GOLPH3 promotes cell proliferation and malignancy in Hypopharyngeal squamous cell carcinoma via AKT-mTOR axis activation

Jianmin Liu
Department of otolaryngology, head and neck surgery, People's hospital of Deyang city
Tao Zhou
Department of otolaryngology, the Sixth People's Hospital of Deyang city
Yongkuan Wang
Department of otolaryngology, head and neck surgery, People's hospital of Deyang city
Shuwei Cao
Department of otolaryngology, head and neck surgery, People's hospital of Deyang city
Meng Zhang (ameng0838@126.com)
Department of otolaryngology, head and neck surgery, People's hospital of Deyang city

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Abstract

Background Hypopharyngeal squamous cell carcinoma (HSCC) is a type of the head and neck squamous cell carcinoma (HNSCC), and it tends to have malignant transformation and result in poor prognosis effected by oncogene. Studies have shown that Golgi phosphoprotein 3 (GOLPH3) is highly expressed in HNSCC, which can activate AKT-mTOR signaling pathway. However, the functions of GOLPH3 in HSCC was unclear.

Methods Based on the analysis of 520 patients from The Cancer Genome Atlas (TCGA) database, we employed GOLPH3 stable knockdown and overexpression of FaDu cell lines to investigated that function and impact of GOLPH3 on HSCC in vitro and vivo. In addition, we verifying the functions and pathway of genes that positively related to GOLPH3 via enrichment analysis and western blot.

Results GOLPH3 promotes HSCC cells proliferation, migration and subcutaneous tumor in vivo. We confirmed the positive correlation between GOLPH3 and AKT-mTOR signal activation in vitro. Enrichment analysis indicated that GOLPH3 is critical for HSCC development.

Conclusion GOLPH3 promotes tumor cells proliferation and malignancy via AKT-mTOR signal activation, and this mechanism may be a key factor for HSCC tumor malignancy.

Highlight GOLPH and it activating the mTOR pathway is important mechanism for the tumorigenesis and development of HSCC, which may provide an important target for clinical treatment of HSCC.

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is one type of the head and neck squamous cell carcinoma (HNSCC) (Johnson et al., 2020; Newman et al., 2015). Most patients were diagnosed at an advanced stage and have a poor prognosis in HSCC (Kwon et al., 2019; Bova et al., 2005). To better find a diagnosis and treatment target of HSCC, further exploration of the cellular and molecular mechanisms of the tumorigenesis and development for HSCC are needed.

Golgi phosphoprotein 3 (GOLPH3) has been demonstrated as an oncogene that has a powerful transforming activity, and is involved in the development and leads to poor prognosis of multiple cancers (Scott et al., 2009; J. H. Wang et al., 2017a; Chen et al., 2020). Recent studies demonstrated that GOLPH3, as a key factor, can activate and enhance the mTOR pathway during tumorigenesis and development of HNSCC (Chen et al., 2020; J. H. Wang et al., 2017a; Cui et al., 2015). However, the correlation between GOLPH3 and HSCC remains unclear.

To gain a deeper understanding of the functions of GOLPH3 in HSCC tumorigenesis, we preliminarily demonstrated that GOLPH3 can promote tumor cell proliferation and metastasis via activating mTOR targets based on the experimental validation, enrichment analysis and correlation analysis. The results indicated that the mechanism mainly involved in AKT/mTOR axis.

Materials And Methods

GOLPH3 Expression Analysis

The RNA-seq transcriptome data and related clinical information were obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). There are 520 HNSCC samples and 44 adjacent normal samples included in this study. Statistical analyses were performed using R software v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria), $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

Cell Culture

WUS-HN30 and FaDu cells were cultured in DMEM (Gibicol) supplied with 10% fetal bovine serum (FBS, Gibicol) and 1% penicillin – streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

Real-Time qPCR

Total RNA from cells or tissues was extracted using TRIzol reagent (Invitrogen). Quantitative PCR (qPCR) was applied using SYBR Green PCR Master Mix (Takara Bio) on a Quant Studio 3 (Thermo fisher). Relative expression was calculated by normalization to β-actin. The primers for GOLPH3 were designed by online tool Primer Bank (https://pga.mgh.harvard.edu/cgi-bin/primerbank/new_search2.cgi), and the primer sequences (Primer Bank ID: 14140240a1) are as followed:

Forward primer: TGGTAGAAAAGGGTGTACTGACG,

Reverse primer: TGATGAGACGCTGCTTAATGTTG.

Western Blot Assay

25 μg protein per sample extract were separated by 12% SDS-PAGE. After transferring to a membrane and blocking, the primary antibodies were incubated overnight at 4 ℃. After incubation with the respective secondary antibodies for 1 h at room temperature, the protein bands were visualized using enhanced chemiluminescence reagents (Millipore). Antibodies used in the experiment included rabbit polyclonal antibody GOLPH3 (ab236296, Abcam), mouse monoclonal antibody α-Tubulin (YM3035, Immunoway) and Goat anti-rabbit/Mouse HRP (HA1001, HA1006, HUABIO).

Colony Formation Assay

About 200 cells per well were seeded and incubated for 14 days. The colonies were fixed using 100% methanol for 10 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature.

Trans-Well Assay

Migration assessed using Trans-well plate. About 5×10^4 cells were resuspended in 250 μL of serum free medium in the upper chamber (8-μm pore size, Corning) while the lower chambers were filled with 750 μL of complete medium. After incubating for 24 h at incubator, the upper chambers were fixed with 100% methanol for 10 min and stained with 0.1% crystal violet at room temperature. The number of transmembrane cells was calculated under a microscope at three random perspectives (Nikon).

Wound Healing Assay

FaDu cells were seeded into the 6-well plate. After incubation for overnight, the cell density reached 100%. Equal wounds were made by 10 μL tips. The images of wound were obtained under a microscope (Nikon) at 100 × magnification.

Cell Counting Kit-8 (CCK-8) Assay

500 cells per well were seeded into 96-well plates. The viability of cells was determined using a CCK-8 assay (Bimake) everyday by measuring the absorbance at 450 nm (BioTek). The absorbance was normalized to the baseline.

Immunohistochemistry (IHC)

Tissue sample was fixed in 4% neutral formaldehyde solution for 48 hours, then embedded in paraffin, section, dewaxing, and antigen retrieval, and the rest of steps were completed according to the immunohistochemical protocol (Hofman and Taylor, 2013). The images were collected by light microscope (200 ×, Nikon). Antibodies used in the experiment included rabbit polyclonal antibody GOLPH3 (ab236296, Abcam), Donkey anti rabbit HRP (bs-0295D, BIOSS).

Statistical Analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The results of data are displayed as the mean ± SD. Student's t - test was applied to determine the difference in data. All experiments were repeated at least three times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Enrichment Analysis and Correlation analysis

The genes that positively correlated with GOLPH3 in HNSC were gathered from UALCAN database (http://ualcan.path.uab.edu/index.html). Enrichment analysis was performed by Metascape online (https://metascape.org). $p < 0.01$ and an enrichment factor > 1.5 indicate statistical significance of the results.

Results

GOLPH3 is upregulated in HNSCC tissue and FaDu cells

From The Cancer Genome Atlas (TCGA) database, comparing HNSCC and adjacent normal tissues, the result showed that GOLPH3 was upregulated in tumor sites (**Fig. 1a, b, *$p < 0.05$, **$p < 0.01$**). In addition, we found that the prognostic survival time was significantly and negatively correlated with the expression level of GOLPH3 in cancer tissues (R = 0.211, P = 0.008, n = 158) (**Fig. 1c**). Then, we explore whether the expression level of GOLPH3 is consistent with tumor tissue in vitro and we investigated WSU-HN30 and FaDu cells by qPCR and western blot. As expected, the expression of GOLPH3 was significantly upregulated in FaDu cells in mRNA and protein level (**Fig. 1d, e, *$p < 0.05$**). Therefore, it suggests that the expression level of GOLPH3 was closely related to HSCC.

GOLPH3 promotes proliferation of HSCC

To explore the effect of up-regulation of GOLPH3 on the development of HSCC, we constructed a FaDu cell line that has stable overexpression and knock-down effect of GOLPH3. This two stable FaDu cell lines have been verified by qPCR and western blot (**Fig. 2a, b, ***$p < 0.001$, **$p < 0.01$**). The results of CCK8 and cells colony assay indicated that, compared with NC group, overexpression of GOLPH3 could significantly promote the proliferation and colony formation while GOLPH3 knockdown showed opposite effects (**Fig. 2c and d - e, **$p < 0.01$, *$p < 0.05$**). Taken together, the results of this part demonstrated that GOLPH3 was a necessary factor for cell proliferation process and development of HSCC.

GOLPH3 promotes migration of HSCC

To further explore the effect of GOLPH3 on migration of HSCC tumor cells, we performed the wound healing and trans-well migration assay. As expected, the results of wound closure (%) and number of cell migration showed that oeGOLPH3 group had a significant promotion on the migration of FaDu cells. Conversely, the two groups of knock-down (shGOLPH3#1 and #2) all displayed that the significant inhibition of migration ability (**Fig. 3a and b, 3c and d, **$p < 0.01$, *$p < 0.05$**). Taken together, the GOLPH3 expression level was positively correlated with migration ability of HSCC tumor cells.

GOLPH3 promotes HSCC tumor growth in vivo

To investigate whether the level of GOLPH3 will affect tumorigenesis in vivo, we injected nude mice with three FaDu cell lines: stable knockdown (shGOLPH3#1), overexpression (oeGOLPH3) and control (blank vector mix, CTL) respectively. Tumor was observed continuously for 15 days after injection, and the weight and volume of tumors were measured in this process. Our results showed that the weight and volume of tumors in shGOLPH3#1 group were significantly lower than that in group oeGOLPH3 and CTL (**Fig. 4a - c, **$p < 0.01$, ***$p < 0.001$**). The results of immunochemistry showed that the expression trend of GOLPH3 was corresponded to tumorigenic experiments (**Fig. 4d**). Taken together, these results indicated that GOLPH3 significantly promotes tumorigenesis of HSCC in vivo.

GOLPH3 activates the AKT/mTOR axis in HSCC in vitro

Related studies have shown that GOLPH3 can activate mTOR signaling pathway in the process of promoting tumor growth (Scott et al., 2009; Liu et al., 2018; J. H. Wang et al., 2012; Yu et al., 2020). To

verify and explore the correlation between GOLPH3 and mTOR signaling pathway in HSCC, we conducted an enrichment analysis online via Metascape system based on the genes positively correlated with GOLPH3 in HNSC (Pearson-CC ≥ 0.6) and the genes were gathered from TCGA database. The results showed that GOLPH3 was significantly associated with HNSCC and mTOR signaling pathway (**Fig. 5a**). We further investigated the expression trend of proteins AKT1, mTOR in HNSCC, and their correlation with GOLPH3 expression respectively. The results showed that the expression of AKT1 and mTOR was significantly upregulated with the tumor progression grade of HNSCC (**Fig. 5b, *$p < 0.05$**). Besides, correlations between the expression of GOLPH3 and AKT1, mTOR were significant and positive (**Fig. 5c**). It showed that the expression level of GOLPH3 was positively correlated with the expression and phosphorylation level of AKT and mTOR via western blot (**Fig. 5d**). Taken together, our results demonstrated that GOLPH3 can activates the AKT/mTOR axis in development of HSCC.

Discussion

Many studies have found that the proto-oncogene GOLPH3 is highly expressed in different tumors, and its expression level is related to clinical stage and tumor grade (Kuna and Field, 2019; Zeng et al., 2012; Tokuda et al., 2014). At present, it has been confirmed that GOLPH3 activates mTOR signaling and downstream pathways in development of some tumors (Scott et al., 2009; Sechi et al., 2020; Hara et al., 2002). Mammalian mTOR is a serine/tyrosine kinase associated with PI3K, and the phosphorylated mTOR (p-mTOR, S2448) and AKT (p-AKT, S473) are characteristic markers in this pathway, which play important roles in cell growth, proliferation and survival (Sarbassov et al., 2005; Hay, 2005). Studies have shown that the AKT-mTOR axis is the most frequently altered oncogenic pathway in HNSCC tumorigenesis(Cancer Genome Atlas, 2015; Z. Wang et al., 2017b). However, this mechanism of GOLPH3/AKT-mTOR has not been elucidated in HSCC.

The high expression level of GOLPH3 was closely related to tumor cells proliferation and poor prognosis in epithelioid tumor tissues (Sun et al., 2017; R. Wang et al., 2015). Analysis of TCGA data showed that the expression level of GOPLH3 was significantly higher than that in normal tissues from 520 HNSCC patients (Cancer Genome Atlas, 2015) (Fig. 1a). Meanwhile, we verified the differential expression of GOLPH3 based on squamous carcinoma cells in vitro (Fig. 1d **- e**). Studies have shown that GOLPH3 is highly expressed in esophageal squamous cell cancer (ESCC) and promotes tumorigenesis and progression (J. H. Wang et al., 2012; J. H. Wang et al., 2017a). We explored the phenotype and subcutaneous tumorigenesis via the FaDu cells of stable knockdown and overexpressing of GOLPH3. We found that GOLPH3 significantly affected the growth, proliferation and migration of HSCC cells (Fig. 2–4). Therefore, it indicated that GOLPH3 was an important factor in HSCC tumorigenesis and progression.

The phenotype of GOLPH3 capable for activating the mTOR signaling pathway is well confirmed (Rizzo et al., 2017; Makowski et al., 2017), and PI3K-AKT-mTOR pathway activity is enhanced in most HNSCC (Z. Wang et al., 2017b). However, GOLPH3 can also affect mTOR signaling to promote tumorigenesis and transformation via JAK2-STAT3 and Wnt/β-catenin axis (Wu et al., 2018; Sun et al., 2017). Therefore, the molecular mechanism of GOLPH3 activates the AKT-mTOR pathway needs further study. In this study, we

analyzed the data of HNSCC patients from the TCGA database, and to further confirm the correlation between GOLPH3 and axis in HSCC (Fig. 5a **-** **c**). The expression levels of protein verified positive correlation between GOLPH3 and AKT-mTOR pathway in vitro (Fig. 5d). This part demonstrated that GOLPH3-AKT-mTOR axis was one of the key mechanisms for tumorigenesis and development of HSCC.

In conclusion, our study confirmed that GOLPH3 was highly expressed in HSCC tumors and promotes tumorigenesis and development. We further revealed the important correlation and mechanism between GOLPH3 and AKT-mTOR pathways in HSCC. However, we need to further explore the physiological and biochemical effects of the GOLPH3 on HSCC tumor cells, which is conducive to a deeper understanding of the function and mechanism. To summarize, this study provides a potential target for the treatment and research of HSCC.

Declarations

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Author contributions

Study concept and design: JM.L, T.Z and M.Z; Acquisition of data: JM.L, T.Z; Analysis and interpretation of data: JM.L, T.Z, YK. W; Statistical analysis: YK.W, SW.G; Drafting of the manuscript: JM.L, T.Z, M.Z; Critical revision and final approval of the manuscript: M.Z. All authors contributed to the article and approved the submitted version.

Declaration

Conflict of interests

The authors declare that they have no competing interests.

Ethics Statement

The studies involving animal experiments were reviewed and approved by the Animal Care and Use Committee of People's Hospital of Deyang city (Sichuan, China).

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Figures

Figure 1

GOLPH3 is upregulated in HNSCC and FaDu cells. **a** GOLPH3 was significantly upregulated in HNSCC relative to normal tissues from TCGA database (*$p < 0.05$, **$p < 0.01$). **b** The correlation between prognostic survival time of patients and expression level of GOLPH3 in HNSCC from TCGA database (R = 0.211, $p = 0.008$, n = 158). **c** Relative mRNA expression level of golph3 in WUS-HN30 and FaDu cells by qPCR (*$p < 0.05$). **d** Expression level of WUS-HN30 and FaDu cells by western blotting (*$p < 0.05$).

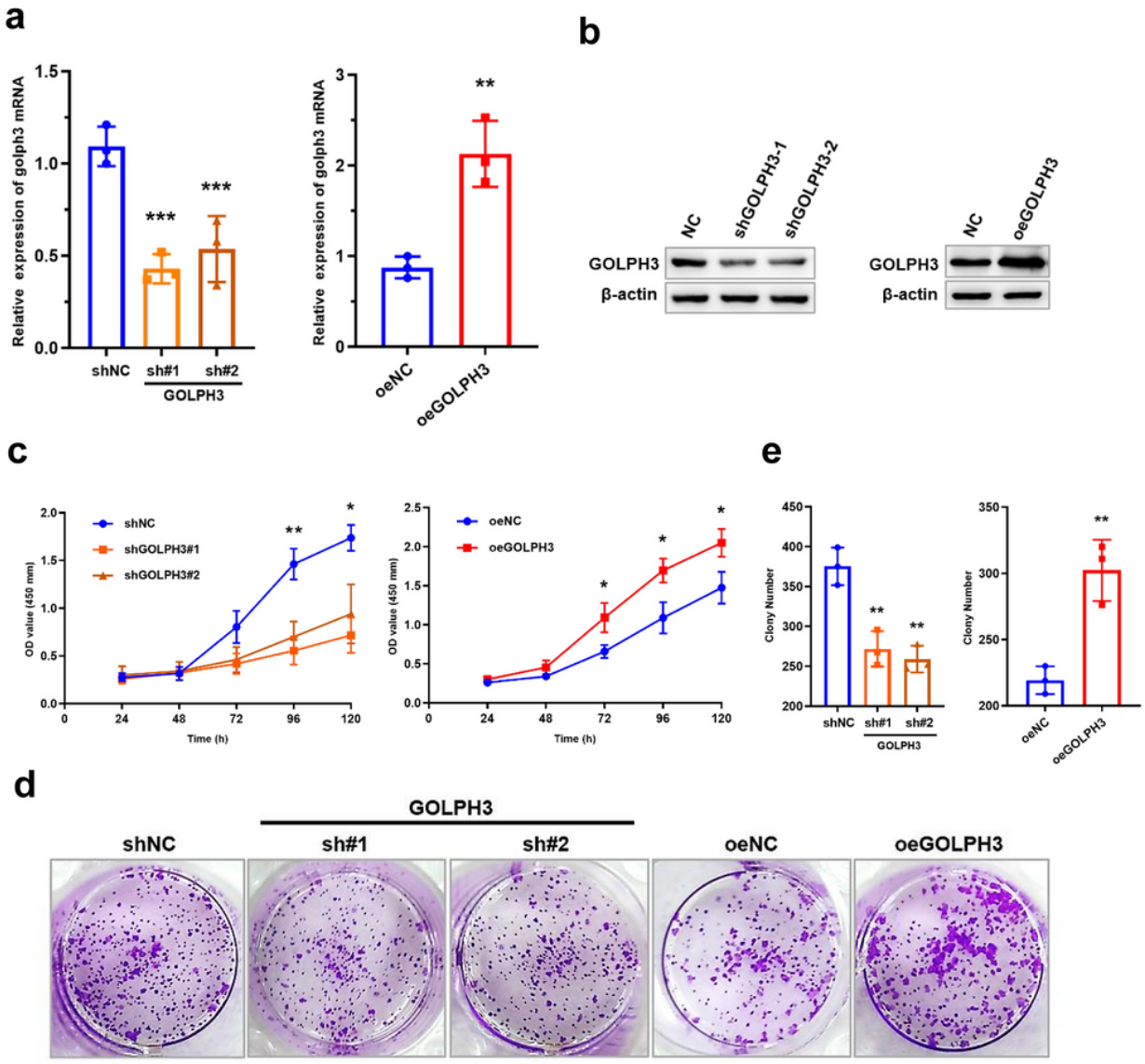

Figure 2

GOLPH3 promotes the proliferation of HSCC cells in vitro. **a** and **b** qPCR and western blotting showed FaDu cell lines with GOLPH3 stable knockdown and over expression after constructing with lentivirus (**p

< 0.01, ***p < 0.001). **c** - **e** CCK8 and colony formation assay determined the proliferation ability of GOLPH3 knockdown and over expression cell lines (Data are displayed as the mean ± SD, *p < 0.05, **p < 0.01).

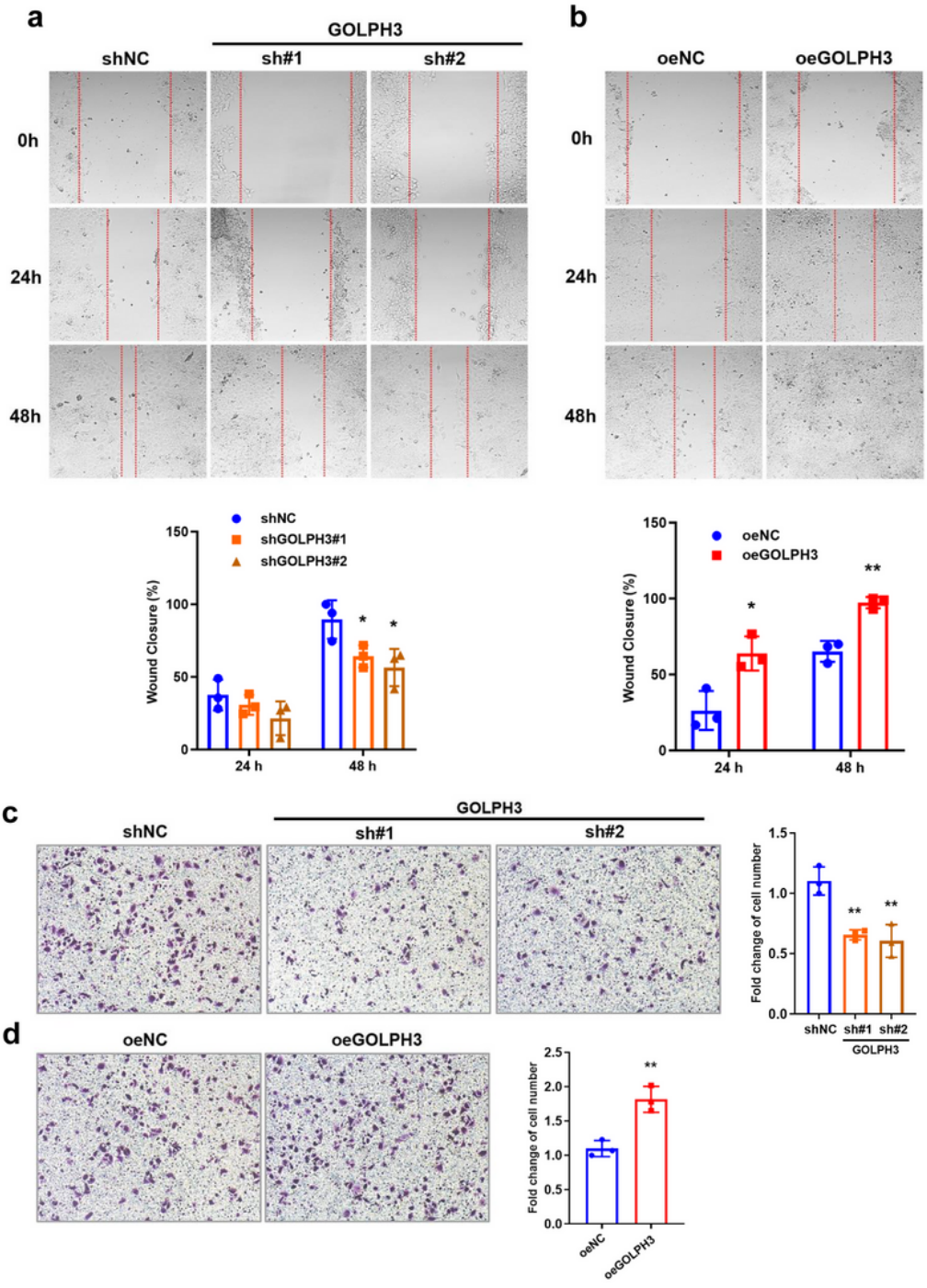

Figure 3

GOLPH3 promotes migration of HSCC in vitro. **a** and **b** Wound healing assay determined migration ability of the GOLPH3 stable knockdown and over expression FaDu cell lines (Data are displayed as mean ± SD, *$p < 0.05$, **$p < 0.01$). **c** and **d** Trans-well assay determined migration ability of the GOLPH3 stable knockdown and over expression FaDu cell lines (Data are displayed as mean ± SD, **P < 0.01).

Figure 4

GOLPH3 promotes HSCC tumor growth in vivo. **a** Display of solid tumors from tumorigenic assay of subcutaneous injection with stable GOLPH3 knockdown and over expression FaDu cell lines. **b** and **c** show the weight and volume of tumors at 15 days after injection respectively (**$p < 0.01$, ***$p < 0.001$). **d** The results of immunochemistry for tumors of CTL, oeGOLPH3 and shGOLPH3 respectively.

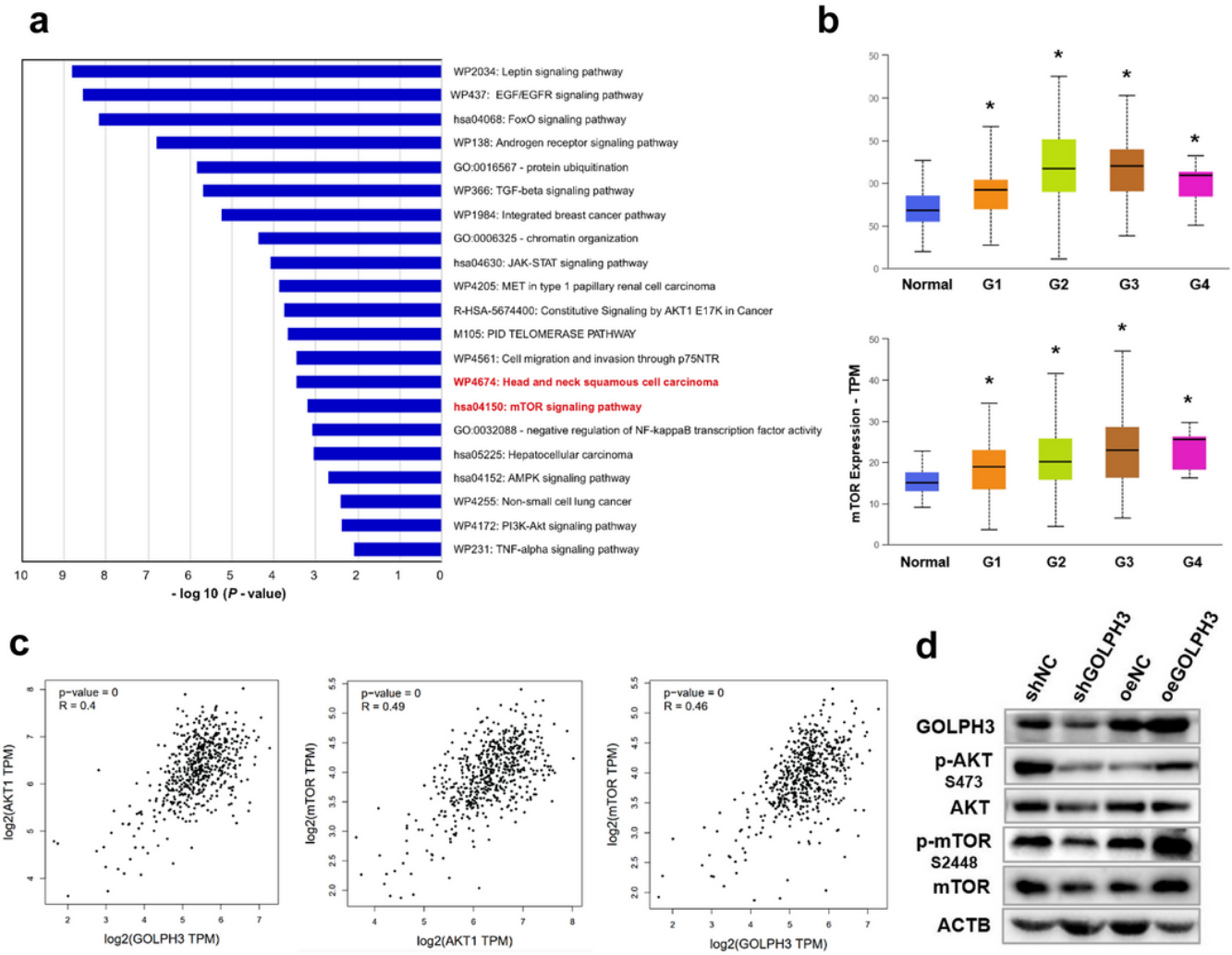

Figure 5

GOLPH3 activates the AKT - mTOR signaling pathway in HSCC in vitro. **a** The results of enrichment analysis of GOLPH3 positively correlated with genes in HNSCC from TCGA database. **b** and **c** Expression level of AKT1 and mTOR in HNSCC and correlation between GOLPH3 and AKT1, mTOR expression from TCGA database respectively (*$p < 0.05$). **d** Western blot confirmed relationship between GOLPH3 and AKT - mTOR signaling pathway via GOLPH3 stable knockdown and over expression FaDu cell lines.