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GLI1 blockade potentiates the antitumor activity of PI3K antagonists in lung squamous cell carcinoma

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Abstract

Lung squamous cell carcinoma (SCC), strongly associated with smoking, is treated primarily with traditional cytotoxic chemotherapy due to a lack of FDA-approved targeted agents available. Here we identify the Hedgehog pathway transcription factor GLI1 as a critical driver of lung SCC. Analysis of human lung cancer datasets showed that GLI1 mRNA was highly expressed in human lung SCC and portended a poor prognosis. Inhibition of GLI1 in human lung SCC cell lines suppressed tumor cell clonogenicity and proliferation in culture and in vivo. Addition of SHH ligand, SMO antagonists, or other Hedgehog pathway agonists did not affect GLI1 expression in lung SCC cells. However, GLI1 expression was modulated by either inhibition or activation of the PI3K and MAPK pathways. Furthermore, in vivo growth of SCC harboring amplifications of the PI3K gene PIK3CA was attenuated by antagonizing GLI1 and PI3K. Thus, a combinatorial therapeutic strategy that targets the PI3K-mTOR pathway and GLI1 may lead to effective outcomes for PI3K pathway-dependent cancers, in contrast to recent results of human trials with single-agent PI3K antagonists.

Keywords

Lung squamous cell carcinoma; GLI1; arsenic trioxide; PI3K pathway; BKM120

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INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality in the U.S. (1). For lung squamous cell carcinoma (SCC), chemotherapy remains the standard treatment in contrast to available targeted therapies for adenocarcinomas (AD). Recent genome-wide analyses of lung SCC (2,3) revealed a number of frequent genetic aberrations in *FGFR1*, *DDR2*, components of the PI3K/mTOR and CDKN2A/RB1 pathways that represent promising targets for the development of small molecule antagonists.

The classical Hedgehog (Hh) pathway is critical for development, adult tissue repair and homeostasis, and cancer (4). Upon binding of a Hh ligand (SHH, IHH, DHH), Patched (PTCH) releases its inhibition of Smoothened (SMO) that in turn, activates the GLI transcription factors to initiate transcription of target genes including pathway members, *PTCH1*, *GLI1*, and *HHIP*. The transcription of these target genes can be used to monitor pathway activation.

Non-classical activation of GLI1 and GLI2 by pathways orthogonal to the classical Hh pathway has been reported in a number of tumors (5) including loss of SMARCB1 (SNF5) in malignant rhabdoid tumors (6), K-ras and TGF β in pancreatic cancer (7), TNF α via mTOR in esophageal cancer (8), PI3K in glioblastoma (9), and aPKC ζ/λ in drug-resistant basal cell carcinomas (10). Thus, GLI1 and GLI2 also act as terminal foci for numerous other oncogenic pathways.

The precise role of the Hh pathway in NSCLC is unclear. Reports have ranged from no Hh pathway activation (11), classical pathway activation (12–14) and non-classical SMO-independent pathway activation (15). Thus, we sought to examine the role of the Hh pathway through GLI1 in NSCLC.

MATERIALS AND METHODS

SPORE and TCGA databases

Lung cancer patient tumor specimens (n = 263) from the UT Lung Cancer Specialized Program of Research Excellence (SPORE)/MD Anderson PROSPECT Tissue Bank at MD Anderson Cancer Center were profiled for mRNA expression using Illumina HumanWG-6 V3 BeadArrays (16). Bead-level data were obtained and pre-processed using the R package mbcb for background correction and probe summarization. The data has been deposited in the Gene Expression Omnibus repository (GSE41271). The Kaplan-Meier plot for the overall survival of NSCLC samples compared the overall survival of patients in the top (high GLI1) and bottom (low GLI1) tertiles of *GLI1* mRNA expression levels by a log-rank (Mantel-Cox) test. For SCC and AD survival curves, patients within the bottom and top tertiles of *GLI1* mRNA expression from the NSCLC data set were stratified based upon squamous cell and adenocarcinoma histology. Kaplan-Meier plots were then generated by comparing the survival difference between the high and low expression of *GLI1* mRNA expression groups within each histological subset and analyzed by a log-rank (Mantel-Cox) test. Additionally, we downloaded mRNA expression levels extracted from RNAseq data (RPKM) from 404 lung cancer specimens collected at The Cancer Genome Atlas (TCGA).

Comparison of adenocarcinomas and squamous carcinomas were performed with Student's *t*-test and corrected for multiple testing using the Benjamini-Hochberg method.

Cell culture studies

All cell lines were obtained from the Hamon Cancer Center Collection (University of Texas Southwestern Medical Center (UTSW)). They were DNA fingerprinted with a PowerPlex 1.2 kit (Promega, Madison, WI) and were tested for mycoplasma with an e-Myco kit (Boca Scientific, Boca Raton, FL). The cell lines used in this study were generated between 1979 and 2007. HCC95 and HCC2814 cell lines were generated in 1992 and 1997, respectively. Cells lines were thawed and used for these studies starting in 2013. Cells were maintained under humidified conditions (37°C, 5% CO₂) in RPMI-1640 (Life Technologies, Grand Island, NY) with 5% FBS. For drug treatment studies, RPMI-1640 with 0.5% FBS media was used except when cells were treated with BEZ235, LY294002, and U01265 where 5% FBS was used. For cell culture studies with arsenic trioxide (ATO, Sigma-Aldrich, St. Louis, MO), culture media was buffered with HEPES 2 mM. Arsenic trioxide was prepared as previously described (17). Briefly, ATO powder was dissolved at 250 mM concentration in 1N sodium hydroxide and diluted to working concentrations with PBS. Growth factors EGF, bFGF, IGF-1 (Life Technologies), and recombinant human N-terminal SHH (C24II) (R & D Systems, Minneapolis, MN) were purchased and prepared according to manufacturer instructions. BEZ235, LY294002, UO126 (Sigma-Aldrich), BKM120 (Selleckchem, Houston, TX), GDC-0449 (LC Labs, Woburn, MA), LDE225 (Collagen Technology, San Diego, CA), KAAD-cyclopamine (Millipore, Billerica, MA), and SAG (Xcessbio, San Diego, CA) were prepared in DMSO.

Gene silencing

Endogenous GLI1 in lung SCC lines was knocked down using three GLI1 siRNAs (Dharmacon, Pittsburgh PA) as previously described (18). Briefly, cells were transiently transfected with siRNAs by reverse transfection using Lipofectamine RNAiMax (Life Technologies) for 2.5 hours. Transfected cells were suspended in RPMI-1640 medium with 5% FBS and plated on to tissue culture plates. The silencing efficiency was validated using qPCR and western blot. Two TRC2-pLKO-puro lentiviral shGLI1 constructs were obtained from Sigma-Aldrich. Lentiviruses were packaged in 293T cells, which were cultured in DMEM/10% FBS and transiently transfected with shRNA vector together with pMDG-VSVG and pCMV- R8.91 plasmids using Eugene6 (Roche, Indianapolis, IN). The viral supernatant was collected, filtered, and used for the transduction of lung cancer cells in the presence of 8 µg/ml polybrene (Sigma-Aldrich). Stable shRNA expressing lung SCC lines were generated after a two-week selection in 1 µg/ml puromycin.

Colony formation assay

Anchorage-dependent (liquid) colony formation assays were performed as previously described (18). Briefly, 2000 cells per well were plated in a 6-well plate and cultured for 10 days. The cells then were washed twice with PBS and stained with 1 mL of 0.5% crystal violet (Sigma-Aldrich) for 2 minutes before images were obtained. Experiments were performed in triplicate.

Cell proliferation assay

Lung SCC cells were plated in 96-well plates at a density of 2000 cells/well and incubated in the presence or absence of inhibitors for 4–5 days. CellTiter-Glo (Promega) was performed to measure the viable cells according to manufacturer's instructions.

Quantitative real-time PCR

We generated cDNA using an iScript cDNA synthesis kit (BioRad) or Superscript III First Strand Synthesis System (Invitrogen). Gene specific TaqMan probes (Life Technologies) were utilized for quantitative analyses of mRNA transcripts with *GAPDH* gene as an internal reference gene (Supplementary Table S1). Human reference RNA (Agilent Technologies) served as a control for the comparison of gene expression across a panel of lung cancer lines. Human and murine specific qPCR primers were designed from unique regions of genes and validated (Supplementary Table 2). PCR reactions were run using the ABI 7300 QPCR System (Agilent Technologies, Santa Clara, CA) or CFX96 Touch qPCR System (BioRad, Hercules, CA). The C_T method was used to calculate relative mRNA expression.

Western blot

Cell lysates were generated and analyzed as previously described (19). Briefly, cells were lysed in ice-cold lysis buffer (M-PER Mammalian Protein Extraction Reagent with protease inhibitors and PhosSTOP phosphatase inhibitors (Roche). Cell lysates were centrifuged at 14,000 rpm for 5 min at 4°C and supernatants were collected. Protein concentration was quantified using BCA protein assay kit (Pierce, Rockford, IL) with reference to a BSA standard curve. For GLI1 immunoblots, transfer to membranes was performed at 4 °C overnight at 25 amperes. Primary antibodies (1:000 dilution) used in the study were GLI1, SOX2, phospho-ERK1/2, phospho-Ser473 AKT, GAPDH (Cell Signaling, Beverly, MA), and HSP90 (Santa Cruz Biotechnology, Dallas, TX). Image J software (20) was used to analyze the relative band intensity from scanned images of western blots. The intensity of each protein band was first normalized with the intensity of its corresponding internal loading control band. Then, relative intensities were calculated by comparing the normalized experimental bands to its normalized experimental control band.

In vivo xenograft growth

All *in vivo* studies were approved by and conformed to the policies and regulations of Institutional Animal Care and Use Committees (IACUC) at UTSW. Mice were maintained in a standard facility and conditions of a 12 h light/dark cycle, temperature and humidity. One million HCC95 cells stably transduced with TRC2-pLKO-shNT (non-target control) or TRC2-pLKO-shGLI1 were subcutaneously injected into the flanks of female NOD/SCID mouse. Tumor growth was monitored by caliper measurements. Tumor volume was calculated by: $V = \text{width} \times \text{length}^2 \times \pi/6$. For drug study, 1×10^6 HCC2814 cells in 1:1 PBS:Matrigel (Growth Factor Reduced; BD Biosciences, San Jose, CA,) were injected subcutaneously in flanks of 8 week old female nude mice and tumors grown to $\sim 50 \text{ mm}^3$ (see volume calculation above). NVP-BKM120 suspension was prepared daily in 0.5% methylcellulose and 0.4% Tween 80. Working ATO solution was prepared every 2 days from

stock solution (see “Cell culture studies” above) (17) by dilution with PBS and adjusted to pH 8.3 with hydrochloric acid and sterile filtered. Formalin-fixed and paraffin-embedded tissue samples were examined with Hematoxylin and Eosin stain. Tumor cell lysate was generated using TissueLyser II (Qiagen).

***In vivo* Hair regrowth assay**

Balb-c mice were anesthetized with metered isoflurane. Backs of all mice were shaved with a 0.25 mm clipper blade on a Proclip AGC2 Detachable Blade Clipper (Andis, Sturtevant, WI). In order to depilate hair follicles and stimulate hair regrowth, the shaved area was covered with Nair (Church & Dwight, Ewing, NJ) for ~1 minute and then removed and cleaned with a wet cloth. Any remaining residue was cleaned from the area and surrounding hairs to prevent irritation. First does of ATO or PBS was injected after hair removal and then subsequently dosed at ATO 6.5 mg/kg IP twice per day or equal volume of control PBS for 35 days.

Statistical analysis

Unpaired, two-sided Student's *t*-test was performed using GraphPad Prism 5 software to test for significant differences in qPCR, proliferation, tumor growth, and colony formation assays. For *in vivo* tumor growth studies, unpaired, two-sided Student's *t*-test with Welch's correction was performed.

RESULTS

Hedgehog pathway components are elevated in SCC of the lung

To assess whether the Hh pathway may play a role in NSCLC, we examined the effects of high *GLI1* mRNA expression in the UT Lung Cancer Specialized Program of Research Excellence (SPORE)/MD Anderson PROSPECT Tissue Bank (16). High *GLI1* mRNA expression was significantly correlated with worse overall survival of all NSCLC patients ($P = 0.0141$) with a trend toward worse survival in AD ($P = 0.0561$) and SCC patients ($P = 0.2800$) (Fig. 1A). The lack of statistical significance among individual histologies may be due to the lower number of samples (AD $n=109$; SCC $n=64$) compared to NSCLC patients ($n=173$). *GLI1* mRNA expression of the SPORE dataset was significantly upregulated in SCC compared to AD (Fig. 1B). Similarly in the Cancer Genome Atlas (TCGA), *GLI1* mRNA was also upregulated in lung SCC ($P<0.0001$) (Supplementary Fig. S1A) compared to AD and normal lung. Both *GLI2* and *SMO* mRNA (Supplementary Fig. S1B – D) were upregulated in lung SCCs relative to lung AD, whereas other Hh pathway components showed little difference (Supplementary Fig. S1E–G). These data suggest that *GLI1* may be critical for lung cancer pathogenesis.

Lung SCC cell lines share similar molecular profiles with SCC tumor specimens

As *GLI1* is highly expressed in human SCC (Figs. 1B, S1A), we further investigated the role of *GLI1* in NSCLC cell lines. Lung SCC and non-squamous cell lung cancer (NSCC) cell lines were selected (Fig. 1C) based on recently developed gene expression signatures (21). The NSCC lines consisted of 4 AD, 1 undifferentiated NSCLC, and 1 large cell neuroendocrine lines. The three SCC cell lines expressed substantially higher mRNA

transcripts of SCC markers, *SOX2*, *TP63*, and *KRT5* (22) compared to lung AD lines as detected by real-time PCR (qPCR) (Supplementary Fig. S2A–C). SCC cells also expressed substantially higher levels *GLI1* mRNA and protein than AD cells (Fig. 1C). To verify the reliability of an anti-*GLI1* antibody, we used Hh pathway-responsive NIH-3T3 cells (23). *GLI1* levels robustly increased with active N-terminal SHH conditioned medium (CM) (24) treatment and then decreased to baseline levels after the addition of KAAD-cyclopamine 200 nM (23), a potent SMO antagonist (Supplementary Fig. S2D). mRNA expression of other Hh pathway genes (*GLI2*, *PTCH1*, *SMO*, and *SHH*) was also increased in lung SCC compared to lung AD (Supplementary Fig. S2E–H). Subcutaneous xenografts of SCC HCC2814 cells in NOD-SCID mice showed sheets of large malignant cells with keratinization, atypical mitosis and areas of necrosis, resembling features of SCC, whereas AD HCC2450 xenografts showed a solid pattern of malignant cells with mucus production suggestive of poorly differentiated AD (Fig. 1D). SCC xenografts showed increased expression of *SOX2*, *TP63*, and *KRT5* mRNA compared to AD xenografts, further verifying the SCC histology of the xenografts (Fig. 1E). Analogous to cultured cells (Fig. 1C), SCC xenografts expressed substantially higher levels of *GLI1* mRNA and protein than AD xenografts (Fig. 1E).

GLI1 is critical for lung SCC growth and clonogenicity *in vitro*

We tested the requirement of *GLI1* for SCC growth. After *GLI1* siRNA transduction to HCC95 and HCC2814 cells, *GLI1* mRNA and protein expression (Fig. 2A) and mRNA of *GLI1* target genes, *CCND1*, *MYCN*, and *BCL2* (25,26) (Fig. 2B, Supplementary Fig. S3A), were significantly decreased. *GLI1* knockdown also significantly decreased *GLI2* mRNA (Supplementary Fig. S3A) suggesting that, here, *GLI2* expression is dependent on *GLI1*. To validate the siRNAs that targeted the *GLI1* coding regions, C911 oligonucleotides were used where the core nucleotides 9–11 of a siRNA is replaced with its complementary sequence. C911 oligonucleotides lose the siRNA's "on-target" effects while retaining many of its "off-target" effects (27). C911 oligonucleotides of si*GLI1*-2 and si*GLI1*-3 had little effect on *GLI1* mRNA and protein levels of HCC2814 cells whereas C911-si*GLI1*-1 reduced *GLI1* mRNA by almost 70% compared to controls (Supplementary Fig. S3B, C), suggesting that si*GLI1*-2 and si*GLI1*-3 demonstrate "on-target" effects but not si*GLI1*-1. Knock down of *GLI1* significantly inhibited anchorage-dependent liquid colony forming ability in both HCC95 and HCC2814 cells (Fig. 2C) compared to scrambled siRNA controls suggesting that *GLI1* is critical for lung SCC clonogenicity.

Loss of GLI1 suppresses SCC tumor growth *in vivo*

For *in vivo* studies, we utilized lentiviral-mediated shRNAs to generate stable cell lines with reduced *GLI1* expression. sh*GLI1*-2 and sh*GLI1*-3 significantly reduced *GLI1* mRNA and protein expression in HCC95 cells (Fig. 3A) with significantly decreased cell proliferation (Fig. 3B) and liquid colony formation (Fig. 3C) compared to control (no shRNA) or scrambled shNT-expressing cells. Subcutaneous xenograft tumors of sh*GLI1* HCC95 cells grew significantly slower than tumors expressing shNT and exhibited decreased expression of *GLI1* mRNA (Fig. 3D). Thus, *GLI1* is critical for the growth of lung SCC growth *in vivo*.

GLI1 is regulated by PI3K/AKT and MAPK pathways rather than classical Hh pathway

SCC cell lines expressed *SHH* mRNA (Supplementary Fig. S2H) thus, autocrine classical Hh signaling may regulate GLI1. SCC cells treated with recombinant SHH or SAG (28), a potent SMO agonist, had little effect on *GLI1*, *GLI2*, and *PTCH1* mRNA transcription (Supplementary Fig. S4A–D) unlike NIH-3T3 cells (Supplementary Fig. S4E). Treatment with SMO antagonists, GDC-0449 (vismodegib, Genentech) (29), NVP-LDE225 (sonidegib, Novartis) (30), and KAAD-cyclopamine (23), did not significantly alter GLI1 mRNA or protein (Fig. 4A, B), liquid colony formation or cell proliferation (Fig. 4C, D) compared to controls. These findings strongly suggest that GLI1 expression is independent of Hh ligands and SMO in lung SCC.

We thus sought to identify other pathways that may regulate GLI1 expression (5). PI3K (8,9) and MAPK (31,32) pathways were considered first as genetic aberrations in these pathways are frequently found in lung SCC (33). Using a pharmacologic approach, SCC cells were treated with BEZ235 (PI3K/mTOR dual inhibitor) (34,35), LY294002 (PI3K inhibitor) (36,37), and U0126 (MEK1/2 inhibitor) (38,39). Both PI3K inhibitors and U0126 effectively inhibited their targets as evidenced by diminished levels of pAKT and pERK, respectively (Fig. 5A). The drugs also decreased GLI1 protein levels, although greater effects were seen with PI3K inhibitors (Fig. 5A and Supplementary Fig. S5). The drugs also decreased *GLI1* mRNA expression in these cells (Fig. 5B) except for U0126 in HCC2814 cells. GLI1 protein is destabilized by U0126 in skin keratinocytes (40) and pancreatic adenocarcinoma cells (31). Thus, GLI1 stabilization by MAPK pathway activity may account for the discrepancy of GLI1 protein and mRNA expression in HCC2814 cells. Induction of the PI3K pathway with IGF and MAPK pathway with EGF and FGF in HCC95 cells increased GLI1 expression although the effects were greatest with IGF (Fig. 5C). Further investigation of the SCC cell lines revealed that HCC95 and HCC2814 cells expressed high levels of *FGFR2* mRNA (Supplementary Fig. S6) and have *PIK3CA* amplifications with upregulated pathway activity (41); HCC2814 also has PTEN loss (41). As GLI1 expression was more sensitive to PI3K pathway modulation, we tested the requirement of PI3K pathway for proliferation and clonogenicity of SCC cells. Indeed, BEZ235 significantly inhibited clonogenicity and proliferation (Fig. 5D, E) of lung SCC cells. Thus, PI3K and MAPK pathways regulate GLI1 expression independent of the Hh pathway in these lung SCC cells.

Arsenic trioxide reduces GLI1 expression and inhibits lung SCC proliferation

We next tested the effects of pharmacologic inhibition of GLI1 with arsenic trioxide (ATO), an FDA-approved drug for acute promyelocytic leukemia (APL) with sub-micromolar potency against GLI1/2 (42). HCC95 cells treated with ATO 5 and 10 μ M significantly reduced GLI1 mRNA and protein expression (Fig. 6A). ATO also strongly inhibited HCC95 colony forming efficiency (Fig. 6B) and cell proliferation (Fig. 6C) compared to controls. Thus, ATO inhibits lung SCC growth through inhibition of GLI1.

Combination of PI3K inhibitor and ATO inhibited xenograft tumor growth *in vivo*

Next, we examined the therapeutic strategy of combinatorial PI3K and GLI1 inhibition *in vivo*. During our studies, the development of BEZ235 was discontinued for clinical use (<http://tinyurl.com/ycd5967b>). We thus tested NVP-BKM120 (Novartis) (43,44), a pan-PI3K

inhibitor in current clinical trials. Titration of BKM120 revealed 8 and 10 μ M as the effective concentrations to inhibit the PI3K pathway as evidenced by loss of pAKT (Supplementary Fig. S7A) in HCC2814 cells. Inhibition of PI3K pathway by BKM120, as evidenced by decreased pAKT and pS6K protein (45,46), led to decreased GLI1 protein and mRNA expression (Fig. 7A) with no decrease of MAPK pathway as tested by pERK (Fig. 7A, left panel). ATO dose was optimized with an *in vivo* Hh-pathway dependent hair regrowth assay (47,48) (Supplementary Fig. S7B). We then treated established HCC2814 subcutaneous xenograft tumors with vehicle control, ATO, BKM120, or the combination. ATO or BKM120 alone had little effect on tumor growth compared to control. However, combination ATO+BKM therapy significantly suppressed tumor growth with regression of tumors between days 6–12 and then a slow growth (Fig. 7B). Further examination revealed that all treated tumors except one in the combination arm showed either no growth or regression (Fig. 7C). The one tumor (bold line, Fig. 7C) that progressively grew accounted for the overall slow growth of the combination arm (Fig. 7B). Tumor weights at the end of the study showed a significant decrease in the combination drug arm compared to control (Fig. 7D) with the resistant tumor (bold line, Fig. 7C) having the highest weight (dashed circle, Fig. 7D). Using human specific qPCR primers (Table S2) to detect mRNA from tumor cells only, *GLI1* mRNA transcription was significantly reduced compared to vehicle (Fig. 7E) only in tumors treated with combination BKM120 and ATO. No significant changes in animal body weights were measured during the study period (Fig. 7F).

DISCUSSION

We have demonstrated that lung SCCs expressed high levels of GLI1 mRNA and that lung SCC cell lines and their derived xenografts recapitulated the biomarkers and histology associated with human SCC. GLI1 expression in these *PIK3CA*-amplified SCC cell lines was critical for tumor growth, was regulated primarily by PI3K pathway instead of the classical Hh pathway, and combinatorial inhibition of PI3K and GLI1 led to tumor regression *in vivo*.

Two recent reports have noted the involvement of the Hh pathway and lung SCC (14,15). PKC α and SOX2 have been reported to cooperatively activate cell autonomous classical Hh pathway in lung SCC oncospheres but not in parental cells (14). In another set of SCC cell lines, SMO-independent activation of GLI2 has been reported (15). In our studies, the classical Hh pathway is not activated despite the expression of SHH ligand (Supplementary Fig. S2H). GLI1 is regulated by the PI3K and MAPK pathways, and in turn, regulates GLI2 expression (Supplementary Fig. S3A). SHH ligand expressed from the cells in our study may function to activate paracrine classical Hh pathway in stromal cells. Thus, we have identified the PI3K pathway, a pathway that harbor genetic aberrations with high frequency in numerous cancers, as a key driver of GLI1 expression independent of classical Hh pathway. We also demonstrated the *in vivo* efficacy of combinatorial GLI1 and PI3K antagonism as an effective therapeutic strategy for PI3K-activated lung cancers.

Many other tumor types have high frequency of genetic aberrations in the PI3K pathway (Supplementary Fig. S8). As of this writing, there are >60 clinical trials with PI3K pathway inhibitors ([Clinicaltrials.gov](https://clinicaltrials.gov)). However, early clinical data suggest that single agent PI3K

inhibition may be insufficient. A phase II trial with BKM120 in endometrial carcinoma, where genetic aberrations in the PI3K pathway occur in >90% of cancers ((49), Supplementary Fig. S8), showed no objective responses (50). The phase II BASALT-1 trial used BKM120 as second-line therapy for metastatic lung SCC and AD patients with *PIK3CA* or *PTEN* mutations (51). Interim analysis revealed a 12-week progression-free survival (PFS) of 23.3% and 20% and overall response rates of 3.3% and 3.0% for SCC and AD, respectively. The trial was considered futile (PFS<50%) and halted. The authors concluded that BKM120 combined with other agents are warranted.

Analogous to the human trials, we did not see tumor responses with ATO or BKM120 alone whereas the combination significantly inhibited tumor growth (Fig. 7B). The lack of single agent efficacy may be due to several factors. HCC2814 xenografts contain both *PIK3CA* amplifications and PTEN loss (41). The loss of PTEN removes the primary restraint to PI3K activity in HCC2814 cells. Thus, higher concentrations of antagonist may be required. Indeed, we did not see inhibition of PI3K activity as indicated by decreased pAKT until BKM120 5 μ M and nearly complete loss of pAKT until BKM120 10 μ M (Supplementary Fig. S7A, Fig. 7A) - ~63 $\times$ and ~125 $\times$ IC₅₀ for pAKT suppression in A2780 cancer cells (43). For ATO, we identified an optimal dose schedule based on a Hh-dependent hair regrowth assay (Supplementary Fig. S7B). Hair follicles may require less ATO for GLI1 inhibition than HCC2814 cells. Furthermore, as the tumors were transplanted subcutaneous xenografts, the tumors may have had decreased vascularity compared to normal skin and other murine autochthonous tumors, leading to decreased drug exposure. We have seen similar differential responses between autochthonous and subcutaneous allografts of basal cell carcinoma that were treated with itraconazole (19,52). Thus, BKM120 and ATO concentrations in the tumors may not have reached adequate levels to completely inhibit their targets as single agents.

ATO and BKM120 are readily available as they are in active use or clinical testing, respectively. Furthermore, as GLI1 may be a general target of the PI3K pathway in cancers, our therapeutic strategy to combine clinically available PI3K pathway antagonists with ATO (or other future GLI antagonists) may be applicable and effective for a broad array of tumor types with activated PI3K pathway and can be rapidly introduced into clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

GLI1 mRNA expression is increased in human squamous cell lung cancer. (A) Kaplan-Meier plot of patient overall survival in the SPORE dataset (Illumina V3 microarrays) based on tumors expressing high (upper tertile) and low (lower tertile) *GLI1* mRNA of NSCLC (n=173) (left panel; $P = 0.0141$) is shown. Overall survival Kaplan-Meier curves of SCC (n=64; middle panel; $P = 0.2600$) and AD (n=109; right panel; $P = 0.0561$) patients of upper and lower tertile expression of *GLI1* mRNA from NSCLC set (left panel) are plotted. (B) Analysis of *GLI1* mRNA expression in patient tumor specimens collected by SPORE is

shown. AD n=183 and SCC n=80. $P<0.0001$. (C) *GLI1* mRNA was analyzed by qPCR in lung SCC and non-squamous cell carcinoma (NSCC) cell lines. All data were normalized to human reference (HR). HBEC34KT is a normal lung epithelial cell line. Inset: Western blot analysis of SOX2 and GLI1 in SCC and NSCC cells. (D) Representative hematoxylin and eosin (H&E) images of xenograft tumors derived from SCC (HCC2814) and AD (HCC2450) cells. Scale bars represent 20 μ m. (E) mRNA expression of *SOX2*, *TP63*, *KRT5*, and *GLI1* and western blot of GLI1 from xenograft tumors of SCC (HCC95, HCC2814) and AD (H1869, HCC2450) cells. mRNA levels were measured by qPCR. Line over histogram bars in panel E represent SCC cell lines. mRNA data from panels C, E are mean of triplicates $\pm$ S.D.

Fig. 2.

Depletion of *GLI1* reduces the colony forming ability of lung SCC cells. (A) *GLI1* mRNA and protein levels of HCC95 and HCC2814 SCC cells treated with three different siRNAs against *GLI1* were measured by qPCR and western blot, respectively. (B) Expression of mRNA levels of *GLI1* target genes, *CCND1*, *MYCN*, and *BCL2* in HCC95 SCC cells treated with siRNAs against *GLI1* was measured by real time PCR. (C) Representative images and quantification of liquid colony formation assays are shown. HCC95 and HCC2814 SCC cells were treated with siRNAs against *GLI1*. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

Fig. 3.

Knockdown of *GLI1* impairs lung SCC proliferation and xenograft tumor growth. All data refer to HCC95 and HCC2814 SCC cells with stable knockdown of *GLI1* by shRNA. (A) *GLI1* mRNA and protein expression of HCC95 SCC cells after stable knockdown of *GLI1* were analyzed by qPCR and western blot, respectively. (B) Cell proliferation of HCC95 cells with stable knockdown of *GLI1* was monitored. 'Control' represents cells with no shRNA transduction, and 'shNT' represents cells transduced with scrambled shRNA. (C) Formation of liquid colonies was quantified of HCC95 and HCC2814 cells after stable knockdown of *GLI1*. (D) Growth of subcutaneous xenograft tumors derived from shNT or sh*GLI1*-expressing HCC95 cells was monitored (left; n = 3) after injection of 10⁶ cells in flanks of NOD-SCID mice. Data represent mean of triplicates $\pm$ S.E.M. *GLI1* mRNA levels of the xenograft tumors were measured by qPCR (right). Data in panels A-C represent mean of triplicates $\pm$ S.D. **P*<0.05, ***P*<0.01.

Fig. 4.

GLI1 expression is not regulated by the classical Hh pathway. (A, B) HCC95 cells were treated with indicated concentrations of SMO antagonists (GDC-0449, KAAD cyclopamine, and LDE225) for 48 hours. *GLI1* mRNA (A) and protein (B) were monitored by qPCR and western blot, respectively. (C) For liquid colony formation assays, HCC2814 and HCC95 cells were treated with increasing doses of KAAD-cyclopamine. (D) Cell proliferation of HCC2814 and HCC95 cells was monitored in the presence of control DMSO or KAAD-cyclopamine 1 μ M. Data for panels A and D are mean of triplicates $\pm$ S.D.

Fig. 5.

GLI1 expression is regulated by MAPK and PI3K pathways in lung SCC. (A) HCC2814 and HCC95 cells were treated with the indicated concentrations of BEZ235 (a PI3K-mTOR antagonist), LY294002 (a PI3K antagonist) and U0126 (a MEK1/2 antagonist) for 48 hours. Protein levels of the indicated proteins were detected by western blots. (B) HCC2814 and HCC95 cells were treated with vehicle control, BEZ235 200 nM, LY294002 20 μ M, U0126 20 μ M for 32 hours. *GLI1* mRNA was monitored by qPCR. (C) HCC95 cells were stimulated with EGF, bFGF, and IGF ligands and indicated proteins were detected by

western blot (left panel). Right panel shows quantification of relative GLI1 immunoblot band intensity of the left panel. (D) Representative images of liquid colony formation assays are shown of HCC2814 and HCC95 cells treated with increasing concentrations of BEZ235. (E) HCC2814 and HCC95 cells were treated with indicated concentrations of BEZ235 and monitored for cell proliferation. Data for panels B and E are mean of triplicates $\pm$ S.D. ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0005$.

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Fig. 6. Arsenic trioxide (ATO) inhibits GLI1 expression and cell viability in lung SCC. (A) GLI1 mRNA and protein levels of HCC95 cells treated with the indicated concentrations of ATO or KAAD-cyclopamine for 48 hours were detected by qPCR and western blots, respectively. (B) Liquid colony formation assays are shown of HCC2814 and HCC95 cells treated with increasing concentrations of ATO. (C) Cell proliferation of HCC95 and HCC2814 cells treated with indicated concentrations of ATO was monitored. Data for panels A and C are mean of triplicates $\pm$ S.D. * $P < 0.05$, *** $P < 0.001$.

Fig. 7. Combinatorial antagonism of PI3K and GLI1 inhibits the growth of lung SCC *in vivo*. (A) Cultured HCC2814 cells were treated with two different doses of BKM120 for 24 hours. Immunoblot (left panel) for indicated proteins was performed. *GLI1* mRNA expression (right panel) was assessed by qPCR. Data represent mean of triplicates $\pm$ S.D. (B) Established subcutaneous xenografts of HCC2814 in female nude mice were treated with control vehicle, ATO 6.5 mg/kg i.p. twice daily, BKM120 15 mg/kg oral gavage daily, or combination ATO and BKM120. Tumor measurements were made every 3 days. Control

n=13, ATO n=12, BKM120=12, ATO+BKM n=10. Data represent mean of group $\pm$ S.E.M. (C) Growth curves of individual tumors of the ATO+BKM120 arm from panel B is shown. Bold line represents tumor that did not respond to drug treatment. (D) Weight of treated tumors at the end of the drug study from panel (B) is shown. Tumor with dashed circle represents the tumor that grew on combination treatment (bold line from panel C). (E) In a separate experiment, *GLI1* mRNA was measured by qPCR from HCC2814 subcutaneous xenograft tumors treated for 5 days with control vehicle, ATO 6.5 mg/kg i.p. twice daily, BKM120 15 mg/kg oral gavage daily, or ATO+BKM120. (F) Body weight measurements of mice from the drug study in panel (B) are shown. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.