Silencing glioma-associated oncogene homolog 1 suppresses the migration and invasion of hepatocellular carcinoma in vitro

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Abstract. Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-associated death worldwide. Glioma-associated oncogene homolog 1 (Gli1) is a key component and functions as a reliable marker of Hedgehog signaling pathway activation. Previous studies have demonstrated that Gli1 serves important roles in the progression of various types of cancer, including HCC. However, its effect on HCC invasion and metastasis and the underlying mechanism remain to be elucidated. Small interference RNA was employed to silence the Gli1 gene in liver cancer cells. Reverse transcription-quantitative PCR and western blot analysis were performed to evaluate the mRNA and protein expression of Gli1, respectively. A series of assays, including Cell Counting Kit-8, adhesion, wound healing and Matrigel invasion were performed to investigate cell viability, adhesive, migratory and invasive capabilities of liver cancer cells, respectively. In addition, immunofluorescence staining was performed to determine the cellular localization of focal adhesion kinase (FAK), phosphorylated (p-)FAK and p-AKT. The mRNA and protein expression of Gli1 in liver cancer cells (HepG2 and SK-Hep1) were markedly decreased in a dose-dependent manner following Gli1-knockdown. Gli1 silencing significantly inhibited the adhesion, migration and invasion of SK-Hep1 cells. Additionally, knockdown of Gli1 markedly suppressed the expression of metalloproteinase (MMP)-2 and MMP-9. Furthermore, downregulation of Gli1 blocked the FAK/AKT signaling pathway. Gli1 serves significant roles in the migration and invasion of HCC cells through activation of the FAK/AKT signaling pathway and subsequent upregulation of MMP-2 and MMP-9 expression. Thus, Gli1 may be a potential protein target for the regulation of HCC migration and invasion.

Introduction

Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-associated death worldwide (1,2). Due to the limited improvements in the diagnosis and treatment of HCC over the past two decades, the prognosis and survival rate of patients with HCC remain poor (3,4). The high mortality rate of HCC, with ~782,000 deaths annually, is largely caused by its metastases and recurrence (5). Therefore, an understanding of the mechanisms underlying the invasion and metastasis of HCC is key to improving the treatment of this disease.

Previous studies have demonstrated that aberrant activation of the Hedgehog (Hh) signaling pathway serves a critical role in the tumorigenesis and progression of various types of cancer, including pancreatic, breast, lung, ovarian, gastrointestinal cancer and HCC (6-8). As a key component of the Hh signaling pathway, glioma-associated oncogene homolog 1 (Gli1) is a member of the family of zinc finger transcription factors that functions as a downstream protein in the Hh signaling pathway. Gli1 is a reliable activation marker of the Hh signaling pathway (9). Emerging evidence has demonstrated that Gli1 is overexpressed in a number of types of cancer, including ovarian (10), prostate (11), gastric (12), esophageal squamous cell (13), pancreatic (14) and liver cancer (15). A recent study concluded that hyperactivation of Gli1 is sufficient to trigger the uncontrolled progression of cancer features such as proliferation, migration and invasion (16). However, the underlying mechanisms of Gli1 in the invasion and metastasis of HCC remain to be elucidated.

The focal adhesion kinase-phosphoinositide 3-kinase-AKT (FAK/Pi3K/AKT) axis is a critical pathway that regulates the invasion and metastasis of HCC (17). Briefly, FAK, a non-receptor protein tyrosine kinase, is an essential factor that regulates the PI3K/AKT signaling...
pathway, which is associated with a poor prognosis in different types of cancer (18). Activation of FAK can phosphorylate PI3K and subsequently lead to the phosphorylation of AKT (19). Phosphorylation of FAK is important for invasion and metastasis as it regulates the expression of matrix metalloproteinase (MMP)-2 and MMP-9 in HCC (20). Overexpression of FAK has been detected in various malignant tumors, including breast, prostate, colorectal and ovarian cancer (21-24). Ectopic expression of FAK and phosphorylated (p-)FAK are associated with the invasion and metastasis of HCC (20,25). Furthermore, phosphorylation of FAK can activate the PI3K/AKT signaling pathway, leading to regulation downstream of the pathway that transduces a β1 integrin viability signal in collagen matrices (26).

The present study evaluated the roles of Gli1 in liver cancer cells. Gaining an understanding of the mechanism underlying the function of Gli1 in the invasion and metastasis of HCC will provide a potential target to control the invasion and metastasis of HCC.

Materials and methods

Cell lines and culture. Human liver cancer cell lines (HepG2 and SK-Hep1) were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 (Biological Industries) supplemented with 10% fetal bovine serum (FBS; Biochrom, Ltd.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 µg RNA was reverse transcribed to complementary DNA (cDNA) using a RevertAid First Strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified 5% CO₂ atmosphere at 37°C.

RNA interference. Small interfering (si)RNA specific for Gli1 (si-Gli1) sequences and scrambled negative control (NC) siRNA with Gli1 analogue nonsense sequences were obtained from Sangon Biotech Co., Ltd. and were as follows: Gli1 (sense: 5'-CCAGUGUCGUCACUUAGAdTdT-3'; antisense: 5'-UGUGAAUCGAGACUGGtTd-3') and NC (sense: 5'-UUC UCGCGACUGUCACUGTT-3'; antisense: 5'-ACGUACGACGUUCCGAGAA-3'). Cells were seeded into 6-well plates and cultured to 80% confluence and then transfected with 50 nmol/l NC siRNA, and 50 or 100 nmol/l si-Gli1 for 24 h at 37°C using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, cells were divided into four groups: A blank control (CTRL) group (only treated with serum-free medium), a NC group (transfected with NC siRNA), a 50 nmol/l si-Gli1 group and a 100 nmol/l si-Gli1 group. All subsequent experiments were performed 24 h post-transfection.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from liver cancer cells (HepG2 and SK-Hep1) using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 µg RNA was reverse transcribed to complementary DNA (cDNA) using a RevertAid First Strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, qPCR was performed using FastStart Universal SYBR-Green Master (cat. no. 31168620; Roche Diagnostics GmbH), according to the manufacturer's protocol, and performed on an ABI 7500 Real-time PCR system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 95°C Denaturation for 15 min and then 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The relative mRNA expression levels of target genes were normalized to β-actin and calculated by use of the 2-ΔΔCT method (27). The specific sequences of primers were as follows: Gli1 forward: 5'-AGGGCTGCTGACAGCCTCTA-3', Gli1 reverse: 5'-CCTGACATGTGTTCGACAGG-3'; MMP-2 forward: 5'-GACAAACCCGCCATACAC-3', MMP-2 reverse: 5'-CATCTCGCCCGCTGTGTTAGT-3'; MMP-9 forward: 5'-AGCGACATCGTCATCCAGT-3', MMP-9 reverse: 5'-GGACCACAACCTGCTATCGTCACTC-3'; β-actin forward: 5'-GGTGACATCGCAGAAGCA-3', β-actin reverse: 5'-GAAAGGGTTGTAACGCAGACT-3'. All experiments were performed in triplicate.

Western blotting. Briefly, protein was extracted from cells (5x10⁶/well) using 6X sample buffer (1 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 0.5% Bromophenol Blue and 2-mercaptoethanol) for 10 min at 100°C and then centrifuged at 10,000 x g for 15 min on ice. The total protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The protein samples (10 µg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore). Subsequently, the membranes were incubated with blocking buffer (5% non-fat dry milk and 0.05% Tween-20 in PBS) for 1 h at 37°C and the following primary antibodies overnight at 4°C: Rabbit anti-Gli1 (Cell Signaling Technology, Inc.; cat. no. 3538S; 1:1,000 dilution), rabbit anti-MMP-2 (Cell Signaling Technology, Inc.; cat. no. 40994S; 1:1,000 dilution), rabbit anti-MMP-9 (Abcam; cat. no. ab83889; 1:1,000 dilution), rabbit anti-FAK (Abcam; cat. no. ab40794; 1:1,000 dilution), rabbit anti-p-FAK (Abcam; cat. no. ab39967; 1:1,000 dilution), rabbit anti-P13K (Cell Signaling Technology, Inc.; cat. no. 4257S; 1:1,000 dilution), rabbit anti-p-P13K (Cell Signaling Technology, Inc.; cat. no. 4228S; 1:1,000 dilution), mouse anti-AKT (Cell Signaling Technology, Inc.; cat. no. 2920S; 1:1,000 dilution), rabbit anti-p-AKT (Abcam; cat. no. ab81283; 1:1,000 dilution) and mouse anti-β-Actin (Abcam; cat. no. ab8226; 1:2,000 dilution). Finally, the membranes were incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich; Merck KGaA; cat. nos. A8919 and AP186P; 1:10,000 dilution) for 1 h at room temperature. The immunoblot bands were visualized using an enhanced chemiluminescence kit (cat. no. E1328; Beijing Lillye Science & Technology Co., Ltd., http://www.micro-helix.com) and exposed on the gel imaging analysis system (Amersham Imager 600; Cytiva). The intensity of bands was quantified using ImageJ software (version 1.48; National Institutes of Health).

Immunofluorescence staining. The cells transfected with or without si-Gli1 were fixed in 4% paraformaldehyde/PBS for 15 min at -20°C and then washed three times using PBS containing 0.1% Triton X-100 (PBST) for 15 min at room temperature. Following blocking in PBS containing 5% BSA for 30 min at 37°C, cells were incubated with the primary antibodies rabbit anti-FAK (Abcam; cat. no. ab40794; 1:1,000 dilution), rabbit anti-p-FAK (Abcam; cat. no. ab39967; 1:1,000 dilution) and rabbit anti-p-AKT (Abcam; cat. no. ab81283; 1:1,000 dilution) for 1 h at room temperature. The labeled
cells were washed three times with PBST and then incubated with Alexa Fluor 594 (goat anti-rabbit IgG; Abcam, cat. no. ab150088; 1:1,000 dilution) for 1 h at room temperature in the dark. After washing three times with PBST, the cell nuclei were stained with DAPI (1 µg/ml, Abcam, cat. no. ab228549) for 10 min at room temperature in the dark and detected with a confocal laser scanning microscope (LSM 880; Zeiss AG) at x63 magnification. Cell clusters were counted in five randomly selected fields using a fluorescence microscope and the mean intensity was calculated using ImageJ software.

**Cell viability assay.** Cell viability was determined by Cell Counting Kit (CCK)-8 assay (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. CA-1210-500) following the manufacturer’s instructions. Briefly, liver cancer cells with or without transfection of si-Gli1 were incubated in 96-plates for 24 h at 37°C. Then, cells were added to 10 µl CCK-8 staining reagent and then incubated for another 4 h at 37°C. Absorbance at 450 nm was measured using a microplate reader (Varioskan Lux; Thermo Fisher Scientific, Inc.).

**Adhesion assay.** Cell adhesive abilities of SK-Hep1 cells in different groups were determined using an adhesion assay. In brief, 50 µl Matrigel (BD Biosciences; cat. no. 8036008) was added to each well in a 96-well plate for 30 min at 37°C and then 5x10⁴ cells were seeded in the coated plates for incubation for 4 h at 37°C. Finally, cells were washed with PBS to wash away the unattached cells. The attached cells were analyzed using the CCK-8 assay as described above.

**Wound healing assay.** Cell migratory abilities were detected using wound healing assay. The prepared cells treated with or without si-Gli1 were cultured in 6-well plates to ~90% confluence using serum-free medium. The confluent monolayer cells were scratched gently with a 100 µl pipette tip and images captured using an inverted microscope (DMi8; Leica Microsystems GmbH; magnification, x100) at 0 and 24 h. The wound area was quantified using ImageJ software.

**Transwell invasion assay.** Cell invasion assay was performed using 24-well Transwell plates with 8-µm pore filters (Corning Life Sciences; cat. no. 3422) precoated with Matrigel Basement Membrane Matrix (BD Biosciences) at 37°C for 1-4 h according to the manufacturer’s protocols. Briefly, 1x10⁴ cells were suspended in 100 µl of serum-free medium and added to the upper chamber and then 600 µl RPMI-1640 supplemented with 20% FBS was loaded into the lower chamber. After 24 h of incubation, the non-invaded cells in the top chamber were removed with a cotton swab and the cells fixed with 100% methanol for 20 min at room temperature and stained with 0.1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. G1063) for 30 min at room temperature. The stained cells were imaged under an inverted light microscope (DMi8; Leica Microsystems GmbH; magnification, x100) and analyzed using ImageJ software.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Unpaired Student’s t-test was used to compare differences between two groups, whilst one-way analysis of variance followed by
Knockdown of Gli1 inhibits Gli1 expression in HepG2 and SK-Hep1 cells. To understand the potential function of Gli1 in liver cancer cells, Gli1 expression was downregulated by RNA interference. The knockdown efficiency of Gli1 in liver cancer cells was determined using RT-qPCR and western blotting. The results demonstrated that the mRNA and protein expression levels were significantly decreased in a dose-dependent manner and the most efficient concentration for Gli1 silencing was 100 nmol/l in both HepG2 and SK-Hep1 cells (P<0.05 and P<0.01; Fig. 1).

To investigate whether the efficiency of Gli1 transfected with siRNA was dependent on its cellular cytotoxicity, CCK-8 assay was performed to examine the viability of HepG2 and SK-Hep1 cells. The results demonstrated that the 50- and 100-nmol/l si-Gli1 groups were not significantly different compared with the CTRL group (P>0.05; Fig. 2), which indicated that the effect of Gli1 transfection and the following experiments using liver cancer cells was independent of its cellular cytotoxicity.

Reduced Gli1 reduces the adhesion, migration and invasion of SK-Hep1 cells. Our previous findings demonstrated that the aberrant activation of Gli1 is correlated with invasion and metastasis in HCC tissues (28). Therefore, the effects of Gli1-knockdown on the adhesive, migratory and invasive abilities of hepatoma cells in vitro were determined. First, a cell adhesion assay was performed to test the adhesive ability of the SK-Hep1 cells. The results demonstrated that the 50- and 100-nmol/l si-Gli1 groups were not significantly different compared with the CTRL group (P>0.05; Fig. 2), which indicated that the effect of Gli1 transfection and the following experiments using liver cancer cells was independent of its cellular cytotoxicity.

Figure 3. Gli1 interference inhibits cell adhesion, migration and invasion in SK-Hep1 cells. (A) Cell adhesive ability of hepatoma cells was significantly suppressed in the si-Gli1 groups compared with the CTRL group using cell adhesion assay. (B and C) Images and (D and E) histograms showing that Gli1 siRNA markedly decreased the migration and invasion abilities of SK-Hep1 cells as demonstrated by the wound healing and Matrigel invasion assays, respectively. Scale bar, 100 µm. *P<0.05, **P<0.01 and ***P<0.001 vs. CTRL group. Gli1, glioma-associated oncogene homolog 1; si, small interference; CTRL, blank control group; NC, negative control siRNA group.

Dunnett's post-hoc test were performed to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of Gli1 inhibits Gli1 expression in HepG2 and SK-Hep1 cells. To understand the potential function of Gli1 in liver cancer cells, Gli1 expression was downregulated by RNA interference. The knockdown efficiency of Gli1 in liver cancer cells was determined using RT-qPCR and western blotting. The results demonstrated that the mRNA and protein expression levels were significantly decreased in a dose-dependent manner and the most efficient concentration for Gli1 silencing was 100 nmol/l in both HepG2 and SK-Hep1 cells (P<0.05 and P<0.01; Fig. 1).

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Dunnett's post-hoc test were performed to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.
Knocked down in hepatoma cells compared with the CTRL group (P<0.05 and P<0.01; Fig. 3B and D), suggesting that downregulation of Gli1 expression may suppress the motility of SK-Hep1 cells. Finally, the results of the invasion assay revealed that the invasive capability of the cells was also significantly inhibited after Gli1 was knocked down (P<0.01 and P<0.001; Fig. 3C and E). In addition, NC siRNA had no significant effects on the cell adhesion, migration and invasion of SK-Hep1 cells.

Knockdown of Gli1 decreases the expression levels of MMP-2 and MMP-9. Accumulating evidence has elucidated that MMPs serve a critical role in the invasion and metastasis of HCC by degrading the extracellular matrix (ECM) components (29,30). Therefore, to determine if Gli1 silencing-mediated inhibition of cell migration and invasion was associated with MMP-2 and MMP-9, RT-qPCR and western blot analyses was performed to detect their expression. The results revealed that the mRNA and protein expression levels of MMP-2 and MMP-9 were significantly decreased in both si-Gli1 groups compared with the CTRL group (P<0.05, P<0.01 and P<0.001; Fig. 4), indicating that inhibition of the migration and invasion of hepatoma cells by Gli1 depletion may be associated with the downregulation of MMP-2 and MMP-9.

Silencing Gli1 inhibits FAK/AKT signaling. To explore the crosstalk between Gli1 and the FAK/AKT pathway, FAK, p-FAK, PI3K, p-PI3K, AKT and p-AKT protein expression was detected in SK-Hep1 cells following Gli1-knockdown. The western blotting results demonstrated that the protein levels of p-FAK, p-PI3K and p-AKT were markedly decreased in both si-Gli1 groups compared with the CTRL group, whereas those of FAK, PI3K and AKT were not significantly different from the CTRL group (P<0.05, P<0.01 and P<0.001; Fig. 5A and B). The results of immunofluorescence staining were in agreement and demonstrated that the fluorescence intensity of p-FAK localized in the cell membrane was significantly weaker compared with the CTRL group (P<0.05; Fig. 5C and E) and that of p-AKT located in the nucleus was also significantly weaker in both si-Gli1 groups compared with the CTRL group (P<0.01; Fig. 5D and F). Notably, the intensity of total FAK localized in the cell membrane did not reveal any significant change (P>0.05; Fig. 6), which was consistent with the results of western blotting above, suggesting that the observed decrease in p-FAK protein level was not due to a decrease in the amount of total FAK protein.

Discussion

The results of the present study demonstrated that the efficiency of Gli1 transfected with siRNA was feasible and was independent of its cellular cytotoxicity. Loss of function experiments were performed to elucidate the effects of Gli1 on the biological functions of hepatoma cells. By performing cell adhesion, wound healing and invasion assays after knockdown of Gli1, the data revealed that knockdown significantly reduced the motility and invasion of hepatoma cells. These results indicated that Gli1 serves a crucial role in cell migration and invasion, and provides a potential therapeutic target for HCC.

Cancer invasion and metastasis are complicated multi-step processes in which tumor cells separate from the original site, intravasate into blood vessels, extravasate to a systemic location and colonize at a secondary site (31). Degradation of ECM and proteolysis of the basement membrane are early events in cancer invasion and metastasis (32,33). It has been found that MMPs contain three domains: A catalytic domain, pro-peptide domain and hemagglutinin-like C-terminal domain (34). Tumor cells undergo invasion and metastasis during the early stages of cancer based on their ability to degrade ECM components (35). Among MMPs, MMP-2 and MMP-9 are the two most important factors that degrade type IV collagen, which is the main component of the basement membrane, and serve an essential role in tumor invasion.
and metastasis (36). Chen et al (15) found that overexpression of MMP-2 and MMP-9 is observed in HCC tissues and is associated with the invasion and metastasis of malignant hepatoma. The present study found that silencing of Gli1 markedly inhibited the mRNA and protein expression of MMP-2 and MMP-9, suggesting that low expression of Gli1 may suppress HCC migration and invasion by regulating the expression of MMP-2 and MMP-9.

A previous study demonstrated that blockade of the FAK/AKT signaling pathway inhibits head and neck cancer metastasis (37). Another study demonstrated that activating the FAK/AKT pathway promotes the epithelial-mesenchymal transition and invasion of HCC (38). Consequently, identifying the crosstalk between Gli1 and FAK/AKT pathway may provide a further understanding of the molecular mechanism of HCC invasion and metastasis. In the present study, Gli1 interference decreased the expression of p-FAK, p-PI3K, and p-AKT as known as the active ingredients of the FAK/AKT signaling, while total FAK, PI3K, and AKT protein expression was unaltered in the HCC cells. The
data from the present study indicated that siRNA-mediated depletion of Gli1 may significantly inhibit the FAK/AKT signaling pathway in vitro. In addition, a previous study demonstrated that phosphorylation of FAK promotes the invasion and metastasis of HCC by upregulating the expression of MMP-2 and MMP-9 (20). Abnormal activation of the PI3K/AKT signaling pathway can promote the proliferation and invasion of tumor cells by regulating the expression of MMP-2 and MMP-9 (39-41). Thus, it was hypothesized that the downregulation of Gli1 suppressed the invasion and metastasis of HCC cells, which may be achieved by blocking the expression of MMP-2 and MMP-9 mediated through the FAK/AKT signaling pathway.

The present study is not without limitations. First, the present study only assessed the effects of Gli1 knockdown on the biological functions of hepatoma cells. Prospective studies will upregulate the Gli1 gene to determine the effects of Gli1 on the migratory and invasive abilities of liver cancer cells.

Secondly, the molecular mechanism in the present study is not fully elucidated. Whether downregulation of Gli1 blocks the FAK/AKT signaling pathway, directly or indirectly, requires further investigation via co-immunoprecipitation. Furthermore, understanding protein interactions between the Gli1 and FAK/AKT pathway components may help determine the underlying molecular mechanism presented here.

In conclusion, the results of the present study demonstrated that abnormal Gli1 expression resulted in the significant repression of hepatoma cell migration and invasion via down-regulating MMP-2 and MMP-9 by blocking the FAK/AKT signaling pathway. These observations revealed novel biological insights into the mechanisms underlying HCC invasion and metastasis, and highlight Gli1 as a potential therapeutic target for HCC.

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Availability of data and materials

All data generated or analyzed during the present study are available from the corresponding authors upon reasonable request.

Authors’ contributions

JNZ and BC conceived and designed the study. ZH, FX, AH and MX performed the experiments and collected the data. YL and JKZ analyzed the data and ZH wrote the manuscript. JX and YS replicated the experiments and revised the figures. JNZ and BC critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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