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Inhibition of Gli1-mediated prostate cancer cell proliferation by inhibiting the mTOR/S6K1 signaling pathway

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Abstract. Ectopic activation of the canonical Hedgehog signaling pathway is involved in the development and progression of prostate cancer, which is one of the leading causes of cancer-associated mortality in males worldwide. However, the role of the non-canonical Hedgehog signaling pathway in prostate cancer remains generally unexplored. In the present study, it was identified that Gli (glioma-associated oncogene)1 and Gli2 were highly expressed at the protein level in the androgen-independent prostate cancer cell lines PC3 and DU145, but not in the androgen-dependent cancer cell line LNCaP. Silencing of Gli1 using small interfering RNA markedly decreased PC3 cell viability and liquid colony formation *in vitro*. The Gli1/2-specific inhibitor GANT61 markedly decreased cell viability by inducing cell apoptosis in PC3 and DU145 cells. GANT61 also alleviated liquid colony formation efficiency in PC3 and DU145 cells, suggesting that the activity of Gli1 is required for prostate cancer cell survival. To explore further the upstream signaling pathway involved in the regulation of Gli1 expression, it was identified that tumor necrosis factor α-triggered mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase 1 (S6K1) activation was required for Gli1 expression. Pharmacological and genetic inhibition of S6K1 activation markedly decreased Gli1 and its downstream target gene mRNA expression. In addition, the phosphoinositide 3-kinase/mTOR inhibitor BEZ235 markedly decreased *in vitro* PC3 cell proliferation. The results of the present study indicate that the non-canonical Hedgehog pathway (mTOR/S6K1/Gli1) contributes to the development and progression of prostate cancer and that Gli1 is a potential therapeutic target in the treatment of prostate cancer.

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Introduction

Prostate cancer is the second most common malignancy in males worldwide, characterized by its high mortality and poor prognosis (1). In spite of early detection techniques and multidisciplinary therapeutic approaches, the overall 5-year survival rate remains <30% in China (2). Anti-androgen treatment remains the first-line therapy for patients with prostate cancer, among whom the majority will eventually develop highly metastatic androgen-independent prostate cancer (3). Prostate cancer is a complex and heterogeneous disease characterized by various molecular signatures in patients, which provides opportunities to explore targeted therapies.

Glioma-associated oncogene 1 (Gli1) is highly expressed in the prostate and serves various functions in prostate development (4,5). Ectopic activation of the Hedgehog (HH) signaling pathway has been demonstrated to be involved in the initiation as well as the progression of prostate malignancy (3,6). The HH signaling pathway was first identified in *Drosophila* as a central organizer for proper embryonic patterning and development. Three HH ligands have been identified in vertebrate organisms: Sonic Hedgehog (SHH), Indian Hedgehog and Desert Hedgehog, which are all able to initiate signaling by binding and inactivating the HH receptor Patched 1 (PTCH1) (5). In the canonical HH signaling pathway, inactivation of PTCH1 releases the seven-pass transmembrane protein Smoothened (SMO). SMO then transduces the signal to downstream effectors (Gli proteins) via blocking the inhibitory partner, suppressor of fused. Activated Gli proteins eventually translocate into the nucleus and trigger the transcription of downstream target genes (5,7). In addition to classical HH signal transduction, the non-canonical HH pathway, in which Gli proteins are regulated by phosphoinositide 3-kinase (PI3K)/V-Akt murine thymoma viral oncogene (Akt), mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK), nuclear factor κB (NF-κB) and/or transforming growth factor β (TGFβ) pathways as well as the key tumor suppressors tumor protein p53 (TP53) and phosphatase and tensin homolog (PTEN) in a ligand-independent manner, has been the focus of previous research (8-10). Although the canonical pathway has been well-investigated, how Gli proteins are regulated in a SMO-independent manner in prostate cancer remains largely unknown.

The Akt/mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase 1 (S6K1) signaling pathway is involved in numerous aspects of molecular and cellular biology, such as mRNA translation, ribosome biogenesis, cell proliferation, metabolism, immunosuppression, development, aging and malignancies (8). As a serine/threonine protein kinase, the activation of mTOR leads to the phosphorylation of S6K1 and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (11). S6K1 is also a serine/threonine kinase and serves a role in target gene translation following its phosphorylation by mTOR. More importantly, tumor necrosis factor α (TNFα) is capable of activating the mTOR/S6K1 signaling pathway to promote angiogenesis, which mediates chronic inflammation-induced cancer, including breast and prostate cancer (8,12).

In order to explore the potential therapeutic targets for prostate cancer, an initial siRNA screen was performed (data not shown) and it was identified that Gli1 and Gli2 are critical in prostate cancer survival rates. Combined with other published data, it was hypothesized that Gli1 and/or Gli2 contributes to prostate cancer cell proliferation. In the present study, it was demonstrated that androgen-independent prostate cancer cell lines are dependent on Gli1 expression for proliferation and that its activation by mTOR/S6K1 is required for this function.

Materials and methods

Cell culture. The human prostate cancer cell lines PC3 (CRL-1435™), DU145 (HTB-81™) and LNCaP (CRL-1740™) were purchased from the American Type Culture Collection (Manassas, VA, USA). The prostate cancer cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; cat. no. 10082147 Thermo Fisher Scientific, Waltham, MA, USA) and cultured at 37˚C in a humidified atmosphere containing 5% CO_2. All cell lines were confirmed to be *Mycoplasma*-free using an e-Myco kit (Boca Scientific Inc., Boca Raton, FL, USA). The inhibitors used were GANT61 (Tocris Bioscience, Bristol, UK), rapamycin (Sigma-Aldrich; Merck KGaA) and BEZ235 (Selleck Chemicals, Houston, TX, USA).

Reverse transfection in prostate cancer cells with small interfering RNA (siRNA) targeting Gli1 or S6K1. SiRNA screening was performed as follows. The genome wide siRNA library was purchased from Dharmacon (Lafayette, CO, USA). The library contained a mixture of 4 individual siRNA oligos for each gene. PC3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C for three days to reach 60-70% confluency. A total of 10 pmol of each siRNA pool (5 μl) was reverse transfected to 95 μl serum-free media in empty 96-well assay plates. Firstly, 5 μl siRNA was diluted in 25 μl OptiMEM (cat. no. 31985-062; Thermo Fisher Scientific, Inc.) serum-free medium and incubated for 5 min at room temperature. Then, 0.13 μl RNAiMax (cat. no. 13778075; Thermo Fisher Scientific, Inc.) was diluted in 10 μl OptiMEM medium. Secondly, siRNA was mixed with Lipofectamine® RNAiMax (cat. no. 13778075; Thermo Fisher Scientific, Inc.) transfection reagent, incubated for 15 min at room temperature and transferred to 96-well plates. Thirdly, PC3 cells were harvested by incubation with trypsin for 2-4 min at 37˚C and centrifugation for 5 min at 100 x g and 4˚C. A total of ~2,500 cells in 60 μl cell suspension were seeded in each well and cultured for 96 h at 37˚C. CellTiter-Glo Assay kit (Promega Corporation, Madison, WI, USA) was used to measure the cell viability. Cells were plated onto a 96-well plate at a density of 2,500 cells/well. After 96 h incubation at 37˚C, CellTiter-Glo reagent was added to the culture medium, and plates were agitated at room temperature for 10 min. The luminescent signal was determined using a GloMax absorbance plate reader at a wavelength of 560 nm. siUBB and siTMEM114 were used as positive and negative controls, respectively. Each screening was triplicated and repeated three times. For Z scores, -3 was a cut-off value. The siRNAs targeting human Gli1 or S6K1 were synthesized by Sigma-Aldrich; Merck KGaA (siGli1: NM_005269, siS6K1: NM_003161). A scrambled siRNA (SIC001, Sigma-Aldrich; Merck KGaA) was used as a negative control. Transient knockdown of Gli1 or S6K1 with these siRNAs in prostate cancer cells was carried out using Lipofectamine RNAiMax (Thermo Fisher Scientific, Inc.) together with negative or positive controls (GAPDH siRNA, NM_002046; Sigma; Merck KGaA), according to the manufacturer's protocol. The cells were then cultured for 72 h at 37˚C in 5% CO_2. Silencing efficiency was determined using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting.

RT-qPCR. Total RNA was extracted from cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). cDNA was generated with oligo-dT primers from 0.5 μg RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. TaqMan probes (Thermo Fisher Scientific, Inc.) of Gli1, Gli2, N-Myc, PTCH1 and CCND1 genes were used to quantitatively analyze mRNA transcript levels with the 18S ribosomal RNA gene as an internal reference. PCR was performed using the ABI 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were 95˚C for 3 min, 45 cycles at 95˚C for 15 sec and 60˚C for 45 sec. The primers were Gli1 (Hs00171790_m1), Gli2 (Hs01119974_m1), N-Myc (Hs00190768_m1), PTCH1 (Hs00181117_m1), CCND1 (Hs00765553_m1), and reference gene GAPDH (Hs002786624_g1). All primers were purchased from Thermo Fisher Scientific, Inc. The results were analyzed using SDS Software v1.4.1 (Thermo Fisher Scientific, Inc.). The comparative C_q method was used to calculate relative mRNA expression levels (13).

Cell viability assays. Cell viability was examined using the CellTiter-Glo Assay kit (Promega Corporation), according to the manufacturer's protocol. Cells were plated onto a 96-well plate at a density of 2,000 cells/well. At 24, 48, 72 and 96 h, CellTiter-Glo reagent was added to the culture medium, and plates were agitated at room temperature for 10 min. The half-maximal inhibitory concentration was the concentration of GANT61 required for 50% inhibition of the cell viability in the curve. The luminescent signal was determined using a GloMax absorbance plate reader at a wavelength of 560 nm. Each individual experiment was performed at least three times independently.

Liquid colony formation assays. To evaluate anchorage-dependent liquid colony formation efficiency, 500 cells were

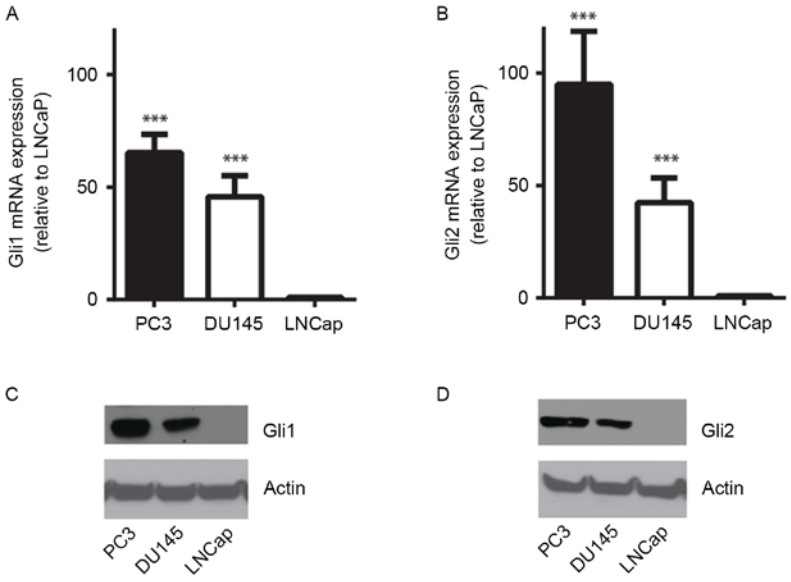

Figure 1. Gli1 and Gli2 are overexpressed in androgen-independent prostate cancer cell lines. (A) Gli1 and (B) Gli2 mRNA expression in prostate cancer cell lines PC3, DU145 and LNCaP was detected using the reverse transcription-quantitative polymerase chain reaction (fold expression compared with LNCaP). (C) Gli1 and (D) Gli2 protein expression was detected by immunoblotting lysates from PC3, DU145 and LNCaP cells. β-actin was used as a loading control. Results are presented as the mean ± standard deviation. ***P<0.001 vs. control. Gli, glioma-associated oncogene.

suspended in 3 ml RPMI-1640 medium with 10% FBS and plated in a 6-well plate to grow for 2-3 weeks. The cells were fixed and stained with 1 ml 0.05% crystal violet (Sigma-Aldrich; Merck KGaA) to count the colonies. Each individual experiment was performed three times independently.

Western blotting. Treated cells were lysed in Protein Extraction Reagent Type 4 (Sigma-Aldrich; Merck KGaA) with a PhosStop phosphatase inhibitor and cOmplete protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA). The protein concentration of cell lysates was measured using the Bradford reagent (Bio-Rad Laboratories, Inc.). Equal amounts of total protein (30 μg) were separated by SDS-PAGE on an 8-10% gel, and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked in 5% milk followed by incubation with primary antibodies in Tris-buffered saline with 0.1% Tween-20 solution at 4˚C overnight. The following day, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies: Goat anti-mouse immunoglobulin (Ig)G (cat. no. ab6789; Abcam, Cambridge, MA, USA; 1:3,000) and goat anti-rabbit IgG antibodies (cat. no. ab97051; Abcam; 1:3,000) for 1 h at room temperature. Membranes were exposed to LucentBlue X-ray film (Advansta, Menlo Park, CA, USA) at room temperature for between 30 sec and 5 min. Antibodies used for western blotting were as follows: Anti-Gli1 (cat. no. 2643), anti-poly(ADP-ribose) polymerase (PARP)/cleaved PARP (cat. no. 9546) and anti-phospho-S6K1 (Thr421/Ser424; cat. no. 9204; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-S6K1 (cat. no. ab14708; Abcam); anti-Gli2 (cat. no. ABN506; EMD Millipore, Billerica, MA, USA); and anti-β-actin (cat. no. sc-4778; Santa Cruz Biotechnology, Dallas, TX, USA).

Statistical analysis. Student's t-test (two-tailed) was used to examine the significance of *in vitro* cell viability and quantitative real-time PCR data between different groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of Gli1 and Gli2 in androgen-independent prostate cancer cell lines. Kim *et al* (4) demonstrated that the expression of certain HH signaling proteins was significantly associated with poor prognosis, including larger tumor size, increased level of prostate-specific antigen (PSA), higher Gleason score and poorer invasiveness. In the present study, it was examined whether Gli expression is associated with androgen dependency in cultured prostate cancer cells. RT-qPCR revealed that mRNA expression of Gli1 and Gli2 in the androgen-independent prostate cancer cell lines PC3 and DU145 was significantly increased compared with that in LNCaP cells, which is an androgen-sensitive cell line (P<0.001; Fig. 1A and B). Immunoblotting of the cell lysates using anti-Gli1 or -Gli2 antibody identified that Gli1 and Gli2 were overexpressed at the protein level in PC3 and DU145 cells compared with LNCaP cells, in which both Gli1 and Gli2 protein were barely detected (Fig. 1C and D). These results indicate that the HH signaling pathway may serve a critical role in androgen-independent prostate cancer cells.

Gli1 depletion decreases prostate cancer cell viability and liquid colony formation. To investigate whether endogenous Gli1 serves a role in prostate cancer cell proliferation, an siRNA targeting Gli1 (siGli1) was used to transiently knock down the expression of Gli1 in the PC3 cell line. RT-qPCR and western blotting demonstrated that siGli1 significantly decreased Gli1 mRNA and protein expression in PC3 cells (P<0.01; Fig. 2A and B). *In vitro* liquid colony formation assays revealed that knocking down Gli1 resulted in a

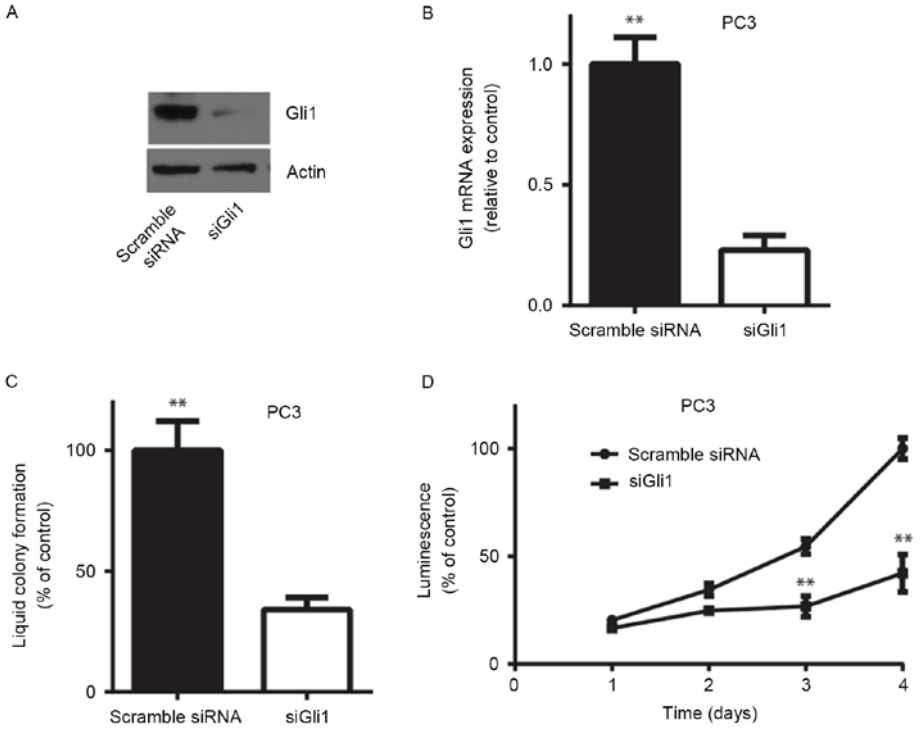

Figure 2. Knocking down of Gli1 leads to cell proliferation arrest and decreased cell clonogenicity in PC3 cells *in vitro*. (A) PC3 cells were transiently transfected with siRNA targeting Gli1 or a scrambled siRNA. Depletion of Gli1 protein expression in PC3 cells was examined by western blotting. β-actin was used as a loading control. (B) The reverse transcription-quantitative polymerase chain reaction revealed that siGli1 significantly decreased Gli1 mRNA levels in transfected PC3 cells. (C) The capability of PC3 cells to form colonies in liquid medium following depletion of Gli1 expression was significantly decreased. The number of colonies is expressed as a percentage of the control. (D) PC3 cells were seeded and cultured for 4 days, and cell viability was assessed using the CellTiter-Glo assay. The proliferation rate is presented as a percentage of the scramble control. **P<0.01 vs. control. Gli, glioma-associated oncogene 1; siRNA, small interfering RNA; siGli1, siRNA targeting Gli1.

significant decrease in anchorage-dependent colony formation efficiency in PC3 cells (P<0.01; Fig. 2C). Similarly, depletion of Gli1 expression led to a significant decrease in cell viability determined by measuring the ATP level in PC3 cells (P<0.01; Fig. 2D). These results indicated that Gli1 is required for androgen-independent prostate cancer cell survival *in vitro*.

Gli-specific small molecule inhibitor GANT61 suppresses prostate cancer cell proliferation. Inhibiting Gli1 function may be a potential therapeutic strategy for the treatment of prostate cancer. A number of small-molecule inhibitors targeting Gli family proteins have been developed (14,15). In the present study, it was examined whether the Gli1/2-specific inhibitor GANT61 was able to suppress prostate cancer cell viability. Cell viability assays revealed that GANT61 inhibited both PC3 and DU145 cell viability *in vitro*. The underlying molecular mechanisms by which GANT61 causes cell death are not fully understood, although a previous study demonstrated that GANT61 was able to inhibit the binding of Gli1/2 to the target gene promoter regions (16). PC3 cells exhibited slightly increased sensitivity to GANT61 compared with DU145 cells, and their half-maximal inhibitory concentration values were ~5 μM (Fig. 3A). Immunoblotting of the cell lysates indicated that GANT61 treatment led to PARP cleavage, suggesting that PC3 and DU145 cells underwent apoptosis (Fig. 3B). In addition, GANT61 treatment significantly decreased the efficiency of PC3 and DU145 cells to form liquid colonies (P<0.05 or P<0.01; Fig. 3C and D). These results indicate that the Gli inhibitor GANT61 may be used as a potential targeted therapy for androgen-independent prostate cancer.

mTOR/S6K1 signaling pathway is involved in the regulation of Gli1 expression in prostate cancer cells. Wang *et al* (8) reported that the activated TNFα/mTOR/S6K1 signaling pathway promotes Gli1 transcriptional activity and oncogenic function in esophageal adenocarcinoma. To study the upstream pathway further, rather than the canonical HH pathway, involved in the regulation of Gli1 expression, it was investigated whether mTOR/S6K1 regulates Gli1 and its downstream target gene expression in prostate cancer cells. The documented Gli1 target genes include *Gli1* and *PTCH1*, of which the corresponding proteins are important regulators of the canonical HH pathway itself. Other validated target genes include the cell cycle regulator cyclin D1 (*CCND1*), epithelial-mesenchymal transition regulator *SNAIL*, and self-renewal-associated molecules *NANOG* and *OCT4*. PC3 cells were treated with 5 ng/ml TNFα for 24 h and it was identified that the *PTCH1*, *CCND1*, *N-MYC* and *Gli1* mRNA expression levels significantly increased, whereas the presence of rapamycin, an mTOR inhibitor, significantly decreased gene expression (Fig. 4A-D). Western blotting confirmed that TNFα stimulation induced the activation of S6K1 and increased Gli1 protein expression, and that rapamycin inhibited TNFα-induced S6K1 phosphorylation and decreased Gli1 expression in PC3 cells (Fig. 4E). Similarly, depletion of S6K1 expression using siRNA, which was confirmed using RT-qPCR, markedly decreased

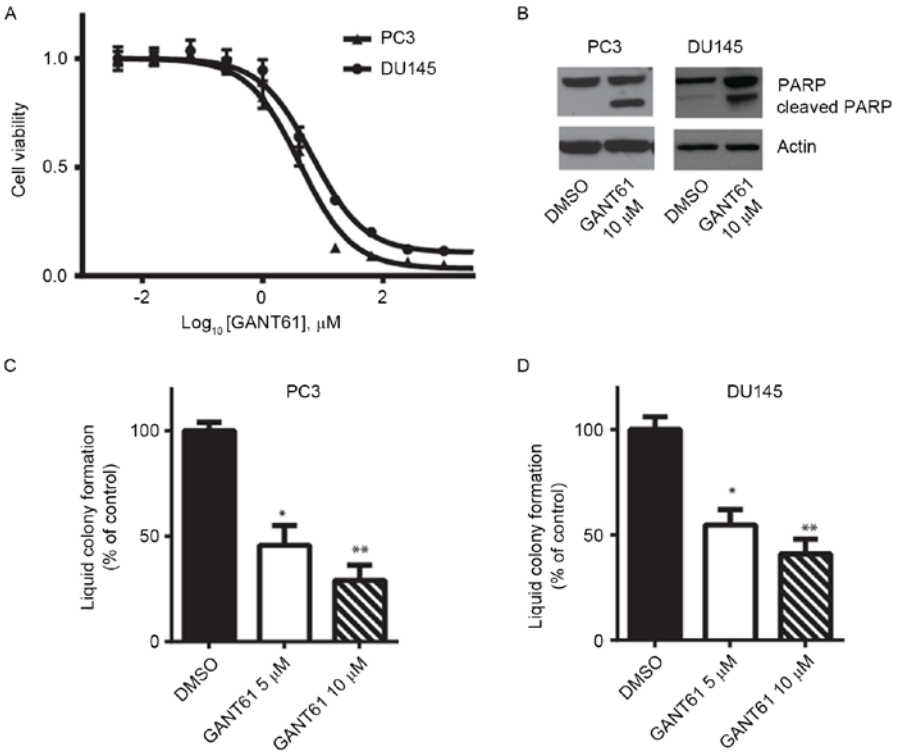

Figure 3. Gli inhibitor GANT61 decreases prostate cancer cell viability and colony formation *in vitro*. (A) PC3 and DU145 cells were seeded in 96-well plates and treated with the indicated concentration of GANT61 for 5 days. Cell viability was measured using CellTiter-Glo assays. (B) Immunoblotting for cleaved PARP revealed that GANT61 treatment for 48 h led to prostate cancer cell apoptosis compared with vehicle control. GANT61 significantly decreased (C) PC3 and (D) DU145 to form colonies in liquid medium. *P<0.05, **P<0.01 vs. control. PARP, poly(ADP-ribose) polymerase; DMSO, dimethyl sulfoxide.

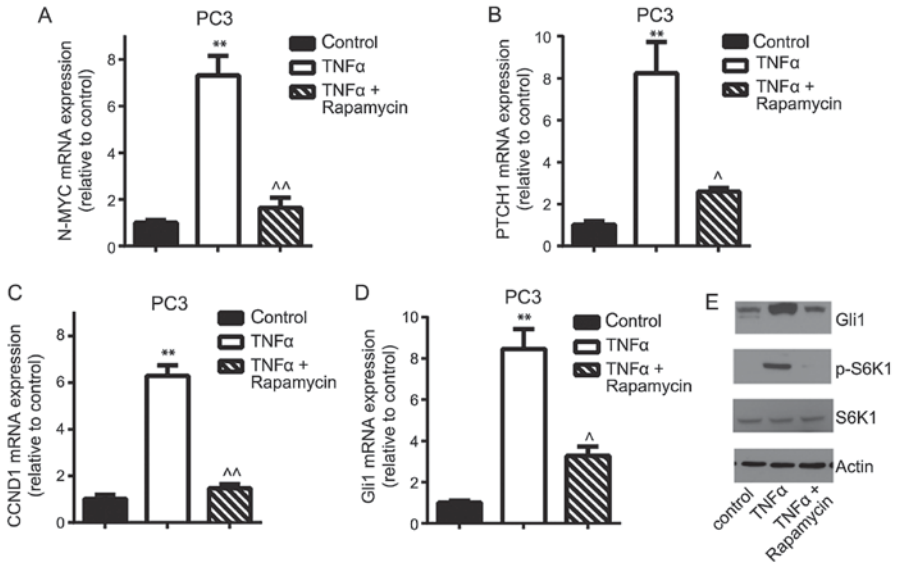

Figure 4. mTOR/S6K1 signaling pathway is involved in the regulation of Gli1 expression in PC3 cells. The reverse transcription-quantitative polymerase chain reaction demonstrated that 5 ng/ml TNFα treatment for 24 h stimulated the Gli1 downstream target genes and the Gli1 gene (A) *N-MYC*, (B) *CCND1*, (C) *PTCH1* and (D) *Gli1* mRNA expression compared with controls, whereas the mTOR inhibitor rapamycin decreased TNFα-induced mRNA expression. (E) Western blotting confirmed TNFα-triggered phosphorylation of S6K1 and increased Gli1 expression. However, rapamycin inhibited TNFα-induced S6K1 activation and decreased Gli1 expression in PC3 cells. **P<0.01 vs. control; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 compared with TNFα treatment. mTOR, mammalian target of rapamycin; S6K1, p70 ribosomal S6 kinase 1; Gli, glioma-associated oncogene; PTCH1, Patched 1; CCND1, cyclin D1; TNFα, tumor necrosis factor α; p-, phosphorylated.

TNFα-induced Gli1 mRNA and protein expression (Fig. 5A and B). Consistent with genetic manipulation, pharmacological inhibition of mTOR/S6K1 pathway using the PI3K/mTOR dual inhibitor BEZ235 markedly decreased PC3 cell viability and Gli1 protein expression (Fig. 5C and D). These results suggest that the TNFα/mTOR/S6K1 signaling pathway contributes to the regulation of Gli1 gene expression and its transcriptional activity in prostate cancer cells.

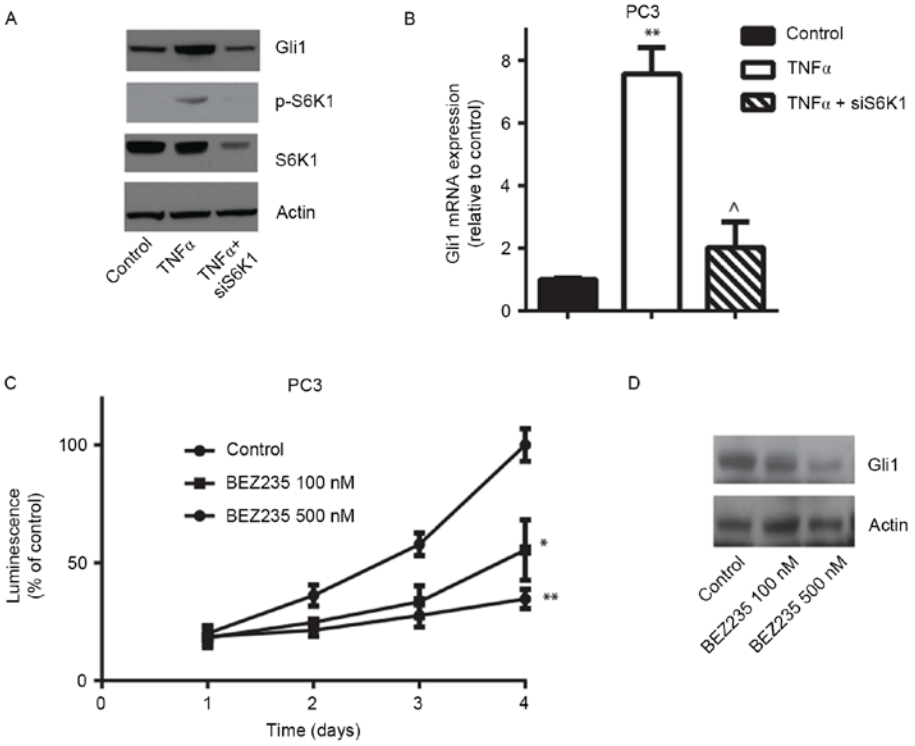

Figure 5. Inhibiting the mTOR/S6K1 signaling pathway suppresses Gli1 expression and PC3 cell viability *in vitro*. (A) Reverse transcription-quantitative polymerase chain reaction demonstrated that siS6K1 significantly decreased S6K1 mRNA expression in the presence of TNFα. β-actin was used as a loading control. (B) Transient transfection of PC3 cells with siS6K1 significantly decreased TNFα-induced Gli1 mRNA expression compared with scrambled siRNA control. (C) Similarly, the phosphoinositide 3-kinase/mTOR dual inhibitor BEZ235 treatment at the indicated concentrations suppressed PC3 cell viability. (D) BEZ235 decreased endogenous Gli1 protein expression in PC3 cells. β-actin was used as a loading control. *P<0.05, **P<0.01 vs. control; $^{\wedge}$P<0.05 vs. TNFα treatment. mTOR, mammalian target of rapamycin; S6K1, p70 ribosomal S6 kinase 1; Gli1, glioma-associated oncogene; TNFα, tumor necrosis factor α; siRNA, small interfering RNA; p-, phosphorylated; siS6K1, siRNA targeting S6K1.

Discussion

The results of the present study identified that Gli1 is overexpressed at the protein level in androgen-independent prostate cancer cell lines compared with androgen-dependent prostate cancer cells. Silencing of Gli1 inhibited prostate cancer cell proliferation and liquid colony formation *in vitro*. The Gli1/2-specific inhibitor GANT61 markedly decreased prostate cancer.cell viability by inducing cell apoptosis. Pharmacological and genetic manipulation of the TNFα/mTOR/S6K1 signaling pathway altered Gli1 expression and cancer cell survival. These results suggest that Gli1 is a suitable target for drug development for androgen-independent prostate cancer.

Previous studies have indicated that the HH pathway contributes to the initiation as well as the progression of prostate cancer (17-19). Previous studies have assessed the protein expression of the HH components in tissue microarrays and identified that the expression of SHH, SMO and PTCH1 in the tumor was upregulated compared with adjacent normal tissue. However, stromal PTCH1, SMO and Gli1 expression were downregulated in the tumor compared with normal tissue (20,21). Canonical HH pathway activation appears to be more marked in late-stage prostate cancer (22,23). In addition, results of the present study and previous studies observed a potential association between HH signaling component expression and androgen-independent prostate cancer cells (1,15). Long-term androgen deprivation may induce an upregulation of HH signaling in human specimens and in cell lines. Inhibition of HH signaling led to a decrease in androgen receptor activation, partly because of the interaction between Gli1/2 and the androgen receptor (24). The present study provides experimental evidence for the application of Gli1/2 inhibitors in the treatment of androgen-insensitive prostate cancer.

In the present study, the main focus was on Gli1 activity and function in androgen-independent prostate cancer lines and it was identified that the Gli1/2-specific small-molecule inhibitor GANT61 significantly impaired prostate cancer cell proliferation. Further studies are required to elucidate the function of Gli1 and pharmacological effect of GANT61 *in vivo*. Gli2 has been demonstrated to be involved in the malignant transformation of prostate cancer cells. Thiyagarajan *et al* (25) reported that knockdown of Gli2 in prostate cancer cells suppressed tumor growth *in vitro* and *in vivo*. The mechanisms by which the HH signaling pathway serves a role in the initiation and progression of prostate cancer include the anti-apoptotic effect and the inhibition of invasiveness and metastasis. Karlou *et al* (26) also demonstrated that the SMO inhibitor GDC-0449 exhibited the ability to inhibit prostate cancer xenograft tumor growth. Despite the promising results of HH inhibition in prostate cancer cell lines and mouse models, this has not been fully translated into the clinic.

Previously, aberrant HH signaling in prostate tumors was reported to be ligand-dependent, although it is controversial whether this is in a paracrine or autocrine manner (3). In fact, the HH pathway is part of a complex signaling network that remains incompletely understood (9). The PI3K/Akt and

Ras/MAPK/ERK kinase (MEK)/ERK signaling pathways have been demonstrated to activate Gli1 in a SMO-independent manner (10,11). In prostate cancer, alterations in the PI3K/Akt signaling pathway are common in primary and metastatic lesions (27). The Ras/MEK/ERK pathway is also constitutively activated in a number of prostate tumor tissues and appears to exhibit an association with advanced and androgen-independent prostate cancer (27). Furthermore, the tumor suppressor PTEN, which is a negative regulator of the signaling pathway, is lost in <80% of prostate cancers, leading to constitutive activation of Akt pathway (22); however, the underlying molecular mechanism is not fully understood. In the present study, it was identified that the TNFα/mTOR/S6K1 signaling pathway was partly responsible for the aberrant overexpression of Gli protein. Inhibiting mTOR/S6K1 activity markedly blocked Gli1 function in androgen-independent prostate cancer cells. The next step is to determine the *in vivo* effect of inhibiting the mTOR/S6K1 signaling pathway on prostate xenograft tumor growth. Additionally, Narita *et al* (28) have demonstrated that inhibition of HH signaling may increase the chemosensitivity of prostate cancer cells.

The results of the present study indicate that Gli1 transcriptional activity is critical in androgen-independent prostate cancer cell proliferation. The PI3K/mTOR/S6K1 signaling pathway is implicated in the regulation of Gli1 expression and functions in a SMO-independent manner. Blocking the non-canonical HH pathway or directly inhibiting Gli1 transcriptional activity may open a new avenue for targeted therapies. Further study using mouse models may lead to a better understanding of the role of Gli1 in prostate cancer growth and eventually to inhibitors that may be used as tools for research or treatment.

Acknowledgements

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