Transcriptional Regulation of Serine/Threonine Protein Kinase (AKT) Genes by Glioma-Associated Oncogene Homolog I*

Nitin K. Agarwal§, Changju Qu§, Kranthi Kunkulla, Yadong Liu and Francisco Vega#

From the Department of Hematopathology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

*Running title: AKT1 direct downstream target of GLI1

#Corresponding author: Francisco Vega, M.D., Ph D. Department of Hematopathology, Unit 72, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX, 77030, USA. Telephone: (713)-794-1220; Fax: (713)-563-3166. E-mail: fvegava@mdanderson.org

Keywords: AKT1; DLBCL; GLI1; Hedgehog Signaling Pathway; Transcription

§contributed equally in this manuscript

Background: Little is known regarding the transcriptional regulation of AKT.

Results: GLI1 contributes to the survival of DLBCL cells by promoting transcription of AKT genes.

Conclusion: AKT1 is a novel direct downstream target of the Hedgehog transcriptional factor GLI1.

Significance: Identifying target genes of GLI1 provide insights into the contribution of Hedgehog signaling in the pathobiology of malignant tumors.

SUMMARY

Diffuse large B-cell lymphoma (DLBCL) is one of the most common non-Hodgkin lymphoma in adults (1). Despite overall improvements in outcomes of DLBCL, approximately 30-40% of patients have disease that is either refractory or relapses after standard therapy (2). Therefore, new advances in our understanding of the molecular pathobiology of DLBCL are needed. These advances are expected to contribute to the development of new therapeutic approaches.

Hedgehog (Hh) signaling is evolutionary conserved signaling pathway that serves several physiological and development processes (3) and is deregulated in several cancers including DLBCL (4,5). In fact, inhibition of Hh signaling
has been proposed as a useful therapeutic target for some cancers (6-9).

The Hh family of proteins comprises of three distinct ligands, Sonic Hh, Indian Hh and Desert Hh, which appear to be processed intracellularly by autoproteolysis from a precursor peptide (~ 45 kDa) to generate a N-terminal secreted peptide that is retained at or near the cell surface (10). Secreted N-terminal Hh peptides interact with a receptor complex composed of two major proteins, a 12 transmembrane domain protein, patched 1 (PTCH1), and a 7 transmembrane domain protein, smoothened (SMO). In the absence of Hh ligands, PTCH1 inhibits SMO. Once Hh ligands bind to PTCH1, this inhibition is released allowing SMO to transduce the Hh signal mediated by the five-zinc finger transcription factors, GLI1, GLI2 and GLI3 (11-13).

The GLI family members contain a conserved C2-H2 zinc-finger DNA-binding domain that can specifically interact with target DNA sequences encompassing a GACCACCCA-like motif (14,15). Because the full length GLI1 transcription factor does not contain a repressor domain, it consequently acts as a strong transcriptional activator and participates in the regulation of the expression of numerous Hh target genes including itself (16-20). Although inappropriate activation of GLI1 has been shown in many cancers, the assessment of the contribution of GLI1 has not been thoroughly examined in hematological malignancies.

We previously showed that GLI1 mediated canonical Hh signaling is active in DLBCL (5,21). This activation of GLI1 is not due to amplifications of GLI1 (22) or mutations of Hh related genes (23,24). Our data support that Hh pathway in DLBCL is aberrantly activated as its activation is in part mediated by external Hh ligands (autocrine and paracrine Hh signaling loops) (5,16) but also intrinsically by crosstalks with other oncogenic pathways (22).

AKT (protein kinase B) is a serine/threonine kinase involved in the regulation of cell survival signals in response to growth factors or cytokine stimulation. AKT is one of most frequently hyperactivated kinase in cancer and it has been shown to play critical roles in the tumorigenesis of many neoplasms (25-27). In mammalian cells, three major isoforms of AKT, termed AKT1, AKT2, and AKT3, encoded by three separate genes have been identified (28). Among the three isoforms, AKT1 is ubiquitously expressed and constitutively activated in several cancers (29,30). Whereas post-translational regulation of AKT signaling is being extensively studied, there are few data available regarding the transcriptional regulation of AKT1 (31,32) and its transcriptional regulation remains largely unknown.

It has been reported that activation of AKT predicts poor outcome in patients with DLBCL (33). Multiple mechanisms have been proposed to the activation of AKT in cancer such as mutations of PTEN, amplification of AKT genes and mutations of genes coding the regulatory and catalytic subunits of PI3K (e.g. PIK3CA) (34). In DLBCL, however, causative factors that might explain the constitutive activation of AKT are not completely known. Functional loss in the p110 catalytic subunit-α (PIK3CA) and inactivation or deletion of PTEN has been reported in a small subset of DLBCL (35-37).

In this report, we provide evidence that canonical Hh signaling regulates the transcription of AKT genes, and that AKT1 is a novel direct downstream target of the transcriptional factor GLI1. We also provide evidence that GLI1 contribute to the survival of DLBCL cells by promoting the transcription of AKT genes. Moreover, by finding a strong correlation between AKT1 and GLI1 in DLBCL patient samples, our in-vitro data may be extrapolated to DLBCL tumor samples.

**EXPERIMENTAL PROCEDURES**

**Cell lines, Cell culture and Patient Samples**

DOHH2 and OCI-LY19 cell lines were purchased from DSMZ (Braunschweig, Germany). HBL1 and 293T cell lines were obtained from ATCC (Manassas, VA). LP cells were established from a diagnostic specimen from a DLBCL patient (38) and characterized as a DLBCL cell line of ABC type (39). LP cells were kindly gifted by Dr. Richard J Ford (Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA). DLBCL cell lines
were exclusively maintained at 37°C in RPMI 1640 (ATCC, Manassas, VA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), 1% L-glutamine and 1% penicillin streptomycin in a humidified atmosphere containing 5% CO2. 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin streptomycin at 37°C in an atmosphere of 5% CO2. When mentioned, cells were treated with recombinant Shh N-terminal peptide and cyclopamine-KAAD for indicated time periods. All frozen and paraffin embedded DLBCL patient specimens, reactive lymph nodes and DLBCL cells from pleural fluids were provided by the Hematopathology Tissue Bank of University of Texas MD Anderson Cancer Center, Houston, TX, USA.

**RNA Extraction and Quantitative Real Time PCR Analysis**-Quantitative (q) real-time PCR analysis was performed according to the described protocol (16). The primers for GLI1 (Hs01110766), SMO (Hs01090243), AKT1 (Hs00178289), AKT2 (Hs01086102), AKT3 (Hs00178533), BCL2 (Hs00608023), and 18S (Hs03928985) were obtained from Applied Biosystems (Carlsbad, CA, USA). Each target was amplified in duplicate and data analyses were done using 2-(ΔΔCT) method (40).

**Cell Lysates and Immunoblotting**-Cells were rinsed with ice-cold phosphate buffer saline and lysed in buffer containing 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 50 mM NaF, 1% Triton X-100, and protease inhibitor cocktail (Roche, Basel, Switzerland). The cell lysates were incubated for 20 min at 4°C to complete lysis and processed for immunoblotting, as described (41). Antibodies used were obtained as follow: Histone H3, GLI1 (C6H83), AKT and P-AKT (Ser473) (Cell Signaling Technology, Danvers, MA, USA), GLI1 (H70) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) SMO (Abcam, Cambridge, MA, USA) and β-actin-HRP (Sigma-Aldrich Corp., St. Louis, MO, USA).

**Chromatin Immunoprecipitation (ChIP)** Assay-ChIP assay was conducted using SimpleChIP Enzymatic Chromatin IP kit as per manufacturer protocol (Cell Signaling Technology). GLI1 (C6H83; Cell Signaling) and GLI1 (H300; Santa Cruz Biotechnology) antibodies were used for ChIP analysis. The sequences of primers used in ChIP assays are as follows: binding sites1 (BS1) forward (-4293/+1), 5’-GTACCTAGGTGAATGGTTGACTTCAG-3’, reverse 5’-GTCGGCTTAGGTGACTTCAG-3’, binding sites 2 (BS2) forward (-325/+1888), 5’-GATCAATGGATAAAGTGTGCTCAG-3’, reverse 5’-ACAAAAAGAGGAGTCAGACAAAGTCC-3’. PCR product was purified using PCR purification kit (Invitrogen, Grand Island, NY, USA) and sequenced at gene sequencing core facility of University of Texas MD Anderson Cancer Center, Houston, TX, USA.

Luciferase Reporter Gene Assay-Luciferase reporter plasmids with luciferase gene under transcriptional control of AKT1 gene regulator chromatin were obtained by Dr. Jin Q. Cheng (Departments of Pathology and Interdisciplinary Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, USA) as described previously (32). Flag tagged full length GLI1 plasmid was kindly gifted by Dr. Peter Zaphiropoulos (Department of Bioscience and Nutrition, Karolinska Institutet, Sweden). The AKT1 driven luciferase reporter assay was performed as described previously (16).

**Site Directed Mutagenesis**-Site directed mutagenesis was performed as described previously (16). The sequences of primer used for mutagenesis are as follows: BS1- forward (-4293/+1), 5’-GCCGCCCTCCACGGGGCCAGAGTTGGG-3’ and reverse 5’-CCCACCTCTGGCCCCGTGGAGGGGC-3’; BS2- forward (-325/+1888), 5’-GTAAATTAGGGTCGGTTACAAGGGTGGACTTCAG-3’, reverse 5’-AGGAGCACCAGTCACCTTTGTTACEGACCATAATTAC-3’.

Transfection, Lentiviral particles formation and Infections-Lentiviral human GLI1 shRNAs GLI1#1: TRCN0000020484, GLI1#2:
TRCN000002 0488 and Luciferase (control) plasmids (Puromycin selection) were obtained from Sigma Mission shRNA (Sigma-Aldrich Corp.). Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible control and GLI1 shRNA plasmids were custom made by Sigma Mission shRNA (Sigma-Aldrich Corp.). Precision Lenti AKT1 (blasticidin selection) ORF clone (PLOHS-100067520) was obtained from shRNA core facility of University of Texas MD Anderson Cancer Center, Houston, TX, USA. Transfections, lentiviral particles production and infections were done as previously described (42). Briefly, 293T cells (1.2 × 10⁶) were cotransfected with control or GLI1 shRNAs together with the delta VPR and VSVG plasmids into actively growing cells. Lentiviral particles were harvested 48 h after transfection and centrifuged at 3000 g for 15 min to eliminate any remaining HEK 293T cells. DLBCL cells were transduced with the collected viral supernatant in the presence of polybrene (8 μg/ml) and incubated for 48 h. After 48 h, infected cells were selected with puromycin (2-5 μg/ml) or blasticidin (10 μg/ml) for 7 days.

For IPTG inducible GLI1 knock down experiments, puromycin selected control and GLI1 OCI-LY19 cells were treated with 1mM IPTG for 3-4 days. Upon removal of IPTG from the culture media, the GLI1 expression could be recovered. To recover GLI1 expression, OCI-LY19 cells were washed at least thrice with PBS, centrifuged and cultured in new RPMI medium (without IPTG) for 7 days.

Cell Viability, Cell Cycle Arrest and Apoptosis Analysis-Cell viability of DLBCL cell lines was determined using the Vi-CELL cell viability analyzer (Beckman Coulter, Miami, FL, USA). Cell cycle analysis and Annexin-V and Propidium Iodide (PI) staining (BD Biosciences PharMingen, San Jose, CA, USA) was performed by flow cytometry as described previously (5).

Double Immunofluorescence labeling-Immunofluorescence assays were performed as previously described (43). Routine histologic tissue sections of DLBCL involving lymph nodes and reactive lymph nodes were used. Briefly, tissue sections were deparaffinized, hydrated and underwent heat-induced epitope antigen retrieval in a steamer for 20 minutes as previously described (21). Tissue sections were permeabilized in PBS containing 0.3% triton X-100 and 0.3% Sodium deoxycholate for 30 min, blocked with Image IT FX signal enhancer (Invitrogen) and incubated in mouse monoclonal GLI1 and rabbit AKT primary antibodies (Cell Signaling Technology) overnight at 4°C. Unbound antibodies from tissue specimens were removed by washing three times with PBS for 5 minutes. The secondary antibodies, Alexa Fluor 544 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen), were applied at 1:1000 in PBS for 45 minutes followed by three PBS washes for 5 minutes. The sections were incubated in 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen) at 1:2000 in PBS for 5 minutes for nuclear staining and washed twice with PBS for 5 minutes. The prolong-Gold antifade reagent (Invitrogen) was used for the mounting the slides. Images were acquired with the deconvolution microscope using 40x/1.30 objectives with oil immersion. The dot profile analysis of the immunofluorescence images were performed with the Image J software (National Institutes of Health, Bethesda, MD) as described previously (44).

Statistical Analysis-To calculate the statistical significance of the changes in the response of DLBCL cell lines to drugs the paired Student t-test was used. Student test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego CA, USA). To evaluate the significance of AKT1 and GLI1 mRNA expression in tumor specimens and circulating DLBCL tumor cells, the Spearman correlation coefficient was used.

RESULTS

Activation status of Hh signaling modulates the transcriptional expression of AKT genes-We previously observed that silencing of SMO in lymphoma cell lines resulted in decrease of total protein levels of AKT. Because of this finding we decided to explore whether canonical Hh signaling pathway has a role in the regulation of the expression of AKT genes.
We first inhibited Hh signaling using the classical SMO inhibitor, cyclopamine-KAAD (45). Treating two DLBCL cell lines (DOHH2 and LP) that are sensitive to Hh signaling inhibition with cyclopamine-KAAD (2.5 μM for 24 h) resulted in significant decrease in the mRNA levels of AKT1, AKT2 and AKT3 as compared to controls (Fig. 1A). Cyclopamine-KAAD (2.5 µM) treatments did not affect significantly the number of viable cells as indicated by a cell viability assay (data not shown). Concomitant decrease of GLI1 mRNA confirmed the inhibition of canonical Hh signaling pathway (Fig. 1A). Expression levels of BCL2 gene, known direct target of GLI1, were used as an additional positive control (46).

Similarly, we observed decreased in AKT phosphorylation at Ser473 and total AKT protein levels from lysates obtained from cyclopamine-KAAD (2.5 µM for 48 h) treated DOHH2 and LP cells as compared with those collected from the control cells (Fig. 1A, Right panels).

We have previously shown that DLBCL cells are responsive to recombinant Shh N-terminal peptide (R&D Biosystems, Minneapolis, MN, USA) or conditioned culture medium (CCM) obtained from HS5 cells (human stromal bone marrow cells) resulting in activation of the canonical Hh signaling pathway (5,16). To determine whether AKT mRNA expression is induced with the activation of Hh signaling pathway, we treated DOHH2 cells with recombinant Shh N-terminal peptide or CCM for 24 h and analyzed the expression levels of AKT genes by quantitative RT-PCR. Expression levels of AKT1, AKT2 and AKT3 mRNA were significantly increased in DOHH2 cells after treatment with recombinant Shh N-terminal peptide or CCM (Fig. 1B and C). Increased mRNA levels of GLI1 and BCL2 in response to recombinant Shh N-terminal peptide or CCM confirmed activation of Hh signaling pathway (Fig. 1A). To address whether phosphorylation status of AKT at Ser473 and total protein levels of AKT are modulated with recombinant Shh N-terminal peptide or CCM, we performed immunoblotting analysis. We observed that phosphorylation of AKT at Ser473 and total AKT protein levels were increased in recombinant Shh N-terminal peptide (250 nM for 48 h) or CCM treated cell lysates as compared with control (untreated) cell lysates (Fig. 1B and C, Right panels).

To further confirm the transcriptional regulation of AKT genes by canonical Hh signaling, we established LP and OCI-LY19 cells with stable knockdown expression of SMO gene using a lentiviral shRNA system (Fig. 2). The mRNA levels of AKT1, AKT2 and AKT3 were significantly decreased in SMO depleted LP cells in comparison with the control cells harboring luciferase shRNA (Fig. 2A). Decreased level of SMO and GLI1 mRNA confirmed the suppression of Hh signaling pathway (Fig. 2A). As expected, we observed decreased in AKT phosphorylation at Ser473 and total AKT protein levels from lysates collected from SMO knocked down LP cells as compared with those collected from the control cells (Fig. 2B). Because GLI1 is constitutive activated in DLBCL cells, these experiments were done without Hh stimulation.

Similar results were obtained in SMO depleted OCI-LY19 cells (Fig. 2C). Although GLI1 is constitutive activated in DLBCL cells, adding recombinant Shh N-terminal peptide resulted in further increased expression levels of AKT1 and AKT2 in cells harboring luciferase shRNA. However, a significantly decreased in the expression of AKT1 and AKT2 was noticed in those cells with silenced SMO despite of the presence of Shh N-terminal peptide (Fig. 2C) further supporting a role of SMO in the expression of AKT genes.

These findings indicate that canonical Hh signaling modulates the expression of AKT genes. However, these data do not clarify if the modulation of AKT expression is due to GLI1, the main transcriptional activator of Hh signaling.

**AKT1 gene expression is transcriptionally regulated by GLI1**—Having shown that SMO played a role in the transcriptional regulation of AKT genes; we decided to investigate if these changes are mediated by its downstream transcriptional factor, GLI1. We knocked down GLI1 in OCI-LY19 cells using RNAi system (Fig. 3) and performed qRT-PCR. Silencing GLI1 resulted in a decrease of GLI1 expression associated with decreased levels of BCL2, AKT1.
and AKT2 (Fig. 3A). We could not detect mRNA levels of AKT3 in OCI-LY19 indicating very low expression of AKT3 in these cells. Similarly, we observed decreased phosphorylation of AKT at Ser473 in parallel with decrease levels of total AKT protein in GLI1 knocked down OCI-LY19 cells as compared with the control cells harboring luciferase shRNA (Fig. 3B).

To confirm the regulatory role of GLI1 in the transcription of AKT genes, we established an inducible GLI1 knock down lymphoma cell line (OCI-LY19) using lentiviral IPTG-inducible system. Initial characterization confirmed that, upon IPTG addition, OCI-LY19 cells decreased the expression of GLI1, BCL2, AKT1 and AKT2 genes (Fig. 3C). Removal of IPTG from the culture medium resulted in recover of the expression levels of AKT1 and AKT2 (Fig. 3C). Similarly, we observed decreased in AKT phosphorylation at Ser473 and total protein levels of AKT from lysates obtained from IPTG inducible GLI1 knock down OCI-LY19 cells as compared with IPTG inducible control cells. Removal of IPTG from the culture medium resulted in recover of the expression levels of phospho AKT and total AKT (Fig. 3D).

These experiments were also performed in the presence of recombinant Shh N-terminal peptide (Fig. 3E). Adding Shh N-terminal peptide resulted in increased expression levels of AKT1 and AKT2 genes in cells harboring luciferase shRNA but not in those with silenced GLI1 further confirming a role of GLI1 in the transcriptional regulation of AKT genes. Altogether, these data confirm that GLI1 regulates the transcription of AKT genes.

**GLI1 binds to the AKT1 promoter** - AKT is one of the hyperactivated kinases in cancer and plays an essential role in tumorigenesis (25-27). In mammalian cells, three major isoforms of AKT (AKT1, AKT2, and AKT3) have been identified (28). Using MATINSPектор professional version 7.2 (47), we identified several potential GLI1 binding sites (GLI1 binding motif 5'-GACCACCCA-3') in the gene promoters of the three AKT genes (Fig. 4A and Table 1). We identified two putative GLI1 binding sites (BS1 & BS2) located upstream of the transcriptional start site of AKT1 gene (Fig. 4A), three and two potential GLI1 binding sites were identified in the promoters of AKT2 and AKT3, respectively (Table 1). The homology of each GLI1 binding site to the consensus sequence was of 67% for BS1 and of 78% for BS2.

To confirm a direct interaction between GLI1 and the AKT1 promoter in DLBCL cells, we performed ChIP studies in DOHH2 cells using two independent control and GLI1-specific antibodies (Fig. 4B). These studies demonstrated the precipitation of AKT1 promoter encompassing BS1 and BS2 regions and therefore the direct binding of GLI1 with the AKT1 promoter.

To validate the functionality of both BS, a luciferase reporter assay was performed in 293T cells cotransfected with vector or full length GLI1, renilla and AKT1 promoter luciferase construct (pGL3-4293/+1888) containing BS1 and BS2. As shown in Fig. 4C, expression of GLI1 significantly stimulated the luciferase activity of AKT1 promoter (13 fold), implying that the AKT1 response elements reside in this region. To test the functionality of individual BS, we performed the same luciferase reporter experiments in two deleted constructs (pGL3-AKT1-4293/+1 and pGL3-AKT1-325/+1888) of the AKT1 promoter, each containing one putative GLI1 BS. The luciferase assay showed that GLI1 significantly increased the luciferase activity of both AKT1 deleted constructs as compared to the empty vector control (Fig. 4C). Similarly, activation of GLI1 using recombinant Shh N-terminal peptide increases the luciferase activity in DOHH2 (Fig. 4D) and 293T cells (data not shown) transfected with the AKT1 constructs as compared to untreated cells. On the other hand, inhibition of GLI1 activity with cycloamine-KAAD (2.5 μM for 24 h) considerably decreases the AKT1 luciferase activity as compared to untreated DOHH2 (Fig. 4E) and 293T cells (data not shown).

To further confirm the functionality of these BS, we mutated three cytosines (C) to guanines (G) in BS1 and BS2 (Fig. 5A) to see whether these mutations could abrogate the ability of GLI1 to increase AKT1 luciferase activity. When we cotransfected mutated BS1 and BS2 luciferase, renilla constructs cotransfected with full length GLI1 in 293T cells, we observed an
approximately 4-fold less luciferase activity in comparison with the wild-type constructs indicating a decreased binding of GLI1 to both mutated BSs (Fig. 5B).

GLI1 contributes to DLBCL cell survival through the transcription of AKT1 gene—Previously, it has been reported that AKT1 knock out mouse embryonic fibroblasts are more susceptible to apoptosis upon exposure to genotoxic stress suggesting the important role of AKT1 in cell survival (48). To address if GLI1 is important for DLBCL cell survival we established stable GLI1 knock down DLBCL cell lines (OCI-LY19 and HBL-1) using a lentiviral shRNA system (Fig. 2A and 6A) and performed cell viability (trypan blue) assays. Cell viability assays demonstrated that GLI1 knockdown DLBCL cells experienced a statistically significantly decrease in the number of viable cells in comparison with control cells harboring luciferase shRNA (Fig. 6B).

To examine how depletion of GLI1 suppresses number of viable DLBCL cells, we performed apoptosis and cell cycle analysis. Annexin V and PI assays demonstrated marked increase of apoptosis in GLI1 depleted DLBCL cells versus controls (Fig. 6C). In addition, cell cycle analysis demonstrated that DLBCL cells with GLI1 knockdown experienced mild to modest decreased cell proliferation, due to G1 cell cycle arrest, in comparison with the control cells harboring luciferase shRNA (data not shown).

To address whether decrease cell survival in GLI1 depleted lymphoma cells is associated with the AKT1 expression, full-length AKT1 has been expressed in control (harboring luciferase) and GLI1#2 knock down HBL1 cells based on the lentiviral expression system (Fig. 6D). Constitutive expression of AKT1 in GLI1 knock down cells partially rescued cell viability of lymphoma cells in comparison with GLI1 knock down cells without transduced AKT1 (Fig. 6E). Altogether, these data suggest that GLI1 contributes to cell survival in part through the transcription of AKT1 gene.

GLI1 and AKT1 levels are positively correlated in human DLBCL tumors—To investigate whether there is any correlation between GLI1 and AKT protein levels in human DLBCL tumors, we performed double immuno fluorescence analyses in paraffin embedded DLBCL tumor and reactive lymph node (non-neoplastic) specimens. We found higher nuclear expression of the GLI1 and cytoplasmic AKT expression in DLBCL specimens as compare to reactive lymph nodes (control; Fig. 7, lower panels). There was a correlation between GLI1 and AKT protein levels as shown in plot profile analysis (Fig. 7, upper panels).

Next, we performed quantitative real-time PCR analyses in 11 frozen DLBCL tumor specimens. The real time PCR analysis revealed a strong Spearman correlation coefficient (R²=0.88) between GLI1 and AKT1 mRNA expression (Fig. 8A). We additionally analyzed expression of AKT1 and GLI1 in four DLBCL samples collected from aphaeresis samples from pleural effusions (samples lacking of stromal component). In the aphaeresis samples a positive correlation between the mRNA expression of GLI1 and AKT1 was also noted (R²=0.90) (Fig. 8B). Similarly, we also found a significant Spearman correlation coefficient (R²=0.74) between AKT1 and GLI1 protein expression in 50 neoplastic non-lymphoma cell lines available in the human protein atlas database (http://www.proteinatlas.org) (Fig. 8C).

DISCUSSION

GLI1, a full length transcriptional activator, has been shown to be involved in the intracellular signal transduction controlled by the Hh family of secreted ligands (49). Although, a comprehensive analysis of direct transcriptional targets of GLI1 on a genomic level is lacking, a number of studies have shown that GLI1 directly regulates the transcription of various genes that are known to be involved in cell proliferation, survival and chemotolerance (16-20).

Previously, we have shown that canonical Hh signaling is a key factor behind high ABCG2 expression in DLBCL through direct up regulation of ABCG2 gene transcription (16). Now, we provide evidence of the contribution of GLI1 in the survival of DLBCL cells and demonstrate, for the first time, that GLI1 mediated canonical Hh signaling pathway modulates the transcriptional expression of
AKT1, AKT2 and AKT3 genes. In particular, we demonstrated that AKT1 promoter possesses two GLI1 binding sites and that the expression of AKT1 is regulated, at the transcriptional level, by GLI1. These findings are important because they contribute to understand the transcriptional regulation of AKT1, which is known to provide survival signals to the cells (48).

The AKT signaling pathway is activated in DLBCL cell lines as well as in a subset of DLBCL primary tumor samples independent of the molecular subtype (27,33). Several mechanisms have been proposed for the activation of AKT in DLBCL such as inactivation or deletion of PTEN and mutations of PIK3CA, the gene coding for the catalytic subunit p110α of PI3K (35-37). Some DLBCL are also characterized by the over expression and secretion of cytokines such as IL-6 and IL-10 that result in the activation of AKT pathway among other pathways (50). Although, the AKT signaling is extensively studied at the kinase activity levels, there are few data available regarding AKT transcriptional regulation and the overall transcriptional regulation of AKT remains largely unknown. Transcription factors that have been found to contribute to the transcription of AKT genes are β-catenin and signal transducer and activator of transcription (STAT3) (31,32).

In our study, we observed that the total levels of AKT run in parallel with its phosphorylation levels at Ser473. Similar correlation between total AKT and its phosphorylation levels was also seen by Dihlmann and colleagues using colon cancer cells (31). These authors found that the phosphorylation levels of AKT at Ser473 correlated with the total levels of AKT, supporting that up-regulation or down-regulation of AKT genes on the transcriptional level result in changes of AKT activity. It is known that the activity of a kinase is not necessary a reflection of its total protein level as extra- and intra-cellular factors such cytokines, growth factors or activated oncogenic signals contribute to modulate the kinase activity independently of its transcriptional level. However, the presence of high kinase levels likely contributes to oncogenesis by establishing the basis for an enhanced kinase signaling activity.

Bidirectional interconnectivity between Hh signaling with PI3K/AKT pathway at the post-transcriptional level has been well documented (41,51,52). For example, Riobo et al have reported that AKT activation is important for activation of Hh signaling, effects that were mediated by AKT controlling PKA-mediated GLI proteosomal degradation (51). Several other groups have also reported that adenoviral delivery of activated AKT in non-neoplastic and neoplastic cells is associated with activation of Hh signaling and increase expression of GLI1 (41,53). Recently, Wang et al showed that activation of GLI1 by AKT requires S6K1 and that S6K1 phosphorylates GLI1 and enhances GLI1 activity (53). Moreover, mice studies have also found a synergistic effect between PI3K/AKT and Hh signaling in medulloblastoma tumorigenesis (52).

This view of interconnectivity between Hh and PI3K/AKT at multiple levels, including at the transcriptional level as here reported, has potential clinical implications as inhibitors of both pathways are currently available and the use of combination of inhibitors of both pathways may result in synergistic cytotoxicity effects or in decreased chemotolerance of the tumor cells to current chemotherapeutic protocols.

In conclusion, we demonstrate that canonical Hh signaling pathway regulates the transcription of AKT genes, and that AKT1 is a novel direct downstream target of the transcriptional factor GLI1. We also provide evidence that GLI1 contributes cell survival of DLBCL cells through the expression of AKT in DLBCL and likely also in other malignant tumors.

Conflict of Interest
The authors declare no competing financial interests.
AKT1 direct downstream target of GLI1

REFERENCES

1. Armitage, J. O., and Weisenburger, D. D. (1998) New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol* **16**, 2780-2795

2. Larouche, J. F., Berger, F., Chassagne-Clement, C., Ffrench, M., Callet-Bauchu, E., Sebban, C., Ghesquieres, H., Broussais-Guillaumot, F., Salles, G., and Coiffier, B. (2010) Lymphoma recurrence 5 years or later following diffuse large B-cell lymphoma: clinical characteristics and outcome. *J Clin Oncol* **28**, 2094-2100

3. McMahon, A. P., Ingham, P. W., and Tabin, C. J. (2003) Developmental roles and clinical significance of hedgehog signaling. *Curr Top Dev Biol* **53**, 1-114

4. Taipale, J., and Beachy, P. A. (2001) The Hedgehog and Wnt signalling pathways in cancer. *Nature* **411**, 349-354

5. Singh, R. R., Kim, J. E., Davuluri, Y., Drakos, E., Cho-Vega, J. H., Amin, H. M., and Vega, F. (2010) Hedgehog signaling pathway is activated in diffuse large B-cell lymphoma and contributes to tumor cell survival and proliferation. *Leukemia* **24**, 1025-1036

6. Tang, T., Tang, J. Y., Li, D., Reich, M., Callahan, C. A., Fu, L., Yauch, R. L., Wang, F., Kotkow, K., Chang, K. S., Shpall, E., Wu, A., Rubin, L. L., Marsters, J. C., Jr., Epstein, E. H., Jr., Caro, I., and de Sauvage, F. J. (2011) Targeting superficial or nodular Basal cell carcinoma with topically formulated small molecule inhibitor of smoothened. *Clin Cancer Res* **17**, 3378-3387

7. Yauch, R. L., Dijkgraaf, G. J., Alicke, B., Januario, T., Ahn, C. P., Holcomb, T., Pujara, K., Stinson, J., Callahan, C. A., Tang, T., Bazan, J. F., Kan, Z., Seshagiri, S., Hann, C. L., Gould, S. E., Low, J. A., Rudin, C. M., and de Sauvage, F. J. (2009) Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. *Science* **326**, 572-574

8. Rudin, C. M., Hann, C. L., Laterra, J., Yauch, R. L., Callahan, C. A., Fu, L., Holcomb, T., Stinson, J., Gould, S. E., Coleman, B., LoRusso, P. M., Von Hoff, D. D., de Sauvage, F. J., and Low, J. A. (2009) Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. *N Engl J Med* **361**, 1173-1178

9. Von Hoff, D. D., LoRusso, P. M., Rudin, C. M., Reddy, J. C., Yauch, R. L., Tibes, R., Weiss, G. J., Borad, M. J., Hann, C. L., Brahmer, J. R., Mackey, H. M., Lum, B. L., Darbone, W. C., Marsters, J. C., Jr., de Sauvage, F. J., and Low, J. A. (2009) Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med* **361**, 1164-1172

10. Johnson, R. L., and Scott, M. P. (1998) New players and puzzles in the Hedgehog signaling pathway. *Curr Opin Genet Dev* **8**, 450-456

11. Kalderon, D. (2000) Transducing the hedgehog signal. *Cell* **103**, 371-374
AKT1 direct downstream target of GLI1

12. Taipale, J., Cooper, M. K., Maiti, T., and Beachy, P. A. (2002) Patched acts catalytically to suppress the activity of Smoothened. *Nature* 418, 892-897

13. Varjosalo, M., and Taipale, J. (2008) Hedgehog: functions and mechanisms. *Genes Dev* 22, 2454-2472

14. Kinzler, K. W., Bigner, S. H., Bigner, D. D., Trent, J. M., Law, M. L., O'Brien, S. J., Wong, A. J., and Vogelstein, B. (1987) Identification of an amplified, highly expressed gene in a human glioma. *Science* 236, 70-73

15. Kinzler, K. W., and Vogelstein, B. (1990) The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. *Mol Cell Biol* 10, 634-642

16. Singh, R. R., Kunkalla, K., Qu, C., Schlette, E., Neelapu, S. S., Samaniego, F., and Vega, F. (2011) ABCG2 is a direct transcriptional target of hedgehog signaling and involved in stroma-induced drug tolerance in diffuse large B-cell lymphoma. *Oncogene* 30, 4874-4886

17. He, S., Wang, F., Yang, L., Guo, C., Wan, R., Ke, A., Xu, L., Hu, G., Xu, X., Shen, J., and Wang, X. (2011) Expression of DNMT1 and DNMT3a are regulated by GLI1 in human pancreatic cancer. *PLoS One* 6, e27684

18. Wang, F., Xu, L., Guo, C., Ke, A., Hu, G., Xu, X., Mo, W., Yang, L., Huang, Y., He, S., and Wang, X. (2011) Identification of RegIV as a novel GLI1 target gene in human pancreatic cancer. *PLoS One* 6, e18434

19. Laner-Plamberger, S., Kaser, A., Paulischta, M., Hauser-Kronberger, C., Eichberger, T., and Frischauf, A. M. (2009) Cooperation between GLI and JUN enhances transcription of JUN and selected GLI target genes. *Oncogene* 28, 1639-1651

20. Kuphal, S., Shaw-Hallgren, G., Eberl, M., Karrer, S., Aberger, F., Bosserhoff, A. K., and Massoumi, R. (2011) GLI1-dependent transcriptional repression of CYLD in basal cell carcinoma. *Oncogene* 30, 4523-4530

21. Kim, J. E., Singh, R. R., Cho-Vega, J. H., Drakos, E., Davuluri, Y., Khokhar, F. A., Fayad, L., Medeiros, L. J., and Vega, F. (2009) Sonic hedgehog signaling proteins and ATP-binding cassette G2 are aberrantly expressed in diffuse large B-cell lymphoma. *Mod Pathol* 22, 1312-1320

22. Ramirez, E., Singh, R. R., Kunkalla, K., Liu, Y., Qu, C., Cain, C., Multani, A. S., Lennon, P. A., Jackaky, J., Ho, M., Dawud, S., Gu, J., Yang, S., Hu, P. C., and Vega, F. (2012) Defining causative factors contributing in the activation of hedgehog signaling in diffuse large B-cell lymphoma. *Leuk Res* 36, 1267-1273

23. Pasqualucci, L., Trifonov, V., Fabbri, G., Ma, J., Rossi, D., Chiarenza, A., Wells, V. A., Grunn, A., Messina, M., Elliot, O., Chan, J., Bhagat, G., Chadburn, A., Gaidano, G., Mullighan, C. G., Rabad, R., and Dalla-Favera, R. (2011) Analysis of the coding genome of diffuse large B-cell lymphoma. *Nat Genet* 43, 830-837
24. Morin, R. D., Mendez-Lago, M., Mungall, A. J., Goya, R., Mungall, K. L., Corbett, R. D., Johnson, N. A., Severson, T. M., Chiu, R., Field, M., Jackman, S., Krzywinski, M., Scott, D. W., Trinh, D. L., Tamura-Wells, J., Li, S., Firme, M. R., Rogic, S., Griffith, M., Chan, S., Yakovenko, O., Meyer, I. M., Zhao, E. Y., Smailus, D., Moksa, M., Chittaranjan, S., Rimsza, L., Brooks-Wilson, A., Spinelli, J. J., Ben-Neriah, S., Meissner, B., Woolcock, B., Boyle, M., McDonald, H., Tam, A., Zhao, Y., Delaney, A., Zeng, T., Tse, K., Butterfield, Y., Birol, I., Holt, R., Schein, J., Horsman, D. E., Moore, R., Jones, S. J., Connors, J. M., Hirst, M., Gascoyne, R. D., and Marra, M. A. (2011) Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature 476, 298-303

25. Bacus, S. S., Altomare, D. A., Lyass, L., Chin, D. M., Farrell, M. P., Gurova, K., Gudkov, A., and Testa, J. R. (2002) AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. Oncogene 21, 3532-3540

26. Testa, J. R., and Bellacosa, A. (2001) AKT plays a central role in tumorigenesis. Proc Natl Acad Sci U S A 98, 10983-10985

27. Uddin, S., Hussain, A. R., Siraj, A. K., Manogaran, P. S., Al-Jomah, N. A., Moorji, A., Atizado, V., Al-Dayel, F., Belgaumi, A., El-Solh, H., Ezzat, A., Bavi, P., and Al-Kuraya, K. S. (2006) Role of phosphatidylinositol 3'-kinase/AKT pathway in diffuse large B-cell lymphoma survival. Blood 108, 4178-4186

28. Alessi, D. R., and Cohen, P. (1998) Mechanism of activation and function of protein kinase B. Curr Opin Genet Dev 8, 55-62

29. Staal, S. P. (1987) Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A 84, 5034-5037

30. Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V., and Cheng, J. Q. (2001) AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am J Pathol 159, 431-437

31. Dihlmann, S., Kloor, M., Fallsehr, C., and von Knebel Doeberitz, M. (2005) Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells. Carcinogenesis 26, 1503-1512

32. Park, S., Kim, D., Kaneko, S., Szewczyk, K. M., Nicosia, S. V., Yu, H., Jove, R., and Cheng, J. Q. (2005) Molecular cloning and characterization of the human AKT1 promoter uncovers its up-regulation by the Src/Stat3 pathway. J Biol Chem 280, 38932-38941

33. Hasselblom, S., Hansson, U., Olsson, M., Toren, L., Bergstrom, A., Nilsson-Ehle, H., and Andersson, P. O. (2010) High immunohistochemical expression of p-AKT predicts inferior survival in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. Br J Haematol 149, 560-568
34. Gonzalez, E., and McGraw, T. E. (2009) The Akt kinases: isoform specificity in metabolism and cancer. *Cell Cycle* **8**, 2502-2508

35. Baohua, Y., Xiaoyan, Z., Tiecheng, Z., Tao, Q., and Daren, S. (2008) Mutations of the PIK3CA gene in diffuse large B cell lymphoma. *Diagn Mol Pathol* **17**, 159-165

36. Sakai, A., Thieblemont, C., Wellmann, A., Jaffé, E. S., and Raffeld, M. (1998) PTEN gene alterations in lymphoid neoplasms. *Blood* **92**, 3410-3415

37. Lenz, G., Wright, G. W., Emre, N. C., Kohlihammer, H., Dave, S. S., Davis, R. E., Carty, S., Lam, L. T., Shaffer, A. L., Xiao, W., Powell, J., Rosenwald, A., Ott, G., Muller-Hermelink, H. K., Gascoyne, R. D., Connors, J. M., Campo, E., Jaffé, E. S., Delabie, J., Smeland, E. B., Rimsza, L. M., Fisher, R. I., Weisenburger, D. D., Chan, W. C., and Staudt, L. M. (2008) Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. *Proc Natl Acad Sci U S A* **105**, 13520-13525

38. Pham, L. V., Tamayo, A. T., Yoshimura, L. C., Lin-Lee, Y. C., and Ford, R. J. (2005) Constitutive NF-kappaB and NFAT activation in aggressive B-cell lymphomas synergistically activates the CD154 gene and maintains lymphoma cell survival. *Blood* **106**, 3940-3947

39. Pham, L. V., Fu, L., Tamayo, A. T., Bueso-Ramos, C., Drakos, E., Vega, F., Medeiros, L. J., and Ford, R. J. (2011) Constitutive BR3 receptor signaling in diffuse, large B-cell lymphomas stabilizes nuclear factor-kappaB-inducing kinase while activating both canonical and alternative nuclear factor-kappaB pathways. *Blood* **117**, 200-210

40. Schmittgen, T. D. (2001) Real-time quantitative PCR. *Methods (San Diego, Calif)* **25**, 383-385

41. Singh, R. R., Cho-Vega, J. H., Davuluri, Y., Ma, S., Kasbidi, F., Milito, C., Lennon, P. A., Drakos, E., Medeiros, L. J., Luthra, R., and Vega, F. (2009) Sonic hedgehog signaling pathway is activated in ALK-positive anaplastic large cell lymphoma. *Cancer Res* **69**, 2550-2558

42. Boulbes, D., Chen, C. H., Shaikenov, T., Agarwal, N. K., Peterson, T. R., Addona, T. A., Keshishian, H., Carr, S. A., Magnuson, M. A., Sabatini, D. M., and Sarbassov dos, D. (2010) Rictor phosphorylation on the Thr-1135 site does not require mammalian target of rapamycin complex 2. *Mol Cancer Res* **8**, 896-906

43. Li, W., You, L., Cooper, J., Schiavon, G., Pepe-Caprio, A., Zhou, L., Ishii, R., Giovannini, M., Hanemann, C. O., Long, S. B., Erdjument-Bromage, H., Zhou, P., Tempst, P., and Giancotti, F. G. (2010) Merlin/NF2 suppresses tumorigenesis by inhibiting the E3 ubiquitin ligase CRL4(DCAF1) in the nucleus. *Cell* **140**, 477-490

44. Guillaud, L., Setou, M., and Hirokawa, N. (2003) KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons. *J Neurosci* **23**, 131-140

45. Chen, J. K., Taipale, J., Cooper, M. K., and Beachy, P. A. (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev* **16**, 2743-2748
46. Bigelow, R. L., Chari, N. S., Unden, A. B., Spurgers, K. B., Lee, S., Roop, D. R., Toftgard, R., and McDonnell, T. J. (2004) Transcriptional regulation of bcl-2 mediated by the sonic hedgehog signaling pathway through gli-1. *J Biol Chem* **279**, 1197-1205

47. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* **23**, 4878-4884

48. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., Kadowaki, T., and Hay, N. (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev* **15**, 2203-2208

49. Hui, C. C., and Angers, S. (2011) Gli proteins in development and disease. *Annu Rev Cell Dev Biol* **27**, 513-537

50. Voorzanger, N., Touitou, R., Garcia, E., Delecluse, H. J., Rousset, F., Joab, I., Favrot, M. C., and Blay, J. Y. (1996) Interleukin (IL)-10 and IL-6 are produced in vivo by non-Hodgkin's lymphoma cells and act as cooperative growth factors. *Cancer Res* **56**, 5499-5505

51. Riobo, N. A., Lu, K., Ai, X., Haines, G. M., and Emerson, C. P., Jr. (2006) Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *Proc Natl Acad Sci U S A* **103**, 4505-4510

52. Rao, G., Pedone, C. A., Del Valle, L., Reiss, K., Holland, E. C., and Fults, D. W. (2004) Sonic hedgehog and insulin-like growth factor signaling synergize to induce medulloblastoma formation from nestin-expressing neural progenitors in mice. *Oncogene* **23**, 6156-6162

53. Wang, Y., Ding, Q., Yen, C. J., Xia, W., Izzo, J. G., Lang, J. Y., Li, C. W., Hsu, J. L., Miller, S. A., Wang, X., Lee, D. F., Hsu, J. M., Huo, L., Labaff, A. M., Liu, D., Huang, T. H., Lai, C. C., Tsai, F. J., Chang, W. C., Chen, C. H., Wu, T. T., Buttar, N. S., Wang, K. K., Wu, Y., Wang, H., Ajani, J., and Hung, M. C. (2012) The crosstalk of mTOR/S6K1 and Hedgehog pathways. *Cancer Cell* **21**, 374-387
AKT1 direct downstream target of GLI1

Acknowledgements—We thank Dr. Jin Q. Cheng (Departments of Pathology and Interdisciplinary Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, USA) for providing the AKT1 promoter luciferase constructs. We also thank Dr. Peter Zaphiropoulos (Department of Bioscience and Nutrition, Karolinska Institutet, Sweden) for providing full length Flag-tagged GLI1 plasmid. We also thank Dr Richard Ford (Department of Hematopathology, MDACC) for providing LP cells.

FOOTNOTES
This work was supported by funds from The Translational Grant of The Leukemia & Lymphoma Society (to FV), and K08 Physician-Scientist Award 1 K08 CA143151-01 (NIH) (to FV). The primary tumor samples were provided by the Hematopathology Tissue Bank of the UT MD Anderson Cancer Center (supported by the NCI/NIH Grant CA16672).

FIGURE LEGENDS

FIGURE 1. Hh signaling activity regulates the expression of AKT genes. A. DLBCL cells (DOHH2 and LP) were treated with or without the classical SMO inhibitor, cyclopamine-KAAD (CY) 2µM for 24h. Treated cells were subjected to qRT-PCR to analyze the mRNA expression of GLI1, BCL2, AKT1, AKT2 and AKT3. As a control we used DMSO (vehicle for cyclopamine-KAAD). DLBCL cells as described in A were used for immunoblotting to detect the phosphorylation levels of AKT (Ser473) or total AKT after treatment with 2µM of cyclopamine-KAAD for 48h. B. DOHH2 cells were treated with or without recombinant Shh N-terminal peptide (250 nM) for 24h. Treated cells were subjected to qRT-PCR to analyze the mRNA expression of GLI1, BCL2, AKT1, AKT2 and AKT3. As a control we used PBS (vehicle for recombinant Shh N-terminal peptide). C. DOHH2 cells were exposed to conditioned culture medium (CCM) obtained from HS5 cells (24h). Cells were then subjected to qRT-PCR to analyze the mRNA expression of GLI1, BCL2, AKT1, AKT2 and AKT3. As a control we used DOHH2 cells cultured in 2% FBS medium. DOHH2 cells as described in B-C were used for immunoblotting to detect the phosphorylation levels of AKT (Ser473) or total AKT after treatment with 250 nM recombinant Shh N-terminal peptide for 48h or CCM. Results shown in A-C are normalized to 18S mRNA level and expressed as fold change in mRNA expression compared with control. Data represent the mean and standard deviation of three independent experiments. * P<0.05, ** P<0.005, *** P<0.0005

FIGURE 2. SMO participates in the expression of AKT genes. A. LP cells were infected with lentiviruses expressing shRNA-targeting luciferase (control) and SMO. The transduced cells were selected with puromycin and the expression levels of SMO, GLI1, AKT1, AKT2 and AKT3 mRNA was analyzed by qRT-PCR. B. The same cells as described in A were used for immunoblotting to detect the phosphorylation levels of AKT (Ser473) or total AKT after silencing of SMO. C. Similarly, OCI-LY19 cells were transduced with lentiviruses expressing shRNA-targeting luciferase (control) and SMO, and treated with or without recombinant Shh N-terminal peptide (250 nM) for 24h. Treated cells were subjected to qRT-PCR to analyze the mRNA expression of SMO, GLI1, AKT1 and AKT2. As a control we used PBS (vehicle for recombinant Shh N-terminal peptide). Results shown in A and C are normalized to 18S mRNA level and expressed as fold change in mRNA expression compared with control. Data represent the mean and standard deviation of three independent experiments. * P<0.05, ** P<0.005, *** P<0.0005

FIGURE 3. Silencing of GLI1 decreases the expression of AKT genes. A. qRT-PCR analysis of GLI1, BCL2, AKT1 and AKT2 mRNA expression levels in OCI-LY19 cells lentivirally transduced with shRNAs targeting GLI1 or luciferase (control). B. The same cells as described in A were used for immunoblotting to detect the phosphorylation levels of AKT (Ser473) and total AKT after silencing of GLI1. C. OCI-
LY19 cells were transduced with lentiviruses expressing IPTG-inducible shRNA-targeting luciferase (control) and GLI1. The transduced cells were selected with puromycin and treated with 1mM IPTG for 3-4 days to suppress the expression of GLI1. qRT-PCR analysis of GLI1, BCL2, AKT1 and AKT2 mRNA expression levels in IPTG-inducible control, GLI1 depleted and GLI1 recovered OCI-LY19 cells (IPTG-GLI1-R) were performed as described in Experimental Procedures. D. The same cells as described in C were used for immunoblotting to detect the phosphorylation levels of AKT (Ser473) and total AKT. E. The same cells as described in C were treated with or without recombinant Shh N-terminal peptide (250 nM) for 24h. Thereafter, cells were subjected to qRT-PCR to analyze the mRNA expression of GLI1, BCL2, AKT1 and AKT2. As a control we used PBS (vehicle for recombinant Shh N-terminal peptide). Results shown in A, C and E are normalized to 18S mRNA level and expressed as fold change in mRNA expression compared with control. Data represent the mean and standard deviation of three independent experiments. * P<0.05, ** P<0.005, *** P<0.0005

FIGURE 4. GLI1 transactivates AKT1 promoter activity. A. Schematic diagram of the luciferase constructs that includes two potential GLI1 binding sites (BS1 and BS2) in the AKT1 promoter. The 9 base pairs sequence of the GLI-binding site is shown along with the sequence of two closely spaced GLI1 binding sites identified in the AKT1 promoter. B. ChIP assays were performed with two independent control or GLI1-specific antibodies. These studies resulted in the precipitation of AKT1 promoter chromatin containing the GLI1-binding sites in DOHH2 cells. The human RPL30 promoter region serves as positive control, while IgG serum Monoclonal (Mono) and Polyclonal (Poly) antibodies were used as controls. C. A series of AKT1 luciferase constructs were transfected into 293T cells together with or without full length GLI1 plasmid and subjected to luciferase reporter assay. D. AKT1 luciferase constructs, as indicated, were transfected into DOHH2 cells. After 24h, cells were treated with or without recombinant Shh N-terminal peptide for 24h and subjected to luciferase reporter assay. As a control we used PBS (vehicle for recombinant Shh N-terminal peptide). E. DOHH2 cells were also treated with or without cyclopamine-KAAD (CY) (2.5 μM for 24h) and subsequently subjected to luciferase reporter assays. As a control we used DMSO (vehicle for cyclopamine-KAAD). Results shown in C-E are normalized to renilla luciferase and expressed as fold change in relative luciferase activity compared with control. Data represent the mean and standard deviation of three independent experiments. Expression of GLI1 was confirmed using qRT-PCR assays. * P<0.05, ** P<0.005, *** P<0.0005

FIGURE 5. Mutation analysis of GLI1 coding sequences on AKT1 promoter region. A. Three cytosines (C) were mutated to 3 guanines (G) in BS1 and BS2 region. B. Mutated and wild-type AKT1 luciferase constructs were transfected into 293T cells together with or without full length GLI1 plasmid and subjected to luciferase reporter assays. Results are normalized to renilla luciferase and expressed as fold change in relative luciferase activity compared with control. Data represent the mean and standard deviation of three independent experiments. * P<0.05, ** P<0.005, *** P<0.0005

FIGURE 6. AKT1 mediates GLI1 survival signals in DLBCL. A. Two DLBCL cell lines (OCI-LY19, and HBL1) were infected with lentiviruses expressing shRNA-targeting luciferase (control) and GLI1. The transduced cells were selected with puromycin and the expression level of GLI1 mRNA was analyzed by qRT-PCR (results for OCI-LY19 were presented in Fig 2A, left panel). Results are normalized to 18S mRNA level and expressed as fold change in mRNA expression compared with control. B. The same cells as described in A were seeded onto six-well plates and incubated for indicated time periods. Cells were harvested and viable cell numbers were counted by trypan blue exclusion. Relative cell viability (Fold change) was calculated as follows: relative cell viability = viable cell numbers in control or CY treated at indicated time periods/control at 0h. C. Control and GLI1 knock down HBL1 and OCI-LY19 cells were seeded onto six-well plates and incubated for 24h. Annexin V and propidium iodide (PI) staining were analyzed by flow cytometry. Percentage of Annexin V/PI positive cells was calculated as follows: % of Annexin V/PI positive cells = (AnnexinV/PI positive cells/Total cell numbers) x100. Results shown in A-C represent the mean and standard deviation of three
AKT1, a direct downstream target of GLI1

D. Full-length AKT1 was expressed in control (harboring luciferase) and GLI1#2 knockdown HBL1 cells using a lentiviral expression system. The AKT1 transduced cells were selected with blasticidin and subjected to qRT-PCR to analyze the expression levels of GLI1 and AKT1 mRNA. Results are normalized to 18S mRNA level and expressed as fold change in mRNA expression compared with control. E. The same cells as described in D were seeded onto six-well plates and incubated for indicated time periods. Cells were harvested and viable cell numbers were counted by trypan blue exclusion. Percentage of relative cell viability was calculated as follows: % relative cell viability = (viable cell numbers in control or GLI1 shRNA group/ control at 0h) x100. * P<0.05, ** P<0.005, *** P<0.0005

FIGURE 7. Expression levels of AKT and GLI1 in non-neoplastic lymph nodes and DLBCL tumors. Reactive lymph node (control) and DLBCL tissue sections were stained in anti mouse GLI1 (green) and anti rabbit AKT (red) antibodies. Nuclear staining was done with 4’,6-diamidino-2-phenylindole (DAPI) (blue). Plot profile analyses of GLI1 and AKT fluorescence signal intensities were done using Image J software.

FIGURE 8. Correlation between AKT and GLI1 expression levels in cancer cells. A. qRT-PCR analysis of GLI1 and AKT1 mRNA expression levels in DLBCL tumor specimens. Results are normalized to 18S mRNA level and expressed as % change in mRNA expression compared with control. B. qRT-PCR analysis of GLI1 and AKT1 expression in primary DLBCL cells obtained from aphaeresis samples from pleural effusions from 4 patients with DLBCL. Results are normalized to 18S mRNA level and expressed as % change in mRNA expression compared with control. C. Protein expression levels of AKT1 and GLI1 in 50 cell lines available in the human protein atlas database.
Table 1.

Prediction of potential GLI1 binding sites (GLI1 binding motif 5'-GACCACCCA-3') on AKT2 and AKT3 promoter using MATINSPECTOR professional version 7.2

| Gene Name | Matrix Family | Detailed Family Information | Matrix | Opt. | Start Position | End Position | Anchor Position | Strand | Core sim. | Matrix sim. | Sequence         |
|-----------|---------------|------------------------------|--------|------|----------------|--------------|-----------------|--------|-----------|-------------|------------------|
| AKT2      | V$GLIF        | GLI zinc finger family       | V$GLI1.01 | 0.87 | 730            | 744          | 737             | -      | 1         | 0.876       | cagtcctCCCaata    |
| AKT2      | V$GLIF        | GLI zinc finger family       | V$GLI1.01 | 0.87 | 379            | 393          | 386             | -      | 1         | 0.904       | gccacctCCCAgcca   |
| AKT2      | V$GLIF        | GLI zinc finger family       | V$GLI1.01 | 0.87 | 137            | 151          | 144             | +      | 1         | 0.944       | ttgacctCCCaaagt   |
| AKT3      | V$GLIF        | GLI zinc finger family       | V$GLI1.01 | 0.87 | 3              | 17           | 10              | -      | 1         | 0.911       | tccacctCCCaggtt   |
| AKT3      | V$GLIF        | GLI zinc finger family       | V$GLI1.01 | 0.87 | 336            | 350          | 343             | -      | 1         | 0.911       | tccacctCCCaggtt   |
FIGURE 1.

A

DOHH2

Relative mRNA expression (Fold)

GLI1 BCL2 AKT1 AKT2 AKT3

C CY

GLI1
P-Akt (Ser473)
AKT
Actin

B

DOHH2

Relative mRNA expression (Fold)

GLI1 BCL2 AKT1 AKT2 AKT3

C Shh

GLI1
P-Akt (Ser473)
AKT
Actin

C

DOHH2

Relative mRNA expression (Fold)

GLI1 BCL2 AKT1 AKT2 AKT3

C CCM

GLI1
P-Akt (Ser473)
AKT
Actin
**FIGURE 2.**

A. Relative mRNA expression (Fold).

B. shSMO, shLuc.

C. Relative mRNA expression (Fold).

- **SMO**
- **GLI1**
- **AKT1**
- **AKT2**

- **Control**
- **Actin**

- **SMO**
- **P-Akt (Ser473)**
- **Akt**

**AKT1 direct downstream target of GLI1**
**Figure 3.**

**A.** Relative mRNA expression (Fold) of GLI1 knockdowns on AKT1 and downstream targets.

**B.** Western blot analysis showing GLI1, P-Akt (Ser473), Akt, and Actin levels.

**C.** Relative mRNA expression (Fold) under different experimental conditions.

**D.** Additional Western blot analysis showing GLI1, P-Akt (Ser473), Akt, and Actin levels.

**E.** Relative mRNA expression (Fold) with Shh intervention.
**FIGURE 4.**

A

AKT1 promoter

\[-4293\] \[-325\] \[+1\] \[+1888\]

AKT1 Gene

GLI BS1
CACGCCCCCCAGAGT

GLI BS2
GTAACCAACCTGGAC

GLI-binding sequence
GACCACCCCA

B

DNA ladder

IP

Input
Neg. PCR reaction
IgG (Mono)
IgG (Poly)
GLI1 (C6H83)-Mono
GLI1 (H300)-Poly

Akt1 (-4293/+1)
Akt1 (-325/+1888)
DOHH2

RPL30

C

Relative mRNA expression (Fold)

-20
0
defect
Flag-GLI1

D

Relative AKT1 luciferase activity (Fold)

-3
0

DOHH2

Control
Shh

E

Relative AKT1 luciferase activity (Fold)

-1.2
0

DOHH2

Control
CY
FIGURE 5.

A

cagGCGccccagct (wt)
cagGCGccccagct (mut)
gtaacccCCttgac (wt)
gtaacccGCGtgcac (mut.)

B

Relative AKT1 luciferase activity (Fold)
FIGURE 6.

A. Relative GLI1 mRNA expression (Fold) for shLuci and shGLI1#2 in HBL1 cells.

B. Relative Cell Viability (Fold) over time for OCI-LY19 and HBL1 cells with shLuci, shGLI1#2, and shGLI1#1.

C. Percentage of Annexin/V+ positive cells for OCI-LY19 and HBL1 cells with shLuci, shGLI1#2, and shGLI1#1.

D. Relative mRNA expression (Fold) for AKT1, GLI1, and BCL2 in HBL1 cells with shLuci, shLuci + AKT1, shGLI1#2 + AKT1.

E. % Relative Cell Viability (Trypan blue) for shLuci, shLuci + AKT1, shGLI1#2, and shGLI1#2 + AKT1 over 0 h, 48 h, and 96 h.
AKT1 direct downstream target of GLI1
FIGURE 8.

A.

![Graph showing relative mRNA changes for GLI1 and AKT1 across DLBCL tumor samples with a regression line and $R^2 = 0.88$.]

B.

![Graph showing relative mRNA changes for GLI1 and AKT1 across DLBCL cell samples from pleural fluid with $R^2 = 0.90$ and $P = 0.04$.]

C.

![Graph showing the relationship between AKT1 and GLI1 with $R^2 = 0.7424$.]
Transcriptional Regulation of Serine/Threonine Protein Kinase (AKT) Genes by Glioma-Associated Oncogene Homolog 1
Nitin K. Agarwal, Changju Qu, Kranthi Kunkalla, Yadong Liu and Francisco Vega

J. Biol. Chem. published online April 10, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.425249

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts
Transcriptional regulation of serine/threonine protein kinase (AKT) genes by glioma-associated oncogene homolog 1.

Nitin K. Agarwal, Changju Qu, Kranthi Kunkalla, Yadong Liu, and Francisco Vega

Dr. Kunkalla’s last name was misspelled. The correct spelling is shown above.