

GOLPH3 promotes cell proliferation and tumorigenicity in esophageal squamous cell carcinoma via mTOR and Wnt/ β -catenin signal activation

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Abstract. The authors' previous study demonstrated that Golgi phosphoprotein 3 (GOLPH3) was significantly overexpressed in esophageal squamous cell carcinoma (ESCC), correlating with poor patient survival. In the present study, GOLPH3 stable overexpression and knockdown KYSE-140 cell lines were constructed. Cell proliferation, colony formation, cell cycle progression and tumorigenesis assays were performed. The results revealed that GOLPH3 promoted ESCC cell growth and proliferation. The effects of GOLPH3 on the mechanistic target of rapamycin (mTOR) and Wnt/ β -catenin signaling pathways were investigated using western blot analysis and dual-luciferase reporter assays, and were observed to be activated in cells with GOLPH3 overexpression. Furthermore, overexpression of GOLPH3 resulted in the

downregulation of p21 protein, upregulation of cyclin D1 and increased retinoblastoma-associated protein phosphorylation, consequently leading to accelerated cell cycle progression. In addition, GOLPH3 knockdown resulted in reversed effects. The results of the current study suggest that GOLPH3 serves an important role in promoting tumorigenicity of ESCC via mTOR and Wnt/ β -catenin signaling pathway activation.

Introduction

Esophageal cancer is one of the most common cancers in the world, whose cases are most prevalent in developing countries. The North-Central part of China is often referred to as part of the 'esophageal cancer belt', which has the highest incidence of esophageal cancer in the world. And over 90% of the newly diagnosed cases were esophageal squamous cell carcinoma (ESCC) (1). Although recent development of screening technology contributed to the early detection of ESCC, the decrease of ESCC morbidity remains slight (2,3). Surgical resection is considered to be the main treatment of ESCC, followed with adjuvant chemotherapy and radiotherapy (4). Although targeted therapy of ESCC has been widely studied (5,6), clinical trials were mostly concentrated on relapse or metastasis cases until now, and only a few agents had entered phase III clinical trial stage. As the result, much more researches will be needed to reveal the mechanisms involved in ESCC tumorigenesis, in order to devise new schemes for therapy.

The Golgi phosphoprotein 3 (GOLPH3), also named GPP34/GMx33/MIDAS was discovered as a Golgi-associated protein (7) which was conserved from yeast to human (8). GOLPH3 binds to Golgi-localized glycosyltransferase, and contributes to their steady-state Golgi membrane localization (9,10). Furthermore, the main functions of GOLPH3 are also achieved via binding to phosphatidylinositol-4-phosphate [PtdIns(4)P] on the trans-Golgi membranes, which will simultaneously connect to myosin XVIII A (MYO18A) to provide Golgi a tensile force to form tubule and vesicle (11,12). In sum, GOLPH3 is involved in vesicle budding and retromer cargo

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; cDNA, complementary DNA; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; EDTA, ethylene diamine tetraacetic acid; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gelelectrophoresis; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PI, propidium iodide; pS6, ribosomal protein S6; shRNA, short hairpin RNA; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1

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transport (13-18); and all these procedure also contributes to DNA-damage repairing (19).

GOLPH3 was first considered as a potent oncogene by Scott *et al* (20). Further studies regarding the role of GOLPH3 in several cancers, such as rhabdomyosarcoma, tongue cancer, breast cancer, glioblastomamultiforme, gastric cancer and ovarian cancer (21-27), all illustrated that high expression of GOLPH3 indicated patients' poor prognosis. Our previous work also showed that GOLPH3 was overexpressed in ESCC cancer tissues, and correlated with ESCC patients' poor prognosis (28). In order to gain deeper insight into the role of GOLPH3 play in ESCC tumorigenesis, we conducted a series of gain- and loss-of-function analyses and preliminarily demonstrated the activation of mechanistic target of rapamycin (mTOR) and Wnt/ β -catenin signaling pathway by GOLPH3 overexpression.

Materials and methods

This research was approved by the Ethics Committee of Guangdong Science and Technology Department. All animal work had been conducted according to relevant national and international guidelines. ESCC cell suspension was injected subcutaneously into the left flank of 4-week old female BALB/c-nu mice. Mice were sacrificed 30 days after injection by cervical dislocation method.

Cell lines and culture. ESCC cell line KYSE-140, constructed KYSE-140 cell lines and the human kidney cell line HEK-293T were cultured under 37°C with 5% CO₂ and 95% humidity, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (both from Life Technologies, Carlsbad, CA, USA).

A GOLPH3 stable knockdown KYSE-140 cell line, labeled KYSE-140-GOLPH3-RNAi, was constructed by infection of retrovirus expressing shRNA targeted GOLPH3. The negative control cell line was constructed with scramble sequence and labeled KYSE-140-scramble. The viruses expressing shRNAs were packaged with pSuper.retro.puro backbone plasmids.

A GOLPH3 stable overexpressed KYSE-140 cell line was constructed with retrovirus expressing GOLPH3 cDNA sequence, and an empty vector packaged retrovirus was used to construct negative control, which were labeled KYSE-140-GOLPH3 and KYSE-140-vector, respectively. The viruses expressing cDNA and control were packaged with pBaBe.retro.puro backbone plasmids.

KYSE-140-GOLPH3-RNAi, KYSE-140-scramble, KYSE-140-GOLPH3 and KYSE-140-vector cell lines were selected with puromycin (EMD Millipore, Billerica, MA, USA) and maintained in 10% FBS DMEM with 0.5 μ g/ml puromycin.

Quantitative real-time PCR (qPCR). Cultured cells were digested with 0.25% trypsin with EDTA (Life Technologies) and washed with PBS twice. Total RNA of cells was extracted with TRIzol reagent (Life Technologies). Total RNA (2 μ g) was used as the template of reverse transcription in 2.0 μ l system, M-MLV reverse transcriptase (Promega, Madison, WI, USA) was applied following the manufacturers' instruction. qPCR was carried out by GoTaq qPCR Master Mix (Promega).

Western blot analysis. Total protein from cells or tissues was extracted with RIPA lysis buffer. Protein samples were treated with protein loading buffer, with reducing agent maintained at 100°C for 5 min after protein quantification with bicinchoninic acid (BCA). Protein samples were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. Subsequently, Protein samples were incubated with primary antibody for over 12 h at 4°C, and HRP-conjugated second antibody for 1 h at room temperature. Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was exposed to Kodak X-OMAT BT XBT-1 Film (Kodak, Rochester, NY, USA).

Cell proliferation assay. Growth curve was drawn based on the cell viability tested by MTT assay. Cells were digested and washed, and suspended to 0.5x10⁴/ml with 10% FBS DMEM. 200 μ l per well cell suspension was seeded to 96-well plate. Cell viability of each group of cells was analyzed by MTT assay, and the OD values of the first day were used as standards; cell viabilities were measured at the following 4 days.

Cell cycle assay. Cells were synchronized by 24 h serum starvation and then cultured with 10% FBS DMEM for 20 h. Cells were digested with trypsin without EDTA (Life Technologies) and washed twice, and then were fixed with 75% ethanol at 20°C for 1 h, suspended with PBS. Prior to flow cytometry analysis, cells were treated with RNase A at 37°C for 30 min and stained with propidium iodide (PI) on ice for 30 min.

Plate colony formation assay. Cultured cells were digested and washed with PBS once. After suspension with 10% FBS DMEM to 0.5x10³ cells/ml, 2 ml cell suspension per well was added to 6-well plate and cultured for 10 days. Cells were fixed with 75% ethanol at room temperature for 1 hr and stained with crystal violet. Cell colonies larger than 50 cells were counted.

Dual-luciferase reporter assay. The Dual-Luciferase® Reporter Assay system (Promega) was used to perform the TOP-Flash and FOP-Flash assays. The TOP-Luciferase vector and FOP-Luciferase vector were separately transfected cultured cells which were co-transfected with a pRL-TK control vector. 48 hr after transfection, the activity of firefly luciferase and *Renilla* luciferase were measured following manufacturers' instructions. The ratio of firefly and *Renilla* luciferase activity was recorded as relative luciferase activity; the relative luciferase activity of the TOP-Luciferase vector transfected cell was standardized with data FOP-luciferase vector transfected cells.

Tumorigenesis. Cultured cells were digested and washed, cells were suspended in 2.5x10⁶/ml with PBS, and 200 μ l of cell suspension were injected subcutaneously into the left flank of 4-week old female BALB/c-nu mice. Tumor volumes were measured every 3 to 4 days and calculated via following: Volume = (length x width²)/2. Mice were sacrificed after 30 days, tumor tissues were excised and photographed. Tumor tissues were preserved in liquid nitrogen.

Statistic analysis. All experiments were triplicated, and data shown as mean \pm SE. Statistical analyses were performed with

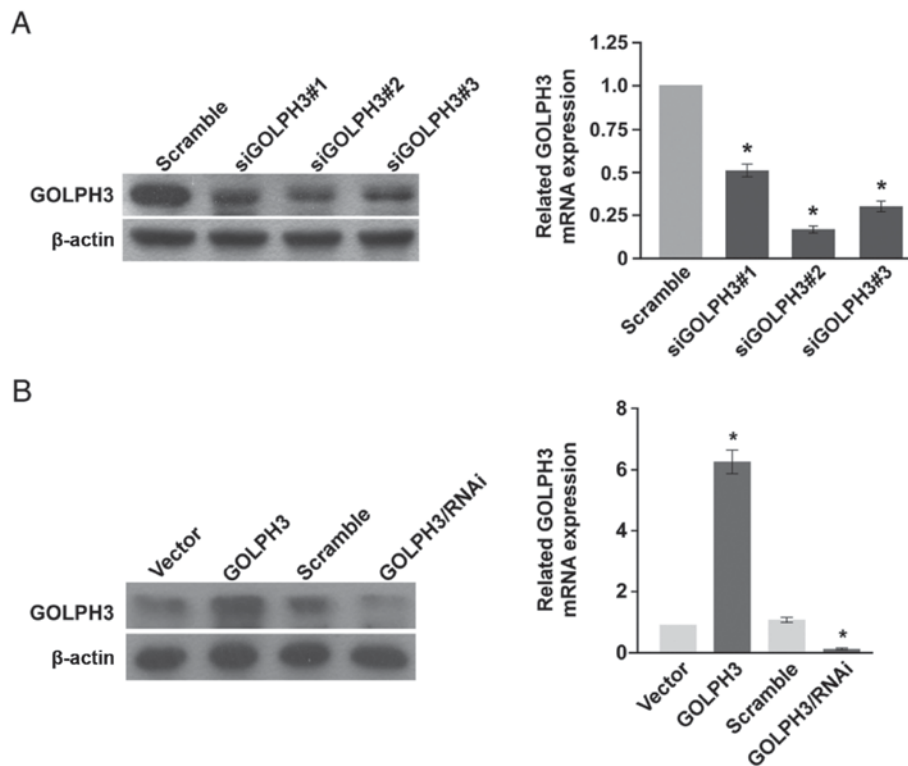


Figure 1. GOLPH3 stable overexpression and knockdown KYSE-140 cell lines. (A) Three different siRNAs targeted GOLPH3 were designed and transfected to KYSE-140 cell line. GOLPH3 protein expression levels were analyzed by western blot analysis (left panel), and the relative mRNA expression were revealed by qPCR (right panel). (B) GOLPH3 protein expression levels of stable GOLPH3 overexpression cell line KYSE-140-GOLPH3 and the negative control KYSE-140-vector, stable knockdown GOLPH3 cell line KYSE-140-GOLPH3/RNAi and the negative control KYSE-140-scramble (left panel), the relative mRNA levels were tested by qPCR (right panel). * $P < 0.05$.

PASW Statistics 18 (IBM, Armonk, NY, USA). P-values in graph were depicted as follows: $P < 0.05$ (significant), $P < 0.01$ (very significant), and $P < 0.001$ (highly significant).

Results

GOLPH3 stable overexpression and knockdown KYSE-140 cell lines were constructed. GOLPH3 stable overexpression and knockdown ESCC cell lines were first constructed in order to investigate the function of GOLPH3 in ESCC (Fig. 1). KYSE-140 ESCC cell lines were chosen, and stable cell lines were constructed via retrovirus.

GOLPH3 CDs were cloned and inserted into retrovirus backbone vector and subsequently packed into retrovirus particles. KYSE-140 cells were transfected and selected by puromycin. GOLPH3 protein expression of KYSE-140-GOLPH3 was substantively higher than the negative control KYSE-140-vector cell line (Fig. 1B, left). *GOLPH3* mRNA level was also 5 times higher in KYSE-140-GOLPH3 than in KYSE-140-vector (Fig. 1B, right).

GOLPH3 knockdown was performed via RNA interference (RNAi). Three different GOLPH3-targeted siRNAs were transfected to KYSE-140 cells, following which the protein levels and mRNA levels of GOLPH3 in transfected cells were analyzed (Fig. 1A). SiGOLPH3#2 demonstrated the best knockdown efficacy at both the protein and mRNA levels, and the GOLPH3 mRNA expression level was suppressed by 83.33% (Fig. 1A, right). The sequence of siGOLPH3#2 was then chosen to construct the retrovirus backbone vector expressing

shRNA. The retrovirus particles were packed and infected KYSE-140 to construct the GOLPH3 stable knockdown cell KYSE-140-GOLPH3/RNAi. A negative control cell line was constructed by scramble sequence labeled KYSE-140-scramble. GOLPH3 protein and mRNA levels were significantly down regulated in KYSE-140-GOLPH3/RNAi compared with KYSE-140-scramble, while no difference was observed between KYSE-140-vector and KYSE-140-scramble (Fig. 1B).

GOLPH3 promoted ESCC cell growth and proliferation in vitro. The GOLPH3 stable overexpression cell line KYSE-140-GOLPH3, the negative control KYSE-140-vector; the GOLPH3 stable knockdown cell line KYSE-140-GOLPH3/RNAi, and the negative control KYSE-140-scramble were all analyzed for cell viabilities for five days. Growth curves revealed that the proliferation of GOLPH3 overexpressed cell line KYSE-140-GOLPH3 was accelerated compared with KYSE-140-vector, cell viability was elevated significantly from the 3rd day (day 2 $P = 0.15$, day 3 $P = 0.0002$, day 4 $P < 0.0001$, day 5 $P < 0.0001$). Simultaneously, proliferation of GOLPH3 downregulated cell line KYSE-140-GOLPH3/RNAi was visibly hindered compared to KYSE-140-scramble, cell viability was decreased significantly from the 2nd day (day 2 $P = 0.017$, day 3 $P = 0.0001$, day 4 $P = 0.0010$, day 5 $P = 0.0005$). At the same time, cell curves indicated near-equal proliferation ability between KYSE-140-scramble and KYSE-140-vector (Fig. 2A).

The cell cycle of each cell line was further examined to determine the reason for proliferation influence by GOLPH3.

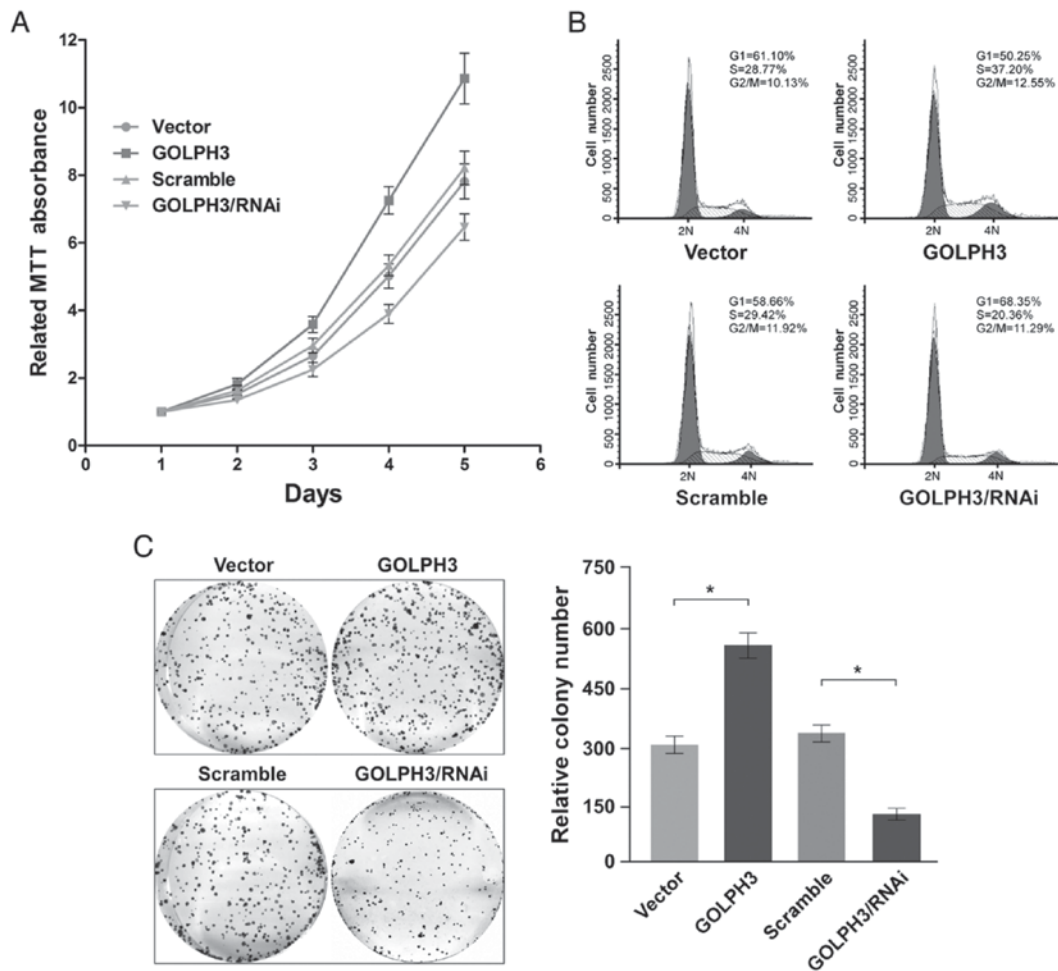


Figure 2. GOLPH3 promoted KYSE-140 cell proliferation growth. (A) MTT assays were performed to portray the growth curve of KYSE-140-GOLPH3, KYSE-140-vector, KYSE-140-GOLPH3/RNAi and KYSE-140-scramble cell lines. (B) Cell cycle of GOLPH3 stable overexpressed and knockdown KYSE-140 cell lines were analyzed by flow cytometry after PI staining. (C) Cell growth of GOLPH3 stable overexpressed and knockdown KYSE-140 cell lines were analyzed by plate colony formation assay (left panel), colony formation rates were calculated and shown by column graph (right panel). * $P < 0.05$.

The cell cycle of KYSE-140 with GOLPH3 overexpression was accelerated compared to the negative control, with the S Phase cell proportion increased from 28.77 to 37.20%, and the G1 Phase cell proportion decreased from 61.10 to 50.25%, simultaneously. Reversely, GOLPH3 knockdown resulted in cell cycle arrest, with S phase cell decreased from 29.42 to 20.36% and G1 Phase increased from 58.66 to 68.35% (Fig. 2B).

GOLPH3 could promote cell growth in addition to cell proliferation; plate colony formation assay demonstrated the colony of KYSE-140-GOLPH3 to be nearly twice the number of KYSE-140-vector ($P = 0.0003$). Meanwhile, the colony formed of KYSE-140-GOLPH3/RNAi was only half the number of KYSE-140-scramble ($P = 0.0003$) (Fig. 2C).

In general, GOLPH3 overexpression promoted ESCC cell proliferation and growth, while GOLPH3 knockdown inhibited cell proliferation and growth *in vitro*.

GOLPH3 activated mTOR signaling pathway. Compared with the KYSE-140-vector cells, the phosphorylation of the key proteins of mTOR signaling pathway, mTOR pS6 and 4E-BP, were enhanced in the KYSE-140-GOLPH3 cells. At the same time, the phosphorylation of the key proteins of mTOR signaling

pathway were suppressed in the KYSE-140-GOLPH3/RNAi cell compared with KYSE-140-scramble cell (Fig. 3A). As the results determine, GOLPH3 plays a tumorigenesis role in ESCC cells through mTOR signaling activation.

GOLPH3 accelerated cell cycle through Wnt/ β -catenin signaling pathway activation. In order to investigate the mechanisms GOLPH3 played in cell cycle acceleration when GOLPH3 is overexpressed in the KYSE-140 cell, the proteins involved in G1-S Phase transition was analyzed. Cyclin D1 and c-Myc were overexpressed when GOLPH3 was upregulated, while p21 expression was down regulated. At the same time, pRb phosphorylation was increased. In KYSE-140 cell with GOLPH3 knockdown, the trends reversed (Fig. 3B).

The Wnt/ β -catenin signaling pathway is the upstream of all above proteins. Western blot analysis revealed that more β -catenin progressed to the nucleus of KYSE-140-GOLPH3 compared to KYSE-140-vector cell, but less β -catenin localized to the nucleus of KYSE-140-GOLPH3/RNAi than the KYSE-140-scramble cell (Fig. 3B).

TOP-Flash and FOP-Flash assays were performed to confirm the activity of TCF4 and Wnt/ β -catenin signaling

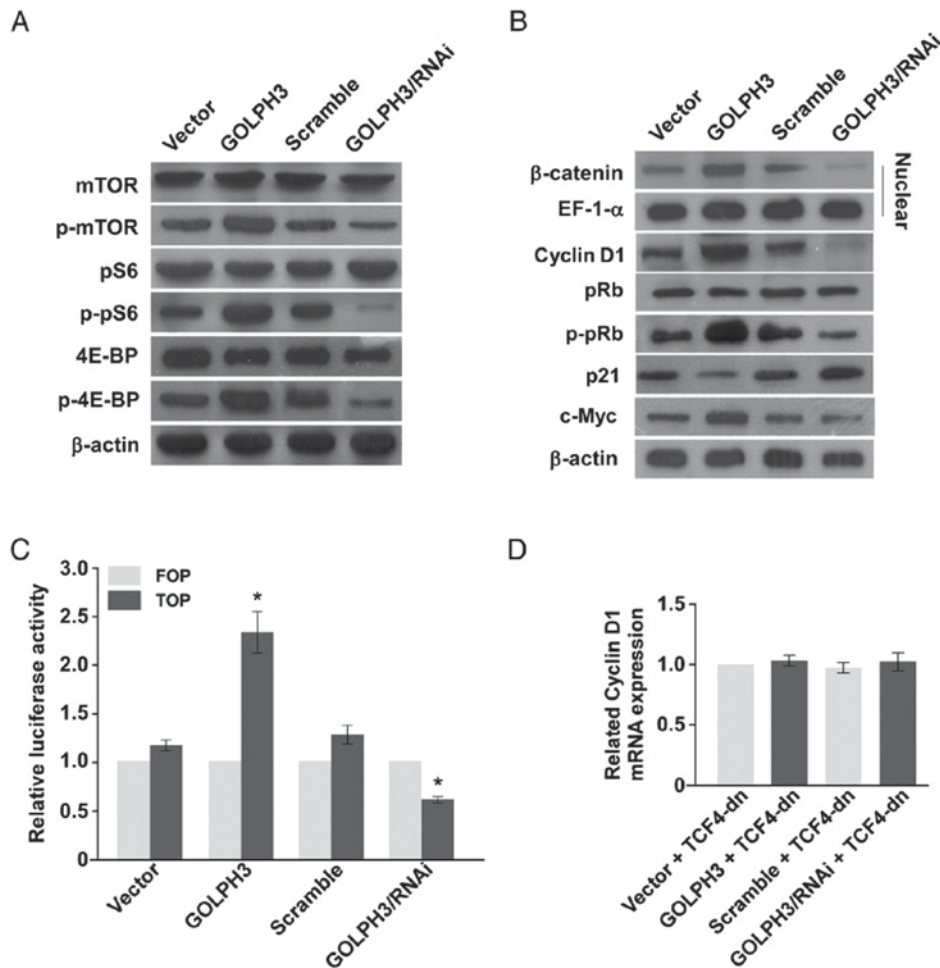


Figure 3. GOLPH3 activated mTOR and Wnt/β-catenin signaling pathway. (A) The phosphorylation of key proteins of mTOR signaling pathway, mTOR pS6 and 4E-BP, were tested by western blot analysis. (B) Wnt/β-catenin signaling pathway activity and the downstream protein were analyzed by western blot, nuclear protein level of β-catenin were detected independently with EF-1-α as the internal standard. (C) Dual-luciferase reporter assay with TOP-Luciferase and FOP-Luciferase revealed the Wnt/β-catenin signaling effect protein TCF4 activity. (D) TCF4-dn hindered Wnt/β-catenin signaling and cyclin D1 mRNA expression of GOLPH3 stable overexpressed and knockdown KYSE-140 cell lines were tested by qPCR. *P<0.05.

activity. In the KYSE-140-GOLPH3 cell, TCF4 activity was increased compared to KYSE-140-vector cell. Contrastingly, TCF4 activity was decreased in KYSE-140-GOLPH3/RNAi cell compared to KYSE-140-scramble cell (Fig. 3C). When the Wnt/β-catenin signaling was fully blocked with TCF4-dn, which lacked the β-catenin binding domain and antagonized Wnt/β-catenin signal, the GOLPH3 overexpression or knockdown had no effect on cyclin D1 (Fig. 3D).

GOLPH3 promoted ESCC tumorigenesis *in vivo*. Nude mice tumorigenesis assays demonstrated that the KYSE-140-GOLPH3 cell-formed tumors were larger than KYSE-140-vector cells. While KYSE-140-GOLPH3/RNAi cell-formed tumors were smaller than KYSE-140-scramble cells (Fig. 4A).

Further analysis of protein expression of tumor tissues formed *in vivo* by western blot indicated that GOLPH3 in generated tumor tissues remained stable. Cell proliferation related protein Ki-67 was found upregulated in KYSE-140-GOLPH3 generated tumor tissues, but down regulated in KYSE-140-GOLPH3/RNAi generated tumor tissues. Interestingly, migration related protein MMP9 was also upregulated when GOLPH3 was overexpressed, while down regulated when GOLPH3 was knockdown (Fig. 4B).

In general, GOLPH3 can also play a tumorigenesis role *in vivo*. And the tumor progression may due to cell proliferation and invasion promoting abilities of GOLPH3.

Discussion

Our previous study evidenced that GOLPH3 high expression in ESCC cancer tissues correlated with patients' clinical stage, TNM classification, histological differentiation and shorter overall survival time (28). In this study, gain- and loss-of-function analysis were performed based on the construction of the GOLPH3 stable overexpressed cell line KYSE-140-GOLPH3 and the negative control KYSE-140-vector, and the GOLPH3 stable knockdown cell line KYSE-140-GOLPH3/RNAi and the negative control KYSE-140-scramble.

Our both *in vitro* and *in vivo* experiments demonstrated that GOLPH3 promoted cell growth and proliferation, and in the end triggered tumorigenesis. The underlying mechanisms may attribute to cell cycle acceleration.

GOLPH3 has already been reported as a potent oncogene which is able to promote cell growth and activate mTOR signaling (20). mTOR signaling pathway was reported to be activated in many other cancers, which played a role as cell

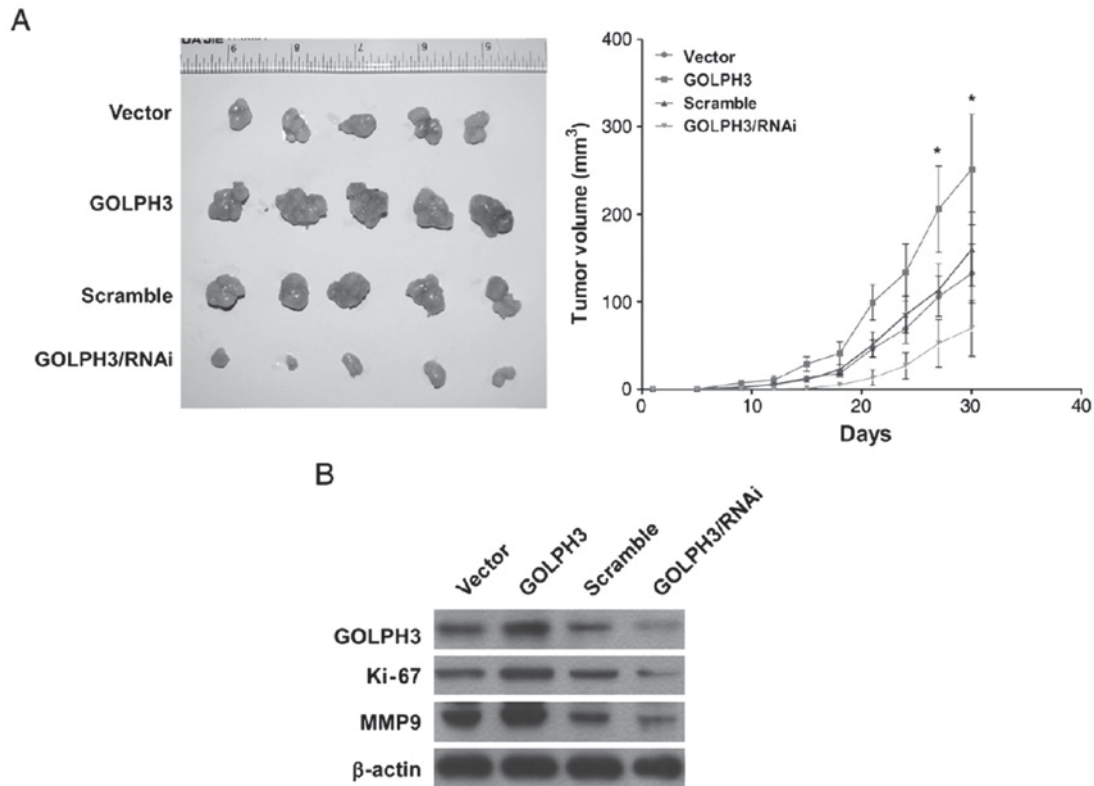


Figure 4. GOLPH3 promoted ESCC cell growth *in vivo*. (A) Cells were injected subcutaneously into the left flank of 4-weeks old female BALB/c-nu mice, and growth curves were plotted (right panel). Thirty days after injection, mice were euthanized and tumor tissues were excised (left panel). (B) GOLPH3, Ki-67 and MMP9 protein expressions were analyzed by western blot analysis. *P<0.05.

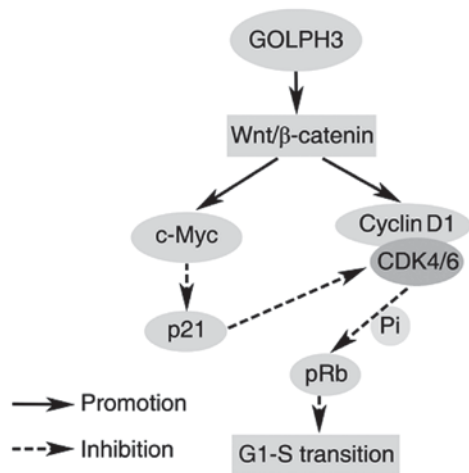


Figure 5. GOLPH3 regulated cell cycle via activation of Wnt/β-catenin signaling pathway

growth accelerator and cell survival protector. The results of our study corroborated this through confirmation of the activation of mTOR attributing to GOLPH3 overexpression in ESCC. Aside from mTOR signaling, GOLPH3 was also reported to be involved in cell autophagy regulation and coordination of the tumor cell and the fibroblasts in the surrounding microenvironment, which culminates in the promotion of tumorigenesis (29). Another study suggested that GOLPH3 overexpression protected cells from DNA damage and contributed to tumor growth (24). However, the cell cycle

acceleration function of GOLPH3 could not be explained adequately until now.

Our analysis of the expression level of cell cycle G1-S phase checkpoint associated protein showed that overexpression of GOLPH3 led to down regulation of the p21 protein and upregulation of cyclin D1 at the same time, which also increased pRb phosphorylation. The total effect was G1-S phase transition and cell cycle acceleration. Cyclin D1 is one of the most specific effector of the Wnt/β-catenin signaling pathway (30), thus this pathway became the priority of our following study. We inspected another effector, c-Myc of Wnt/β-catenin signal, which also regulates p21 expression (31-33), and found c-Myc upregulation followed by GOLPH3 overexpression. TOP-Flash and FOP-Flash assays and western blot of the nuclear β-catenin protein expression levels both demonstrated the activation of Wnt/β-catenin signal by GOLPH3 overexpression. Thereafter, elimination of the Wnt/β-catenin signal by TCF4-dn confirmed that the overexpression of GOLPH3 promoted tumorigenesis via Wnt/β-catenin signaling activation (33). Oppositely, stable knockdown of GOLPH3 led to a complete reversed effect. In addition, studies from the other researchers indicated that the overexpression of cyclin D1 and p21 also correlated with poor prognosis of ESCC patients (34,35); our study elucidated the relationship between GOLPH3, cyclin D1 and p21. Considering the expression of cyclin D1 and p21 in ESCC influenced by GOLPH3, which also dedicates to poor prognosis of the patients, the role of GOLPH3 in ESCC may also be defined to some extent.

In our study, another protein matrix metalloproteinase 9 (MMP9), which was involved in cancer metastasis (36,37),

was also detected in nude mice tumorigenesis assays. Tumors originated from KYSE-140-GOLPH3 cell line also showed a variation of MMP9, indicating a new research direction of GOLPH3 and metastasis.

In summary, GOLPH3/Wnt/ β -catenin signaling plays an important role in ESCC tumorigenesis (Fig. 5). In this study, mechanisms relating to the tumorigenesis role of GOLPH3 in ESCC cells were analyzed, and a new signaling in ESCC is proposed, providing a new target for ESCC therapy.

Acknowledgements

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