RESEARCH ARTICLE

Lentivirus Mediated GOLPH3 shRNA Inhibits Growth and Metastasis of Esophageal Squamous Cancer

Qiang Wang* , Xian Wang, Can-Bin Zhang

Abstract

Aim: To investigate the role of Golgi phosphoprotein 3 (GOLPH3) in tumour growth and metastasis of esophageal squamous cancer. Methods: A lentiviral shRNA-vector was utilized to stably knockdown GOLPH3 in Eca-109 esophageal squamous cancer cells. mRNA transcription and protein expression of GOLPH3 were examined by real-time quantitative PCR and Western blotting, respectively. Cell proliferation activity was assessed by MTT assay and invasion and migration potentials by matrigel invasion and transwell motility assays. Results: Stable knockdown in the GOLPH3 cell line was established. PD-A gene expression was significantly suppressed by lentivirus-mediated RNAi, which resulted in reducing the capacity for cell proliferation, migration, invasion and adhesion *in vitro*. *In vivo*, GOLPH3 depletion resulted in inhibition of tumour growth, with stable decrease in the expression of GOLPH3 in tumor xenografts. Conclusions: Our findings suggest that lentivirus mediated silencing of the GOLPH3 gene has a significant anti-tumour effect on esophageal squamous cancer *in vitro* and *in vivo*. In addition, the results indicate that GOLPH3 might be an effective molecular target for gene therapy in esophageal squamous cancer.

Keywords: GOLPH3 - esophageal squamous cancer - shRNA - lentivirus-mediated treatment

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Introduction

Esophageal cancer is often refractory to current therapeutic approaches and has poor outcomes. Worldwide, almost 400,000 new cases of esophageal cancer are diagnosed annually- it is the eighth most common cancer and the sixth most common cause of cancer-related mortality (Higuchi et al., 2009; Parkin et al., 2009; Wan et al., 2013). The overall 5-year survival rate for esophageal cancer is approximately 15% (Polednak, 2003). Therefore, it is an urgent task that seeking a safe and effective therapeutic method to inhibit the malignant behaviors of ovarian cancer.

Recently, Golgi phosphoprotein 3 (GOLPH3) has been demonstrated as a novel oncogene involved in the development of cancer of the lung, breast, ovary, prostate, colon, melanoma, rhabdomyosarcoma and glioma (Romanuik et al., 2009; Scott et al., 2009; Kunigou et al., 2011; Li et al., 2011). GOLPH3 was initially detected as a phosphorylated protein localized to the Golgi apparatus (Dippold et al., 2009; Scott and Chin 2010). Recently, the cell biological, biochemical and functional analyses have shown that GOLPH3 can increase cell transformation and tumor growth by enhancing the activity of mTORC (Dippold et al., 2009; Wood et al., 2009; Scott and Chin 2010; Graham and Burd, 2011). Moreover, the preliminary researches shown that GOLPH3 is relative with poor prognosis of some tumors, including gastric cancer (Hu et al., 2013) and esophageal squamous cell carcinoma (Wang et al., 2012). However, it is unclear whether inhibition of GOLPH3 can reduce tumor growth of esophageal squamous cell carcinoma.

Due that Lentivirus vector-based transgenes can be incorporated into the host cell genome and maintain long-term expression without inducing a host immune response against the transduced cells (Dropulic, 2011), lentivirus vector system can be considered as the most efficient gene therapy tool and may act as an in vivo gene delivery system for gene therapy application.In the present study, using lentivirus mediated RNAi technology, we first established a stable GOLPH3-knockdown cell line, which derived from high invasive epithelial esophageal squamous cancer Eca-109 cell line, in order to investigate the role that reducing of GOLPH3 inhibit esophageal squamous cell cancer (ESCC) growth.

Materials and Methods

Cell Line and Cell Culture

The human esophageal squamous cancer cell line Eca-109 and human embryonic kidney cell line 293FT were purchased from ATCC. Human esophageal squamous

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cancer Eca-109 cells were grown in Roswell Park Memorial Institute 1640 medium (Gibco, USA), supplemented with 10% of fetal bovine serum (Hyclone, USA). 293FT were cultured in Dulbecco's modified Eagle medium containing 4.5g/l glucose (PAA Laboratories, Pasching, Austria), supplemented with 10% heat inactivated fetal calf serum (Hyclone, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (both Invitrogen,USA) .Both two cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2. The cells were always detached using 0.25% trypsin with 0.02% ethylene diamine tetra acetic acid (EDTA).

Experimental Animals

Twenty-eight athymic nude female BALB/c mice (6~8weeks old), obtained from Beijing HFK Bioscience CO.,LTD (Beijing, China). Animal breeding, care and experimental procedures were approved by the ethical and humane Committee of Henan University and carried out strictly in accordance with the related regulations on administration of experimental animals.

Establishment of stable GOLPH3-knockdown tumor cells by lentiviral vector mediated RNAi

The specific lentiviral expression vector containing the GOLPH3 shRNA sequence (OriGene, Rockville, USA) was selected for specifically targeting GOLPH3 silence, and classified as Lenti-GOLPH3-sh. As control, the negative control vector (OriGene, Rockville, USA) was classified as Lenti-GOLPH3-nc. These two lentiviral expression vectors contain the reporter gene of eGFP (enhanced green fluorescent protein). Then the recombinant lentivirus and the control lentivirus were produced by co-transfecting 293FT cells with Lenti-Pac™ HIV Expression Packaging Kit (GeneCopoeia, USA), The virus-containing supernatant was harvested 48h and 72 h post-transfection. The prepared Eca-109 cells were transduced with the lentiviral expression vectors (Lenti-GOLPH3-sh or Lenti-GOLPH3-nc). Transfection efficiency was detected directly by testing the expression ratio of eGFP by fluorescence microscopy. Stable cell lines were obtained after selection by long-term culture in medium containing 1.5 ug/mL puromycin (Invitrogen) for 20 d. The clones derived from Eca-109 cells stably transfected with Lenti-GOLPH3-sh was classified as GOLPH3-KD (DP1-knockdown) cells, whereas the derived Eca-109 cells stably transfected with Lenti-GOLPH3-nc as mock cells. The untreated Eca-109 cells was classified as parental Eca-109 cells. The knockdown of GOLPH3 was evaluated by Real-time Quantitative PCR and Western blot analysis.

Real- time quantitative polymerase chain reaction (real-time qPCR)

The total RNA were extracted using Trizol (Life Technologies, Carlsbad, USA), and reverse transcripted into cDNA using the Prime Script RT-PCR kit (TaKaRa Bio Inc, Shiga, Japan),respectively, according to the manufacturer's protocol. The primers for the human GOLPH3 gene were as follow: 5'- GGGCGACTCCAAGGAAAC -3' (forward) and 5'- CAGCCACGTAATCCAGATGAT -3' (reverse); The GAPDH was used as the internal control with the specific primers: forward primer: 5'-GCACCGTCAAGGCTGAGAAC-3',reverse prime: 5'-TGGTGAAGACGCCAGTGGA-3'. PCR amplification and fluorescent signals real-time monitoring was performed by Stratagene fluorescence quantitative PCR instrument.

Western blot assay

Protein Extracts were prepared by using RIPA lysis buffer and the protein concentrations were measured by the Bradford method using BCA Protein Assay Kit (Pierce Biotechnology, USA). Equal amounts of protein extracts (30 ug) were separated by 12% SDS-PAGE and then transferred onto 0.45um PVDF membranes (Millipore, USA) in wet condition. The membranes were blocked in 1×TBST containing 5% non-fat dry milk for 1h and then incubated at 4°C overnight with rabbit anti-GOLPH3 polyclonal (sc-179, Santa Cruz Biotechnology, USA) ,or rabbit anti-β-actin (AP0060, Bioworld Technology, USA) both diluted 1:1000 in 1×TBST containing 5% non-fat dry milk, followed by secondary HRP-conjugated anti-rabbit Antibody (BS13278, Bioworld Technology, USA) diluted 1:5000 in 1×TBST containing 5% non-fat dry milk for 1h at room temperature, and detection was by chemiluminescence (ECL) system (Pierce Biotechnology, USA) in a darkroom. Western band densities were qualified using ImageJ 1.44p software (National Institutes of Health).

Cell proliferation assay

Cell proliferation activity of the three group cells were assessed by MTT assay (Feng et al., 2002).. For measurement of cell surviving rate, the collected cells, which were parental, mock or GOLPH3-KD cells, were plated into 96-well plates in 5×10^3 cells/100 μl medium/well.10ul of MTT solution (Sigma,USA) was added into each well daily from the 1st to 5th day, and plates were incubated for 4 h at 37°C. Then medium of each well was abandoned and 100 μl DMSO was added to dissolve formazan and shaken for 10min. Absorbance values (A) were measured at a wavelength of 492 nm with a microplate reader.

Cell invasion and migration assays

The invasion and migration assays was performed (Kelly et al., 2006a; Kelly et al., 2006b) by using a 24-well transwell chamber with 8- um pore size polycarbonate membrane (Corning, USA) as recommended by the supplier. For invasion assay, the upper side of polycarbonate filter was coated with matrigel (30 ul, diluted in 1:3, BD, Biosciences). The chambers were incubated at 37°C in 5% CO2 for 30 min to allow the matrix to form a continuous thin layer. The parental Eca-109, mock, and GOLPH3-KD cells were starved for 24 hours and then were harvested. The 5×10^4 cells in 200 μl of 0.5% bovine serum albumin (Sigma, USA) were placed in the upper chamber. The lower chamber was filled with 600 ul of 20% fetal bovine serum-medium. Cells were cultured at 37°C with 5% CO_2 for 48 h. Cells on the upper surface of the filter were removed using a cotton swab. Cells invading through the Matrigel and filter on

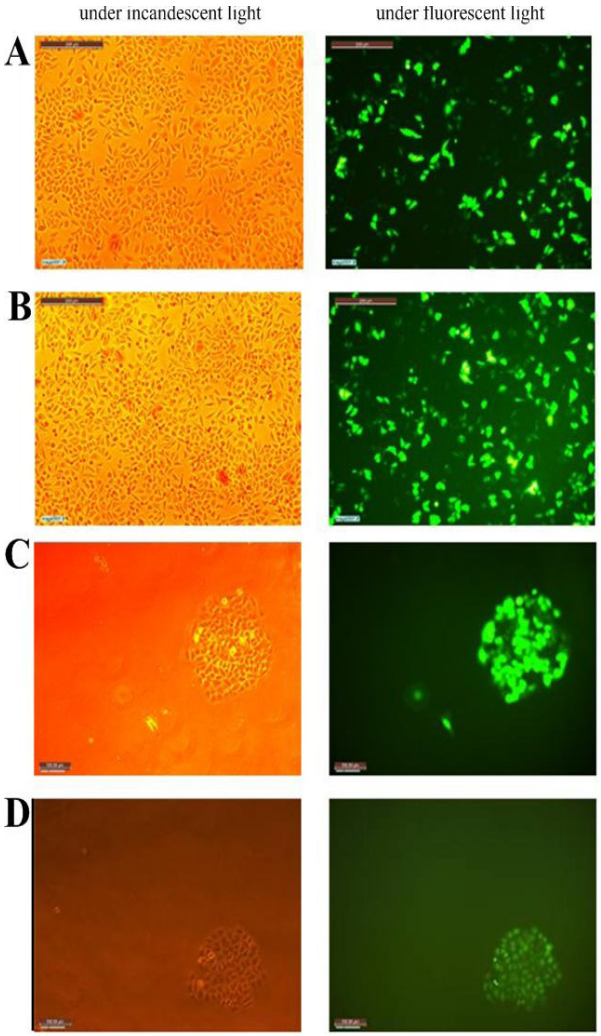

Figure 1. Eca-109 Cells Transfected with Lentiviral Vectors and Stable Clones Established. (A) The Eca-109 cells transfected with Lenti-GOLPH3-sh for 48 h (×100); (B) The Eca-109 cells transfected with Lenti-GOLPH3-nc for 48h(×100); (C) GOLPH3-KD clones derived from the Eca-109 cells stably transfected with Lenti-GOLPH3-sh were established (×200); (D) Mock clones derived from the Eca-109 cells stably transfected with Lenti-GOLPH3-nc were established (×200)

the lower surface were fixed with 4% neutral-buffered formalin and stained in 0.01% crystal violet solution. The cell numbers in five fields (up, down, median, left, right. ×200) were counted for each chamber, and results were expressed as mean value ± SD. Migration assay was done by the same procedure, except that the membrane was not coated with Matrigel. After 48h-incubation, the number of migrated cells (lower side of the membrane) was counted as described above. Each experiment was repeated three times with the similar result.

Cell adhesion assay

Cell adhesion assay was performed by using a 96-well plate, which was coated with matrigel 30 ul (diluted in 1:3) and incubated overnight with 0.1% BSA. The three group Cells were washed three times in serum-free medium, resuspended at the concentration of 1×10^6/ml in serum-free medium and added to the wells (100 ul/well), respectively. The assay was incubated at 37°C for 1h, and then the unattached cells were washed away by phosphate

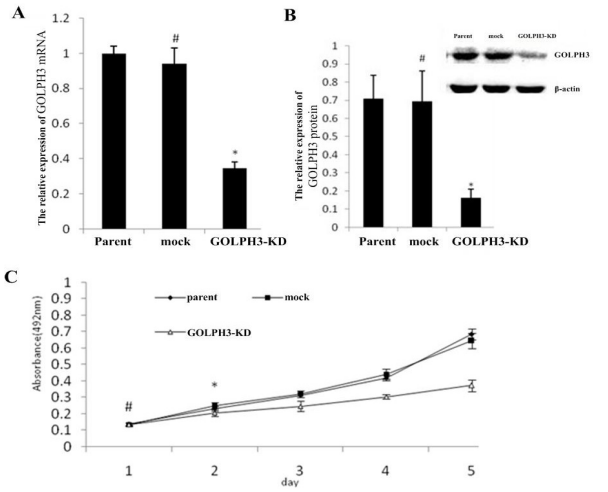

Figure 2. GOLPH3 Expression and MTT Assay. (A) shows dramatic decrease of GOLPH3 mRNA in the GOLPH3-KD cells. (B) show significant reduction of GOLPH3 protein in the GOLPH3-KD cells. (C) A492 values of GOLPH3-KD cells were significantly decreased from 2nd day. Parent, parental Eca-109 cells; Mock, mock cells derived from Eca-109 cells stable transfected with Lenti-GOLPH3-nc; GOLPH3-KD, GOLPH3-KD cells derived from Eca-109 cells stable transfected with Lenti-GOLPH3-sh .Data were expressed as mean value ± SD, $^*P < 0.05$ versus Parent and Mock; $^\#P > 0.05$ versus Parent

buffered saline (PBS), 10 μl of MTT solution was added into each well and plates were incubated at 37°C for 4 h. Then 100 μl DMSO was added to dissolve formazan and shaken for 10min. Absorbance values (A) were measured at a wavelength of 492 nm with a microplate reader. Results were expressed as mean value ± SD and the adhension rate was calculated: the relative adhension rate (%) = (A492 of experimental group/A492 of the control group) ×100%. Each experiment was repeated three times with the similar result.

Statistical analysis

Statistical analysis was performed with SPSS 13.0 statistical software (SPSS Inc, Chicago, Illinois). Differences among three groups were assessed using one-way ANOVA and further differences between the two groups were assessed using Student-Newman-Keuls (SNK). A *P* value less than 0.05 was considered statistically significant.

Results

Stable GOLPH3-knockdown tumor cells by lentiviral vector-based RNAi system were successfully established The eGFP expression ratio of 293FT cells were both more than 85% 24 h post-cotransfection. It indicates that the recombinant lentivirus (Lenti-GOLPH3-sh) and the control lentivirus (Lenti-GOLPH3-nc) were packed. Then Eca-109 cells were transfected with the lentivirus (Lenti-GOLPH3-sh or Lenti-GOLPH3-nc) for 48h, transfection efficiency observed by the fluorescent microscope were 60% (Figure 1A and B). Therefore, the lentivirus-transduced cells were selected by puromycin (1.5 ug/ml). After continuous culture for 20 d, the GOLPH3-KD and mock stable clones which derived from Eca-109 cell lines

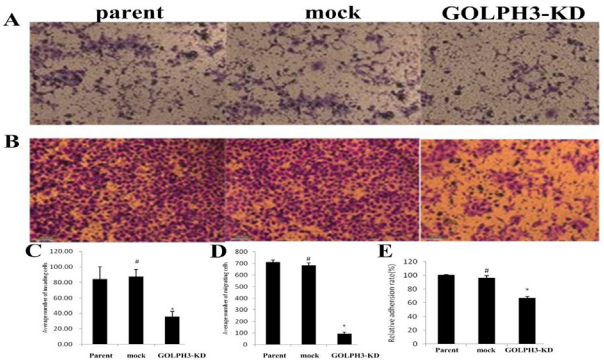

Figure 3. The Impact of GOLPH3 Silencing on the Metastatic Phenotypes and Adhension Capability of Eca-109 Cells. (A) Matrigel invasion assay; (B) Transwell migration assay. The numbers of invading or migrating cells were determined by counting the cells stained with 0.01% crystal violet solution in the lower side of the membrane. The decrease in the number of invading and migrating cells in the KD cells compared to those of the parental and mock cells was statistically significant (*P<0.05; C, D). (E) The relative adhension rate was significantly lower in the GOLPH3-KD cells than in the parental and mock cells(*P < 0.05). Data expressed as the mean value ± SD of three independent experiments. *P < 0.05, versus Parental and Mock cells. $^{\#}P$>0.05, versus Parent

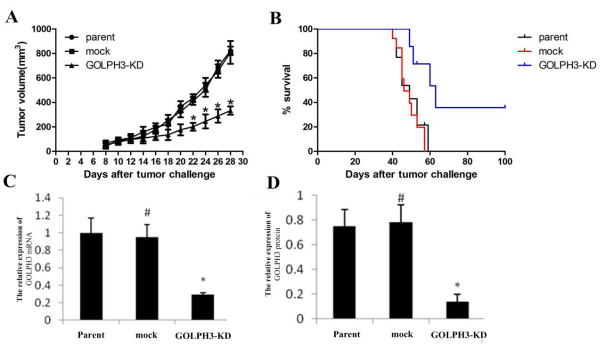

Figure 4. Specific silencing GOLPH3 gene inhibited the tumor growth and prolonged the survival time. (A) From the 22th day, the tumor volume in GOLPH3-KD group was decresed that in other two groups (P<0.05). (B) Within 100 days, the survival time in GOLPH3-KD group was prolonged that in other two groups (P<0.05). (C) Real time quantitive PCR shows dramatic decrease of GOLPH3 mRNA in the GOLPH3-KD group xenograft (*P < 0.05). (D) Western blot quantitive analysis reveal significant reduction of GOLPH3 protein in the GOLPH3-KD group xenograft

transduced with lentivirus were successfully generated (Figure 1C and D). Although these clones cultured and passaged for more than 10 times, the positive eGFP expression in cells was still above 98%. After that, real time qPCR analysis and Western blot assay also confirmed that GOLPH3-KD cells showed a significant decrease of GOLPH3 in both mRNA and protein levels (p<0.05, Figure 2A and B), indicating the successful knockdown of GOLPH3 in the derived clone. Furthermore, there was no difference in GOLPH3 expression levels between the mock cells and the parental Eca-109 cells (p>0.05, Figure 2A and B).

Specific silencing GOLPH3 inhibited cell proliferation activity of Eca-109 cells

We first examined the effects of GOLPH3 silencing on the proliferation activity of Eca-109 cells.The parental, mock and GOLPH3-KD cells were seeded on 96-well plates for a continuous 5-day MTT assay (Figure 2C). The difference in cell proliferation activity among the parental Eca-109 cells, mock and GOLPH3-KD cells was not statistically significant in the first day (P>0.05). However, from the 2nd to the fifth day, cell proliferation ability of GOLPH3-KD cells was significantly attenuated (p<0.05),whereas there is no difference in cell growth between the parental Eca-109 and mock tumor cells (p>0.05). The results revealed that specific GOLPH3 silencing inhibits the capacity of cell proliferation of Eca-109 cells.

Specific silencing of GOLPH3 reduced invasion and migration power of Eca-109 cells

Then we evaluated whether down-regulation of GOLPH3 expression would alter the metastatic phenotype of Eca-109 cells in vitro. The cell motility and invasiveness were analyzed by transwell chamber migration and Matrigel invasion assay (Figure 3 A-D). As expected, GOLPH3-KD cells revealed a pronounced reduction in both cell migration and invasiveness when compared with the mock and parental Eca-109 cells (p<0.05), whereas the mock and parental Eca-109 cells showed similar potential of migration and invasion (p>0.05). These results indicated that silence of GOLPH3 may alter the metastatic potentials of Eca-109 cells.

Specific silencing GOLPH3 suppressed cell adhensive activity of Eca-109 cells

Cancer cell adhesion to peritoneum is a crucial process and the initial step during peritoneal metastasis. Therefore, cell adhension assay was performed and cell adhension capability were assessed (Figure 3E). The results revealed that the capacity of cell adhesion was dramatically decreased in GOLPH3-KD tumor cells, compared with that in the parental Eca-109 or mock cells. Whereas the mock and parental Eca-109 cells showed similar potential of cell adhensive activity. These results indicated that silence of GOLPH3 may attenuate the cell adhensive activity of Eca-109 cells.

Specific silencing GOLPH3 gene inhibited the tumor growth and prolonged the survival time

To further examine the effect of GOLPH3 silence on tumor growth in vivo, the parental Eca-109 cells, mock and GOLPH3-KD cells were injected subcutaneously to the back of nude mice (n=10/group), respectively, and classified as parental xenograft (parent), mock xenograft (mock) and GOLPH3-KD xenograft (GOLPH3-KD) group. The tumor volume and survival time was significantly decreased and increased in GOLPH3-KD group when compared with the other two groups, respectively (p<0.05) (Figure 4A and B). Furthermore, there were no statistically significance in the tumor growth and survival time between Parent and Mock group (p>0.05).

The expression of GOLPH3 were stably attenuated in vivo

Real-time qPCR analysis showed the mRNA level of

GOLPH3 was significantly decreased in GOLPH3-KD group compared with the parent and mock group ($P<0.05$, Figure 4C). Western blot quantitative analysis revealed the relative protein level of GOLPH3 was significantly decreased in GOLPH3-KD group when compared with the parent and mock group ($P<0.05$, Figure 4D). However, there was no significant difference between parent and mock group ($P>0.05$). The results showed that the GOLPH3 gene was specifically and stably silenced by lentivirus mediated shRNA in vivo.

Discussion

In present study, we demonstrated that reducing of GOLPH3 can inhibit the proliferaton, invasion and migration of human esophageal squamous cancer cells Eca-109 in vitro. Moreover, in vivio study revealed that inhibition of GOLPH3 gene can attenuate tumor growth and prolong the survival time.

RNA interference (RNAi), an evolutionarily conserved sequence-specific post-transcriptional gene silencing technology triggered by small double-stranded RNA (dsRNA) with high efficiency and specificity as well as low toxicity, has been widely used for silencing malignant cellular and viral genes (Boudreau et al., 2012). In recent years, lentivirus mediated RNAi technique achieved some preliminary success and provides great promise in the field of cancer therapy (Jiang et al .,2011). In present study, by using lentivirus mediated RNAi technique, we established the stable GOLPH3-KD cell line and a mock cell line derived from human esophageal squamous cancer cells Eca-109, a highly invasive human esophageal squamous cancer cells Eca-109 (Jiang et al., 2010). In our study, founding RNAi-mediated GOLPH3 silencing could significantly and stably down-regulate the expression levels of GOLPH3 gene in GOLPH3-KD cell lines in vitro and the xenograft model of nude mice in vivo. However, the expression levels of GOLPH3 gene are not statistically significant between the parental Eca-109 cells and the mock cells in vitro and in vivo. Our results confirmed that the advantage of stable and long-term gene silencing offered by the lentivirus vector system makes it a powerful tool for gene therapy.

To confirm the effects of GOLPH3 gene silence on cell proliferation, cancer cell proliferation potentials in vitro and tumor growth potentials in vivo were investigated. The finding that cell proliferation potential measured by MTT assay was dramatically decreased in GOLPH3-KD tumor cells compared with those in the parental Eca-109 cells and mock cells in vitro and the intraperitoneal tumorgenicity model in nude mice in vivo demonstrated the tumor growth was significantly inhibited in the GOLPH3-KD xenograft group compared with those in the parental and mock xenograft groups. Therefore, these results suggested GOLPH3 gene silence can effectively suppress tumor growth in vivo, which confirmed the GOLPH3 plays an important role in regulation of cancer cell proliferation (Zeng et al., 2012).

To confirm the effects of GOLPH3 gene silence on malignant behaviors, cancer cell adhesion, migration and invasion capability were investigated in vitro and vivo. Our results indicated the relative adhesion rate, the invasion and migration ability of GOLPH3-KD cells were markedly decreased in GOLPH3-KD tumor cells compared with the parental Eca-109 and mock cells in vitro. Therefore, these results suggested GOLPH3 gene silence can effectively suppress the tumor progression in vivo, which confirmed the GOLPH3 can lead the way in tumor migration and invasion (Zhou et al., 2013).

Taken together, our findings indicate that GOLPH3 plays an important role in the process of human esophageal squamous cancer cells progression, including tumor growth, adhesion, migration and invasion. Further study should be focused on the underlying mechanisms of anti-tumor effects of GOLPH3 signaling pathway, and its potential to be a new strategy in clinical treatment for the human esophageal squamous cancer cells.

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The author (s) declare that they have no competing interests.

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