriptomic data from 179 ESCC patients in the GSE53625 dataset. As shown in Fig. S1C, TROAP expression was markedly elevated in ESCC tissues compared to normal controls. Notably, high TROAP expression positively correlated with higher histological grade and advanced clinical stage in ESCC patients (Fig. S1D-E). Consistently, Kapla–Meier survival analysis showed that patients with high TROAP expression tended to have shorter overall survival than those with low TROAP expression, although the difference was not statistically significance (Fig. S1F). These findings suggests that TROAP might serve as a potential prognostic biomarker in ESCC.

To further investigate whether TROAP exerts biological functions in ESCC, we performed GSEA analysis. As shown in Fig. S1G, high TROAP expression was associated with pathways involved in DNA replication and cell cycle. In consistent, GSVA analysis (Fig. S1H) also showed high TROAP expression correlated with upregulation of proliferative pathways (DNA replication and cell cycle), and downregulation of adhesion-related pathways (gap junction, focal adhesion, adherens junction, and tight junction). These data suggest that aberrant expression of TROAP might contribute to ESCC tumorigenesis by modulating cell proliferation, invasion, and migration.

TROAP might be associated with tumor immune microenvironment

Given the critical role of the tumor immune microenvironment (TME) in cancer progression, we investigated the relationship between TROAP expression and the TME in ESCC. As shown in Fig.S2A, TROAP-high group exhibited lower stromal and estimate scores, along with increased tumor purity, implying a potentially immunosuppressed microenvironment (Tu et al. 2023). CIBERSORT analysis further revealed reduced infiltration of $CD4^+$ memory T cells, mast cells, and eosinophils in TROAP-high group (Fig.S2B). Consistently, single-sample gene set enrichment analysis (ssGSEA) also demonstrated reduced immune cell infiltration in TROAP-high group (Fig.S2C).

Meanwhile, we examined the expression of immune checkpoint molecules between TROAP-high and TROAP-low group. Among these immune checkpoint molecules, the expression of ICOSLG was decreased in TROAP-high group, whereas BTNL9, CD86, and CTLA4 were upregulated (Fig.S2D). We further analyzed the expression of TROAP and immune checkpoint genes (BTNL9, CD86, ICOSLG, and CTLA4) in our ESCC tissue samples. As shown in Fig.S2E, the mRNA levels of BTNL9 and CTLA4 appeared to be higher in TROAP-high group ($n = 4$) than TROAP-low group ($n = 4$), but the differences were not statistically significant. Collectively, these findings indicate a potential relationship between TROAP expression and altered immune status in ESCC. Further studies with large sample size are warranted to clarify the relationship.

TROAP is upregulated in ESCC tissues and cell lines

Initial bioinformatic analysis suggested upregulation of TROAP in ESCC. To further validate this finding, we examined TROAP expression in paired ESCC tumor tissues and adjacent normal tissues obtained from patients at

our hospital. As shown in Fig. 1A-B, RT-qPCR and WB analysis revealed markedly higher TROAP mRNA and protein levels in ESCC tissues compared to adjacent normal tissues. Immunohistochemical staining further confirmed the elevated TROAP expression in ESCC tissues (Fig. 1C). Besides, we also analyzed TROAP expression in human normal esophageal epithelial cell line Het-1A and two ESCC cell lines (KYSE450 and KYSE150). Consistent with the findings in ESCC tissues, the mRNA and protein levels of TROAP were higher in ESCC cell lines than normal esophageal epithelial cell line (Fig. 1D-E). These data indicate that TROAP is upregulated in ESCC tissues and cell lines.

Overexpression of TROAP promotes ESCC cells proliferation, invasion, and migration

Next, we explore the functional role of TROAP in ESCC. Given our previous observation of TROAP upregulation in ESCC, we conducted gain-of-function experiments through the establishment of stable TROAP-overexpressing ESCC cell lines (KYSE450 and KYSE150). As shown in Fig. 2A, CCK-8 assays demonstrated that TROAP overexpression significantly enhanced cell viability in both KYSE450 and KYSE150 cells. Subsequently, colony formation assays revealed TROAP-overexpressing cells formed more colonies than their respective control groups (Fig. 2B). Furthermore, flow cytometry showed that TROAP overexpression reduced G1 phase arrest, characterized by a decreased proportion of cells in G1 phase and a concomitant increase in S phase (Fig. 2C), suggesting that TROAP promotes cell cycle progression and proliferation in ESCC cells. In addition, transwell experiments revealed that TROAP overexpression markedly enhanced both migration and invasion capabilities of ESCC cells (Fig. 2D-E). Collectively, these findings indicate that TROAP promote ESCC cells proliferation, invasion, and migration in vitro.

TROAP knockdown suppresses ESCC cells proliferation, invasion, and migration

Since TROAP is overexpressed in ESCC and promotes tumor progression, we wonder whether TROAP knockdown could modulate ESCC progression. To this end, we established stable TROAP-knockdown ESCC cell lines (KYSE450 and KYSE150). In contrast to TROAP overexpression, knockdown of TROAP significantly suppressed ESCC cell proliferation, as demonstrated by CCK-8, colony formation and flow cytometry assays (Fig. 3A-C). Meanwhile, transwell assays revealed that TROAP knockdown markedly attenuated the migratory and invasive abilities of ESCC cells (Fig. 3D-E). Collectively, both loss-of-function and gain-of-function experiments reveal the oncogenic role of TROAP in ESCC progression.

TROAP facilitated ESCC progression via activating PI3K/AKT pathway

To elucidate the molecular mechanisms underlying the oncogenic role of TROAP in ESCC, we performed RNA-seq on stable TROAP-knockdown KYSE450 cells (shTROAP) and corresponding control cells (shNC). As shown in Fig. 4A, TROAP mRNA levels were markedly reduced in the shTROAP group compared to shNC group. Using a threshold of$|\log2 \text{ fold change}| > 0.5$ and $p < 0.05$, we identified a total of 1,405 DEGs (Fig. 4B). GO analysis revealed that these DEGs were enriched in biological processes critical to cancer progression, such as cell adhesion, epithelial cell proliferation, and extracellular matrix organization (Fig. 4C). Additionally, KEGG pathway enrichment analysis identified the PI3K/AKT signaling pathway as the most significantly enriched pathway (Fig. 4D). Based on these findings, we examined the PI3K/AKT pathway. Consistently, TROAP knockdown markedly reduced the phosphorylation levels of PI3K and AKT (Fig. 4E), whereas TROAP overexpression increased their phosphorylation (Fig. 4F).

To further confirm whether the inhibition of PI3K-AKT pathway and suppression of ESCC progression were specifically due to TROAP knockdown, we performed rescue experiments by re-expressing TROAP in the knockdown cells. As expected, restoration of TROAP expression reversed the inhibitory effects on PI3K-AKT signaling, as well as cell invasion and migration (Fig. 5A-C). To definitively determine whether the PI3K/AKT pathway mediates TROAP's oncogenic function, we treatment TROAP-knockdown ESCC cells with PI3K/AKT pathway activator recilisib (20 μM). As shown in Fig. 5D, recilisib successfully restored AKT phosphorylation. Moreover, recilisib treatment significantly restored the migration and invasion capabilities of TROAP-knockdown ESCC cells compared with control cells (Fig. 5E-F). Taken together, these findings suggest that TROAP promotes ESCC progression by activating the PI3K/AKT pathway.

Knockdown of TROAP suppresses ESCC growth in vivo

To further investigate the impact of TROAP on ESCC growth in vivo, we established a xenograft model using nude mice. KYSE150 cells with stable TROAP knockdown were subcutaneously injected into nude mice. As shown in Fig. 6A-C, compared with the shNC group, shTROAP group exhibited smaller tumor volumes, as well as lighter

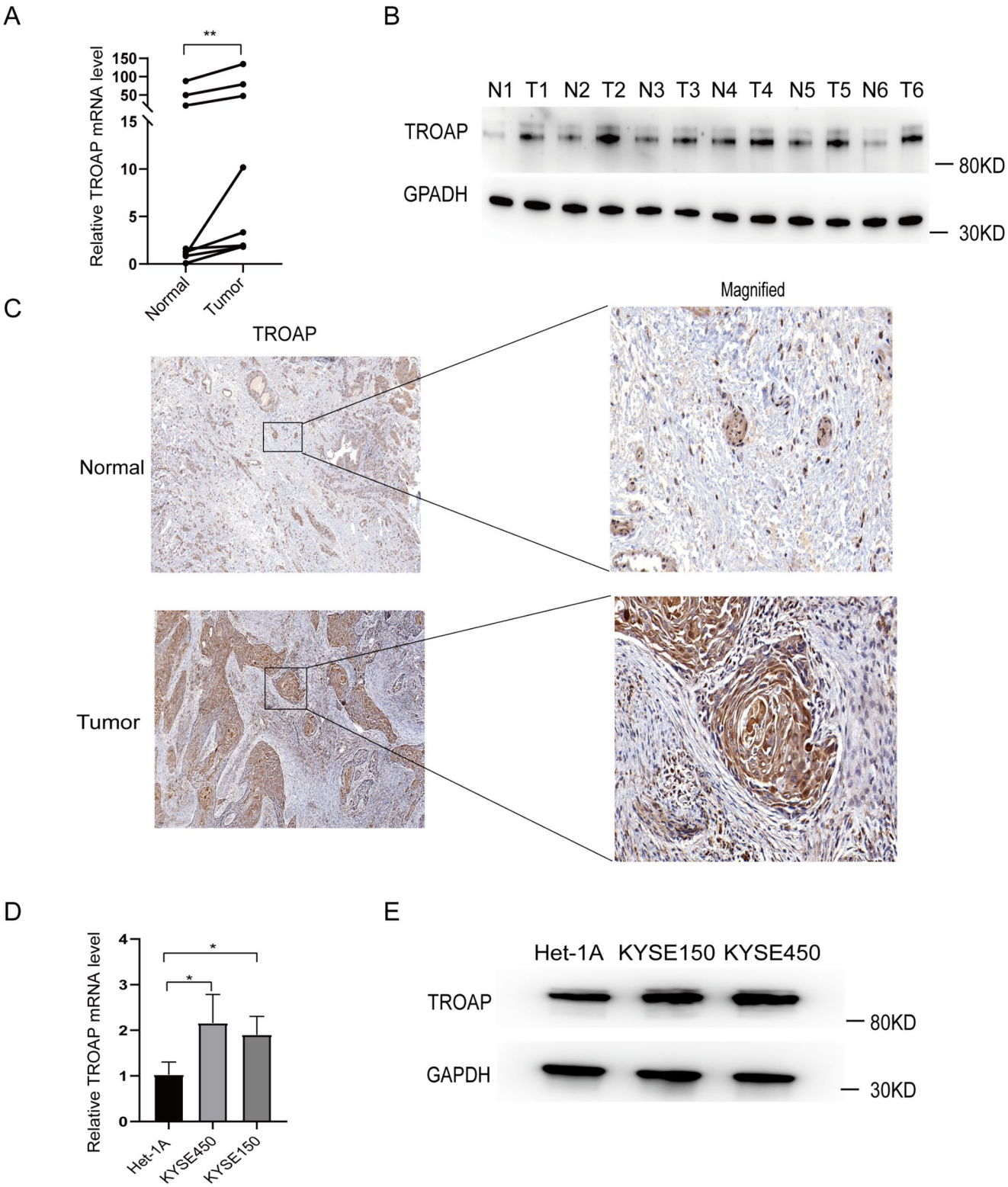

Fig. 1 TROAP expression is upregulated in ESCC. **(A)** Relative TROAP mRNA levels in ESCC tissues and adjacent normal tissues. **(B)** TROAP protein levels in paired ESCC and adjacent normal tissues. **(C)** Relative TROAP protein expression level in human ESCC tissues by IHC. **(D-E)** The mRNA and protein levels of TROAP in ESCC cell lines and normal esophageal epithelial cell line. *$p<0.05$, **$p<0.01$

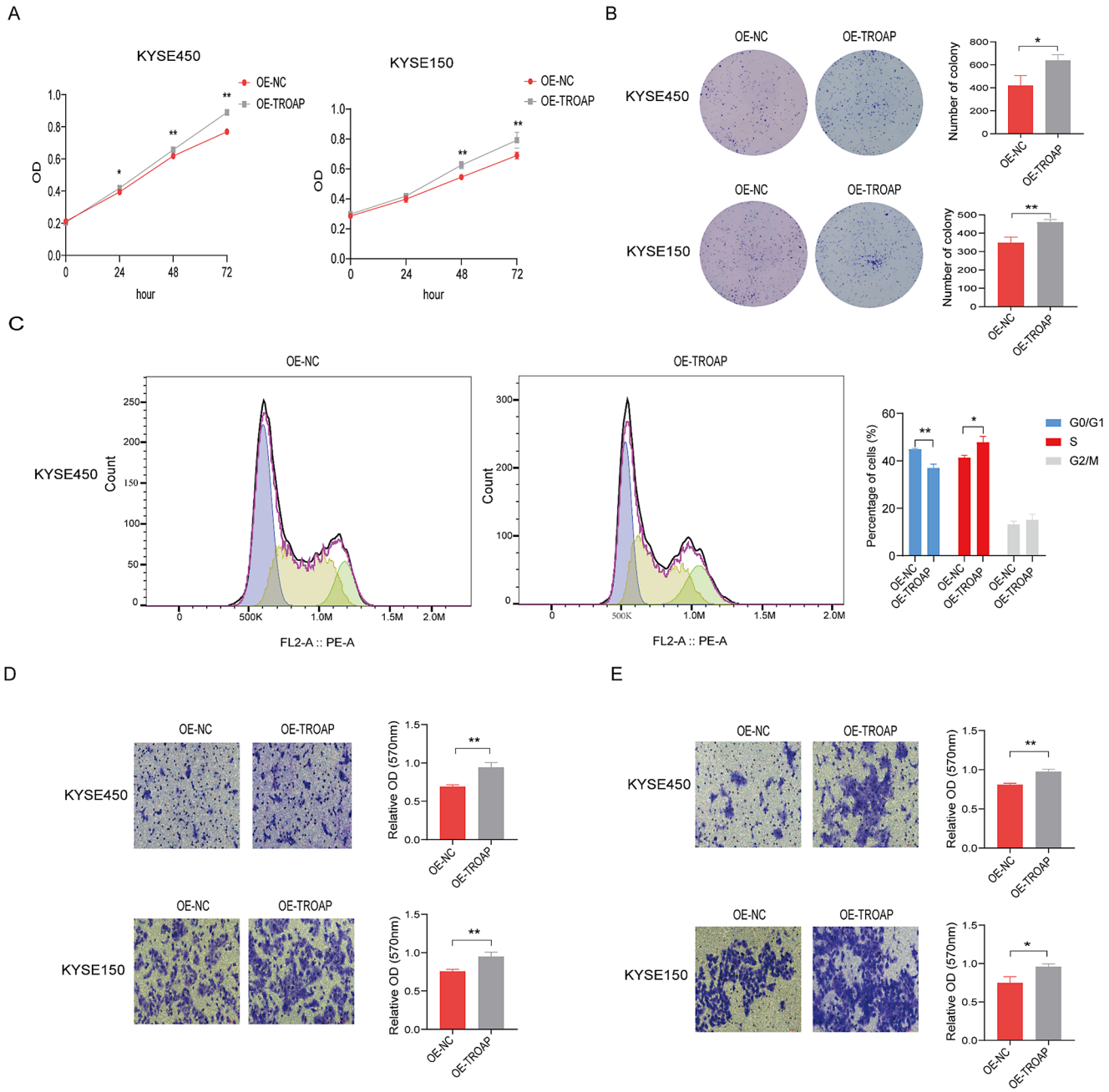

Fig. 2 TROAP overexpression (OE-TROAP) promotes the proliferation, invasion, and migration of ESCC cells. **(A)** The effect of OE-TROAP on the proliferation of ESCC cells. **(B)** The effect of OE-TROAP on the colony formation of ESCC cells. **(C)** PI-FACS detected the effect of OE-TROAP on the ESCC cell cycle. **(D)** The effect of OE-TROAP on the migration of ESCC cells. **(E)** The effect of OE-TROAP on the invasion of ESCC cells. *$p<0.05$, **$p<0.01$

tumor weights. These results indicate that TROAP knockdown effectively suppresses ESCC tumor growth in vivo. Taken together, the in vitro and in vivo experiments consistently support that TROAP promotes the progression of ESCC via PI3K/AKT pathway (Fig. 6D).

Discussion

TROAP is a soluble cytoplasmic protein essential for the function of trophinin as a cell adhesion molecule (Suzuki et al. 1998). It exhibits highly cooperative binding with trophinin and bystin at the interface between the endometrium and trophoblasts (Fukuda et al. 1995). In addition, TROAP is required for bipolar spindle assembly and maintenance

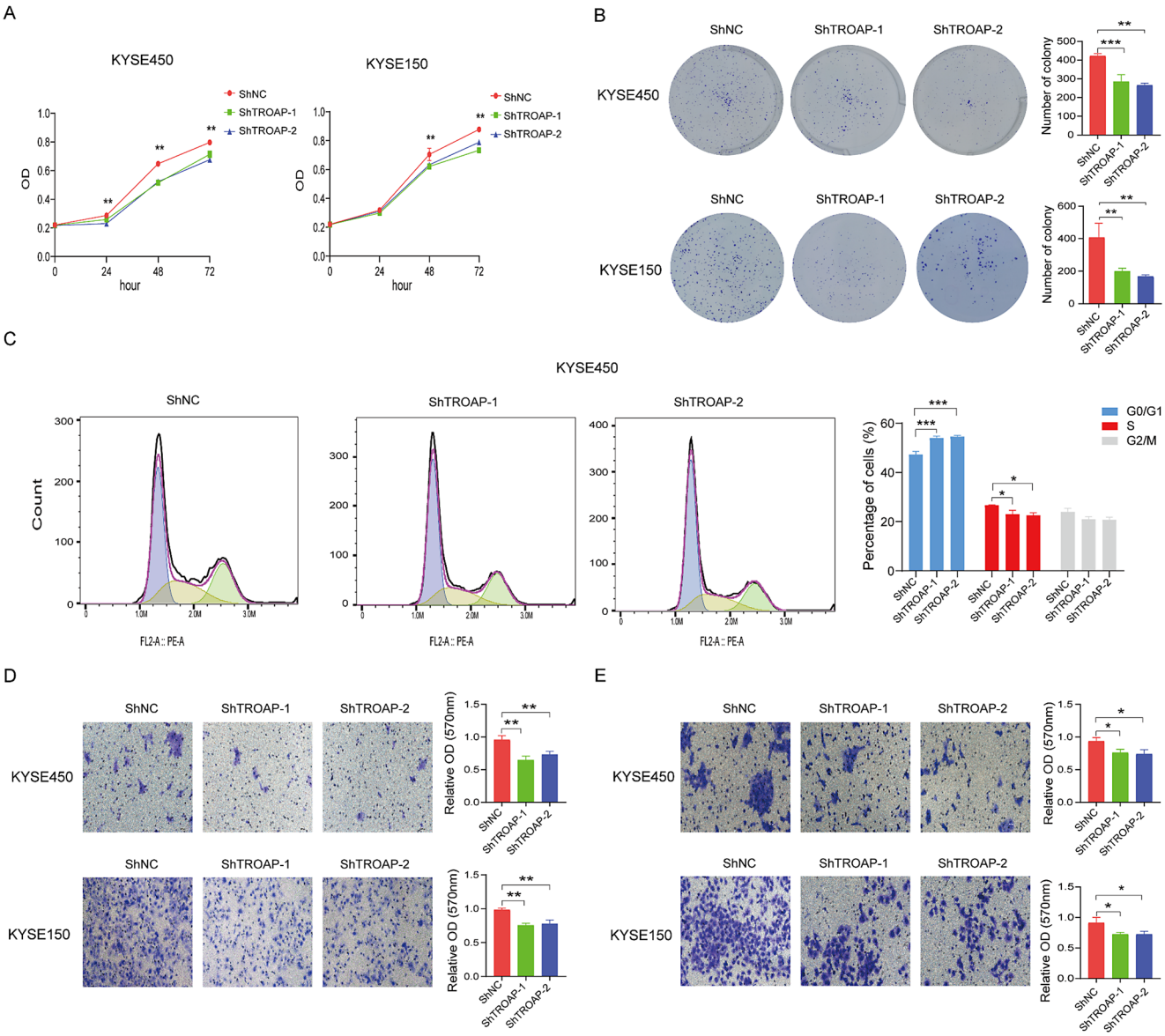

Fig. 3 TROAP knockdown suppresses ESCC cells proliferation, invasion, and migration. **(A)** The effect of shTROAP on the proliferation of ESCC cells. **(B)** The effect of shTROAP on the colony formation of ESCC cells. **(C)** PI-FACS detected the effect of shTROAP on the ESCC cell cycle. **(D)** The effect of shTROAP on the migration of ESCC cells. **(E)** The effect of shTROAP on the invasion of ESCC cells. $*p<0.05$, $**p<0.01$, $***p<0.001$

of centrosome integrity during mitosis, processes that are critical for cell proliferation(Yang et al. 2008). The rapid cellular proliferation and invasion suggest that the associated adhesion binding complex contributes to early embryo implantation.

In this study, both mRNA and protein levels of TROAP were significantly upregulated in ESCC tissues. Bioinformatic analyses revealed that high TROAP expression was positively associated with higher histological grade (G stage) and advanced clinical stage. GSEA and GSVA further indicated that high TROAP expression may be linked to enhanced proliferative, invasive, and migratory capacities in ESCC. Tumor-infiltrating immune cells are widely recognized as independent indicators of immune status and patient prognosis (Tu et al. 2023). Across multiple cancer types, TROAP expression has been negatively correlated with immune, stromal, and ESTIMATE scores within the TME(Li et al. 2022). To further explore this relationship in ESCC, we examined the association between TROAP expression and immune cell infiltration. High TROAP expression was associated with reduced immune cell infiltration, decreased stromal content, and increased tumor purity. Next, we investigated the relationship between TROAP expression and immune checkpoint

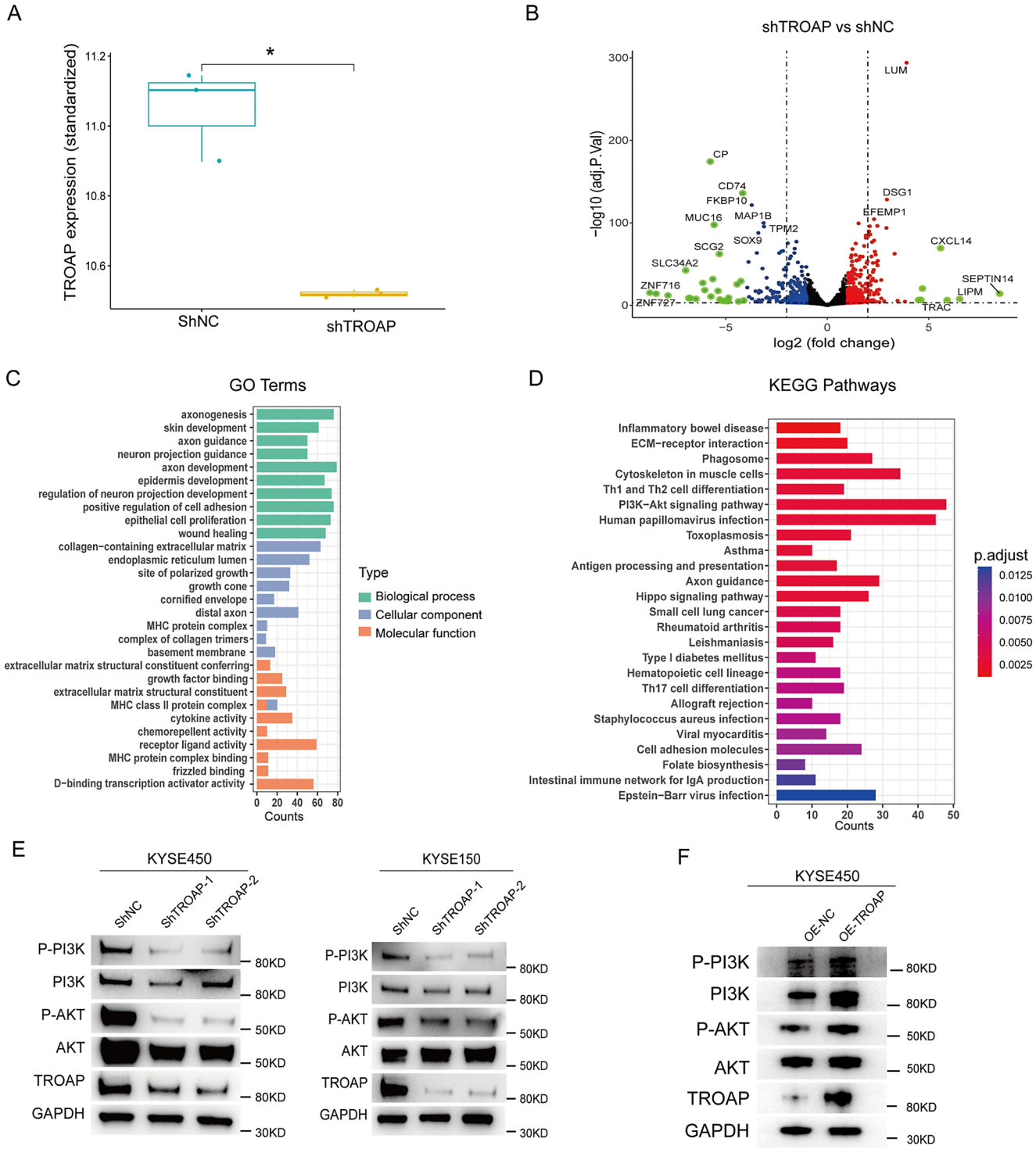

Fig. 4 TROAP regulates PI3K/AKT signaling pathway. **(A)** RNA-Seq analysis of TROAP expression in NC and shTROAP groups. **(B)** Volcano map of DEGs in RNA-Seq. **(C)** GO enrichment analysis of DEGs (shTROAP vs. NC). **(D)** KEGG pathway enrichment analysis of DEGs (shTROAP vs. NC). **(E)** WB analysis of TROAP, AKT, P-AKT, PI3K, P-PI3K in ESCC cells following TROAP knockdown. **(F)** WB analysis of TROAP, AKT, P-AKT, PI3K, P-PI3K in ESCC cells following TROAP overexpression. *$p<0.05$

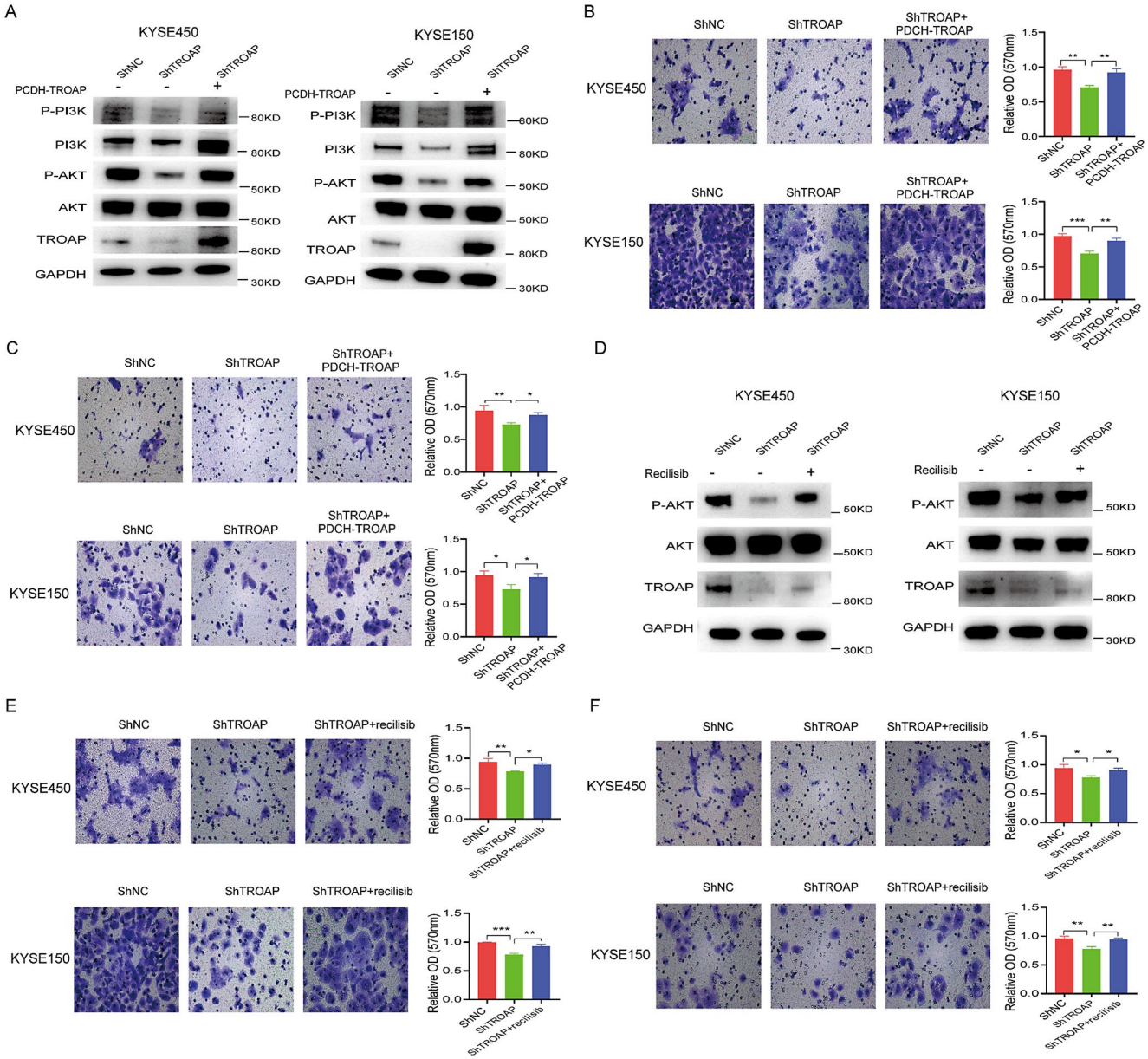

Fig. 5 Rescue experiments in TROAP-knockdown cells. **(A)** Protein expression levels of TROAP, AKT, P-AKT, PI3K, P-PI3K in TROAP-knockdown cells after PCDH-TROAP plasmid transfection; **(B)** The effect on the migration of TROAP-knockdown cells after PCDH-TROAP plasmid transfection. **(C)** The effect on the invasion of TROAP-knockdown cells after PCDH-TROAP plasmid transfection. **(D)** Protein expression levels of TROAP, AKT, P-AKT in TROAP-knockdown cells after recilisib stimulation. **(E)** The effect on the migration of TROAP-knockdown cells after recilisib stimulation. **(F)** The effect on the invasion of TROAP-knockdown cells after recilisib stimulation. *$p<0.05$, **$p<0.01$, ***$p<0.001$

molecules. Several immune checkpoints (BTNL9, CD86, and CTLA4) were up- regulated in TROAP-high group. qRT–PCR analysis confirmed the mRNA levels of BTNL9 and CTLA4 appeared to be higher in TROAP-high group than TROAP-low group, but the differences were not statistically significant. Collectively, these findings indicate a potential relationship between TROAP expression and altered immune status in ESCC. Further studies with large sample size are warranted to clarify the relationship.

Cytoskeletal reorganization is a fundamental mechanism underlying cell motility. TROAP has been identified as a microtubule-associated protein that maintains centrosome dynamics and structural integrity(Yang et al. 2008). Interactions between microtubules and the cytoskeleton contribute to cellular invasion and migration—processes that are critical for tumorigenesis and the acquisition of malignant phenotypes(Akhshi et al. 2014). Therefore, we investigated the impact of TROAP on the biological behavior of ESCC

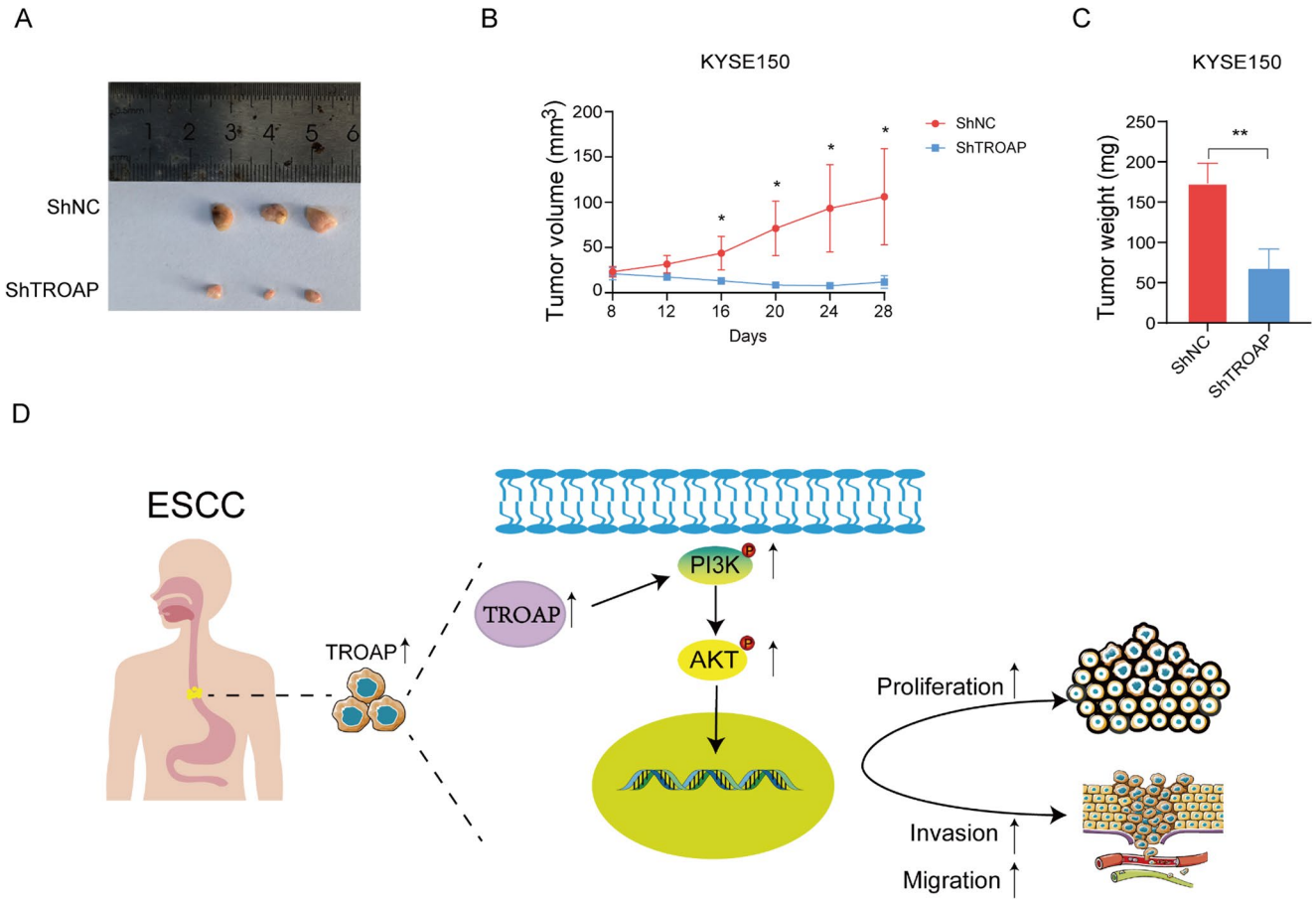

Fig. 6 TROAP knockdown inhibited tumor growth in BALB/c nude mice. **(A)** Images of xenograft-bearing mice 28 days after subcutaneous injection with control or TROAP-knockdown ESCC cells. **(B-C)** Tumor growth was significantly inhibited after TROAP knockdown, as assessed by tumor volume and tumor weight. **(D)** Schematic model illustrating that TROAP is upregulated in ESCC tissues and promotes progression via activation of the PI3K–AKT signaling pathway. *$p<0.05$, **$p<0.01$

cells in our study. Functional assays showed that TROAP knockdown can significantly suppressed cell proliferation, migration, and invasion in ESCC cell lines, whereas TROAP overexpression enhanced them. These findings indicate that TROAP may contribute to the malignant potential of ESCC cells.

Abnormal growth is an important requirement for the tumorigenesis and was closely associated with dysregulation of cell cycle(Qi et al. 2023). TROAP acts as a cycling protein essential for mitosis, with its endogenous expression tightly controlled during cell cycle progression. In this study, TROAP knockdown significantly reduced the proliferation of ESCC cell line by impairing the G1/S phase transition, whereas TROAP overexpression accelerated cell growth by promoting G1/S progression—findings consistent with previous reports(Jing et al. 2018; Li et al. 2021a). Moreover, TROAP knockdown markedly suppressed tumor growth in a mouse xenograft model, further supporting its role in ESCC progression.

Several studies have investigated the mechanisms by which TROAP promotes tumor progression. Ye et al. reported that TROAP regulates prostate cancer progression via the WNT3/survivin signaling pathway(Ye et al. 2019). Zhao et al. demonstrated that TROAP promotes glioma progression by modulating the cell cycle through the Wnt/β-catenin pathway(Zhao et al. 2021). Similarly, Li et al. showed that TROAP enhances hepatocellular carcinoma (HCC) progression by activating the Akt/GSK-3β pathway via modulation of DYRK1 activity(Li et al. 2021a). To explore the mechanism by which TROAP contributes to ESCC progression, we performed RNA-seq analysis following TROAP knockdown. GO analysis revealed that DEGs were primarily involved in cell proliferation, adhesion, and migration. KEGG pathway enrichment analysis further identified the PI3K/Akt signaling pathway as the most significantly enriched pathway. Given the well-established role of the PI3K/Akt pathway in tumor development and progression (He et al. 2021), we next investigated its relationship with TROAP. WB analysis showed that TROAP

knockdown led to decreased phosphorylation levels of PI3K and AKT, whereas TROAP overexpression resulted in pathway activation. These findings suggest that TROAP may promote ESCC progression through activation of the PI3K–Akt signaling pathway.

To further investigate the relationship between TROAP and the PI3K/AKT signaling pathway, rescue experiments were performed in TROAP- knockdown ESCC cell lines. WB showed that the reduced phosphorylation levels of PI3K and AKT induced by TROAP knockdown were restored following transient transfection with PCDH-TROAP plasmid. Similarly, treatment with the PI3K/AKT pathway activator recilisib also rescued AKT phosphorylation levels. These results indicate that TROAP modulates the PI3K/AKT signaling pathway in ESCC. Furthermore, transwell assays demonstrated that the impaired migratory and invasive abilities observed in TROAP-knockdown cells were restored following PCDH-TROAP transfection or recilisib treatment. These results suggest that TROAP promotes tumor progression in ESCC by regulating the PI3K/AKT pathway.

Previous studies reported that TROAP was upregulated in various solid malignancies, such as gastric cancer(Jing et al. 2018), colorectal cancer(Ye and Lv 2018), lung cancer(Xu et al. 2023), breast cancer(Wang et al. 2023), prostate cancer(Ye et al. 2019), and endometrial cancer(Wang et al. 2024). In these cancers, TROAP has been reported to play an oncogenic role and is frequently associated with poor prognosis. However, the role of TROAP in HCC is controversial. Li et al. found that TROAP was upregulated in HCC and drove the growth of different HCC cell lines (Huh7, HepG2, Hep3B and PLC8024) (Li et al. 2021a). But Lian et al. observed that TROAP was downregulated in hepatitis B virus (HBV) infection-related HCC, and TROAP knockdown promotes the proliferation and migration of HCC cell lines (SMMC-7721 and QGY-7703)(Lian et al. 2018). The inconsistency to the previous research may be attributed to the diverse origins of patients and the different cell lines used in these studies. In the present study, we found TROAP was upregulated in ESCC. Furthermore, overexpression of TROAP enhance the proliferation and invasive capabilities of ESCC cell lines. These findings suggest an oncogenic role of TROAP in ESCC.

The study also has certain limitations. Although bioinformatic analysis indicated a correlation between TROAP and immune cell infiltration, as well as immune checkpoint-related gene expression, there is currently no experimental evidence directly supporting the involvement of TROAP in immune cell infiltration. The specific relationship between TROAP expression and various immune cell subtypes remains to be elucidated in future studies. Moreover, preliminary findings indicate possible associations between TROAP and immune checkpoint gene expression, however no significant differences were observed, likely due to the small ESCC sample size. Future studies with larger cohorts are required to clarify this relationship.

In brief, our study showed that TROAP mediates proliferation, invasion, and migration in ESCC cells, at least in part, through the activation of the PI3K/AKT signaling pathway. This research is the first to confirm the role of TROAP and its underlying mechanisms in ESCC. Overall, these findings indicate a potential target for the treatment of ESCC.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate This study was performed in line with the principles of the Declaration of Helsinki. The present study was approved by Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. All patients signed informed consents before surgery. Animal studies were approved by the Animal Care Committee of Shanghai Ruijin Hospital affiliated Shanghai Jiao Tong University School of Medicine.

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