Trop2 as an oncogene in gastric cancer by regulating the PI3K/Akt signaling pathway

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ABSTRACT

High Trop2 expression relates to aggressive tumor behavior and contributes to poor overall survival rates in gastric cancer (GC) patients. However, little is known about the molecular mechanism of Trop2 in the carcinogenesis of GC. We found that over-expressed Trop2 induced cell proliferation and clone formation, inhibited cell apoptosis and induced S cell cycle arrest in GC cell lines, meanwhile, knockdown Trop2 inhibited cell proliferation and clone formation, induced cell apoptosis and inhibits S cell cycle arrest in vitro. Moreover, Trop2 depletion inhibited tumor growth, the anti-tumor rate in this report being 22.53% in vivo. In addition, Trop2 activated the PI3K/Akt signaling pathway to promote GC malignant progression. These results indicated that Trop2 is a critical regulation factor in the progression of GC, which may help to lead a novel insight into understanding the mechanism of the Trop2 in the pathogenesis of GC.

Keywords: Trop2, gastric cancer, signaling pathway

INTRODUCTION

Gastric cancer (GC) is the fourth common cancer and the second leading cancer–related cause of death worldwide[1–3]. Despite GC declining in incidence in Western countries, gastric carcinoma is a major cause of death in numerous parts of the world, especially in East Asia[4]. Fifty-eight percent of global GC deaths occur in China, Korea, and Japan[5]. Although the early detection rate of GC has increased, there are still many patients suffering from distant metastasis. Thus, it is important to identify oncogenes that promote gastric cancer metastasis. These oncogenes may not only serve as biomarkers for gastric cancer progression, but also as molecular targets for gastric cancer therapy.

The human oncogene, trophoblast cell surface glycoprotein (TACSTD2/Trop2) has been given particular attention in our lab since 2009. Trop2, located at chromosome 1q32, was first identified on the surface of human trophoblast cells[6–8]. Our early experimental results indicated highly expressed Trop2 in many solid tumors, moreover its expression related to aggressive tumor behavior[9–11]. Subsequently we prepared several anti–Trop2 antibodies, including human anti–Trop2 antibody IgG, fragment Fab and so on, these antibodies have been found able to bind to specific Trop2 antigen and inhibit the proliferation, migration and invasion of the tumor cells in vivo and in vitro.

The phosphoinositide 3–kinase (PI3K) signal–
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...ing pathway represents the most important signaling pathway relating to cell proliferation, apoptosis, metabolism and is often activated in GC. PI3K catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by phosphorylating phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP2). Growth factors and hormones trigger this phosphorylation event, which in turn coordinates cell growth, cell cycle entry, cell migration, and cell survival.

PI3K is a lipid kinase containing two subunits, catalytic (p110) and regulatory (p85). Akt is activated by phospholipid binding and activation loop phosphorylation within the carboxyl terminus at Ser473 by PDK1. Akt promotes cell survival by inhibiting apoptosis, and moreover phosphorylating and inactivating several targets, including c-Raf. PTEN phosphatase is a major negative regulator of the PI3K/Akt signaling pathway.

In the present study, we aimed to investigate the expression and role of Trop2 in vivo and in vitro and further clarify the role of Trop2 through activating the PI3K/Akt signaling pathway in GC cell lines.

MATERIALS AND METHODS

Cell culture, reagents and transfections

Seven human GC cell lines (MKN45, MKN28, MGC803, SGC7901, HGC27 and BGC823) and a normal human gastric epithelial cell lines (GES–1) were purchased from KeyGEN BioTECH (Nanjing, China). Cells were cultured at 37°C in a 5% CO₂ humidified atmosphere in RPMI–1640 containing 10% fetal bovine serum (FBS). Trop2 were listed in Supplementary Table S1. Transfection into GC cells was carried out using Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. The efficiency of overexpression or Rnock-down were listed in Supplementary Figure S1–S2.

RNA extraction, reverse transcription, and quantitative PCR

Cell RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) in accordance with the manufacturer’s instructions. The primers of Trop2 (All-in-One qPCR Primer) were purchased from GeneCo–poeia (Guangzhou, China), GAPDH (internal control) were purchased from Genscript (Nanjing, China). qRT–PCR was performed on an ABIPRISM 7500HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in 96–well plates. Relative expression levels were calculated as ratios normalized against those of GAPDH. Results were normalized to respective internal controls. The CΔCt-value for each sample was calculated using the ∆ΔCt method, and results were expressed as 2^−ΔΔCt.

Western blot analysis

Cells were lysed using standard methods. Cellular proteins were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with TBS containing 0.1% Triton X–100 and 5% nonfat milk powder for 2 hours at room temperature (RT), then were incubated with anti–Trop2 (R&D Systems, Minneapolis, USA), anti–PTEN(CST, USA), anti–PI3K(CST, USA), anti–Akt(CST, USA), anti–p–Akt(CST, USA), anti–p–c–Raf(CST, USA), anti–GAPDH (CST, USA) antibodies at 4°C overnight. After being washed, the membranes were incubated with HRP–conjugated anti–IgG at RT for 1 hour. Signal detection was carried out with an ECL system (Tanon, Shanghai, China).

CCK–8 assay

Cells at a number of 5 × 10^3 per well were seeded in the 96–well plate and incubated. Cell growth viability was measured with a Cell Counting Kit–8 (Beyotime, Shanghai, China) following the manufacturer’s instructions. Absorbance was then recorded at 450 nm using Elx800 Reader (BioTECH Instruments Inc., Winooski, VT, USA).

 Colony formation assay

The transfected GC cell lines were placed in a fresh 6–well plate and maintained in containing 10% FBS. After 24 hours, the medium was replaced with new medium containing G418 (300 mg/mL) or puro (10 μg/mL). After 14 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Cell cycle and cell apoptosis analyzed by flow cytometry

the trypsinized cells (1 × 10^6) were fixed in 70% ethanol at −20 ℃ for 24 h. The fixed cells were then washed with PBS, and incubated with RNaseA (0.25 mg/mL) for 30 min at 37 ℃, and 5 μL of propidium iodide (PI, KeyGen, Nanjing, China) was then added to the cell suspension; Cells were washed with PBS and incubated with 5 μL Annexin V–APC–7AAD and 10 μL PI for 5 min at 37 ℃. The mixture was incubated at RT for 30 min in the dark. The suspended
cells were analyzed for cell cycle using the FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

**Animal experiments**

Animal experiments were performed with the approval of the Institutional Committee for Animal Research and in conformity with national guidelines for the care and use of laboratory animals. ShRNA–5, shRNA–NC transfected BGC823 cells (1 × 10⁶ cells in 100 μL) were injected subcutaneously into the flanks of each 4-week-old immunodeficiency nude mouse. BCG823–shRNA–NC were injected subcutaneously into the left flank and BCG823–shRNA–Trop2 were injected subcutaneously into the right flank. Tumor growth was evaluated weekly for at least 5 weeks. Then the mice were sacrificed, necropsies were performed, and tumors were evaluated. Tumor volumes were calculated by the following formula: \( V = \frac{\pi A B^2}{6} \), where \( A \) is the largest diameter, and \( B \) is the perpendicular diameter. The tumor tissues were used to perform immuno–staining analysis of Trop2 protein expression.

**Statistical analysis**

The SPSS18.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for general statistical analysis. The differences between groups were estimated by Values of \( P \) less than 0.05 were considered statistically significant.

**RESULTS**

**Over-expression of Trop2 induces cell proliferation and clone formation, inhibits cell apoptosis and induces S cell cycle arrest in GC cell lines**

The expression of Trop2 among 6 types of GC cell lines (MGC803, BGC823, MKN45, MKN28, HGC27, SGC7901) and a normal gastric epithelial cell line (GES–1) were analyzed by qRT–PCR (**Fig. 1A**). We found that Trop2 was relatively high–expressed in MKN45 and BGC823 and showed a relatively low–expression in MKN28 and MGC803. So these four types of GC cell line were used in the following studies.

First, we constructed a plasmid vector, which over–expressed Trop2, named OE–Trop2. The expression efficiency of Trop2 was 1.56±0.06 fold higher than the normal control (NC) and the cloning vector (VE–Trop2) in MKN28, and was 3.86±0.39 fold higher than NC and VE–Trop2 in MGC803 (**Supplementary Fig. S1**).

Then, CCK8 assay was used to assess the cell proliferation 24, 48 and 72 hours after OE–Trop2 plasmid transfection. Compared with NC and VE–Trop2, a significant increase of cell proliferation was detected in MKN28 at 72 h and in MGC803 at 48 and 72 h after being transfected with OE–Trop2. But no difference was observed between NC and VE–Trop2 group (**Fig. 1B**). Colony formation assay was performed to further examine the effect of OE–Trop2 on the proliferation of GC cell lines. As shown in **Fig. 1C**, the cell colony numbers of MKN28 and MGC803 transfected with OE–Trop2 were significantly higher than those transfected with VE–Trop2. Therefore, the results of colony formation assay, consistent with those of

**Fig. S1.** The OE–Trop2 plasmid transfection efficiency of gastric cancer cells were analyzed by fluorescent and qRT–PCR.
Fig. S2. The ShRNA–Trop2 plasmid transfection efficiency of gastric cancer cells were analyzed by fluorescent and qRT–PCR. (A1): BGC823 ShRNA–Trop2 by fluorescent; (A2): BGC823 ShRNA–Trop2 by qRT–PCR; (B1): MKN45 ShRNA–Trop2 by fluorescent; (B2): MKN45 ShRNA–Trop2 by qRT–PCR.

Fig. 1. Trop2 over-expression induces cell proliferation and clone formation, inhibits cell apoptosis and induces S cell cycle arrest in GC cell lines. A: the statistical results of Trop2 expression levels in different gastric cancer cell lines and a normal gastric epithelial cell line (GES-1) by qRT–PCR, and GAPDH was treated as internal control; B: the proliferation ratio of OE–Trop2 transfected MKN28 and MGC803 cells tested by CCK8 at 0, 24, 48, 72 h time point; C: colony formation of OE–Trop2 transfected MKN28 and MGC803 cells; D: the apoptosis cells ratio of OE–Trop2 transfected MKN28 and MGC803 cells analyzed by FACS; E: the cell cycle of OE–Trop2 transfected MKN28 and MGC803 cells analyzed by FACS.
CCK8 assay, suggest that Trop2 can induce the proliferation of GC cells.

Cell cycles of MKN28 and MGC803 transfected with OE-Trop2 or VE-Trop2 were examined using FACS. MKN28 and MGC803 transfected with OE-Trop2 had more cells (nearly 31.25% and 50.58%) arrested in S phase 72 h after transfection compared with VE-Trop2 transfected cells. These results suggested that the S cell cycle progression was induced after the over-expression of Trop2 (Fig. 1E).

All above indicated that Trop2 has oncogenic properties and OE-Trop2 could increase cell proliferation and clone formation, inhibit cell apoptosis and induce S cell cycle arrest in GC cell lines.

**Knockdown of Trop2 inhibits cell proliferation and clone formation, induces cell apoptosis and G1 cell cycle arrest in GC cell lines**

MKN45 and BGC823 were used in subsequent knockdown experiments for their high expression of Trop2. Different vectors (shRNA-5, -6, -7, -8) expressing short hairpin RNAs (shRNA) against Trop2 were tested. The most effective down-regulation of Trop2 was achieved with two sequences: shRNA-5 (65% in BGC823, 35% in MKN45), and shRNA-7 (67% in BGC823, 37% in MKN45) (Supplementary Fig. S2). As a control, cells were transduced with control scrambled shRNA vectors (shRNA-NC).

We examined the cell proliferation at 24, 48, and 72 hours after transfection of shRNA-Trop2 used CCK8 assay. Compared with the non-transfected group (NC) and control scrambled shRNA vectors-transfected group (shRNA-NC), a significant decrease of cell proliferation was detected in MKN45 at 48 h and BGC823 at 48 and 72 h after being transfected with shRNA-5 and shRNA-7 plasmid. Meanwhile, no difference was observed between the NC and shRNA-NC group (Fig. 2A).

Colony formation assay showed that the cell colony numbers of MKN45 and BGC823 transfected with shRNA-5 and shRNA-7 were significantly lower than those transfected with shRNA-NC (Fig. 2B). These results along with the results of CCK8 assay further indicated that down-regulation of Trop2 could inhibit the proliferation of GC cells in vitro.

We next testified the apoptosis cells ratio and the distribution of cell cycles using FACS. The apoptosis ratios of MKN45 transfected with shRNA-5 and shRNA-7 were obviously lower than those transfected with shRNA-NC, and the similar results were also observed in BGC823 (Fig. 2C). In comparison with shRNA-NC group, the shRNA-5 transfected MKN45 group showed cell cycle arrest in G1 phase 72 hours after transfection, characterized by the presence of nearly 68% of cells in the G1 phase, the presence of about 20% of cells in S phase. While in BGC823 cell lines, the shRNA-5 transfected group showed cell cycle arrest in G2/M phase 72 hours after transfection, characterized by the presence of nearly 20% of cells in the G2/M phase, the presence of about 30% of cells in S phase. The results indicated that the down-regulation of Trop2 inhibited S cell cycle arrest (Fig. 2D).

These results indicated that knockdown of Trop2 expression could inhibit cell proliferation and clone formation, induces cell apoptosis and G1 cell cycle arrest in GC cell lines.

**Trop2 depletion inhibits tumor growth in vivo**

Xenograft model was used to evaluate the effects of shRNA-Trop2 on the growth of the GC cells in vivo, in which the BGC823 cells treated with shRNA-5 or shRNA-NC (1 × 10⁶ cells/mouse) were subcutaneously injected into the flanks of immunodeficient nude mice for the developing of tumors. The mice were sacrificed after 5 weeks and the tumors were examined (Fig. 3B). There was no animal dead during the experiment. During the whole tumor growth process, tumors from the shRNA-5 transfected BGC823 cells grew slower than those of shRNA-NC transfected ones (Fig. 3C). After 5-week’s inocula-
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tion, the average weight of tumors developed from shRNA-5 transfected BGC823 cells [(204 ± 116) mg] was significantly smaller than those of control group [(321 ± 152) mg, \(P=0.014\)]. Immunostaining analysis of Trop2 protein was performed in resected tumor tissues. As shown in Fig. 3D, the expression level of Trop2 protein in tumors formed from shRNA-5 transfected BGC823 cells was significantly lower than those of the shRNA-NC group. These results suggest that down-regulated Trop2 expression could inhibit proliferation capacity of GC cells in vivo.

**Trop2 promotes GC malignant progression through PI3K/Akt signaling pathway**

Western blot analysis revealed that compared with the VE-Trop2 transfected groups, the expression levels of PI3K-p110 and p-Akt were markedly increased in the OE-Trop2 transfected MKN28 and MGC803 cells, while no difference was observed in Akt protein expression. Compared with the VE-Trop2 transfected groups, the protein expression levels of PTEN were markedly decreased in the OE-Trop2 transfected MKN28 and MGC803 cells. We found no significant difference on the expression levels of p-c-Raf between VE-Trop2 and OE-Trop2 transfected GC (MKN28 and MGC803) cells (Fig. 4). Next, we detected the activation of the PI3K/Akt signaling pathway after Trop2 was down-regulated by shRNA-5 in GC (MKN45 and BGC823) cells by Western blot, and the results were consistent with those of the OE-Trop2 in GC cells.
Thus, our present results indicate that Trop2 may facilitate GC malignant progression through the PI3K/Akt signaling pathway.

**DISCUSSION**

GC is the most prevalent digestive system malignancy in the world, and China has more GC patients than any other country\(^{[20]}\). Our previous study showed that, Trop2, as an oncogene, highly expressed in GC tissues. The transmembrane protein Trop2 is extensively expressed in most cancers, but not all, and also has a differential expression in certain normal tissues. It has been reported that Trop2 is highly expressed in the following solid tumor cancers: colorectal cancer\(^{[21]}\), breast cancer\(^{[22]}\), pancreatic cancer\(^{[23]}\), cervix cancer\(^{[24]}\), squamous cell carcinoma\(^{[25]}\), gastric cancer\(^{[20]}\), certain lung cancers\(^{[26]}\), prostate cancer\(^{[27]}\), bladder cancer\(^{[28]}\), breast cancer\(^{[9]}\). However, it was down-regulated in non-small lung cancer\(^{[29]}\). Trop2 is also up-regulated in several hematologic malignancies: leukemia, extranodal nasal type lymphoma (ENKTL), and non-Hodgkin’s lymphoma (NHL)\(^{[30]}\).

It has been reported that Trop2 mediates several signaling pathways\(^{[30]}\). High expression of Trop2 can activate MAPK signaling pathway, by increasing Ca\(^{2+}\)\(^{[31]}\), and decreasing AP-1 and p27. But interestingly, Vidmar T\(^{[32]}\) reports that high expression of Trop2 inhibits the IGF–1R signaling pathway, and then attenuates lung cancer proliferation and malignancy. Trop2 is also reported to be able to bind β–catenin to drive stem cell self-renewal\(^{[33]}\).

In the present study, we demonstrated that overexpression of Trop2 in GC cell lines (MKN28 and MGC803) could induce cell proliferation, and clone formation, inhibit cell apoptosis and induce S cycle arrest, and vice versa. These results are consistent with the findings of Lin H et al.\(^{[11]}\), who reported that Trop2 promotes the malignancy functions of ductal breast cancer cells. However, we also found two GC cell lines (MKN45 and MKN28) did not display similar results as for the other GC cell lines (BGC823 and MGC803) in the cell cycle distribution assays. This may partly due to the lower efficiency of plasmid transfection, although we improved several types of transfected ways. Furthermore, we found down-regulation of Trop2 inhibited xenografted tumor’s growth in vivo. This further confirmed the results in vitro.

In addition, we also found Trop2 facilitate GC malignant progression through the PI3K/Akt signaling pathway. Increasing evidences indicate that the PI3K/Akt signaling pathway plays a vital role in the carcinogenesis and development of the malignant tumor, and its different downstream target genes are able to crosstalk with other pathways, therefore displaying a wide range of biological functions such as cell growth, cell cycle, apoptosis, migration and invasion, and so on. Our study shows the PI3K/Akt signaling pathway is likely to play an important anticancer role in various tumors, such as pancreatic cancer, rhabdomyosarcoma,
gastric cancer and others\cite{14}. In this study, we elucidate a novel Trop2–PI3K–Akt signaling pathway regulatory network in GC cells, and we believe our findings provide a new point for understanding the mechanism of Trop2 in the process of GC. Collectively, these results highlight that Trop2 is a critical regulation factor in the progression of GC. Nevertheless, these studies are currently limited, as the down-streaming target genes of PI3K/Akt have not as yet been studied satisfactorily, and there is a lack of clinical evidence to support the results. In order to fully investigate, future studies are required to this aim.

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**References**


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**Fig. 4 Trop2 promotes GC malignant progression through PI3K/Akt signaling pathway.** The representative Western blot results for PI3K/Akt signaling pathway. The relative protein expression levels were obtained from three independent experiments, GAPDH was used as a control, mean ± SD. *P* < 0.01.
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