Hedgehog–GLI Signaling Inhibition Suppresses Tumor Growth in Squamous Lung Cancer

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Abstract

Purpose: Lung squamous cell carcinoma (LSCC) currently lacks effective targeted therapies. Previous studies reported overexpression of Hedgehog (HH)–GLI signaling components in LSCC. However, they addressed neither the tumor heterogeneity nor the requirement for HH–GLI signaling. Here, we investigated the role of HH–GLI signaling in LSCC, and studied the therapeutic potential of HH–GLI suppression.

Experimental Design: Gene expression datasets of two independent LSCC patient cohorts were analyzed to study the activation of HH–GLI signaling. Four human LSCC cell lines were examined for HH–GLI signaling components. Cell proliferation and apoptosis were assayed in these cells after blocking the HH–GLI pathway by lentiviral-shRNA knockdown or small-molecule inhibitors. Xenografts in immunodeficient mice were used to determine the in vivo efficacy of GLI inhibitor GANT61.

Results: In both cohorts, activation of HH–GLI signaling was significantly associated with the classical subtype of LSCC. In cell lines, genetic knockdown of Smoothened (SMO) produced minor effects on cell survival, whereas GLI2 knockdown significantly reduced proliferation and induced extensive apoptosis. Consistently, the SMO inhibitor GDC-0449 resulted in limited cytotoxicity in LSCC cells, whereas the GLI inhibitor GANT61 was very effective. Importantly, GANT61 demonstrated specific in vivo antitumor activity in xenograft models of GLI² cell lines.

Conclusion: Our studies demonstrate an important role for GLI2 in LSCC, and suggest GLI inhibition as a novel and potent strategy to treat a subset of patients with LSCC. Clin Cancer Res; 20(6): 1566–75. ©2014 AACR.
Translational Relevance

Targeted therapeutics for lung squamous cell carcinoma (LSCC) are currently lacking. In this study, we have analyzed molecular subtypes of LSCC and identified overexpression of Hedgehog family members in the classical subtype. In representative LSCC cell lines, genetic deletion of Smoothened (SMO) produced minor effects on cell survival, whereas GLI2 knockdown greatly reduced cell viability and induced extensive apoptosis. Using both in vitro and in vivo approaches, we evaluated therapeutic efficacy of GDC-0449, a clinically available SMO inhibitor, as well as GANT61, a targeted GLI inhibitor. GANT61 was significantly more effective than GDC-0449 in reduction of proliferation and induction of apoptosis. We report SMO-independent regulation of GLI in LSCC, and present a potential strategy of targeting GLIs to treat a subset of patients with LSCC.

Materials and Methods

RNAseq and microarray analysis

RSEM values (35) for 178 tumor samples from The Cancer Genome Atlas (TCGA) LSCC study (3) were converted to expression measurements by replacing values equal to zero with the smallest nonzero value, taking a log-transformation. After median centering by gene, heatmaps of the expression values from TCGA (3) and microarray data from 56 LSCC samples collected at the University of North Carolina (ref. 2; UNC cohort) were produced with R 2.15.1 (36) and the gplots package. Hypotheses were subsequently tested: one-sided Wilcoxon rank sum tests were used to test the null hypothesis that the mean expression levels of PTCH1, GLI1, GLI2, GLI3, and SUFU are the same in the classical subtype as all other subtypes combined. For PTCH1, GLI1, and GLI2, the alternative hypothesis was that the expression levels are higher in the classical subtype, whereas for GLI3 and SUFU, the alternative hypothesis was that the expression levels are lower in the classical subtype. A Bonferroni adjustment was applied to correct for multiple comparisons.

Two-sided Wilcoxon rank sum tests were used to test the null hypothesis that GLI1 and GLI2 expression values were equal in the TCGA and UNC cohorts. Spearman correlation coefficients were computed on the basis of the uncentered expression values of GLI1, GLI2, TP63, PIK3CA, and SOX2 in both cohorts. The resulting unadjusted *P* values were used to assess the significance of these associations (Supplementary Table S2).

Gene expression data from 20 LSCC cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE; ref. 37). After median centering the expression values by gene, the centroid classifier from ref. (2) was used to predict expression subtypes for each line by finding the nearest centroid using a distance metric equal to one minus the Pearson correlation coefficient. Gene expression heatmaps were then produced using R2.15.1 (36) and the gplots package.

Cell culture and reagents

NCI-H520, NCI-H2170, NCI-H226, and SK-MES-1 cells were obtained from the American Type Culture Collection. Cell lines were routinely verified by morphology and growth characteristics, and verified biannually to be mycoplasma-free. NCI-H520, NCI-H2170, and NCI-H226 cells were maintained in the RPMI-1640 medium containing 10% FBS. SK-MES-1 cells were maintained in minimum essential medium (MEM) containing 10% FBS, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium pyruvate. Antibodies used were SHH, PTCH1, and SMO (Santa Cruz Biotechnology); GLI2 and GAPDH (Abcam); GLI1 (Novus Biologicals); cleaved caspase-3 and cleaved PARP (Cell Signaling Technology); and CCND1 (BD Biosciences). Compounds used were GANT61 (Sigma) and GDC-0449 (Chemietek).

Lentiviral production and transduction

Lentiviral short hairpin RNA (shRNA) clones (Sigma; MISSION RNAi) targeting SMO, GLI2, and the nontargeting control (SHC002) were purchased from Sigma-Aldrich. 293T cells were plated in 10-cm plates 24 hours before transfection in Dulbecco’s Modified Eagle Medium (DME
containing 10% FBS without antibiotics; 5 µg of shRNA plasmid, 4 µg pSPAX2, and 1 µg pCI-VSVG packaging vectors (Addgene) were cotransfected into 293T cells using Lipofectamine 2000 Reagent (Invitrogen). Viral supernatants were collected, centrifuged, and filtered with 0.45-µm PES sterile Syringe filter. Target cells were plated and incubated at 37°C, 5% CO₂ overnight, and changed to medium containing lentivirus and 8 µg/ml polybrene. Control plates were incubated with medium containing 8 µg/ml polybrene. Cells were changed to fresh culture medium 24 hours after infection. Puromycin selection (5 µg/mL) was started 48 hours after infection and continued for 4 to 5 days until no viable cells were observed in control plates. Once decreased expression of the targeted gene was confirmed, cells were used for subsequent experiments. Stable expression of non-targeting control, SMO, or GLI2 shRNAs was ensured by culturing cells in the presence of puromycin.

The shRNA sequences were as follows:

SMO sh1 (5’-CCGGCCCTGATGGACAAGACATCGATCGAGATGAGTCTG TGTCACGAGGTGTTTTT-3’)
SMO sh2 (5’-CCGGCACTTTTCTCCTGACTACTCTG ACTGATACAGCA TAGAACCAGGTTG AICTICIATCCACGTTTTT-3’)
SMO sh3 (5’-CCGGGTGAGAGAGAGATGCCTGTGTTTTC TGCTCAGGTGGTTTCTCG-AGAAACAGGTTG ATCTTCTCCACTTTTT-3’)
GLI2 sh1 (5’-CCGGCCCAACGAGAAACCCTACATCTGGCTCAGATGATGGTTGT CTTCGGTGGTTTTT-3’)
GLI2 sh2 (5’-CCGGCACTCAAGAGATCTCCTGCTCAGATGAGAAGAAA CAGGTTG AICTICIATCCACGTTTTT-3’)
GLI2 sh3 (5’-CCGGCCCTGATGGACAAGACATCGATCGAGATGAGTCTG TGTCACGAGGTGTTTTT-3’)

Assessment of cell viability and caspase-3/7 activity

Cell viability and caspase-3/7 activity were determined by using ApoLive-Glo Multiplex Assay (Promega) according to the manufacturer’s instructions. Briefly, cells were seeded in 96-well clear-bottom white plates at a density of 10,000 cells per well and incubated with complete medium overnight at 37°C, 5% CO₂. The following day, cells were changed into 0.5% FBS-containing medium with either dimethyl sulfoxide (DMSO) control or drugs at designated concentrations (0.1% final DMSO concentration) as triplicates and treated for 96 hours. At the end of treatment, viability reagent was added into all wells and gently mixed. After 1.5-hour incubation at 37°C, fluorescence was measured at the wavelength set 355 EX/520 EM by a FLUOstar Omega Microplate reader. Later, Caspase-Glo 3/7 Reagent was added to all wells and gently mixed. Luminescence was measured after 1-hour incubation at room temperature. The reading of the blank control was subtracted from readings of other wells as the background in the data analysis.

RNA isolation and quantitative PCR

Total RNA was isolated using the Qiagen RNeasy Mini Kit, treated with DNase I (Invitrogen), and converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using TaqMan Gene Expression Master Mix on an Eppendorf Mastercycler, and raw data were analyzed by Realplex software. TaqMan probes for SHH, PTCH1, SMO, GLI1, GLI2, HHIP, and GAPDH were purchased from Applied Biosystems.

Western blot analysis

Total cellular lysates were prepared by using RIPA buffer (Sigma) with protease inhibitor cocktail (Sigma) and PhosSTOP (Roche). Protein concentrations were determined by the Micro BCA Protein Assay Kit (Thermo Scientific). Proteins were separated on the NuPAGE 4%–12% Bis-Tris Gel (Life Technologies) and transferred using Invitrolon PVDF/Filter Paper Sandwich. Membranes were blocked with 5% nonfat dry milk or 5% bovine serum albumin (BSA) in 0.1% Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C. They were subsequently washed with 0.1% TBST and incubated with the secondary antibody for 1 hour at room temperature. Western Lightning - ECL (PerkinElmer) was used to develop the membranes.

Xenograft and tumor treatment

Of note, 10⁴ NCI-H520 cells, 10⁵ NCI-H2170 cells, or 5 × 10⁴ NCI-H226 cells were suspended in a total volume of 100 µL of a 1:1 mixture of RPMI-1640 medium: Matrigel (BD Biosciences). Cells were injected subcutaneously in the right posterior flank of 6- to 8-week C.129S7 (B6)−/− mice. Tumors were grown until they reached a median size of approximately 250 mm³ (NCI-H520), approximately 230 mm³ (NCI-H2170), and approximately 150 mm³ (NCI-H226). Animals were randomly divided into groups and treated with solvent only (corn oil: ethanol, 4:1) or GANT61 in solvent (50 mg/kg). Treatments were given every other day for 20 days by intraperitoneal injection. Tumor volumes were calculated by the formula 0.52 × length × width². At the end of treatment, tumors were removed, weighed, and processed for subsequent analysis. All animal experiments were approved by and conformed to the policies and regulations of the Institutional Animal Care and Use Committees at Duke University.

Results

Activation of HH–GLI signaling is associated with the classical subtype of human LSCC

To ascertain whether HH signaling is upregulated in a particular subset of patients with LSCC, the RNA expression data of 178 patient samples from the TCGA LSCC study (3) was queried. As Fig. 1A demonstrates, the expression of HH target genes (PTCH1, GLI1, and GLI2) was significantly higher, whereas expression of negative regulators (GLI3 and SUFI) was substantially lower in the classical subtype in comparison with the other subtypes. The one-sided Wilcoxon rank sum test confirmed these observations (Supplementary Table S1) even after applying a Bonferroni adjustment for multiple comparisons. Similar expression patterns were seen in an independent cohort of 56 LSCC samples collected at the University of North Carolina (ref. 2; UNC cohort: Supplementary Fig. S1A). In both cohorts, GLI2
mRNA level was significantly higher than GLI1 (Fig. 1B and Supplementary Fig. S1B). Samples with high GLI2 expression were mainly found in the classical subtype, although occasionally in other subtypes. When taking the 75th percentile of all GLI2 expression values in a given cohort as the threshold for high GLI2, 55% (TCGA cohort) and 52% (UNC cohort) of all classical subtype samples exhibited high GLI2 expression (Fig. 1C and Supplementary Fig. S1C). Strong positive correlations between GLI2 and the prominent markers for the classical subtype (SOX2, TP63, and PIK3CA) on chromosome 3q were observed in both cohorts (Fig. 1D–F and Supplementary Fig. S1D–S1F). However, GLI1 was only associated with classical chr3q genes in the TCGA cohort (Supplementary Fig. S1G–I), suggesting that GLI2 is highly likely to be the major signaling transducer in LSCC. Spearman correlation coefficients and corresponding P values are provided in Supplementary Table S2.

HH–GLI signaling components are expressed in human LSCC cell lines

The difficulty of growing human LSCC cells in vitro limits available primary cancer cells. Therefore, we chose the four most widely used human LSCC cell lines to analyze active HH signaling: NCI-H520 and NCI-H2170, derived from primary tumors; and NCI-H226 and SK-MES-1, derived from metastatic pleural effusions. By real-time PCR (Fig. 2A) and Western blot analyses (Fig. 2B), high levels of SHH were detected only in NCI-H520, whereas PTCH1 and SMO were expressed universally across all four lines. Neither GLI1 nor GLI2 was detected in NCI-H2170. In the remaining three lines, GLI1 was expressed at a low level, whereas high levels of GLI2 were consistently detected at both the mRNA and protein level. To ascertain whether these cell lines represent different LSCC subtypes by mRNA expression, gene expression profiles from CCLE (37) were analyzed. NCI-H520 and NCI-H2170 were predicted to be classical subtype, and NCI-H226 and SK-MES-1 as secretory subtype. Gene expression of the subtypes between the cell lines and patient tumors is consistent over the validation gene set (Supplementary Fig. S2).

shRNA knockdown of SMO produces minor effects on LSCC survival

The universal expression and the ability to target SMO with multiple available inhibitors prompted us to investigate...
the importance of SMO in LSCC cells. Lentiviral-mediated expression of independent SMO shRNA constructs successfully reduced the SMO mRNA level by 70% to 90% in four cell lines (Fig. 3A). However, only minor effects on cell viability and apoptosis were observed in these cells (Fig. 3B and C). SMO knockdown caused a moderate decrease of PTCH1 mRNA in NCI-H520 and NCI-H226 (Fig. 3D), but no significant reduction of HHIP mRNA in any of four lines (Fig. 3E). These data suggest a minimal role for SMO in regulating LSCC survival via the canonical HH pathway.

Interestingly, loss of SMO did not reduce GLI2 mRNA level in three GLI+ cell lines. Instead, we noted a slight increase of GLI2 mRNA (Fig. 3F), which may be caused by compensatory upregulation of GLI2 by other SMO-independent mechanisms.

Targeting GLI2 with shRNAs inhibits LSCC cell growth and induces extensive apoptosis

Because GLI1 is hardly detectable and high level of GLI2 is consistently expressed in three cell lines and across human LSCC tumors, we focused on GLI2. Independent lentiviral-based GLI2 shRNAs achieved satisfactory knockdown of GLI2 protein in all three GLI2+ cell lines (Fig. 3G). Knockdown of GLI2 reduced the protein level of the GLI target CCND1 (Fig. 3G), corresponding to a strong inhibition of cell proliferation and survival (Fig. 3H). Loss of GLI2 also induced extensive apoptosis, demonstrated by elevated caspase-3/7 activity (Fig. 3I), and the detection of cleaved caspase-3 and cleaved PARP (Fig. 3G). These data suggest an important role of GLI2 in regulating LSCC cell survival, raising the possibility that GLI2 is a therapeutic target in human LSCC.

**GLI inhibitor (GANT61) leads to significant growth inhibition and apoptosis, and demonstrates greater efficacy than the SMO inhibitor (GDC-0449)**

To investigate the feasibility of pharmacologically targeting SMO or GLI proteins in LSCC, we studied the therapeutic potential of a clinically available SMO inhibitor, GDC-0449, and a GLI inhibitor, GANT61. To maintain physiologic relevance and minimize off-target toxicity, we assessed the efficacy of GDC-0449 and GANT61 in four LSCC cell lines at the concentrations of 2.5, 5, and 10 μmol/L. Cells were treated in triplicate with either DMSO control, GDC-0449, or GANT61 for 96 hours, and then assayed for viability and caspase-3/7 activation.

As Fig. 4A and B demonstrates, GDC-0449 showed limited growth inhibition and apoptosis induction only in NCI-H520 and NCI-H226 cells at 10 μmol/L despite the universal expression of SMO. Consistently, GDC-0449 only caused modest reduction of PTCH1 mRNA in NCI-H226 cells (Fig. 5A). Among three GLI+ cell lines, GDC-0449 led to slight decrease of GLI2 mRNA in NCI-H226 and SK-MES-1 (Fig. 5B), again suggesting a minimal role of SMO in mediating HH–GLI signaling in LSCC.

In contrast, GANT61 demonstrated greater efficacy in a dose-dependent manner in all GLI+ cells. The originally reported IC50 of GANT61 to reduce GLI-luciferase reporter...
activity is approximately 5 μmol/L (29), and 5 to 30 μmol/L is commonly used (29–31). In our studies, the IC₅₀ of growth inhibition for three GLI⁺ lines was approximately 5 μmol/L (Fig. 4C). Both NCI-H520 and NCI-H226 showed a 55% reduction at 5 μmol/L and a 90% reduction at 10 μmol/L in cell survival. SK-MES-1 displayed approximately 40% and 60% decrease in viability at 5 and 10 μmol/L, respectively. As expected, GANT61 exhibited little cytotoxicity in GLI⁻ NCI-H2170 cells. Consistently, increased apoptosis was seen in GLI⁺ cell lines at corresponding GANT61 concentrations: NCI-H520 (1.8–2.3 fold), NCI-H226 (2.8–4 fold), and SK-MES-1 (2.4–2.6 fold), but not in GLI⁻ NCI-H2170 (Fig. 4D).

Real-time PCR demonstrated significant reduction of HH downstream targets (GLI2, PTCH1, and HHIP) in NCI-H520 with a greater decrease in NCI-H226 and SK-MES-1 in comparison with DMSO control (Fig. 5C–E). Western blot analysis confirmed the reduction of GLI2 protein in GANT61-treated cells (Fig. 5F). Cleaved caspase-3 and cleaved PARP were detected in cells receiving GANT61 (Fig. 5F). The protein level of CCND1 was also decreased by GANT61 treatment, indicating impaired cell proliferation in addition to increased cell death (Fig. 5I).

Taken together, our results suggest that targeting HH signaling at the level of GLI proteins may be more effective than targeting either the ligand SHH or the receptor SMO in LSCC, potentially due to the existence of the ligand or receptor-independent pathway activation.

GANT61 suppresses GLI⁺ tumor progression in vivo

Currently, there are no available transgenic murine models that faithfully recapitulate human LSCC. Recently, patient-derived xenograft models of LSCC have shown promise, but have not yet achieved satisfactory progress. Therefore, we used a xenograft model of representative human LSCC cell lines to determine the efficacy of GANT61 in vivo. GLI⁺ NCI-H520 or NCI-H226 and GLI⁻ NCI-H2170 cancer cells were injected subcutaneously into the right flank of immune-deficient Rag²⁻/⁻ mice. Mice were randomly divided into two groups when tumors reached a

Figure 3. SMO plays a minor role, whereas GLI2 is required for LSCC survival in vitro. A, change of GAPDH-normalized SMO mRNA level by real-time PCR following lentiviral shRNA knockdown. Nontargeting shRNA control (shNT) or two independent shRNAs (SMO sh1, 2, 3) targeting SMO were used in each cell line. B and C, measurements of viability (B) and apoptosis (C) in cells after SMO knockdown. Data were normalized to shNT control and represent the mean ± SD of three independent experiments. Two-tailed t test, ***, P < 0.01; ****, P < 0.001.
median size of approximately 250 mm$^3$ for NCI-H520 ($n = 8$ for each group), approximately 150 mm$^3$ for NCI-H226 ($n = 5$ for each group), and approximately 230 mm$^3$ for NCI-H2170 ($n = 5$ for each group). We began treatment with either solvent control or GANT61 at a previously described dose of 50 mg/kg (29) by intraperitoneal injection every other day. During a 20-day treatment period, suppression of tumor growth was observed in the groups receiving GANT61 (Fig. 6A and B) for both NCI-H520 and NCI-H226. In contrast, no significant difference of tumor growth was found in the NCI-H2170 xenograft (Fig. 6C). GANT61 led to a significant 40% reduction of tumor weight for both GLI$^+$ cell lines in comparison with solvent control, but had no effects on GLI$^-$ NCI-H2170 tumors (Fig. 6D–F), suggesting specific antitumor efficacy of GANT61. No adverse side effects, such as weight loss, ulcerations, or general illness of the animals, were observed. Real-time PCR analysis confirmed that GANT61 reduced the mRNA level of GLI target genes PTCH1 and HHIP in NCI-H520 and NCI-H226 xenografts, but not in NCI-H2170 tumors (Fig. 6G–I).

Discussion

Aberrant HH signaling has been implicated in a diverse spectrum of human cancers. Previous studies have reported hyperactive HH signaling in a subset of LSCC, but they failed to address the complexity and heterogeneity of the disease. Four distinct molecular subtypes, which have different survival outcomes, patient populations, and biologic processes, were identified by gene expression-subtype signatures (2). In the recent study of TCGA (3), we found that the HH activation is associated with the classical subtype (~36% of LSCC). A consistent pattern was observed in an independent UNC microarray dataset (2). This observation is consistent with previous immunohistochemical studies (19, 20), which showed high activation of HH signaling in approximately 27% of patients with LSCC. Among all four subtypes, the classical subtype (2, 3) has the highest proportion of smokers and the heaviest smoking history, as well as the greatest overexpression of three known oncogenes on 3q26 amplicon: SOX2, TP63, and PIK3CA (2, 3).
While GLI2 was consistently highly expressed in the classical subtype, strong positive correlations between GLI2 and the three best-known markers of the classical subtype on chromosome 3q were observed, together suggesting a critical role of GLI2 on chromosome 3q, as found in SCC in other organs (22–24). Interestingly, the expression of ligand SHH within the classical subtype varied markedly and was not significantly different between subtypes. The expression patterns of other two HH ligands, IHH and DHH, were similar to SHH (Supplementary Fig. S1I). These data indicate the existence of ligand-independent GLI activation in the classical subtype. Genetic alterations, including loss of PTCH within the classical subtype varied markedly and was not significantly different between subtypes. The expression patterns of other two HH ligands, IHH and DHH, were similar to SHH (Supplementary Fig. S1I). These data indicate the existence of ligand-independent GLI activation in the classical subtype. Genetic alterations, including loss of PTCH (27), and GANT61 treatment significantly reduced expression of GLI2 and CCND1 in LSCC cells.

GANT61 is a relatively new member in the HH inhibitor family, as most known HH pathway antagonists focus on the transmembrane activator SMO. Other readily available agents that inhibit GLI2 are rare. Arsenic trioxide (ATO), which is a U.S. Food and Drug Administration (FDA)–approved treatment for acute promyelocytic leukemia, has recently been described as a potent HH inhibitor. ATO has been shown to inhibit HH signaling by inhibiting GLI2 ciliary accumulation and promoting its degradation (38), and inhibit tumor growth in cancers with known drug-resistant SMO mutations and in the context of GLI2 overexpression (39). Because of the likelihood that compounds suppressing HH pathway–dependent proliferation in one cell type may be inactive in others, the clinical relevance of ATO in LSCC treatment is currently under investigation.

A recently emerging idea in clinical treatment is to combine several antitumor agents that specifically target...
different signaling pathways. It has been reported that GLI function can be modulated in a SMO-independent manner by PI3K/AKT signaling (15). Pharmacologic inhibition of PI3K/AKT signaling reduced tumor growth in GDC-0449–by PI3K/AKT signaling (15). Pharmacologic inhibition of different signaling pathways. It has been reported that GLI effectors with high efficacy and selectivity.

Treatment options for LSCC overall are disappointing. Different from standard-of-care chemotherapy or small-molecule inhibition of kinase signaling cascades, we present a potential strategy to treat a subset of patients with LSCC by targeting the GLI transcriptional network. Our studies also highlight the need for agents that suppress GLI effectors with high efficacy and selectivity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Huang, M. Onaitis
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