

Original Article

Knockdown of GOLM1 suppresses proliferation and aggressiveness of non-small-cell carcinoma cells

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Abstract: Golgi Membrane Protein 1 (GOLM1) participates in the regulation of tumor growth and progression. However, the roles of GOLM1 in non-small-cell carcinoma (NSCLC) require further investigation. The present study aimed to explore whether GOLM1 is involved in the progression of NSCLC. Oncomine microarray analysis was applied to detect expression of GOLM1 in NSCLC. Short hairpin RNA (shRNA) targeting GOLM1 was then designed and transfected into NSCLC H1975 cells to decrease expression of GOLM1. Proliferation and apoptosis of H1975 cells were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), colony forming, and flow cytometry assays, respectively. Furthermore, migration and invasion abilities of H1975 cells were determined using wound healing and Transwell assays. It was found that levels of GOLM1 were remarkably higher in NSCLC tissues, compared to normal lung tissues. Knockdown of GOLM1 markedly suppressed the viability and aggressiveness of NSCLC cells. Moreover, flow cytometry analysis demonstrated that GOLM1 knockdown promoted the apoptosis of H1975 cells. Finally, GOLM1 knockdown inhibited levels of Phosphoinositide-3-Kinase (PI3K) and Protein Kinase B (AKT), while increasing expression of BCL2 Associated X (Bax). Taken together, results suggest that downregulation of GOLM1 might be a potential therapeutic target for NSCLC.

Keywords: NSCLC, GOLM1, apoptosis, proliferation, migration, invasion

Introduction

Non-small-cell carcinoma (NSCLC) is currently the fourth most common cause of cancer-related deaths, worldwide. It is characterized by difficult diagnosis, distant metastasis, and aggressive local invasion [1, 2]. In the past two decades, strategies, including surgery, radiation, and chemotherapy, have failed to improve long-term survival [3, 4]. Recent efforts have focused on the application of novel targeted agents based on a better understanding of the mechanisms involved in tumor progression [5].

GOLM1 (Golm 1, NM_016548) is a resident cis-Golgi membrane protein. Its function in tumor progression requires further investigation [6, 7]. GOLM1 is upregulated in the hepatocytes of patients with acute and chronic forms of hepatitis and hepatocellular cancer. GOLM1 has a single N-terminal transmembrane domain and an extensive C-terminal and coiled-coil domain that faces the luminal surface of the Golgi

apparatus [8]. N-terminal cleavage by a furin proprotein convertase results in the release of the C-terminal ectomain. The cleaved form of GOLM1 is detectable in the serum of patients with hepatocellular cancer, suggesting the diagnostic value of GOLM1. GOLM1 can serve as significant predictor of prostate cancer. The GOLM1 protein is released from prostate cell lines, *in vitro*, and is detectable in the urine of patients with prostate cancer [9, 10]. In addition, GOLM1 may promote the metastasis of hepatocellular carcinoma (HCC) through regulating membrane protein trafficking, especially modulating the signaling kinetics of epidermal growth factor receptor/receptor tyrosine kinases (EGFR/RTK) complex recycling [11, 12]. Despite recent advances in understanding the biological function of GOLM1, there are few reports concerning the roles of GOLM1 in the diagnosis and prognosis of NSCLC.

The current study investigated whether GOLM1 acts as potential oncogene in NSCLC. One sta-

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ble knockdown of GOLM1 cell line model was constructed using the lentivirus-mediated RNA interference technique. Based on the GOLM1 silencing cell model, this study further determined the biological function of GOLM1 silencing on the growth and aggressiveness of NSCLC.

Materials and methods

Cell culture

Human NSCLC cell lines and EBAS-2B were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) or 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. A total of 31 pairs of lung cancer tissues and corresponding normal tissues were collected from patients that underwent surgical resection, from February 2002 to November 2016, at Xintai People's Hospital. Clinical tissues were stored in liquid nitrogen following surgical removal from patients. No patients received additional treatment before surgery. The present research was approved by the Ethics Board of Xintai People's Hospital. All participants agreed and provided written informed consent.

Oncomine analysis

To detect levels of GOLM1 in NSCLC, two datasets, Hou Lung [13] and Landi Lung [14] datasets in the Oncomine database (www.oncomine.org), were selected. Expression of GOLM1 was compared between NSCLC tissues and normal tissues, according to standard procedures, previously described [15].

Construction of recombinant lentiviral vectors

Human GOLM1-targeting short hairpin RNA (shRNA) oligonucleotides sequences were cloned into the pFH-L vector at NheI/PacI restriction sites. Lentiviral expression vectors (pFH-L) containing green fluorescence protein (GFP) and packaging vectors (pVSVG-I and pCMVΔR8.92) were co-transfected into 293T cells using Lipofectamine 2000, according to manufacturer instructions. H1975 cells were cultured into 6-well plates at a density of 50,000 cells per well, then transfected with shRNA-expressing lentivirus. GFP expression was observed by fluorescent microscopy 4 days post-transduction.

MTT assay

To detect cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was conducted after lentivirus transduction. Briefly, H1975 cells were cultured into 96-well plates at a density of 2,000 per well. The plates were incubated for 1 day, 2 days, 3 days, 4 days, or 5 days, respectively. On each day, 100 μl of MTT (5 mg/mL) was added into the plate and the cells were incubated for 4 hours. DMSO was added to the plate and cells were incubated at 37°C for 10 minutes. Absorbance at 490 nm was determined using an ELISA reader.

Colony formation assay

Briefly, H1975 cells were cultured into 6-well plates at a density of 500 per well. The culture medium was changed every three days. H1975 cells were cultured for 10 days until the most single colony contained more than 50 cells. Colonies were stained with crystal violet for 15 minutes. Cell colonies were then counted under a microscope.

Wound healing assay

Cells were seeded into 6-well plates and cultured until they reached confluence. Wounds were scratched on the cell monolayer using 100 μl pipette tips. Plates were washed with fresh medium to remove non-adherent cells. Cells were then cultured for 0 hours or 24 hours, then photographed.

Cell invasion assay

Briefly, cells were suspended in serum-free medium and plated into Transwell inserts coated with Matrigel (BD Biosciences, Bedford, MA). Bottom wells were filled with complete medium. After 24 hours, the invaded cells were fixed with methanol and stained with a crystal violet solution. Finally, the number of invaded cells was counted in five random fields under a microscope.

Cell apoptosis

H1975 cells were harvested and stained with FITC-Annexin V and Propidium iodide (PI). Images of cell apoptosis were obtained and analyzed using FACS Calibur (BD Biosciences, USA) and Flowjo software (Tree Star Corp, USA).

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Immunofluorescence staining

H1975 cells transfected with shGOLM1 were cultured on a coverslip. Briefly, H1975 cells were fixed with 3.7% paraformaldehyde in PBS for 20 minutes at room temperature. Next, 0.2% Triton X-100 solutions were used to permeabilize cells for 10 minutes at room temperature. Cells were then incubated with the primary antibody against Bax (1:100, Santa Cruz Biotechnology, USA) overnight at 4°C. After being washed, the fluorescence-conjugated secondary antibody (goat-anti-rabbit-Alexa 594-conjugated antibodies, Life Technologies, USA) was used to incubate the treated coverslips for 1 hour at room temperature. The treated coverslips were then incubated with 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI; Life Technologies, USA) for 10 minutes at room temperature. Images were obtained using a fluorescence microscope (Olympus, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol Reagent and reversely transcribed to cDNA. qRT-PCR products were detected with SYBR Green on BioRad Connect Real-Time PCR platform. Specific cDNAs were then amplified by qRT-PCR using the following primers: GOLM1, 5'-TGGCCTGCATCATCGTCTTG-3' (forward) and 5'-CCCTGGAACCTCGTTCTTCTTCA-3' (reverse); PI3K, 5'-CGAGAGTGTGTCACAGTGTC-3' (forward) and 5'-TGTTTCGCTTCCACAAACACAG-3' (reverse); AKT, 5'-CCTCCACGACATCGCACTG-3' (forward), 5'-TCACAAAGAGCCCTCCATTATCA-3' (reverse); Bax, 5'-CCCAGAGAGGTCTTTTTCCGAG-3' (forward), 5'-CCAGCCATGATGGTTCTGAT-3' (reverse) and GAPDH, 5'-TGGATTTGGACGCA-TTGGTC-3' (forward) and 5'-TTTGCCTGGTAC-GTGTGAT-3' (reverse). The comparative cycle threshold (Ct) method was applied to quantify expression levels by calculating the $2^{(-\Delta\Delta Ct)}$ method.

Western blot analysis

Total protein was extracted with 2 × SDS sample buffer and concentrations of proteins were determined using BCA kit. Proteins were separated by 8% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk in TBST, then incubated with primary antibody (anti-GOLM1, anti-PI3K, anti-

AKT, anti-Bax and anti-GAPDH). Following incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, the membranes were detected using enhanced chemiluminescence (ECL) kit (Amersham).

Statistical analysis

Statistical analysis was conducted with SPSS 13.0 (SPSS Inc, Chicago, IL, USA). Data are expressed as mean ± SD from three independent experiments. Differences in results for two groups were evaluated using either two-tailed Student's *t*-test or one-way ANOVA, followed by post hoc Dunnett's test. *P*-value < 0.05 indicates statistical significance.

Results

GOLM1 is upregulated in NSCLC

The present study investigated mRNA levels of GOLM1 in human NSCLC tissues using two datasets from the publicly available Oncomine database [13, 14]. As shown in **Figure 1A**, each of the two datasets showed significantly higher levels of GOLM1 expression in NSCLC tissues, compared with normal lung tissues. This study then examined expression of GOLM1 in 31 pairs of NSCLC tissues and corresponding normal tissues. qRT-PCR analysis demonstrated that levels of GOLM1 were increased in NSCLC tissues, compared to normal tissues (**Figure 1B**). More importantly, high levels of GOLM1 were significantly associated with poor overall survival in NSCLC (**Figure 1C**). Next, GOLM1 expression was observed in four NSCLC cell lines and normal human bronchial epithelium cell line, BEAS-2B (**Figure 1D**). H1975 cells expressing the highest levels of GOLM1 were chosen for further investigation.

Knockdown of endogenous GOLM1 by shRNA-expressing lentivirus

H1975 cells were transfected with shRNA-expressing lentivirus (shCon: control group or shGOLM1: short hairpin targeting GOLM1). Infection efficiency was confirmed by evaluating GFP expression levels. As shown in **Figure 2A**, over 80% of cells transfected with shGOLM1 expressed GFP, suggesting that lentivirus transduction was successful and highly efficient. The effects of shGOLM1 on expression of GOLM1 in H1975 cells were investigated by

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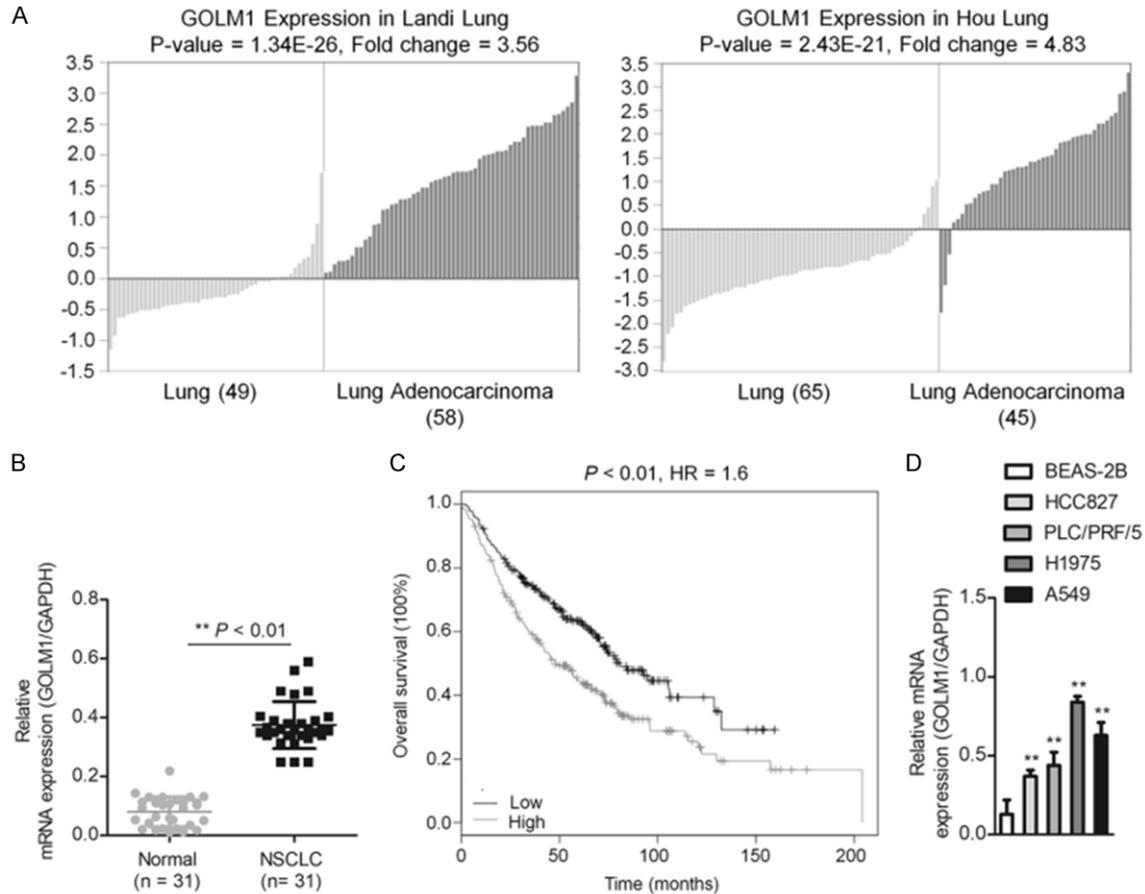


Figure 1. GOLM1 over-expression in NSCLC. A. Oncomine studies analysis. Expression of GOLM1 in NSCLC and corresponding tissues was analyzed using Landi Lung and Hou Lung datasets. B. Levels of GOLM1 mRNA in 31 paired of NSCLC and adjacent normal tissues were determined by qRT-PCR. ** $P < 0.01$ compared to normal. C. Kaplan-Meier analysis of overall survival of NSCLC patients. D. Levels of GOLM1 in four NSCLC cell lines and normal lung epithelial cell line BEAS-2B were detected using qRT-PCR assay. ** $P < 0.01$ compared to BEAS-2B cell.

qRT-PCR and Western blot analysis. As shown in **Figure 2B**, mRNA levels of GOLM1 were significantly downregulated in cells transfected with shGOLM1, compared to cells transfected with shCon. Moreover, protein expression of GOLM1 was also obviously downregulated in cells transfected with shGOLM1, compared to cells transfected with shCon (**Figure 2C**). Results suggest that shGOLM1 could specifically and strongly suppress expression of endogenous GOLM1 in NSCLC cells.

Knockdown of GOLM1 impairs cell viability and colony formation

After NSCLC cells were transfected with GOLM1 shRNA-expressing lentivirus for 4 days, cell viability was examined for five consecutive days by

MTT assay. On day 4, compared with shCon, the number of viable cells infected with shGOLM1 was significantly reduced (**Figure 3A**). Reduction in the growth curve of cells infected with shGOLM1 was more obvious on day 5, indicating that shGOLM1 could strongly decrease the viability of H1975 cells. In addition, colony formation assay was applied to determine cell proliferation *in vitro*. Consistently, colony formation was significantly inhibited in cells transfected with shGOLM1. As shown in **Figure 3B**, the size of a single colony in cells transfected with shGOLM1 was much smaller than that in cells transfected with shCon. The total number of colonies in 6-well plates was remarkably decreased in H1975 cells transfected with shGOLM1, compared with the control group (**Figure 3C**). Present results indicate

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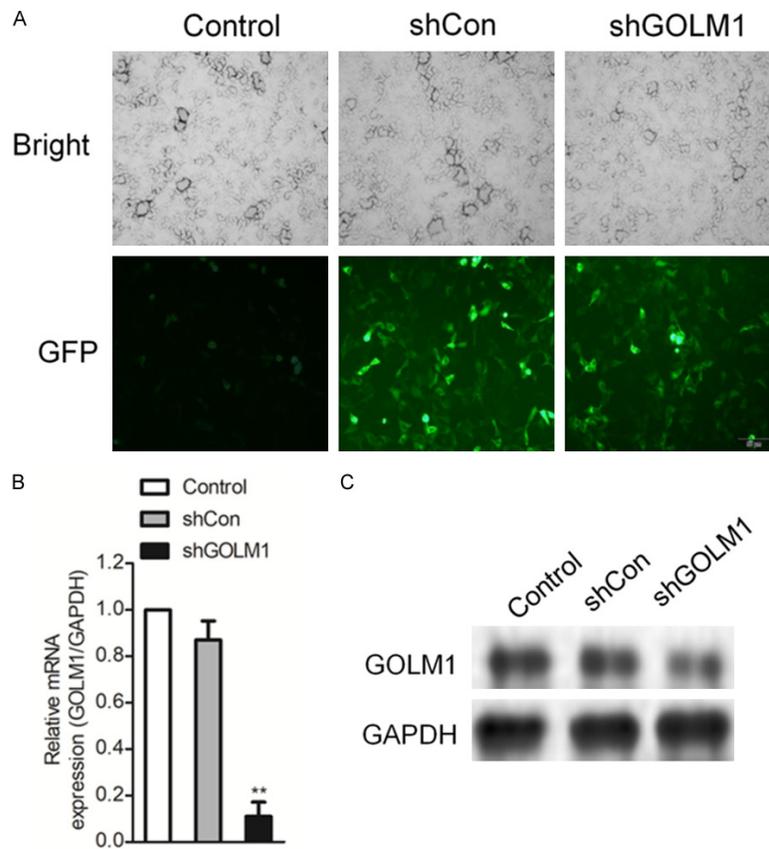


Figure 2. Lentivirus stably expressed shRNA targeting GOLM1 in H1975 cells. A. Lentivirus transfection was detected using fluorescence staining (GFP green fluorescence protein) and light microscopy (Bright). B. Quantitative analysis of GOLM1 knockdown efficiency in H1975 NSCLC cells using by qRT-PCR. ** $P < 0.01$ compared to control cell. C. GOLM1 knockdown efficiency was determined by Western blot. GAPDH protein was used as an internal control.

Knockdown of GOLM1 alters cell growth-associated proteins and inhibits cellular mobility in H1975 cells

To illuminate the molecular basis for cell growth inhibition caused by GOLM1 knockdown, expression levels of cell proliferation regulatory molecules were analyzed by qRT-PCR and Western blot analysis. As shown in **Figure 5A, 5B**, knockdown of GOLM1 significantly suppressed expression levels of PI3K and AKT and enhanced expression levels of Bax in H1975 cells. Results suggest that knockdown of GOLM1 in H1975 cells regulates cell growth and apoptosis probably via regulating cell proliferation-related genes and apoptosis associated-proteins. Furthermore, the present study investigated whether GOLM1 affects migration and invasion abilities of NSCLC cells. As shown in **Figure 5C, 5D**, suppression of GOLM1 significantly inhibited migration and invasion abilities of H1975 cells.

that shGOLM1 efficiently inhibits cell viability and proliferation of NSCLC cells.

Knockdown of GOLM1 promotes cell apoptosis

Flow cytometry was used to determine the effects of shGOLM1 on apoptosis of H1975 cells. Representative images of cell apoptosis are shown as **Figure 4A**. As shown in **Figure 4B**, the percentage of apoptosis was increased from 5.8% in cells transfected with shCon to 25.2% in cells transfected with shGOLM1. As shown in **Figure 4C**, immunofluorescence staining results indicate that protein expression levels of Bax were significantly upregulated in H1975 cells transfected with shGOLM1, compared to the control group. Taken together, results suggest that knockdown of GOLM1 inhibits NSCLC cell proliferation via inducing apoptosis.

Discussion

Despite advancements in diagnosis and treatment, NSCLC still carries the worst prognosis of all solid malignant tumors. Five-year survival rates remain unsatisfactory [16]. Therefore, a better understanding of the molecular mechanisms involved in the progression of NSCLC is particularly important in improving the treatment efficacy for patients with NSCLC [17]. Molecular profiling of primary tumors is helpful for the identification of critical pathways involved in the development of NSCLC [5, 18]. The hope is that this approach will lead to more effective targeted molecular therapies. Recently, studies have indicated the crucial role of Golgi proteins in the development of several cancers. GOLM1, for example, is associated with poor prognosis in hepatocellular carcinoma.

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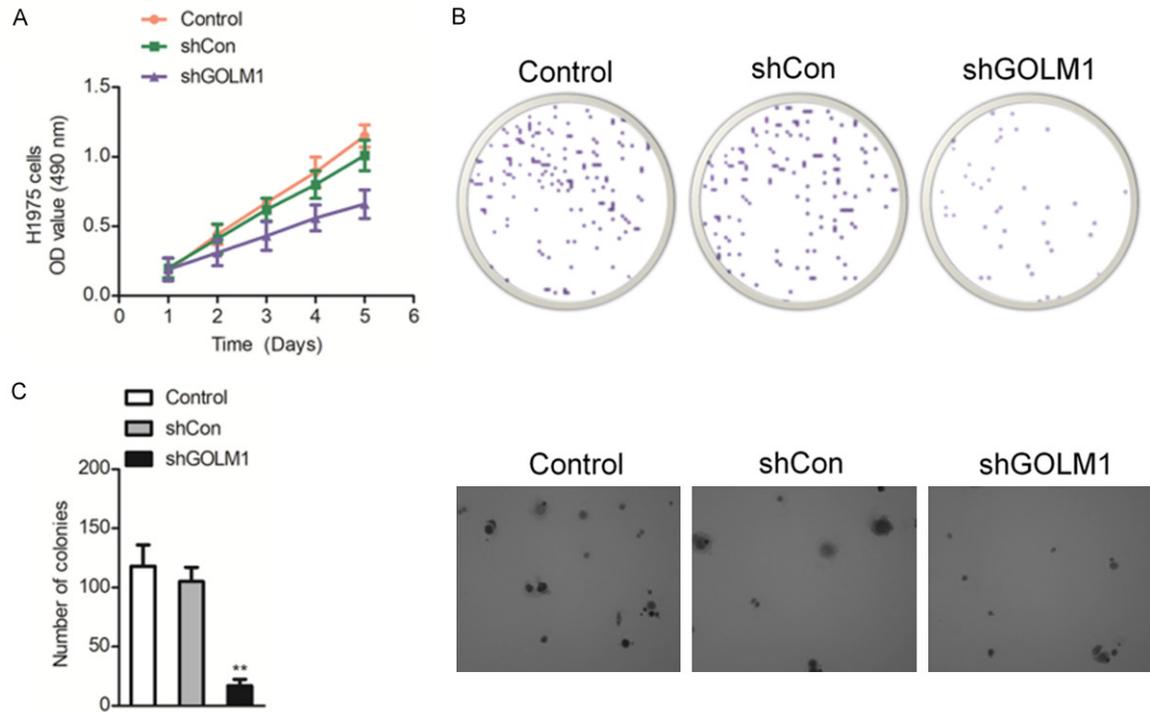


Figure 3. Downregulation of GOLM1 decreases the viability and proliferation of NSCLC cells. A. The growth of H1975 cells was detected using MTT assay. B. H1975 cells were transfected with shCon or shGOLM1 and colony formation assay was conducted. C. The number of colonies was fewer in cells transfected with shGOLM1 than in cells transfected with shCon. ** $P < 0.01$ compared to control cells.

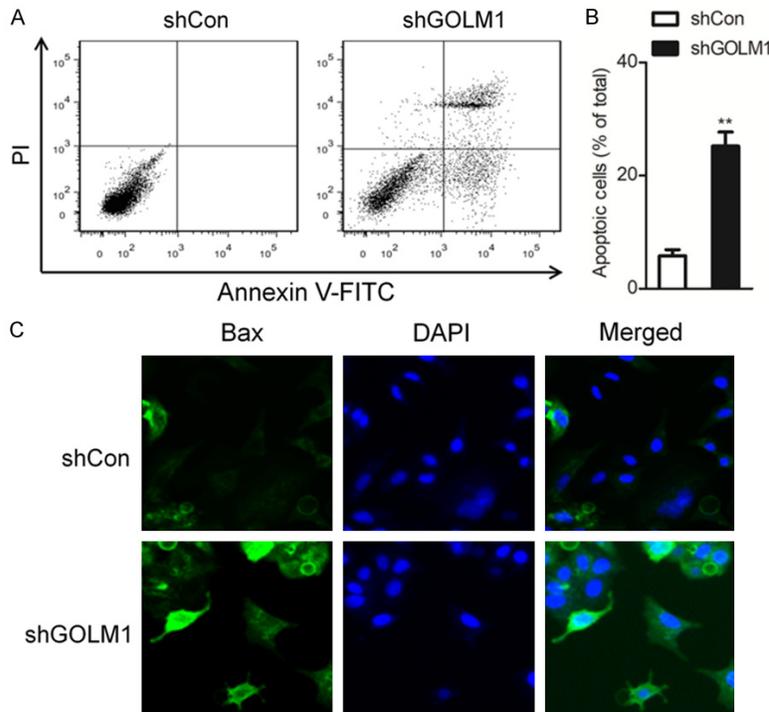


Figure 4. Downregulation of GOLM1 induces apoptosis of NSCLC cells. A. Apoptosis of H1975 cells transfected with shGOLM1 was measured by Annexin V-FITC/PI staining and flow cytometry. B. Percentages of apoptotic cells were calculated. ** $P < 0.01$ compared to shCon transfected cells. C. Immunofluorescence staining of Bax.

noma (HCC) and prostate cancer patients. GOLM1, also known as GP73 and GOLPH2, is a highly-phosphorylated protein, which locates in the cis and medial-Golgi apparatus [19]. GOLM1 processes proteins synthesized in the rough endoplasmic reticulum and assists the transport of protein cargo through the Golgi apparatus. Recently, an increasing number of studies have revealed that GOLM1 serves as a promoter of proliferation, invasion, and migration in diverse human cancers, including hepatocellular carcinoma, prostate cancer, esophageal cancer, gastric cancer, cutaneous melanoma [20].

The present study provides new evidence demonstrating that GOLM1 may be a poten-

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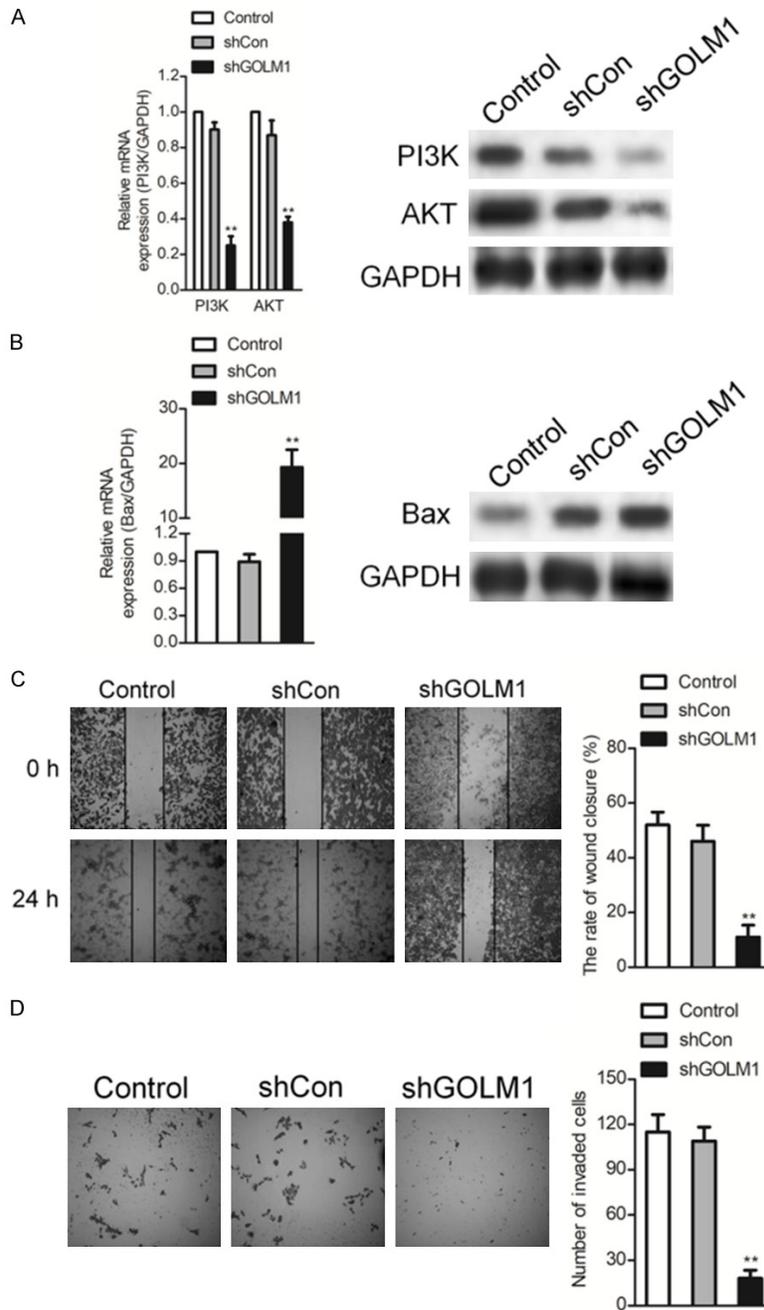


Figure 5. Downregulation of GOLM1 suppresses expression of PI3K and AKT in NSCLC cells. A. qRT-PCR and Western blot analysis of PI3K and AKT in H1975 cells transfected with shGOLM1. GAPDH protein was used as an internal control. B. qRT-PCR and Western blot analysis of Bax in H1975 cells transfected with shGOLM1 or shCon. GAPDH protein was used as an internal control. C. Representative images of wound healing assays. Quantitative analysis of the percentage of H1975 cell wound healing following downregulation of GOLM1. D. Representative images of invasive cells. The number of invaded cells was calculated. ** $P < 0.01$ compared to control cells.

tial tumor marker in human NSCLC. GOLM1 was found to be significantly highly expressed

in NSCLC tissues, compared with normal tissues, using Oncomine database analysis. This study then designed shRNA to specifically block its endogenous expression in human NSCLC cell line, H1975. Functional analysis showed that knockdown expression of endogenous GOLM1 significantly decreased viability and invasion abilities of H1975 cells. Moreover, flow cytometry analysis showed that knockdown expression of GOLM1 induced significant cell apoptosis.

The present study also revealed that growth inhibition by GOLM1 silencing in H1975 cells and expression of PI3K and AKT were decreased in shGOLM1 transfected H1975 cells. Results suggest that knockdown of GOLM1 suppressed the proliferation of NSCLC cells through inhibition of PI3K/AKT signaling pathways. GOLM1-regulated EGFR/RTK recycling is a novel target in combating HCC metastasis [20]. Therefore, it is likely that GOLM1 could modulate the migration and invasion of NSCLC cells. Further investigation demonstrated that knockdown remarkably suppressed migration and invasion abilities of H1975 cells *in vitro*.

In summary, down-expression of GOLM1 by shRNA significantly inhibits the growth of H1975 cells and promotes cell apoptosis. Present findings provide new evidence suggesting that GOLM1 plays an essential role in cell

growth and may be a potent therapeutic target for human NSCLC.

Disclosure of conflict of interest

None.

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