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GOLPH3 is a novel marker of poor prognosis and a potential therapeutic target in human renal cell carcinoma

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Background: Golgi phosphoprotein 3 (GOLPH3) has been reported to be involved in the development of several human cancers. The present study was conducted to investigate the expression of GOLPH3 and its prognostic significance in renal cell carcinoma (RCC). Meanwhile, the function of GOLPH3 in human RCC was further investigated in cell culture models.

Methods: Expression of GOLPH3 was examined in 43 fresh RCC tissues and paired adjacent normal renal tissues by real-time quantitative PCR and western blotting. Immunohistochemistry for GOLPH3 was performed on additional 218 RCC tissues. The clinical significance of GOLPH3 expression was analysed. Downregulation of GOLPH3 was performed using small-interfering RNA (siRNA) in Caki-1 and 786-O cells with high abundance of GOLPH3, and the effects of GOLPH3 silencing on cell proliferation, migration, invasion *in vitro*, and tumour growth *in vivo* were evaluated.

Results: Expression of GOLPH3 was upregulated in the majority of the RCC clinical tissue specimens at both mRNA and protein levels. Clinicopathological analysis showed that GOLPH3 expression was significantly correlated with T stage ($P < 0.001$), lymph-node status ($P = 0.003$), distant metastasis ($P < 0.001$), tumour-node-metastasis (TNM) stage ($P < 0.001$), and Fuhrman grade ($P = 0.001$). Expression of GOLPH3 was inversely correlated with both overall and recurrence-free survival of RCC patients. Multivariate analysis showed that GOLPH3 expression was an independent prognostic indicator for patient's survival. Knockdown of the GOLPH3 expression reduced cell proliferation, anchorage-independent growth, migration, invasion, and tumour growth in xenograft model mice.

Conclusions: These results suggest that GOLPH3 expression is likely to have important roles in RCC development and progression, and that GOLPH3 is a prognostic biomarker and a promising therapeutic target for RCC.

Renal cell carcinoma (RCC) represents ~3% of all adult malignancies (Rini *et al*, 2008), being the most common cancer of the adult kidney (Rini *et al*, 2009). Approximately 60 920 new cases of RCC were diagnosed in the United States in 2011, with estimated 13 120 deaths (Siegel *et al*, 2011). Worldwide, over 200 000 new cases of RCC are diagnosed annually and more than 100 000 deaths occur from this disease (Gupta *et al*, 2008). Because

there are no recommended screening tests for RCC, about 25–30% of patients, at the time of diagnosis, present with metastatic disease (Lam *et al*, 2005). Surgery is the mainstay of therapy for early-stage, localised RCC; however, surgical resection of locally advanced disease is associated with a high recurrence rate (Jiang *et al*, 2008). Furthermore, for metastatic RCC (mRCC), surgery does not usually change the progressive nature of the disease

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(Chan and Marshall, 1998). The clinical outcome of mRCC is poor, with a median survival of only 1.5 years in a recent cohort (Patil *et al*, 2010). Metastatic RCC is typically resistant to conventional chemotherapy, radiotherapy, and hormonal therapy (Lam *et al*, 2008). Cytokine therapies have long been the standard of care in mRCC. However, these agents only provide clinical benefit to a small subset of patients and are associated with a significant toxicity (Motzer *et al*, 2000; Yang *et al*, 2003). Novel therapeutic agents that target angiogenesis, such as sunitinib and axitinib, have been demonstrated to be more effective in treating for mRCC. Objective response rates of 40–50% have been reported with these targeted agents (Rixe *et al*, 2007; Motzer *et al*, 2009; Tomita *et al*, 2011). Nevertheless, durable responses are rare and most patients eventually develop progressive disease (Albiges *et al*, 2012). Therefore, novel alternative molecular therapeutic targets need to be discovered.

Golgi phosphoprotein 3 (GOLPH3), also known as GPP34/GMx33/MIDAS, is a newly identified protein with a molecular weight of 34 kDa in the trans-Golgi matrix, which has a role in anterograde and retrograde Golgi trafficking (Wu *et al*, 2000; Nakashima-Kamimura *et al*, 2005; Snyder *et al*, 2006). It is localised to 5p13, a region frequently amplified in several types of cancer (Böhm *et al*, 2002; Yokoi *et al*, 2002; Gorringer *et al*, 2005). It has recently been recognised as a novel oncogene involved in tumorigenesis *via* enhancing activity of the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase known to regulate cell growth, proliferation, and survival (Abraham, 2009; Scott *et al*, 2009). Downregulation of GOLPH3 abrogates transformation and tumour cell proliferation in GOLPH3-amplified cell lines, whereas ectopic expression of GOLPH3 drives transformation of primary cell lines and enhances mouse xenograft tumour growth *in vivo* (Scott *et al*, 2009; Scott and Chin, 2010).

Recent studies have documented that GOLPH3 is overexpressed in many types of human cancers such as breast cancer (Zeng *et al*, 2012), prostate cancer (Hua *et al*, 2012), oral tongue cancer (Li *et al*, 2012), oesophageal cancer (Wang *et al*, 2012), gastric cancer (Hu *et al*, 2013), and glioma (Zhou *et al*, 2012). More importantly, the aberrant expression of GOLPH3 in these cancers has been indicated to be correlated with clinically aggressive behaviour and patient survival. Silencing of GOLPH3 using small-interfering RNAs (siRNAs) causes reduced tumour cell migration and invasion capacity in glioma-derived cell line, U251 and U87 (Zhou *et al*, 2013). These findings suggest an involvement of GOLPH3 in cancer progression. However, it is still not known about its expression pattern, clinical relevance, and biological function in RCC. With this in mind, we undertook a study with two objectives: (a) to determine the correlations between GOLPH3 expression and various clinicopathologic parameters, and its prognostic value for survival of patients with RCC and (b) to determine whether silencing of GOLPH3 in RCC cell lines affects cell proliferation, migration, invasion, and *in vivo* tumour growth.

Here, we showed that GOLPH3 is frequently overexpressed in RCC tissues, and that this upregulation is significantly associated with worse prognosis of RCC patients. Depletion of GOLPH3 expression using the siRNA technique in 786-O and Caki-1 cells decreased cell proliferation, migration, and invasion. *In vivo* studies further showed that GOLPH3 silencing dramatically retarded xenograft tumour growth in nude mice. Together, our data highlight an important role for GOLPH3 in controlling RCC progression.

MATERIALS AND METHODS

Patients and surgical specimens. A series of 218 patients with histologically confirmed RCC (178 clear cell RCC, 26 papillary

RCC, and 14 chromophobe RCC) were included in the study. The patients underwent nephrectomy in the Department of Urology, First Affiliated Hospital of Gannan Medical University (Ganzhou, China) between 2003 and 2011. None of the patients received chemotherapy or radiotherapy before surgery. After surgical resection, tumour specimens and the corresponding adjacent non-tumour tissues were collected and stored in liquid nitrogen until use. Parts of each sample were fixed in formalin, embedded in paraffin and stored in the Department of Pathology, First Affiliated Hospital of Gannan Medical University. In total, 141 of these 218 patients were men and 77 were women. The median age of the patients was 58 years (range, 31–76 years). All of the cases were staged according to the tumour-node-metastasis (TNM) staging system and nuclear grade was evaluated on the basis of the Fuhrman criteria. The histological subtypes were classified in accordance with the 2002 AJCC/UICC classification system. Detailed information is listed in Table 1. All patients were followed with clinical and radiologic examinations. The median follow-up time was 45.4 months (range, 3–94 months). The study protocol was approved by the Ethics Committee of First Affiliated Hospital of Gannan Medical University (Ganzhou, China), and written informed consent was obtained from each patient.

Real-time quantitative PCR. Total RNA was isolated from tissue specimens or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNAs were synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in a 7900 Real-Time PCR System (Applied Biosystems). The PCR primers used in the study were GOLPH3: 5'-GGGCGACTCCAAGGAAAC-3' (forward) and 5'-CAGCCACGTAATCCAGATGAT-3' (reverse) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-ATTCCACCCATGGCAAATTC-3' (forward) and 5'-ATTCCACCCATGGCAAATTC-3' (reverse). Glyceraldehyde 3-phosphate dehydrogenase was used as the reference gene. Ct values of the samples were calculated, and the relative levels of GOLPH3 mRNA were analysed by the $2^{-\Delta\Delta Ct}$ method.

Western blot assay. Tissues or cells were lysed in lysis buffer containing protease inhibitor cocktail. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). Equivalent amounts of proteins were separated by SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes (Bio-Rad). After being blocked in Tris-buffered saline (TBS) containing 5% non-fat milk, the membranes were incubated with specific primary antibody against GOLPH3 (1:1000; ProteinTech Group, Inc., Chicago, IL, USA) at 4 °C for 12 h and then with horseradish peroxidase-conjugated anti-rabbit antibody (Zhongshan, Beijing, China) for 2 h at room temperature. Proteins were visualised using ECL (Pierce, Rockford, IL, USA) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). The relative protein levels were calculated based on GAPDH as the loading control.

Immunohistochemistry staining. Immunohistochemical staining was done on 4- μ m-thick sections from tissue microarrays (TMAs). The TMA was constructed as described previously (Kononen *et al*, 1998). Sections were dewaxed in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol l⁻¹ of citrate buffer (pH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities for 30 min with methanol containing 0.3% H₂O₂, the sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4 °C with primary polyclonal rabbit anti-human GOLPH3 antibody (1:200; Protein-Tech Group, Inc.). After washing thrice with PBS, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit

IgG for 30 min, followed by reaction with diaminobenzidine and counterstaining with Mayer’s haematoxylin. Negative control was done by omission of the primary antibody and substituting it with non-specific rabbit IgG.

For the immunohistochemical assessment, the product of the scores of staining intensity and quantity of immunoreactive tumour cells was calculated based on the following scoring system: the intensity ranged from 0 = no staining, 1 = weak staining, 2 = moderate staining to 3 = strong staining; the quantity comprised 0 = no expression, 1 = positivity in <10%, 2 = positivity in 10–35%, 3 = positivity in 35–75%, and 4 = positivity in >75%. The final immunohistochemical score (IHS; ranging from 0 to 12) was obtained by multiplication of the intensity score and the quantity score. The cutoff value for high and low expression was determined on the basis of a heterogeneity value measured through log-rank

statistical analysis with respect to overall survival (OS). An IHS of ≥ 6 was defined as high GOLPH3 expression and an IHS of ≤ 4 indicated low GOLPH3 expression. Three board-certified pathologists (HY, SP, and XH) with >10 years of experience in uropathology evaluated the immunostaining in a blinded manner without any knowledge of the clinicopathological data. In the few instances of discrepant scoring, a consensus score was determined with a multiheaded microscope. We defined ‘consensus’ when at least two reviewers were in agreement.

Cell lines. Human RCC cell lines 786-O and Caki-1 were purchased from the American Type Culture Collection (Rockville, MD, USA). Another three human RCC cell lines, A498, ACHN, and OS-RC-2, were preserved in our institute. Immortalised normal human proximal tubule epithelial cell line HK-2 was obtained from Shanghai Cell Bank (Shanghai, China). HK-2 cells

Table 1. GOLPH3 protein expression in 218 RCC tissues determined by immunohistochemistry

Variable	Patients	No. of patients (%)		P-value
		GOLPH3 low	GOLPH3 high	
Age, years (median 58)				
< 58	135	66 (48.9)	69 (51.1)	0.485
≥ 58	83	36 (43.4)	47 (56.6)	
Gender				
Male	141	65 (46.1)	76 (53.9)	0.887
Female	77	37 (48.1)	40 (51.9)	
T stage				
T ₁	125	72 (57.6)	53 (42.4)	<0.001
T ₂	38	17 (44.8)	21 (55.2)	
T ₃	48	12 (25.0)	36 (75.0)	
T ₄	7	1 (14.3)	6 (85.7)	
Lymph-node status				
N ₀	181	93 (51.4)	88 (48.6)	0.003
N ₁₋₂	37	9 (24.3)	28 (75.7)	
Distant metastasis				
M ₀	196	101 (51.5)	95 (48.5)	<0.001
M ₁	22	1 (4.5)	21 (95.5)	
TNM stage				
I	126	73 (57.9)	53 (42.1)	<0.001
II	29	13 (44.9)	16 (55.1)	
III	34	13 (38.2)	21 (61.8)	
IV	29	3 (10.3)	26 (89.7)	
Fuhman grade				
G ₁	103	62 (60.2)	41 (39.8)	0.001
G ₂	61	24 (39.3)	37 (60.7)	
G ₃	40	14 (35.0)	26(65.0)	
G ₄	14	2 (14.3)	12 (85.7)	
Histopathologic subtype				
Clear cell RCC	178	79 (44.4)	99 (55.6)	0.323
Papillary RCC	26	15 (57.7)	11 (42.3)	
Chromophobe RCC	14	8 (57.1)	6 (42.9)	

Abbreviations: GOLPH3 = Golgi phosphoprotein 3; RCC = renal cell carcinoma; TNM = tumour-node-metastasis.

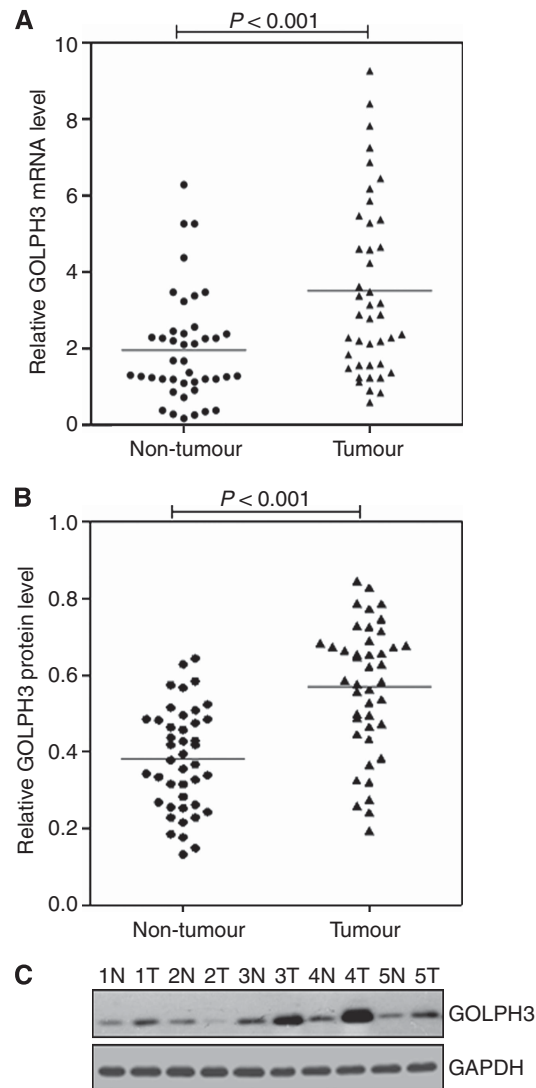


Figure 1. The expression of GOLPH3 mRNA and protein in the human RCC surgical specimens, as evaluated by real-time quantitative PCR and western blotting. (A) The relative mRNA expression of GOLPH3 was higher in 43 RCC tumour tissues than in matched adjacent non-tumour tissues ($P < 0.001$). (B) The GOLPH3 protein expression was higher in the tumour tissues than in matched adjacent non-tumour tissues ($P < 0.001$). (C) Expression of GOLPH3 protein in five representative pairs of RCC tissues is presented. N = non-tumour tissues; T = RCC tissues.

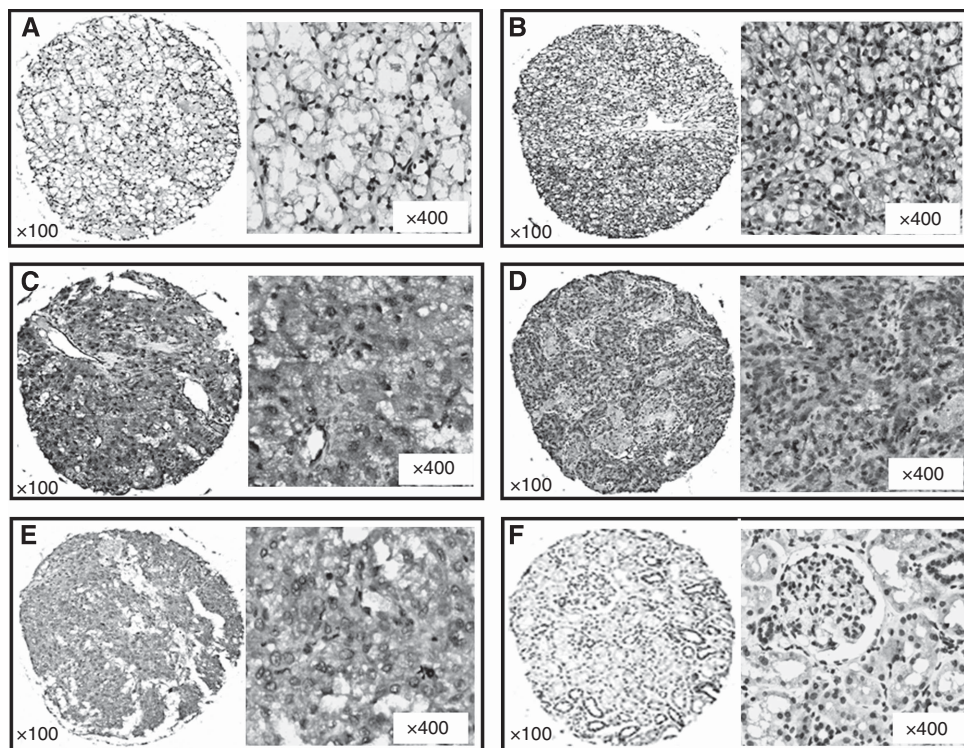


Figure 2. Immunohistochemical staining of GOLPH3 in RCC specimens and normal renal tissues. GOLPH3 immunohistochemical staining was performed on different types of RCCs and normal renal tissues, and representative images are shown. (A) Weak staining of GOLPH3 in clear cell RCC. (B) Moderate staining of GOLPH3 in clear cell RCC. (C) Strong staining of GOLPH3 in clear cell RCC. (D) GOLPH3 staining in papillary RCC. (E) GOLPH3 staining in chromophobe RCC. (F) Extremely weak or negative GOLPH3 staining in renal tubules of normal renal tissues (original magnification: $\times 100$ and $\times 400$).

were cultured in K-SFM medium (Gibco Life Technologies, Grand Island, NY, USA), and other cells were cultured in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) with 10% fetal bovine serum (FBS), 50 U ml^{-1} of penicillin, and $50 \mu\text{g ml}^{-1}$ of streptomycin. All cells were cultured in a sterile incubator maintained at 37°C with 5% CO_2 .

Gene silencing using siRNA. The siRNA targeting GOLPH3 and control siRNA were purchased from Shanghai Genepharma Co. Ltd. (Shanghai, China). The siRNA sequence for GOLPH3 was 5'-GUUA AGAAAUGUACGGGAATT-3'. Cells were transfected with either GOLPH3 or control siRNA using Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Following transfection, the mRNA and protein levels were assessed 48 h later.

MTT assay. Cells were plated in 96-well culture plates at about 5×10^3 cells per well 24 h after transfection. Then, $20 \mu\text{l}$ of 5 mg ml^{-1} MTT solution was added to each well and incubated for 4 h at 37°C , the media was removed from each well, and the resultant MTT formazan was solubilised in $150 \mu\text{l}$ of DMSO. The absorbance values at 490 nm were measured using a microplate reader (Bio-Rad). The experiment was repeated three times and each experiment had six replicate wells.

Colony formation assay. Cells were transfected with control or GOLPH3 siRNA for 48 h and then plated at 1×10^3 cells per well of a 6-well plate in triplicate. After 14 days, the plates were stained with crystal violet, and colonies with > 50 cells were counted.

Anchorage-independent colony growth assay. Cells were transfected with control or GOLPH3 siRNA for 48 h. Cells were then plated on a 0.6% agarose base in 6-well plates (1.0×10^3 cells per well) in 1 ml of RPMI-1640 medium containing 10% FBS and 0.3%

agarose. After 16 days, colonies were stained with crystal violet, and colonies containing at least 50 cells were counted.

Scratch migration assay. Cells were seeded to 12-well plates and transfected with control or GOLPH3 siRNA. At 24 h after transfection, cells were scratched using the tip of a sterile $200\text{-}\mu\text{l}$ pipette in each well. The plates were washed twice with PBS to remove the detached cells, and incubated at 37°C in 5% CO_2 . Wound closure was observed and measured after 24 h. The experiment was done in triplicate.

Matrigel invasion assay. Cell invasion assay was performed using a 24-well transwell chamber with a pore size of $8 \mu\text{m}$ (Costar, New York, NY, USA). The inserts were coated with $50 \mu\text{l}$ Matrigel (dilution at 1:2; BD Bioscience, Franklin Lakes, NJ, USA). Cells were trypsinised after transfection with control or GOLPH3 siRNA for 48 h and transferred to the upper Matrigel chamber in $100 \mu\text{l}$ of serum-free medium containing 1×10^5 cells and incubated for 24 h. The lower chamber was filled with medium containing 10% FBS as a chemoattractant. After incubation, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were stained using crystal violet. The numbers of invaded cells were counted in five randomly selected high power fields under a microscope. This experiment was performed in triplicate.

Cancer xenograft model and tumorigenicity assay. Four-week-old male BALB/c nude mice (Experimental Animal Center of Shanghai, Shanghai, China) were housed in a specific pathogen-free environment at the Animal Laboratory Unit, Gannan Medical University, China. Cells (1.0×10^7) were suspended in $100 \mu\text{l}$ PBS and injected subcutaneously in the right flank region of nude mice. After 2 weeks, the injected nude mice with tumour burden were randomly divided into two groups ($n = 6$ for each group) to receive

intratumoral injection of control siRNA or GOLPH3 siRNA complex every 5 days. Each complex contained 10 μg of siRNA and 7.5 μl Oligofectamine (Invitrogen) in PBS. The tumour volumes were measured two times a week with a caliper and calculated according to the formula: length \times width²/2. All mice were killed humanely after five times of treatment, and the resected tumours were weighed. All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Gannan Medical University, Ganzhou, China.

Statistical analysis. The statistical analyses were performed using the Statistical Package for the Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA). A paired-samples *t*-test was used to compare GOLPH3 mRNA and protein expression in the RCC tissues with that of their paired adjacent non-tumour tissue samples. The relationship between GOLPH3 protein expression and the clinicopathological features was analysed using χ^2 tests. For survival analysis, three end points (cancer relapse, cancer progression, and death) were used to calculate recurrence-free survival (RFS) for non-metastatic patients, progression-free survival (PFS) for metastatic patients, and OS for all patients, respectively. Recurrence-free survival was defined as the time interval between the date of surgery and the date of identification of disease recurrence; PFS was defined as the time interval between the date of surgery and the date of identification of progressive disease or death, and OS was defined as the time interval between the date of surgery and the date of death. The log-rank test and Kaplan–Meier method were used for survival analyses. Univariate and multivariate analyses were done according to Cox proportional hazard model. Unpaired two-tailed Student's *t*-tests were used to analyse comparisons between the two groups. In all tests, a *P*-value of <0.05 was regarded as statistically significant.

RESULTS

Expression of GOLPH3 mRNA and protein in RCC tissues. We first determined the GOLPH3 expression in 43 matched pairs of fresh RCC samples and adjacent non-tumour tissue samples. The results revealed a statistically significant elevation of GOLPH3 mRNA in tumours, as compared with the matched adjacent non-tumour tissues ($P < 0.001$, Figure 1A). Consistent with the mRNA data, GOLPH3 protein in tumour tissues was also significantly higher than that in adjacent non-tumour tissues ($P < 0.001$, Figure 1B). The protein level of GOLPH3 in five representative pairs of samples is shown in Figure 1C.

Immunohistochemical analysis of GOLPH3 expression in RCC samples and its relationship to clinicopathological parameters.

We further analysed GOLPH3 protein level in 218 RCC tissues and 84 adjacent non-tumour tissues using an immunohistochemical approach. Representative examples of GOLPH3 staining in RCC patients are provided in Figure 2A–E. High GOLPH3 expression was observed in 116 patients (53.2%). The adjacent non-tumour tissues showed either no or very weak protein expression (Figure 1F). The association between the expression of GOLPH3 and various clinicopathological parameters is listed in Table 1. The GOLPH3 staining level significantly correlated with T stage, lymph-node status, distant metastasis, TNM stage, and Fuhrman grade (all $P < 0.05$).

GOLPH3 expression and patient survival. The OS rates for patients with high GOLPH3 expression were significantly lower than that for patients with low GOLPH3 expression (Figure 3A, $P < 0.001$). T stage, lymph-node status, distant metastasis, histopathologic subtype, Fuhrman grade, and GOLPH3 expression

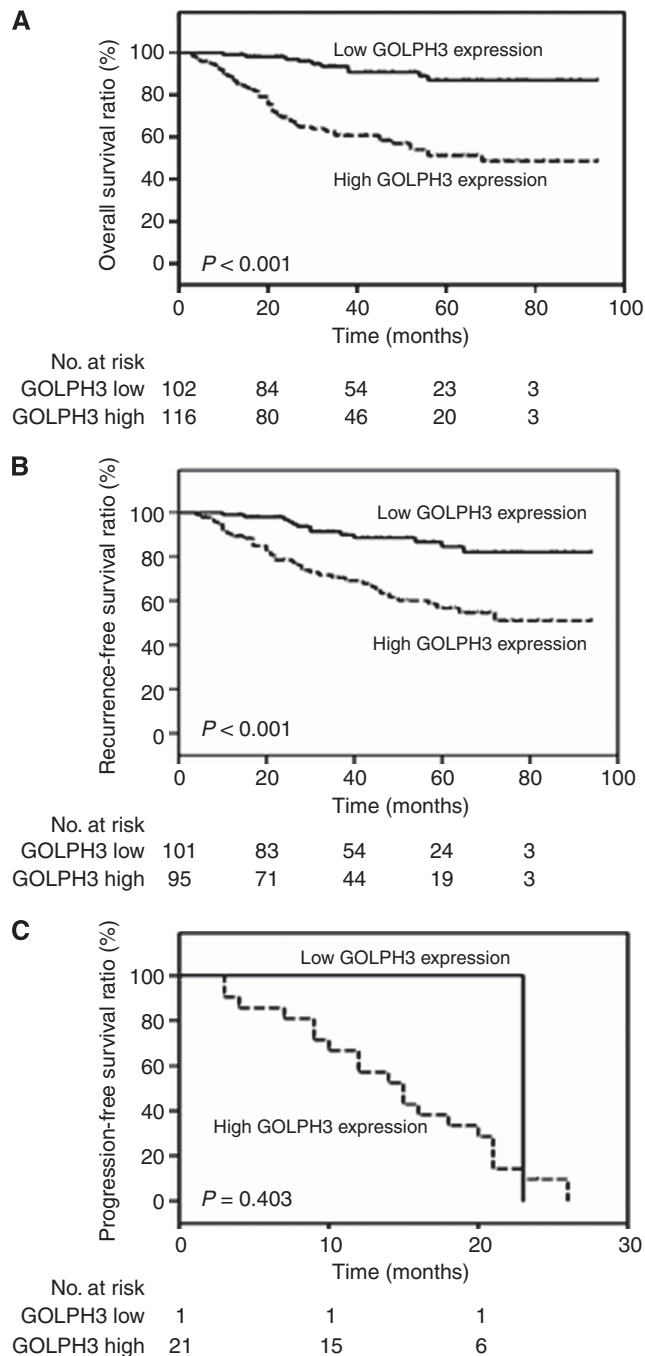


Figure 3. GOLPH3 expression and survival in RCC patients.

(A) Kaplan–Meier survival analysis of overall survival for all patients, recurrence-free survival for non-metastatic patients (B) and progression-free survival for metastatic patients (C). The log-rank test was used to calculate *P*-values.

were significant prognostic factors for OS in univariate analysis (Table 2A). By multivariable analysis, T stage ($P < 0.001$), lymph-node status ($P < 0.001$), distant metastasis ($P = 0.004$), Fuhrman grade ($P = 0.03$), and GOLPH3 expression ($P < 0.001$) were independent prognostic factors for OS (Table 2A). In non-metastasised disease (M0), the RFS rate for patients with high GOLPH3 expression was significantly lower (Figure 3B, $P < 0.001$). T stage, lymph-node status, histopathologic subtype, Fuhrman grade, and GOLPH3 expression were significant prognostic factors for RFS in univariate analysis (Table 2B). By multivariate analysis, T stage ($P < 0.001$), lymph-node status ($P < 0.001$), Fuhrman grade

Table 2. Cox regression analysis for overall survival and recurrence-free survival

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
(A) Overall survival						
T stage						
T ₃₋₄ vs T ₁₋₂	19.627	11.045–34.879	<0.001	8.747	4.249–18.005	<0.001
Lymph-node status						
N ₁₋₂ vs N ₀	15.946	9.294–27.358	<0.001	6.496	3.440–12.269	<0.001
Distant metastasis						
M ₁ vs M ₀	12.775	6.928–23.555	<0.001	2.580	1.350–4.930	0.004
Histopathologic subtype						
ccRCC vs other type	3.144	1.259–7.849	0.014	1.257	0.471–3.351	0.648
Fuhman grade						
G ₃₋₄ vs G ₁₋₂	8.160	4.876–13.656	<0.001	2.006	1.071–3.756	0.030
GOLPH3 expression						
High vs low	5.755	2.923–11.330	<0.001	5.341	2.496–11.428	<0.001
(B) Recurrence-free survival						
T stage						
T ₃₋₄ vs T ₁₋₂	16.920	9.427–30.371	<0.001	7.759	3.671–16.399	<0.001
Lymph-node status						
N ₁₋₂ vs N ₀	21.321	11.580–39.256	<0.001	7.946	3.502–18.032	<0.001
Histopathologic subtype						
ccRCC vs other type	2.824	1.121–7.115	0.028	1.802	0.691–4.703	0.229
Fuhman grade						
G ₃₋₄ vs G ₁₋₂	7.754	4.442–13.535	<0.001	2.260	1.120–4.561	0.023
GOLPH3 expression						
High vs low	3.611	1.9233–6.780	<0.001	4.416	2.193–8.894	<0.001

Abbreviations: 95% CI = 95% confidence interval; ccRCC = clear cell RCC; GOLPH3 = Golgi phosphoprotein 3; HR = hazard ratio.

($P=0.023$), and GOLPH3 expression ($P<0.001$) were independent prognostic factors for RFS (Table 2B). In metastatic disease (M1), GOLPH3 expression was not associated with PFS (Figure 3C, $P=0.403$), possibly due to the limited number of patients ($n=22$).

We further investigated the predictive value of GOLPH3 expression within different subgroups stratified according to the T stage, TNM stage, and lymph-node status. Individuals with high GOLPH3 expression had shorter OS than those with low expression in both the T₁+T₂ subgroup ($n=163$, $P=0.005$; Figure 4A) and the T₃+T₄ subgroup ($n=55$, $P<0.001$; Figure 4B), indicating that GOLPH3 could be a valuable prognostic marker for RCC in all disease stages. Similarly, the OS was significantly shorter in patients with high GOLPH3 expression in TNM stage I + II subgroup ($n=155$, $P=0.014$; Figure 4C) and the TNM stage III + IV subgroup ($n=63$, $P<0.001$; Figure 4D), or in lymph-node status N₀ ($n=181$, $P<0.001$; Figure 4E) and lymph-node status N₁₋₂ subgroup ($n=37$, $P=0.001$; Figure 4F).

GOLPH3 expression in RCC cell lines. We also detect the expression of GOLPH3 mRNA and protein in several human RCC cell lines as well as in an immortalised normal human proximal tubule epithelial cell line. As presented in Figure 5A, Caki-1, 786-O, A498, OS-RC-2, and ACHN showed higher level of GOLPH3 mRNA relative to the HK-2 normal proximal tubule epithelial cell line.

Consistent with the mRNA data, GOLPH3 protein was also elevated in those RCC cell lines compared with the HK-2 cell line (Figure 5B).

Effects of GOLPH3 depletion on cell growth *in vitro*. To further examine the functional role of GOLPH3 in RCC cells, we specifically knocked down its expression using siRNA technique in Caki-1 and 786-O cells expressing high levels of endogenous GOLPH3. We observed that GOLPH3 mRNA and protein levels were significantly reduced in cells transfected with specific siRNA for GOLPH3 compared with those transfected with control siRNA (Figure 5C and D). We next studied the impact of GOLPH3 silencing on cell proliferation *in vitro*. The results of the MTT assay showed that downregulation of GOLPH3 significantly reduced the proliferation rate in both the cell lines tested compared with the control siRNA-transfected cells ($P<0.01$; Figure 6A). Colony formation assay further showed that downregulation of GOLPH3 in two tested cell lines with transfection of GOLPH3 siRNA resulted in a clear reduction in the colony formation capacity compared with the control siRNA-transfected cells ($P<0.01$; Figure 6B).

Effect of GOLPH3 deletion on migration and invasion *in vitro*. In the scratch migration assay, downregulation of GOLPH3 significantly suppressed the migration capability of both

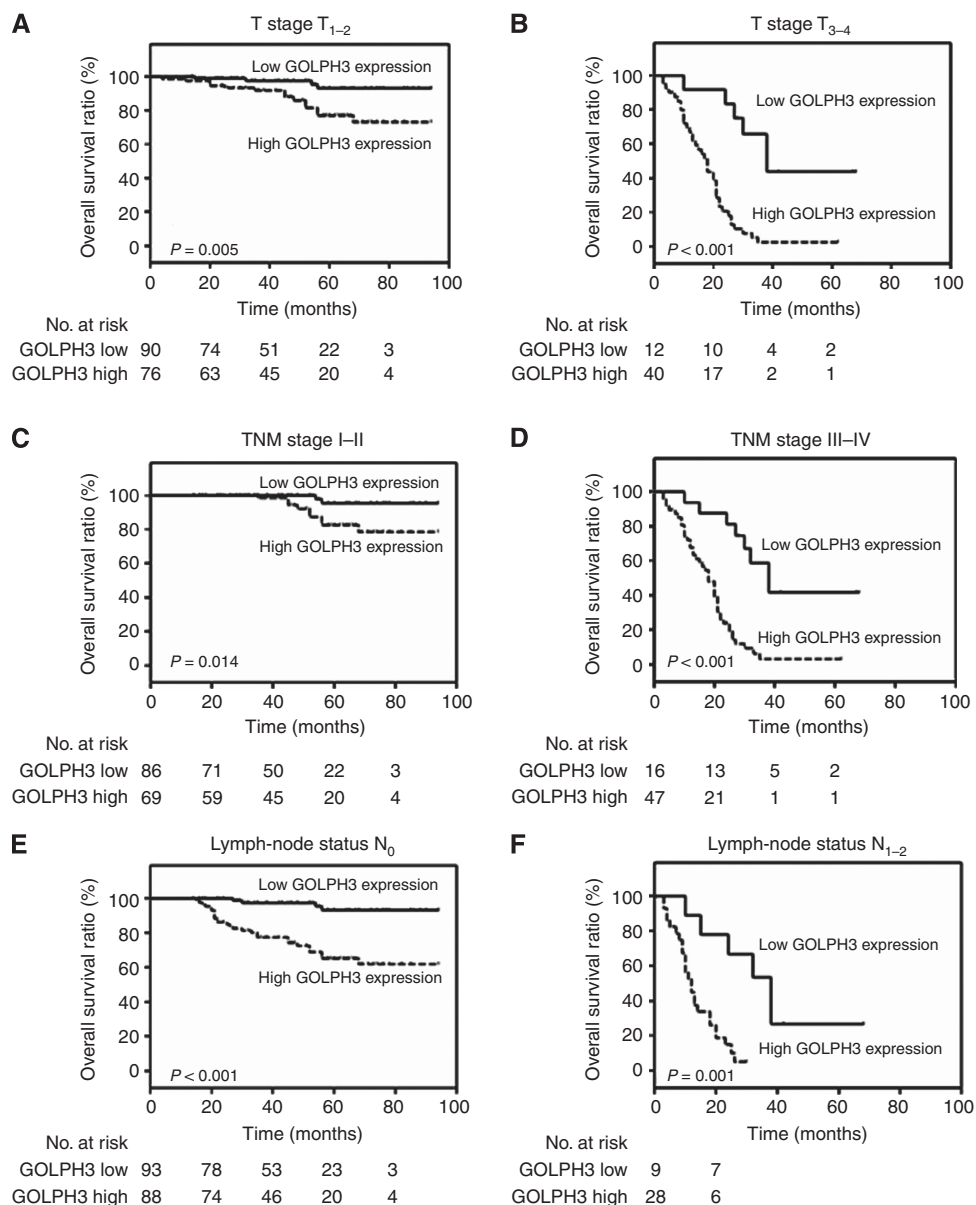


Figure 4. Kaplan–Meier analysis of the overall survival of RCC patients categorised according to the T stage, TNM stage, lymph-node status, and GOLPH3 expression. Statistical significance of the difference between curves of GOLPH3 high-expressing and low-expressing patients was compared in T stage T₁ to T₂ (A) and T stage T₃ to T₄ (B) patient subgroups, TNM stage I to II (C) and TNM stage III to IV (D) patient subgroups, lymph-node status N₀ (E) and lymph-node status N₁₋₂ (F) patient subgroups. P-values were calculated by the log-rank test.

Caki-1 and 786-O cells (Figure 7A). The migrating distance of Caki-1 cells was $294 \pm 20 \mu\text{m}$ in the control siRNA group and $138 \pm 16 \mu\text{m}$ in the GOLPH3 siRNA group ($P < 0.01$). In the 786-O cells, the migrating distance was $296 \pm 22 \mu\text{m}$ in the control siRNA group and $115 \pm 12 \mu\text{m}$ in the GOLPH3 siRNA group ($P < 0.01$). In addition, Matrigel invasion assay showed that downregulation of GOLPH3 significantly suppressed the invasiveness of both cancer cells (Figure 7B). The average cell counts crossing the matrigel-coated membrane in one high power field were 71 ± 5 for the control siRNA group and 23 ± 3 for the GOLPH3 siRNA group of Caki-1 cells ($P < 0.01$); 62 ± 4 for the control siRNA group and 18 ± 4 for the GOLPH3 siRNA group of 786-O cells ($P < 0.01$).

Depletion of GOLPH3 inhibits the tumorigenicity *in vitro* and *in vivo*. Soft agar colony formation assay revealed that GOLPH3 depletion resulted in a significant decrease in the anchorage-independent growth ability of Caki-1 and 786-O cells ($P < 0.01$; Figure 8A). To investigate whether GOLPH3 siRNA had the effect

of inhibition on tumour growth *in vivo*, a nude mice xenograft model of RCC cell line Caki-1 was established, and intratumoral injection with GOLPH3 siRNA or control siRNA complex in the nude mice with tumour burden was performed. As shown in Figure 8B, the tumour volume of GOLPH3 siRNA group from day 22 to the end was significantly smaller than control siRNA groups ($P < 0.01$). Furthermore, GOLPH3 siRNA resulted in a significant decrease in the tumour volume and weight as measured at the end of the experiment at day 42 when compared with control siRNA ($P < 0.01$; Figure 8C and D). In addition, the relative protein expression of GOLPH3 in GOLPH3 siRNA group was decreased (Figure 8E).

DISCUSSION

In the present study, we clearly showed the clinicopathologic significance of the GOLPH3 expression on a relatively large cohort

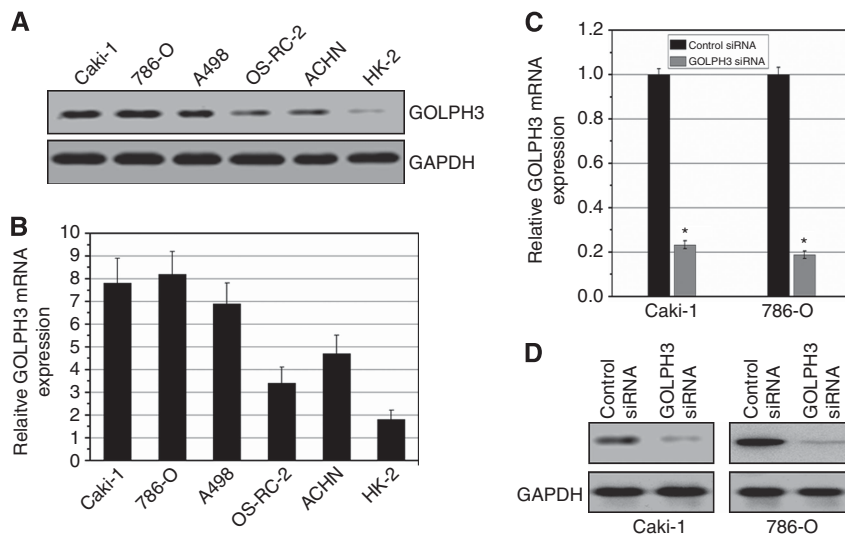


Figure 5. Expression of GOLPH3 protein and mRNA and GOLPH3 knockdown by GOLPH3 siRNA in RCC cell lines. (A and B) GOLPH3 protein and mRNA expression detected in several human RCC cell lines (Caki-1, 786-O, A498, OS-RC-2, and ACHN) as well as in a normal proximal tubule epithelial cell line HK-2 by western blotting and real-time quantitative PCR, respectively. (C) Real-time quantitative PCR analysis of GOLPH3 mRNA expression in Caki-1 and 786-O cells transfected with the specific siRNA targeting GOLPH3 for 48 h. * $P < 0.01$, relative to control. (D) Western blot analysis of GOLPH3 protein expression in Caki-1 and 786-O cells transfected with the GOLPH3 siRNA for 48 h. Efficient depletion of GOLPH3 expression was verified.

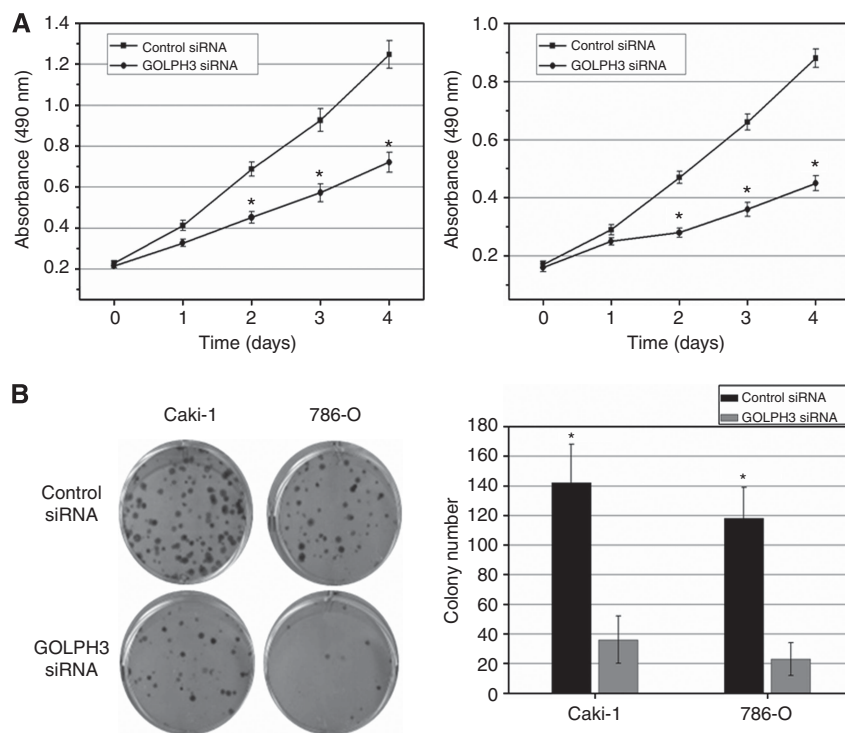


Figure 6. Effects of GOLPH3 depletion on cell growth *in vitro*. (A) Inhibition of Caki-1 and 786-O cell proliferation by GOLPH3 siRNA tested by MTT assay. (B) Inhibition of Caki-1 and 786-O cells colony formation capacity by GOLPH3 siRNA. Values are mean \pm s.d. of three independent experiments. * $P < 0.01$, relative to control.

of RCC specimens. We examined first the GOLPH3 mRNA and protein expression in paired RCC samples. We found that GOLPH3 expression was upregulated at both the transcriptional and translational levels in most RCC tumour tissues. Consistent with these observations, immunohistochemical analysis also confirmed that tumour tissues exhibited abundant GOLPH3 expression compared with adjacent non-tumour tissues. These findings suggest the possibility that upregulated expression of

GOLPH3 may provide a selective advantage in RCC tumorigenic processes.

Our immunohistochemical study also showed that high GOLPH3 expression was correlated significantly with the invasive and aggressive characteristics of RCCs, including T stage, lymph-node metastasis, distant metastasis, TNM stage, and Fuhrman grade. These results indicated that the increased expression of GOLPH3 may have a role in RCC development and progression.

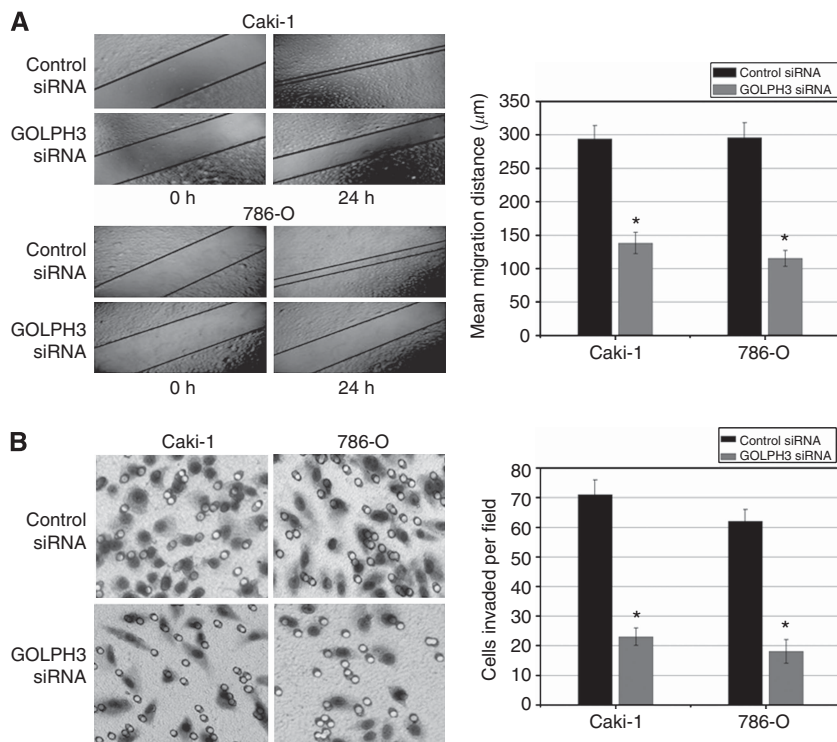


Figure 7. GOLPH3 depletion attenuated the migration and invasion capability of Caki-1 and 786-O cells *in vitro*. **(A)** Scratch migration assay showed that GOLPH3 decreased tumour cell migration. **(B)** Matrigel invasion assay showed that GOLPH3 siRNA-transfected cells resulted in low penetration through the Matrigel-coated membrane, compared with control cells. Values are mean \pm s.d. of three independent experiments. * $P < 0.01$, relative to control.

Consistent with our findings, some previous immunohistochemical studies of GOLPH3 expression on other cancers have also revealed correlations between GOLPH3 expression and clinicopathologic characteristics. In breast cancer (Zeng *et al*, 2012) and oesophageal cancer (Wang *et al*, 2012), GOLPH3 expression was positively correlated with clinical stage, TNM classification, and histological differentiation. In gastric cancers (Hu *et al*, 2013), GOLPH3 expression was associated with tumour size, histological grade, depth of invasion, lymph-node metastasis, distant metastasis, and TNM stage.

High GOLPH3 expression has previously been suggested as a predictor of poor prognosis in different cancers (Hua *et al*, 2012; Li *et al*, 2012; Wang *et al*, 2012; Zhou *et al*, 2012; Zeng *et al*, 2012; Hu *et al*, 2013). In this study, the clinical significance of high GOLPH3 expression was further substantiated by its correlation with shorter OS and RFS time in RCC patients. Univariate survival analyses showed that high GOLPH3 expression was associated with the risk of death from RCC in the whole patient cohort. After excluding cases with initial metastases, high GOLPH3 expression increased the risk of RCC recurrence ($n = 196$). More importantly, multivariate analysis also showed that GOLPH3 expression was an independent prognostic factor for both OS and RFS. Furthermore, when patients were stratified into subgroups according to T stage, TNM stage, and lymph-node status, high GOLPH3 expression also indicated a shorter OS time. Thus, GOLPH3 expression seems to have the potential to predict RCC patient outcome. The examination of GOLPH3 expression by immunohistochemistry may, therefore, be used as an additional tool in identifying those patients at risk of tumour recurrence and/or progression, and it may be a helpful criterion to optimise individual therapy management.

In attempting to determine the possibility of GOLPH3 as a therapeutic target, we employed the siRNA technique for knock-down of its expression and analysed its phenotype. We found that

a transient knockdown of GOLPH3 strikingly inhibited growth and colony formation of RCC cells *in vitro*. Moreover, previous study (Zeng *et al*, 2012) has shown that the deletion of GOLPH3 expression had an antitumorigenic effect *in vivo* in breast cancer, and we similarly showed that the deletion of GOLPH3 expression in RCC cell lines inhibited tumour growth *in vivo* using siRNA technique. Our results were consistent with Zeng *et al* in terms of antitumour activity that the deletion of GOLPH3 expression caused in RCC. Consequently, GOLPH3 seemed to be a critical factor for the proliferation of cancer cells, and therefore, it should be a good therapeutic target for halting proliferation of RCC.

In addition, the findings presented in this study demonstrate that knockdown of endogenous GOLPH3 in RCC cell lines greatly reduces cell migration and invasion capacity *in vitro*, suggesting that GOLPH3 has an important role in RCC invasiveness. These results indicate that overexpression of GOLPH3 may contribute to the malignancy (invasion and metastasis) of RCC. The molecular mechanism by which GOLPH3 increases motile and invasive potential in cancer cells is not fully understood. In an *in vitro* study, GOLPH3 could regulate glioma cell migration and invasion through modulation of RhoA expression (Zhou *et al*, 2013). Although the present study could not prove the mechanism by which GOLPH3 promotes tumour invasion, our finding of a distinct association between GOLPH3 and tumour invasiveness may have therapeutic as well as prognostic implications.

There are several limitations to our study. First, our study was a single hospital-based and retrospective study. Future studies based on a multicentre or community-based prospective study with more extensive collection of potential cofounders are also required. Second, the molecular mechanisms of GOLPH3-induced metastasis in RCC need to be further elucidated. Third, the *in vivo* metastasis assay should be performed to further testify the roles of GOLPH3 in metastasis of human RCC.

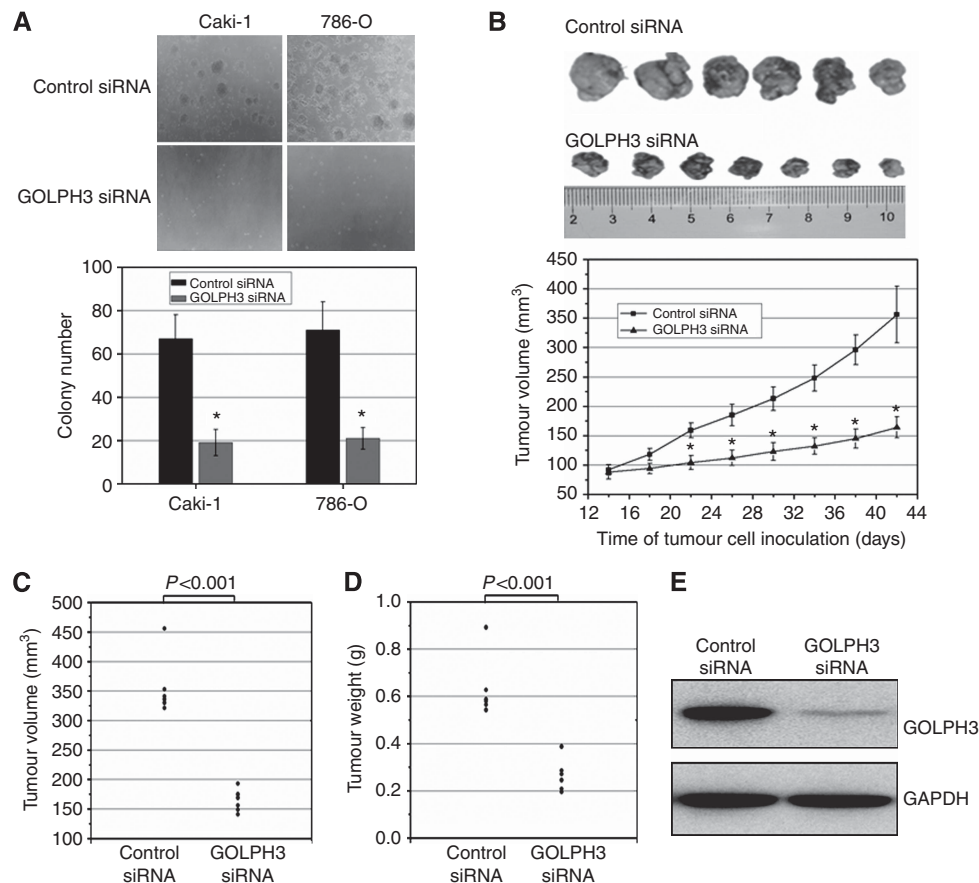


Figure 8. GOLPH3 depletion inhibits the tumorigenicity of RCC cells *in vitro* and *in vivo*. **(A)** Inhibition of Caki-1 and 786-O cells anchorage-independent colony formation capacity by GOLPH3 siRNA. Data are presented as mean \pm s.d. * P <0.01, relative to control. **(B)** Knockdown of GOLPH3 by siRNA inhibits the growth of Caki-1-derived xenografts in nude mice. *Top*, macrographic image showed that the tumour size of GOLPH3 group was markedly smaller on the 42nd day after tumour cells inoculation than that of control group; *bottom*, growth curves of the tumour xenografts. Data are presented as mean \pm s.e. of tumour volumes at the indicated time points. * P <0.01, relative to control. **(C and D)** The final tumour volume and weight of GOLPH3 group were decreased than that of control group. **(E)** GOLPH3 siRNA inhibited the protein expression of GOLPH3 *in vivo* as determined by western blotting.

In summary, the present study first showed that GOLPH3 expression was upregulated in the majority of the RCC clinical tissue specimens. High expression of GOLPH3 positively correlates with the aggressive phenotype of RCCs, and predicts poor survival outcome of patients. We have also presented experimental evidence that depletion of GOLPH3 in RCC cell lines using siRNA inhibited cell proliferation, anchorage-independent growth, migration, invasion, and tumour growth in xenograft model mice. On the basis of these findings, we conclude that GOLPH3 is functionally important in the development and progression of RCC and may serve as a new target for RCC therapy. *In vivo* experiments and further studies on the role of GOLPH3 are very interesting and are an area of active research at our institute.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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